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# FORMATION OF DIFFERENT REACTION PRODUCTS WITH SINGLE- AND DOUBLE-STRANDED DNA BY THE *ORTHO*-QUINONE AND THE SEMI-QUINONE FREE RADICAL OF ETOPOSIDE (VP-16-213)

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**Abstract**—In this report, the types of DNA damage introduced by the *ortho*-quinone and the semi-quinone free radical of 4′-demethylepipodophyllotoxin-9-(4-6-*O*-ethylidene-β-D-glucopyranoside) (etoposide) and their relevance for the inactivation of single-stranded (ss) and double-stranded (ds) replicative form (RF) ΦX174 DNA have been examined *in vitro*. The *ortho*-quinone yielded in both ss and ds DNA only chemical adducts, of which on the average about 1 out of 3 and 1 out of 12 per DNA molecule led to inactivation of ss or RF ΦX174 DNA, respectively. The semi-quinone free radical, on the other hand, generated both frank and alkali-labile strand-breaks in ss and in ds DNA which, however, did not contribute significantly to DNA inactivation. The radical introduced, in addition, chemical DNA adducts. Unlike the *ortho*-quinone adducts, however, each of the semi-quinone adducts was lethal in ss ΦX174 DNA, while more than 40 were required for the inactivation of RF DNA. The excision repair system of *Escherichia coli* did not operate on semi-quinone-modified RF DNA but removed about half of the *ortho*-quinone adducts [van Maanen JMS, Lafleur MVM, Mans DRA, van den Akker E, de Ruiter C, Koostra PR, Pappie D, de Vries J, Retèl J and Pinedo HM, *Biochem Pharmacol* **37**: 3579–3589, 1988]. When *ortho*-quinone-modified ss or ds DNA was subjected to a post-alkaline treatment, the adducts remained stably bound to the DNA and the degree of biological inactivation was not influenced. In contrast, post-alkaline treatment removed about 70 and 60% of the semi-quinone adducts from ss and ds DNA, respectively, which, in the case of ss ΦX174 DNA, resulted in a partial restoration of the biological activity. It is concluded that the *ortho*-quinone and the semi-quinone free radical of etoposide produce different types of damage in DNA which have different effects on the biological activity.

The epipodophyllotoxin etoposide [4′-demethyl-epipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-gluco-pyranoside†); NSC 141540; VP-16-213; Fig. 1] is clinically used in the treatment of several neoplastic disorders [1]. The mechanism of action of this chemotherapeutic agent probably involves the introduction of DNA damage, as demonstrated by the formation of DNA strand-breaks and DNA-protein crosslinks in tumor cells upon incubation with etoposide [2–5]. These phenomena are probably for an important part due to inhibition by the drug of the DNA strand-passing and strand-rejoining activity of DNA topoisomerase II [6–8]. In addition to topoisomerase II-mediated DNA strand-scission, the mechanism of action of etoposide could involve metabolic activation to reactive products which are able to damage DNA (Ref. 9 and references therein). *In vitro* metabolism of etoposide by cytochrome P450-mediated oxidation or via peroxidation (e.g. by prostaglandine synthetase and myeloperoxidase) has been demonstrated. The 3′,4′-dihydroxy (catechol) and *ortho*-quinone intermediates thus produced (Fig. 1) bind more strongly to microsomal proteins and to calf thymus DNA than etoposide itself and, in contrast to the parent compound itself, significantly inactivate biologically active ss and ds RF ΦX174 DNA.

We reported recently [10] that the semi-quinone free radical (Fig. 1), in addition to the *ortho*-quinone and the catechol, may also be an important intermediate of etoposide. Our findings suggested that the radical interacts differently with DNA as compared to the *ortho*-quinone and the catechol and introduces different types of DNA lesions. To verify this assumption, we examined in this study the types of DNA damage which are introduced in ss and RF ΦX174 DNA by the semi-quinone free radical of etoposide in relation to the effects on the biological activity of the DNA. The results were compared with those observed with the *ortho*-quinone. Our findings demonstrate the formation of different types of reaction products with DNA by these two species, which have different effects on the biological activity of ss and RF ΦX174 DNA. Some of the results have been published earlier in abstract form [11].

## MATERIALS AND METHODS

*Drugs and chemicals*. Etoposide was a gift from

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† Abbreviations: etoposide, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside); AP, apurinic and/or apyrimidinic; ds, double-stranded; RF, replicative form; ss, single-stranded.

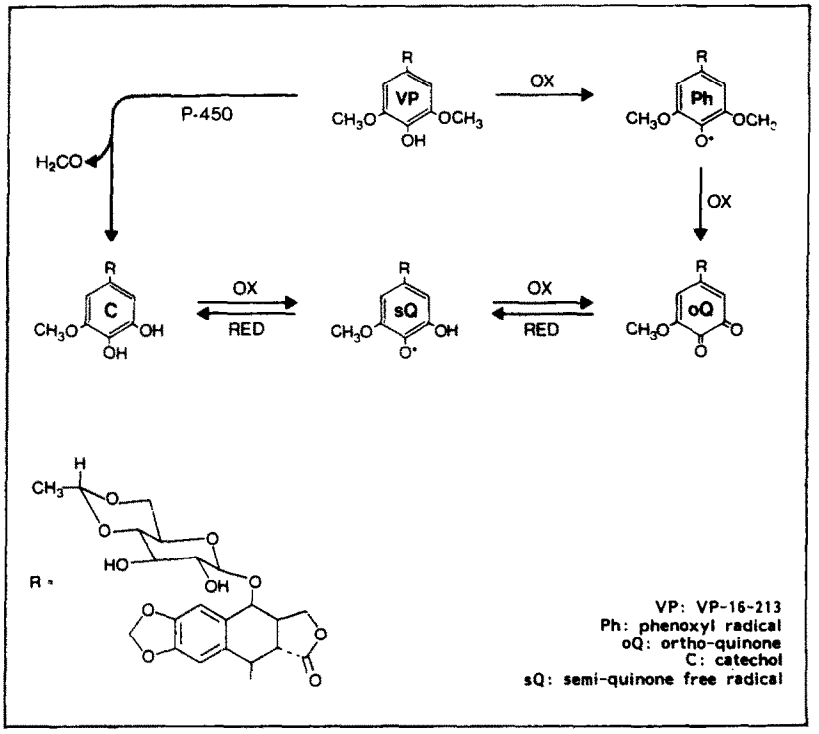


Fig. 1. Possible metabolic conversions of etoposide; for details, see text.

Bristol-Myers Co. (Syracuse, NY, U.S.A.). The *ortho*-quinone of etoposide was synthesized by controlled potential electrolysis of the parent compound at a platinum gauze electrode [12]. The semi-quinone free radical of etoposide was generated from the *ortho*-quinone by incubation at 37° in $5 \times 10^{-2}$ M potassium phosphate pH 7.4 and 9; at pH 4 the *ortho*-quinone is chemically stable [10, 13]. The pH values of all incubation mixtures were checked before and after the experiments to assure that no pH changes had taken place during the incubations. $^3$H-labeled *ortho*-quinone and $^3$H-labeled semi-quinone free radical of etoposide were prepared from a mixture of $^3$H-labeled etoposide (sp. act. 1 Ci/mmol; Moravek Biochemicals, Brea, CA, U.S.A.) and non-labeled etoposide. All other chemicals used were of analytical grade. Only freshly prepared ($^3$H-labeled) *ortho*-quinone of etoposide was used for the experiments.

*DNA preparations*. Biologically active ss and ds RF ΦX174 DNA were isolated from wild-type ΦX174 bacteriophage and ΦX174-infected *Escherichia coli* host bacteria according to earlier published methods [14, 15].

Native, high molecular weight ($1–2 \times 10^7$ daltons) ds calf thymus DNA was purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and dissolved in $2 \times 10^{-2}$ M potassium phosphate pH 7.4 at a concentration of 1 mg/mL. Ss calf thymus DNA was obtained by heating native calf thymus DNA for 5 min at 100°, followed by immediate chilling on ice. Denaturation was confirmed by spectrophotometric measurement of the hyperchromic effect at 260 nm. The prepared ss calf thymus DNA was used immediately for the experiments.

*Simultaneous determination of the number of chemical DNA adducts and of DNA inactivation*. Mixtures of 1.25 μg ss or RF ΦX174 DNA and 2 mg ss or ds calf thymus DNA, respectively, were incubated in open air at 37° with $2.35 \times 10^{-4}$ M $^3$H-labeled *ortho*-quinone (2.70 mCi/mmol) in a total volume of 10 mL $5 \times 10^{-2}$ M potassium phosphate pH 4, 7.4 or 9. At different time intervals during the incubations, fractions of 20 μL were taken to determine the biological activity of ΦX174 DNA (see below); the samples were immediately chilled on ice and diluted 50-fold with $2.5 \times 10^{-2}$ M ice-cold Tris–HCl pH 8.6 to stop the reaction. Additionally, 0.5 mL-DNA fractions were taken to determine the extent of drug-binding. After chilling on ice, the DNA in the 0.5 mL-samples was precipitated by addition of 0.01% (v/v) 5 M NaCl and 2 volumes of cold ethanol, followed by storage for 60 min at −20°. The DNA precipitates were washed twice at 4° with 5 volumes 70% ethanol and twice with 5 volumes 98% ethanol, dried at room temperature under nitrogen and dissolved overnight in 1 mL $10^{-2}$ M sodium citrate pH 7.4. The DNA was then extracted twice at 4° with 2 volumes chloroform/isoamylalcohol/phenol (12/1/12, v/v/w) and twice with 2 volumes diethylether. Finally, the DNA was precipitated, washed, dried and dissolved as described above.

The amounts of DNA recovered were determined spectrophotometrically with a Uvikon 722 LC spectrophotometer (Kontron, Zürich, Switzerland),

assuming that 1 $E_{260\,nm}$ unit measured over 1 cm, equals 40 μg ss DNA/mL or 50 μg ds DNA/mL. The amounts of radioactivity bound to the samples were determined by addition of 5.0 mL Insta-gel II (Packard Instruments BV Chemical Operation, Groningen, The Netherlands) and counting in a Wallac 1214 Rackbeta Liquid Scintillation Counter (LKB, Bromma, Sweden). From the data obtained and from the specific activity of the $^3$H-labeled *ortho*-quinone and semi-quinone free radical used, the number of drug molecules bound per 5386 nucleotides or nucleotide pairs (i.e. per ss or RF ΦX174 DNA molecule, respectively) was calculated.

*Post-incubation alkaline treatment.* Following incubation of the calf thymus DNA–ΦX174 DNA mixtures with $^3$H-labeled *ortho*-quinone of etoposide as described in the previous paragraph, the DNA was assayed for extent of binding and biological inactivation after an alkaline treatment. To this end, 20 μL-ss or -ds DNA samples, taken to determine the extent of DNA inactivation, were post-incubated at pH 12.5 after addition of 0.1 M NaOH. After 0.5 and 3 hr, respectively, samples were neutralized with 0.1 M HCl (pH ± 7.4) and diluted 50-fold with $2.5 \times 10^{-2}$ M ice-cold Tris–HCl pH 8.6. Furthermore, 0.5 mL-DNA samples were taken for determination of the extent of DNA-binding and purified extensively as described in the previous paragraph. After adjusting the pH to 12.5 by addition of 0.1 M NaOH, these samples were post-incubated at 37° for 0.5 and 3 hr, respectively, and neutralized with 0.1 M HCl (pH ± 7.4). Further processing of the DNA was performed as described in the previous paragraph.

*Analysis of frank and alkali-labile strand-breaks in ss and RF ΦX174 DNA.* To investigate whether the reaction of the semi-quinone free radical with DNA causes frank or alkali-labile strand-breaks, DNA strand-break analyses by sedimentation through sucrose gradients were carried out. To this end, 6 μg ss or RF ΦX174 DNA were incubated in open air with $7 \times 10^{-5}$ M *ortho*-quinone and $5 \times 10^{-2}$ M potassium phosphate pH 7.4 or 9 at 37° in a total volume of 2 mL for increasing periods of time. From these incubation mixtures, samples of 0.25 mL containing 0.75 μg DNA were taken and layered on top of neutral linear sucrose gradients. In separate experiments, 0.25 mL-samples of ss or RF ΦX174 DNA which had been incubated with the *ortho*-quinone as described above, were post-incubated at pH 12.5 at 37° for 0.5 or for 3 hr, respectively, and then neutralized (pH ± 7.4). This treatment has been described to convert alkali-labile DNA lesions, including AP sites, into strand-breaks [16]. Sucrose gradient centrifugation of ss and RF ΦX174 DNA and determination of the number of DNA strand-breaks was performed as described previously [17–19].

*Incubations with ss and RF ΦX174 DNA alone.* To examine a possible influence of calf thymus DNA on the biological inactivation of ΦX174 DNA, 12.5 ng ss or RF ΦX174 DNA alone were incubated in open air at 37° with $4.4 \times 10^{-4}$ M or $1.8 \times 10^{-3}$ M *ortho*-quinone (unless indicated otherwise), respectively, in 1 mL $5 \times 10^{-2}$ M potassium phosphate pH 4, 7.4 or 9. At different time intervals during the incubations 20 μL-fractions were taken for which

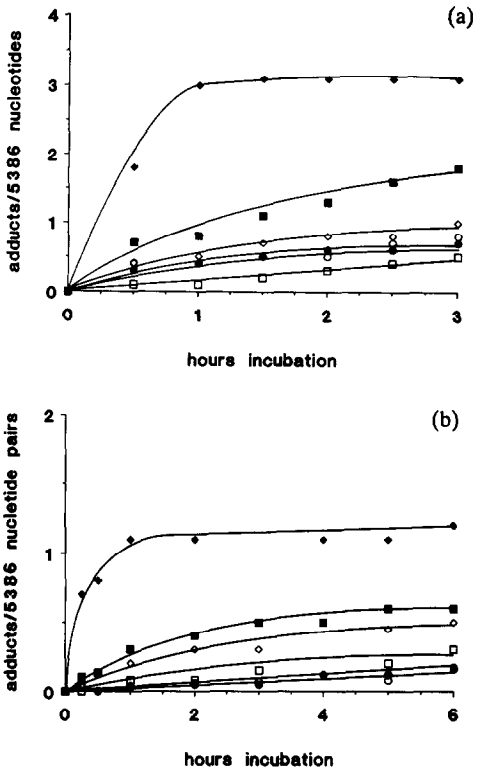


Fig. 2. (a) Introduction of chemical adducts per 5386 nucleotides—i.e. per ss ΦX174 DNA molecule—upon incubation at 37° with $7 \times 10^{-3}$ M *ortho*-quinone of etoposide in $5 \times 10^{-2}$ M potassium phosphate pH 4 (●, ○), 7.4 (■, □) or 9 (◆, ◇) without (closed symbols) and after post-treatment (open symbols) with alkali for 30 min. (b) Introduction of chemical adducts per 5386 nucleotide pairs—i.e. per RF ΦX174 DNA molecule—upon incubation at 37° with $7 \times 10^{-3}$ M *ortho*-quinone of etoposide in $5 \times 10^{-2}$ M potassium phosphate pH 4 (●, ○), 7.4 (■, □) or 9 (◆, ◇) without (closed symbols) and after post-treatment (open symbols) with alkali for 3 hr.

biological activity was determined after dilution to 1 mL with $2.5 \times 10^{-2}$ M ice-cold Tris–HCl pH 8.6.

*Determination of the biological activity.* The biological activity of ss and RF ΦX174 DNA samples in all incubations mentioned in the preceding paragraphs was determined by measuring the bacteriophage production after transfection to spheroplasts prepared from the wildtype *E. coli* strain AB1157, as described in detail elsewhere [20]. In the case of RF ΦX174 DNA the biological activity was also tested on uvrA$^-$ and uvrC$^-$ spheroplasts, prepared from the excision repair deficient *E. coli* strains AB1886 and AB1884, respectively. By plotting semi-logarithmically the surviving fraction of DNA versus incubation time or versus the number of adducts per DNA molecule, $T_{37}$ or $D_{37}$ values, respectively, can be derived, i.e. the incubation time or the mean number of adducts, respectively, leading on the average to one lethal event per DNA molecule. The results presented have been corrected for background effects due to the pH of the incubations alone; these effects were only substantial

in the incubations of ss ΦX174 DNA at pH 4 because of the formation of AP sites (correction factors of about 40%), due to the acidic incubation mixture alone [21].

*Statistics.* All experiments have been performed at least three times in duplicate. Representative data are presented in the figures.

## RESULTS

### *DNA adduct formation*

We showed previously that during incubation at 37° in $5 \times 10^{-2}$ M potassium phosphate pH 4, the *ortho*-quinone of etoposide was chemically stable [13] and was the only DNA-inactivating species in the incubations [10]. At pH values $\geq 7.4$, on the other hand, the semi-quinone free radical was generated from the *ortho*-quinone at increasing rates [13] and was the principal species reacting with DNA [10].

To investigate to what extent formation of chemical adducts plays a role in the observed DNA inactivation by the *ortho*-quinone and the semi-quinone free radical of etoposide, ss or RF ΦX174 DNA was exposed to $^3$H-labeled *ortho*-quinone at pH 4, 7.4 or 9 for increasing periods of time, after which the amount of radioactivity bound to the DNA was determined. Since for such experiments considerable amounts of DNA are required, an excess of ss or ds calf thymus DNA was added to the incubation mixtures. From these experiments the number of *ortho*-quinone and semi-quinone free radical molecules per 5386 nucleotides or nucleotide-pairs, i.e. per ss or RF ΦX174 DNA molecule, respectively, could be accurately calculated. Making the reasonable assumption that the reactivity of both etoposide intermediates towards ss and RF ΦX174 DNA is, on the average, not significantly different from that towards ss and ds calf thymus DNA, this number was taken to represent the mean number of adducts per ΦX174 DNA molecule. Validation of this assumption is strengthened by the comparable kinetics of ss ΦX174 DNA inactivation in the absence and in the presence of ss calf thymus DNA (see below) and by the similarity in guanine–cytosine content of the DNAs.

The results presented in Fig. 2a and b show that exposure of DNA to the *ortho*-quinone led to an increasing rate of binding to both ss and ds (ΦX174) DNA with increasing pH (Fig. 2a and b). Conversion products which are known to be formed upon incubation of the *ortho*-quinone at pH 7.4 and 9 [10] contributed to at the most 3% of the DNA-binding. This could be derived from experiments in which DNA was exposed to $^3$H-labeled *ortho*-quinone which was preincubated at these pH values (data not shown). The DNA-binding observed at pH 7.4 and 9 could thus be largely ascribed to the semi-quinone free radical which, apparently, becomes adducted to DNA at a higher rate than the *ortho*-quinone. Our results show, furthermore, that both intermediates of etoposide react more readily with ss DNA than with ds DNA (Fig. 2a and b).

The chemical adducts formed with DNA after reaction with some drugs or carcinogens can be (partially) released from the DNA by an alkaline

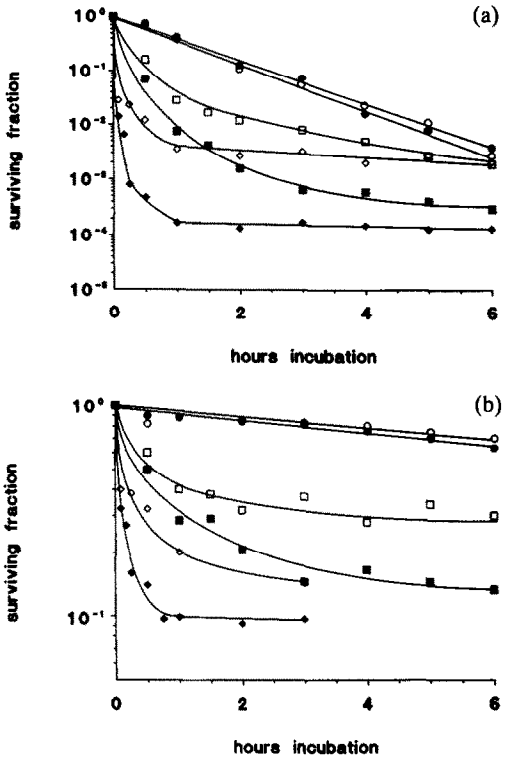


Fig. 3. (a) Representative survival curves of ss ΦX174 DNA (1.25 μg) incubated at 37° in the presence of an excess of ss calf thymus DNA (2 mg) with $2.35 \times 10^{-4}$ M *ortho*-quinone of etoposide in 10 mL $5 \times 10^{-2}$ M potassium phosphate pH 4 (●, ○), 7.4 (■, □) or 9 (◆, ◇) without (closed symbols) and after post-treatment (open symbols) with alkali for 30 min. The survival curves obtained at pH 4 were corrected for inactivation by apurinic sites introduced by the acidic incubation mixture alone (correction factor of about 40%). (b) Representative survival curves of ss ΦX174 DNA (12.5 ng) incubated in the absence of calf thymus DNA at 37° with $4.4 \times 10^{-4}$ M *ortho*-quinone of etoposide in 1 mL $5 \times 10^{-2}$ M potassium phosphate pH 4 (●, ○), 7.4 (■, □) or 9 (◆, ◇) without (closed symbols) and after post-treatment (open symbols) with alkali for 30 min. The survival curves obtained at pH 4 were corrected for inactivation by AP sites introduced by the acidic incubation mixture alone (correction factor of about 40%).

treatment [22–24]. To test whether this situation also applies to the *ortho*-quinone and the semi-quinone adducts, alkaline treatment of the modified DNAs was carried out. Such a treatment had no detectable effect on the binding to ss and ds DNA treated with the *ortho*-quinone (pH 4) whereas the number of semi-quinone adducts in ss and ds DNA (pH 7.4 and 9) was diminished by about 70% and 60%, respectively; (Fig. 2a and b). Thus, the *ortho*-quinone introduces in ss as well as in ds DNA stable adducts (alkali-resistant adducts) only, whereas a considerable part of the semi-quinone adducts in both types of DNA can be removed by post-treatment with alkali (alkali-labile adducts).

### *Inactivation of ss and RF ΦX174 DNA*

When incubated with the *ortho*-quinone of

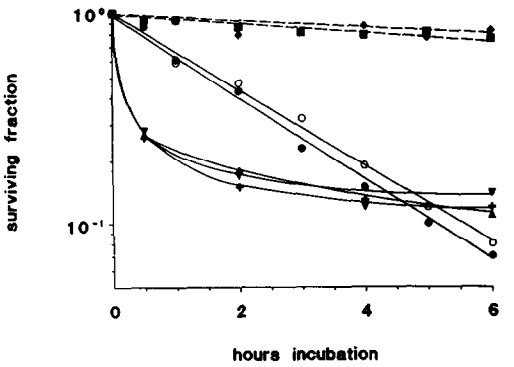


Fig. 4. Representative survival curves of RF ΦX174 DNA (12.5 ng) incubated in the absence of calf thumus DNA at 37° with $1.8 \times 10^{-3}$ M *ortho*-quinone of etoposide in 1 mL $5 \times 10^{-2}$ M potassium phosphate pH 4 (●), 7.4 (■) or 9 (◆), after post-treatment with alkali for 3 hr following incubation with the *ortho*-quinone at pH 4 (○) and upon incubation of 1.5 μg RF ΦX174 DNA at pH 9 with $8.75 \times 10^{-3}$ M *ortho*-quinone (+) compared to those determined with the excision repair deficient spheroplasts uvrA⁻ (▼) and uvrC⁻ (▲). No significant effect of the incubation medium alone on the biological activity of RF ΦX174 DNA was observed during the time course of the measurements.

etoposide, ss ΦX174 DNA was inactivated at an increasing rate upon elevation of the pH from 4 to 7.4 and subsequently to 9, due to increasing formation of the semi-quinone free radical with increasing pH (Fig. 3a and b; Ref. 10). The kinetics of DNA inactivation were not altered in the presence of an excess of ss calf thymus DNA (Fig. 3a and b). The biological inactivation of the DNA by the *ortho*-quinone (pH 4) was not affected by post-treatment with alkali, whereas in the case of the semi-quinone free radical (pH 7.4 and 9) such a treatment led to a partial restoration of the biological activity (Fig. 3a and b). The lower rate and extent of ss ΦX174 DNA inactivation in the presence of an excess of calf thymus DNA compared to those in its absence are probably due to competing reactions with the calf thymus DNA [25].

RF ΦX174 DNA incubated with the *ortho*-quinone was significantly inactivated at pH 4; similarly as for ss ΦX174 DNA, this process was not significantly affected by post-treatment with alkali (Fig. 4). When exposed to the semi-quinone free radical of etoposide (pH 7.4 and 9), no significant inactivation of RF DNA was observed, starting from the same concentrations of *ortho*-quinone as at pH 4 (Fig. 4). When higher concentrations of the semi-quinone free radical were used, appreciable DNA inactivation was observed at pH 9, showing comparable kinetics as found for ss ΦX174 DNA (Fig. 4). Similar DNA survival curves were found after transfection of semi-quinone-modified RF DNA to excision repair-deficient (uvrA⁻ and uvrC⁻) spheroplasts (Fig. 4). This finding indicates that removal of semi-quinone-DNA reaction products via the excision repair pathway is probably not involved in the (relatively small degree of) inactivation of RF ΦX174 DNA by this species.

Inactivation experiments with RF DNA in the presence of calf thymus DNA were not carried out, since from the already very low degree of inactivation observed in its absence (see above) a detectable effect on DNA survival could be expected only after very long periods of time (days). The effect of an alkaline treatment on RF DNA inactivation by the semi-quinone free radical (pH 7.4 and 9) could also not be established due to the fact that this species generates a considerable number of DNA lesions which yield strand-breaks upon an alkaline treatment (see below), thus converting most of the RF DNA into partially degraded ss DNA.

*Introduction of frank and alkali-labile strand-breaks and relationships with DNA inactivation*

Examples of sucrose sedimentation profiles of ss and RF ΦX174 DNA reacted with the semi-quinone free radical of etoposide (at pH 9) are given in Fig. 5a and b. The results from these analyses, as well as those at pH 7.4, show no significant frank strand-breakage in ss ΦX174 DNA (Fig. 6a), whereas in RF ΦX174 DNA a small number of such lesions was introduced (Fig. 6b). Alkaline post-treatment revealed alkali-labile strand-breaks in both types of DNA, particularly in RF ΦX174 DNA (Fig. 6a and b). In contrast, the reaction of the *ortho*-quinone (pH 4) with ΦX174 DNA did not lead to any frank or alkali-labile strand-breaks, as previously shown [20]. Combined with the results from the DNA inactivation studies it can be derived that semi-quinone-induced alkali-labile strand-breakage contributed to at the most 0.3% of the 37% survival level in ss ΦX174 DNA (Figs 3b and 6a), whereas neither frank nor potential (alkali-labile) strand-breaks had a detectable effect on the biological activity of RF ΦX174 DNA (Figs 4 and 6b).

*Relationship between adduct formation and DNA inactivation*

When the surviving fractions of ss ΦX174 DNA, incubated with the *ortho*-quinone or the semi-quinone free radical in the presence of an excess of calf thymus DNA (Fig. 3a), are plotted versus the mean number of adducts per ΦX174 DNA molecule (Fig. 2a), exponential survival curves are obtained (Fig. 7). This means that they represent single-hit functions. From Fig. 7 it can be derived by extrapolation that at 37% survival the *ortho*-quinone (pH 4) introduces, on average, about three adducts per ss ΦX174 DNA molecule and that this value is not affected by post-treatment with alkali. Thus, the *ortho*-quinone introduces in ss ΦX174 DNA stable adducts of which one out of three is inactivating. The inactivation of ss ΦX174 DNA by the semi-quinone free radical (pH 7.4 and 9), on the other hand, required on the average one adduct per DNA molecule, which was reduced to about 0.5 when the DNA was post-treated with alkali (Fig. 7). This indicates that each semi-quinone adduct with a ss ΦX174 DNA molecule represents a lethal event, part of which can be removed by alkali leaving, however, lesions behind which are still inactivating.

Since the inactivation of RF ΦX174 DNA mixed with an excess of ds calf thymus DNA was not examined, only estimations of $D_{37}$ values for its

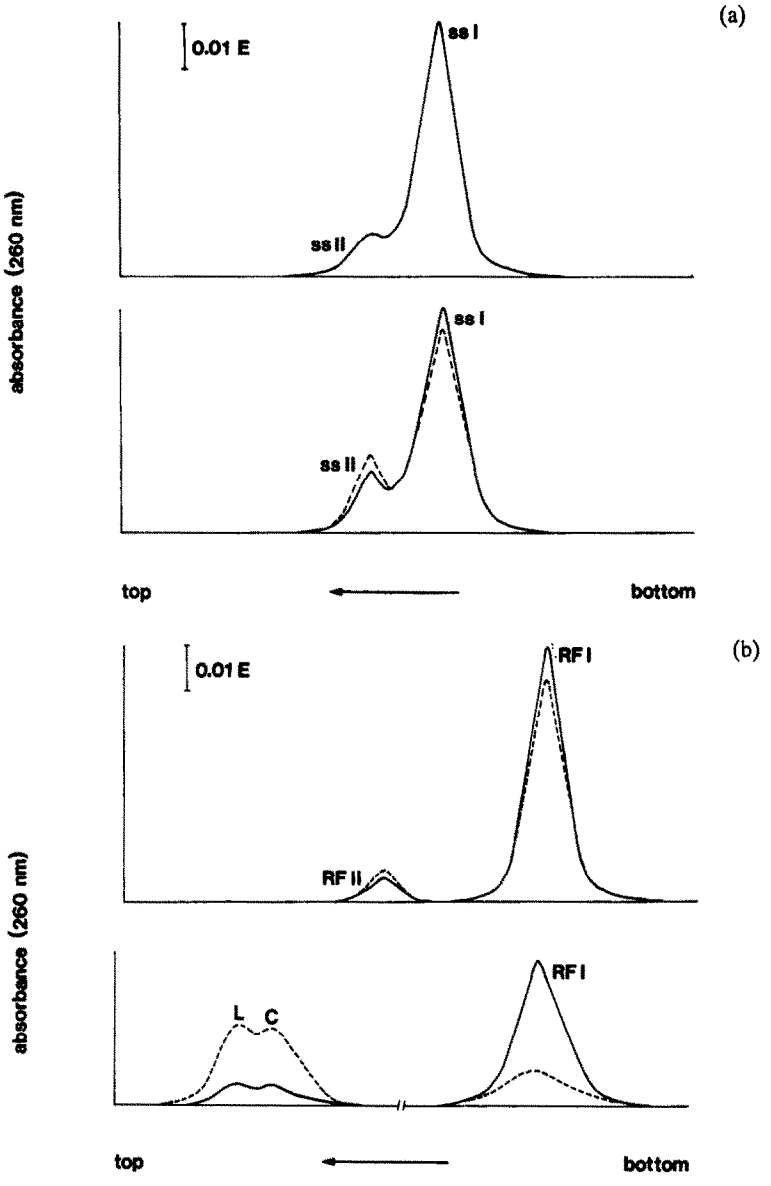


Fig. 5. (a) Sucrose sedimentation profiles of ss ΦX174 DNA (3 μg/mL) alone (——) or after incubation for 2 hr at 37° with $7 \times 10^{-5}$ M *ortho*-quinone of etoposide (– – –) in $5 \times 10^{-2}$ M potassium phosphate pH 9 without (upper panel) and after post-treatment with alkali for 30 min or for 3 hr (lower panel), respectively. Sedimentation profiles were obtained by on line spectrophotometric measurement at 260 nm of the contents of the centrifugation tubes starting from the bottom (direction of arrow). Ss I: covalently closed, circular ss ΦX174 DNA; ss II: ss ΦX174 DNA containing one break. (b) Sucrose sedimentation profiles of RF ΦX174 DNA (3 μg/mL) alone (——) or after incubation for 2 hr at 37° with $7 \times 10^{-5}$ M *ortho*-quinone of etoposide (– – –) in $5 \times 10^{-2}$ M potassium phosphate pH 9 without (upper panel) and after post-treatment with alkali for 30 min or for 3 hr (lower panel), respectively. Sedimentation profiles were obtained by on line spectrophotometric measurement at 260 nm of the contents of the centrifugation tubes starting from the bottom (direction of arrow). RF I: covalently closed, circular, supertwisted RF ΦX174 DNA; RF II: open, circular RF ΦX174 DNA containing one or more single-stranded break(s); C: circular, single-stranded RF ΦX174 DNA; L: linear, single-stranded RF ΦX174 DNA containing one single-stranded break.

inactivation can be given. When corrected for the higher drug concentrations used and for the higher $T_{37}$ values compared to ss ΦX174 DNA (Figs 3b and 4), it can be estimated that about 12 and more than 40 adducts are needed for inactivation of RF ΦX174 DNA by the *ortho*-quinone or by the semi-quinone free radical, respectively.

## DISCUSSION

Based on previous data from DNA inactivation

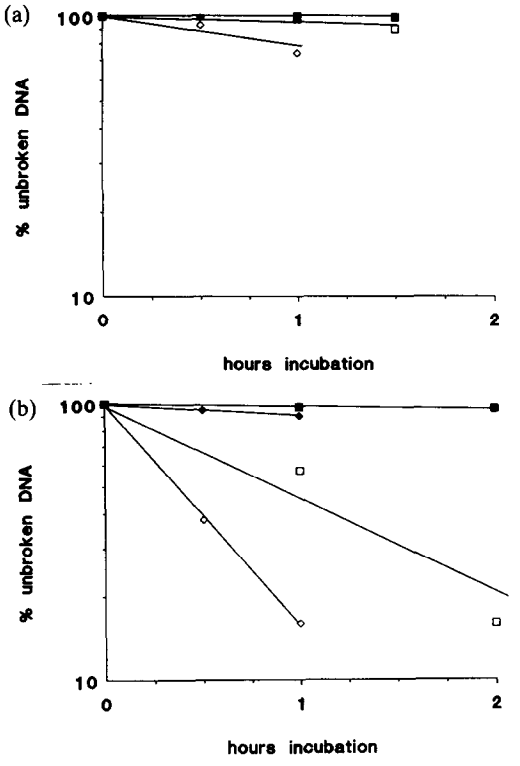


Fig. 6. (a) Introduction of frank strand-breaks in ss ΦX174 DNA (3 μg/mL) incubated at 37° with $0.7 \times 10^{-4}$ M *ortho*-quinone of etoposide in $5 \times 10^{-2}$ M potassium phosphate pH 7.4 (■) or 9 (◆) and of alkali-labile strand-breaks after post-treatment with alkali for 30 min following incubation at pH 7.4 (□) or 9 (◇). (b) Introduction of frank strand-breaks in RF ΦX174 DNA (3 μg/mL), incubated at 37° with $0.7 \times 10^{-4}$ M *ortho*-quinone of etoposide in $5 \times 10^{-2}$ M potassium phosphate pH 7.4 (■) or 9 (◆) and of alkali-labile strand-breaks after post-treatment with alkali for 3 hr following incubation at pH 7.4 (□) or 9 (◇).

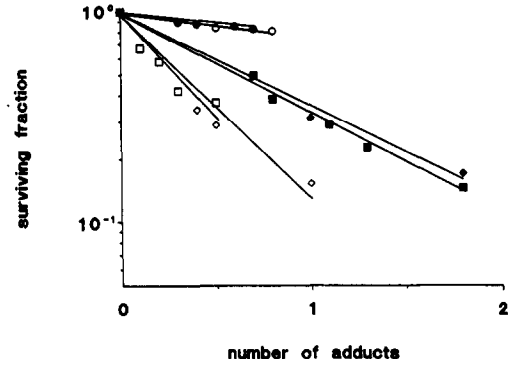


Fig. 7. Relationship between the number of adducts introduced per 5386 nucleotides (see Fig. 2a)—i.e. per ss ΦX174 DNA molecule—and the biological activity of ss ΦX174 DNA as determined in the presence of an excess of ss calf thymus DNA (see Fig. 3a), upon incubation with the *ortho*-quinone of etoposide in potassium phosphate pH 4 (●, ○), 7.4 (■, □) or 9 (◆, ◇) without (closed symbols) and with alkali post-treatment with alkali for 30 min.

studies [10] it could be suggested that the semi-quinone free radical of etoposide interacts differently with DNA as compared to the *ortho*-quinone and probably introduces different types of DNA damage. The results presented in this paper confirm this assumption.

The only detectable type of DNA damage introduced by the *ortho*-quinone (pH 4) in both ss and ds DNA was the formation of chemical adducts (Fig. 2a and b); neither the formation of frank or alkali-labile strand-breaks was observed [20]. In the case of ss ΦX174 DNA, on average one out of three adducts per DNA molecule led to DNA inactivation (Fig. 7). Apparently, a considerable number of the *ortho*-quinone adducts (about 66%) could be easily by-passed during the process of DNA replication. A similar phenomenon has been described before for other types of chemical DNA modification [16, 26, 27]. When RF ΦX174 DNA was treated with the *ortho*-quinone, one lethal hit was estimated (after extrapolation) to correspond to about 12 adducts. About 50% of these lesions were found to be removed via the excision-repair pathway [20]. Thus, in the absence of this process a mean number of six adducts would be required to inactivate a RF molecule, i.e. on average three per DNA strand. These data indicate that the types of *ortho*-quinone adducts formed in ss and ds DNA (probably with the bases) are rather similar.

In contrast to the *ortho*-quinone, the semi-quinone free radical (pH 7.4 and 9) introduced (alkali-labile) strand-breaks in both ss and ds DNA (Figs 5 and 6). These lesions are not due to hydroxyl radicals, since in a previous study [28] the involvement of such species in DNA inactivation was excluded. Moreover, the present results show that the semi-quinone-induced alkali-labile strand-breaks are different in their alkali-catalysed conversion kinetics from those caused by hydroxyl radicals [21]. The most likely candidates for these lesions are AP sites, since such types of DNA damage are characteristically brought to light as strand-breaks by a treatment with alkali [21, 29]. They may have arisen from labilization of the *N*-glycosidic bonds between sugars and bases as a consequence of adduct formation with the bases, as was shown for other DNA base adducts [30, 31]. Although formed in ss DNA in detectable amounts (Fig. 6a), these lesions contributed to the inactivation of ss ΦX174 DNA by at the most 0.3% (Fig. 3b). Involvement of AP sites in the inactivation of RF ΦX174 DNA is probably also negligible—despite their production being brought to light as DNA strand-breaks by alkaline post-treatment (Fig. 6b)—since introduction of such lesions upon reaction with the semi-quinone free radical hardly affected the biological activity of the DNA (Fig. 4).

Thus, the observed DNA inactivation by the semi-quinone free radical could be ascribed largely to the formation of chemical adducts, as was the case for the *ortho*-quinone. In contrast to the *ortho*-quinone adducts, however, each semi-quinone adduct was found to be lethal in ss ΦX174 DNA (Fig. 7) whereas the binding of this species hardly affected the biological activity of RF ΦX174 DNA (Fig. 4). Significant inactivation of RF DNA was only observed upon exposure to relatively high concentrations of

the radical (Fig. 4). In line with this finding is the relatively high number of semi-quinone adducts required to inactivate a RF molecule, which was roughly estimated to be at least 40. Such high numbers are not uncommon and have been reported before for other types of DNA adducts [26]. Excision repair systems, shown previously to operate on *ortho*-quinone-modified RF ΦX174 DNA [20], did not appear to play a role in the (rather small) effect of semi-quinone adducts on the biological activity of RF DNA (Fig. 4).

Other differences between the *ortho*-quinone- and the semi-quinone-DNA reaction products were observed when the modified DNAs were post-treated with alkali. In contrast to the *ortho*-quinone reaction products, a considerable part of the semi-quinone adducts could be removed by alkali (Fig. 2a and b). In the case of ss DNA, about 70% of the adducts became released after such treatment (Fig. 2a) which was accompanied by a considerable restoration of the biological activity of ss ΦX174 DNA (Fig. 3a and b). This indicates that the radical introduced a substantial portion of inactivating adducts in ss DNA which, upon alkali-induced release from the DNA, left behind undamaged sites or damaged sites which were no longer harmful (cf. Refs 22–24). Since, on the other hand, the $D_{37}$ was reduced from one to 0.5 adduct (Fig. 7), it can be deduced that the release of a notable part of semi-quinone-DNA reaction products by alkaline treatment was also accompanied by the formation of DNA lesions which remained lethal. The results can be quantitatively explained by assuming that this latter fraction comprised about 30% of the adducts (Fig. 2a), that the inactivating capacity of the 30% which remained bound to the DNA was unchanged after an alkaline treatment (Fig. 3a and b) and that the release of 37% of the adducts led to reactivation of the DNA (Fig. 7). In the case of ds ΦX174 DNA, alkali-induced removal of DNA adducts (about 60%; Fig. 2b) would probably have no detectable effect on the biological activity, since adduct formation was barely inactivating before the treatment (although a small fraction might cause lethality when AP sites are left behind after alkaline treatment; [21]).

Taken together, the results presented indicate that after reaction of the semi-quinone free radical of etoposide with ss ΦX174 DNA the following types of DNA damage are introduced: (a) inactivating alkali-resistant adducts, (b) inactivating adducts which can be removed by alkali, but leave inactivating DNA lesions behind, (c) inactivating adducts which can be removed by alkali leaving non-lethal sites behind, and (d) inactivating alkali-labile DNA lesions (probably inactivating AP sites) which can be converted into strand-breaks following alkaline treatment but which do not contribute significantly to the process of DNA inactivation. In RF ΦX174 DNA, the semi-quinone free radical introduces also alkali-resistant and alkali-labile adducts as well as frank strand-breaks and alkali-labile lesions; these, however, do not seem to be lethal.

The DNA-binding capacity of the semi-quinone free radical is not confined to that of etoposide. Such an ability has been described for the semi-quinone free radicals of other quinoid compounds as well [32–34]. A possible reaction mechanism could be the formation of carbon-centered radicals which add (probably covalently) to double bonds in the DNA bases [30, 31]. The finding that the introduction of only one semi-quinone adduct per ss ΦX174 DNA molecule was sufficient for inactivation whereas more than 40 were needed to inactivate a RF molecule, suggests, in addition, that the mechanisms involved in the reaction of the radical with ss and ds DNA are rather different.

In a number of studies on the mechanism of action of etoposide, the alkaline elution technique was used to analyse the DNA damage introduced by the drug (Ref. 9 and references therein). The DNA strand-breaks thus detected are associated with the cytotoxicity of etoposide and could be the result of interaction of the drug with the DNA topoisomerase II reaction. The present results indicate, in line with those from previous studies [10, 11, 28, 35], that (alkali-labile) DNA strand-breakage might also be introduced by the semi-quinone free radical of etoposide. As shown in this paper, these lesions may be of little biological relevance, implying that the importance of DNA strand-breaks revealed by the alkaline elution technique can be overestimated.

Recently, we observed binding of radioactivity to isolated and purified DNA of L1210 mouse leukemia cells which were exposed to $^3$H-labeled etoposide (unpublished observations). In a previous study, etoposide-induced DNA strand-scission in A549 human lung adenocarcinoma cells was observed to reach a plateau shortly after addition of the drug while the cytotoxicity of the drug still increased with continued exposure [36]. These findings indicate the involvement of other mechanisms in the cytotoxicity of etoposide in addition to DNA strand-breakage. The data presented in this paper suggest that the formation of chemical DNA adducts by reactive etoposide metabolites may be such an additional mechanism which, moreover, can contribute to the inhibition of the topoisomerase II reaction.

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