

## The effects of counter-ion condensation and co-ion depletion upon the rates of chemical repair of poly(U) radicals by thiols

R. C. FAHEY†, B. VOJNOVIC and B. D. MICHAEL‡

Cancer Research Campaign, Gray Laboratory, P.O. Box 100,  
Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK

(Received 31 August 1990; revision received 19 October 1990;  
accepted 28 October 1990)

Bimolecular rate constants for reactions of poly(U) radicals with a series of thiols of varying net charge ( $Z$ ) were measured by pulse radiolysis with conductivity detection at low ionic strength. At pH 7 and 18°C the values of  $k_2$  ( $M^{-1} s^{-1}$ ) were: reduced glutathione ( $Z = -1$ ),  $< 500$ ; 2-mercaptoethanesulphonic acid ( $Z = -1$ ),  $1.5 \times 10^3$ ; 2-mercaptoethanol ( $Z = 0$ ),  $1.8 \times 10^3$ ; cysteine ( $Z = 0$ ),  $2.0 \times 10^5$ ; cysteamine ( $Z = +1$ ),  $4.1 \times 10^7$ . Values determined at pH 4 were: 2-mercaptoethanol,  $6.1 \times 10^5$ ; cysteamine  $2.2 \times 10^8$ ; *N*-(2-mercaptoethyl)-1,3-diaminopropane (WR-1065,  $Z = +2$ ),  $4.6 \times 10^8$ . The variation in rate with structure could not reasonably be attributed to inherent reactivity differences in the thiols and was ascribed to inhomogeneous distributions of the thiols in solution resulting from electrostatic interactions. Thus, cationic thiols are concentrated  $\sim 100$ -fold near poly(U), relative to neutral thiols, as a consequence of counter-ion condensation, whereas anionic thiols have a  $\sim 100$ -fold lower concentration near poly(U) than neutral thiols as a result of co-ion depletion. These results show that the ability of a thiol to repair radical sites in a polyanion is dramatically influenced by its net charge as a consequence of the counter-ion condensation and co-ion depletion phenomena.

### 1. Introduction

Thiols constitute the most widely studied class of radioprotective compounds and are generally considered to act through limiting damage to DNA by reacting with hydroxyl radicals formed when water is ionized (radical scavenging) and by repairing damaged DNA through hydrogen atom donation (chemical repair) at DNA radical sites in competition with their damage-fixing reaction with oxygen (Alexander and Charlesby 1955, Alper and Howard-Flanders 1956). For protection of transforming DNA in dilute solution by various thiols, the competition between these processes has been demonstrated under steady-state conditions (Held *et al.*, 1981, 1984a) and the rate of chemical repair by dithiothreitol has been determined by the gas explosion technique (Held *et al.* 1984b).

The structural features which make one thiol more radioprotective than another have been the subject of debate for many years (see Klayman and Copeland (1982) for a review). Recently, evidence has been presented suggesting that concentration of cationic thiols near DNA through the counter-ion condensation phenomenon (Manning 1978, Smoluk *et al.* 1986, 1988) and depletion of anionic thiols (co-ions)

†While on sabbatical leave of absence from Department of Chemistry, University of California San Diego, La Jolla, California 92093, USA.

‡To whom correspondence should be addressed.

near DNA via repulsive electrostatic interactions (Le Bret and Zimm 1984, Smoluk *et al.* 1988) may be important factors in determining the radioprotective effectiveness of charged thiols. Support for this view was found in a correlation of the ability of a thiol to protect calf thymus DNA against radiation-induced release of free base with the net charge on the thiol (Zheng *et al.* 1988). The counter-ion condensation and co-ion depletion phenomena can potentially influence the radioprotective action of a charged thiol via both the radical scavenging and the chemical repair mechanisms. That such effects are important in chemical repair is suggested by the finding (Held *et al.* 1984b) that the uncharged thiol, dithiothreitol, appears to repair oxygen-dependent, radiation-induced lesions in bacterial transforming DNA 10-fold faster than does GSH, an anionic thiol. The objective of the present study was to obtain data which would allow a broader test of the effects of counter-ion condensation and co-ion depletion upon the apparent rates of chemical repair reactions.

Chemical repair of poly(U) radicals by dithiothreitol has been measured by Bothe and Schulte-Frohlinde (1982) using pulse radiolysis in combination with conductivity monitoring, and this system appeared to be a promising one for the present study. Like DNA, the poly(U) chains condense cations, but upon introduction of a strand break into the chain, the ability of the poly(U) to condense counterions is reduced, some cations are released, and the conductivity of the solution increases. The rate of conductivity increase measures the lifetime of the poly(U) radical intermediate and in the presence of thiol this lifetime is decreased as a consequence of chemical repair, allowing a determination of the rate for the repair process. This method was used by Bothe and Schulte-Frohlinde (1982) to obtain a value of  $1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  for the second-order rate constant for repair of poly(U) radicals by dithiothreitol. Thus, the poly(U) system is chemically well-defined and it has been well-characterized in previous pulse radiolysis studies.

One feature of the conductivity method as applied to poly(U) is that the solutions must necessarily be of low ionic strength in order to minimize background noise. Under these conditions, ionic thiols present at micro- to milli-molar concentrations can represent a substantial fraction of the total ions present, which means that the counter-ion condensation and co-ion depletion phenomena are expected to have maximal importance. The results presented here confirm this expectation, the measured thiol repair rates varying with net charge on the thiol by nearly six orders of magnitude.

## 2. Methods and materials

### 2.1. Materials

The polyuridylic acid, poly(U), used was obtained from Boehringer but some preliminary experiments were conducted with poly(U) from Sigma. Dithiothreitol (DTT), 2-mercaptoethanol (2-ME), cysteamine (CyA), cysteine (Cys), cystamine, sodium 2-mercaptoethanesulphonate (MESNA), reduced glutathione (GSH), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma.

Monobromobimane was from Calbiochem and *N*-(2-mercaptoethyl)-1,3-diaminopropane (WR-1065) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Methyl chloride was purchased from Greif (Croydon, UK) and nitrous oxide containing < 10 ppm oxygen was obtained from British Oxygen Co., London. Water used in all

preparations was distilled once and purified in a Milli-Q system (Millipore, Inc.) All other chemicals were reagent grade or high purity.

### 2.2. Pulse measurements

The Gray Laboratory's 4 MV Van de Graaff accelerator was utilized to generate electron pulses of 1.5 ns to 1  $\mu$ s duration. A modified glass conductivity cell with platinum black electrodes (cell constant 0.2 cm<sup>-1</sup>; Model CC 4095-00, CP Instrument Co., Bishops Stortford, Herts, UK) was mounted with electrodes perpendicular to the beam and connected to the sample supply system by glass tubing with ground-glass ball joints. The sample supply system was maintained under a positive pressure of N<sub>2</sub>O and permitted solutions which had been previously prepared to be loaded by gravity from a 20–50 ml syringe and to be expelled from the cell by a positive pressure of N<sub>2</sub>O. The flow in the system was controlled by remotely releasing and clamping the syringe so that no valves were used and thus oxygen leakage problems were minimized. The system was not thermostatted but the temperature was monitored at 18  $\pm$  1°C.

Conductivity changes were monitored by symmetrically exciting the cell with a very low distortion balanced sine wave at a frequency of 1 kHz, synchronized with the electron pulse and of amplitude variable from 3 to 100 V peak. A second similar but unirradiated cell was excited by the same source. The currents in the cells were monitored with a differential transformer such that no output was obtained under quiescent conditions, the amplitude and phase of the transformer output thus indicating the magnitude and polarity of the conductance change. The signal from the transformer was passed through a double-balanced phase detector so that information from both half-cycles was used, resulting in the generation of conductivity data every 500  $\mu$ s. This signal was recorded on a Tektronix 7612D digitizer and subsequently stored and analysed. The use of two cells helped to minimize baseline drift at long times, which would otherwise be caused by temperature increases due to the application of the polarizing voltage. Depending upon the signal-to-noise ratio, from 1 to 10 transients were averaged to obtain the final trace. This was fitted with a time-exponential expression,  $G_t = (G_{\text{inf}} - G_0) \exp(k_1^{\text{obs}} t)$ , by a nonlinear least-squares method to yield the value of  $k_1^{\text{obs}}$  and the values of  $G_0$  and  $G_{\text{inf}}$ , the limiting conductances at  $t=0$  and  $t=\infty$ .

The dose was monitored by measuring the pulse charge inductively (Vojnovic 1985) and calibrated using a saturated aqueous solution of methyl chloride. For this purpose a value of  $G = 0.321 \mu\text{mol J}^{-1}$  was used for HCl production (Lilie and Fessenden 1973) and the equivalent conductance of H<sup>+</sup> + Cl<sup>-</sup> at 80°C was taken as 393 S cm<sup>2</sup> mol<sup>-1</sup> (Landolt-Börnstein 1960).

### 2.3. Preparation of solutions

Poly(U) was prepared at least 12 h before use by dissolving it in water at 2 mg ml<sup>-1</sup> and concentrating tenfold to remove low molecular weight contaminants (Bothe and Schulte-Frohlinde 1982) in a Centriflo (CF50A) concentration assembly (Amicon) according to the manufacturer's directions. The concentrate was diluted tenfold with water and the absorbance at 260 nm measured. The actual concentration of poly(U) was calculated from the A<sub>260</sub> value using an extinction coefficient of 9700 M<sup>-1</sup> cm<sup>-1</sup> (Bothe and Schulte-Frohlinde 1982). This poly(U) stock solution was further diluted to the desired concentration and purged with N<sub>2</sub>O for at least 40 min, after which the pH was adjusted if necessary to 7.0 with

10 mM KOH or to 4.0 with 10 mM HClO<sub>4</sub>. The desired aliquot (20–50 ml) of poly(U) was then loaded into a glass syringe and the solution transferred to a gassing tower where it was bubbled with N<sub>2</sub>O for at least an additional 30 min. Thiol was added to the poly(U) solution at the beginning of the latter gassing step from stock solutions in water that were 40 to 20 000 times more concentrated than the final concentration and had been adjusted to the desired pH with KOH if necessary. This lengthy degassing procedure was found to be necessary in order to exclude oxygen completely from the samples. The solutions were then reloaded into the syringe and the latter attached to the sample supply system. The conductivity cell was flushed thoroughly with N<sub>2</sub>O-gassed water and several times with the poly(U) solution before the first pulse measurements were made. After being irradiated, the poly(U) solution was collected and the pH determined; the measured value was normally within 0.2 pH units of the starting value and, if a greater deviation was observed, the measurement was discarded.

#### 2.4. Thiol determinations

Thiol stock solutions were prepared by weight and the thiol level checked by titration with DTNB (Ellman 1959). Cysteamine levels in poly(U) solutions were determined by collecting ~1 ml of solution as it exited from the conductivity cell in a vial containing 20 μl each of 0.25 M Tris (pH 9.0), 50 mM monobromobimane in acetonitrile, and 10 mM 2-mercaptoethanol in 1 mM methanesulphonic acid. The latter was included to deplete any trace amounts of highly reactive bimane contaminants which might otherwise react rapidly with cysteamine but yield products not measured as the cysteamine adduct (M. R. L. Stratford and R. R. Watfa personal communication). The sample was protected from light and allowed to react for 10 min, after which 20 μl of 0.5 M methanesulphonic acid was added. The samples were kept on ice until all samples had been processed. After measurement of the volume, 1 ml of each sample was filtered in a Centricon 10 microconcentrator (Amicon). The filtrate was extracted with 1 ml of methylene chloride to remove non-ionic bimane adducts and unreacted monobromobimane, and then transferred to a brown injection vial for HPLC analysis. HPLC analysis was performed on a Waters Model 840 chromatograph using a method similar to that described by Minchinton (1984). Calibration was performed with solutions prepared as above but using 1 ml of water in place of the poly(U) solution, adding known amounts of cysteamine from concentrated stock solutions (final concentrations 0.2, 2, and 5 μM), and omitting the filtration step. Recovery experiments were conducted using 1 ml of the starting poly(U) stock solutions to which known amounts of cysteamine were added (final concentrations 0.1, 0.3, 1, 2.5 and 5 μM) immediately after the start of the derivatization step; subsequent steps were the same as described above for unknown samples.

### 3. Results

In the initial experiments we tested the ability of our measurement system to reproduce the results obtained by Bothe and Schulte-Frohlinde (1982) with poly(U). The increase in conductivity following an 0.5 μs electron pulse of 41 Gy for poly(U) at pH 7 is shown in Figure 1(a). Also shown is the calculated curve corresponding to a first-order kinetic fit of the results with  $k_1 = 0.7 \text{ s}^{-1}$ . The general features of the curve are in good agreement with the results of Bothe and Schulte-Frohlinde (1982). As noted by them, there appears to be a second slower

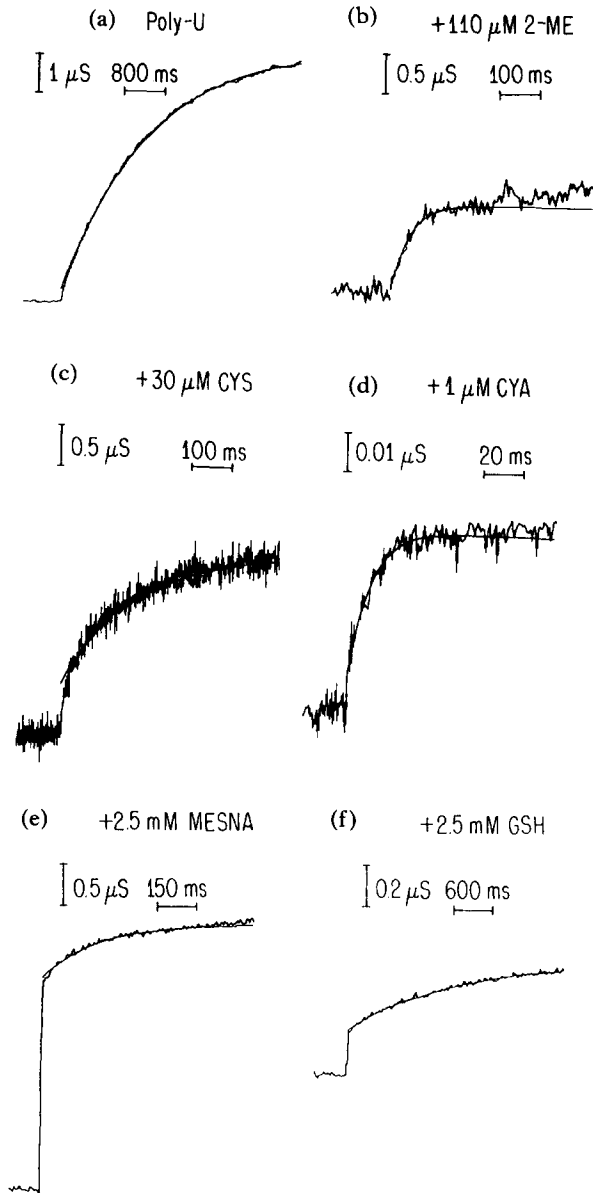


Figure 1. Representative traces of conductance (cell constant =  $0.2 \text{ cm}^{-1}$ ) vs time obtained with poly(U) solutions ( $60 \text{ mg l}^{-1}$ , except as noted) at pH 7 following delivery of a short ( $1.5 \text{ ns}$  to  $1 \mu\text{s}$ ) electron pulse. The calculated line generated using parameters obtained from the first-order least-squares fit of the experimental curves is also shown. (a) Poly(U) alone, 41 Gy, single measurement,  $k_1^{\text{obs}} = 0.67 \text{ s}^{-1}$ . (b) poly(U) plus 2-ME, 32 Gy, average of 7 measurements,  $k_1^{\text{obs}} = 23 \text{ s}^{-1}$ . (c) Poly(U) plus Cys, 8 Gy, single measurement,  $k_1^{\text{obs}} = 7 \text{ s}^{-1}$ . (d) Poly(U) plus CyA, 0.55 Gy, single measurement,  $k_1^{\text{obs}} = 100 \text{ s}^{-1}$ . (e) poly(U) ( $360 \text{ mg l}^{-1}$ ) plus MESNA, 80 Gy, average of six measurements,  $k_1^{\text{obs}} = 4.8 \text{ s}^{-1}$ . (f) Poly(U) ( $360 \text{ mg l}^{-1}$ ) plus GSH, 5.5 Gy, average of seven measurements,  $k_1^{\text{obs}} = 0.6 \text{ s}^{-1}$ .

component to the kinetics which causes the first-order fit at early times to fall below the observed values at long times. We also confirmed their general findings that the conductivity yield plateaued at high doses and that the dose at which the plateau occurred decreased with decreasing poly(U) concentration, but our conductivity yields were generally about half those found by them. The reason for the differences in yield is not clear, but we found a lower yield with Sigma than with Boehringer poly(U) and our method of preparing the poly(U) differs slightly from theirs. As Bothe and Schulte-Frohlinde (1982) point out, hydroxyl radical recombination competes with the reaction between the hydroxyl radical and poly(U) under pulse conditions so that quantitative interpretation of the yield data is complicated. The conductivity yield does not readily provide information about the repair rates and was considered here only in terms of its general consistency with the rate measurements. In the present work, the values of  $k_1^{\text{obs}}$  were of key importance as these provide the basis for determination of thiol repair rates and our value of  $k_1^{\text{obs}}$  for poly(U) is in excellent agreement with that measured by Bothe and Schulte-Frohlinde (1982).

Preliminary measurements of the effect of DTT upon  $k_1^{\text{obs}}$  for poly(U) yielded a second-order repair rate constant  $k_2 \sim 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  which agreed favourably with the value of  $1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  found by Bothe and Schulte-Frohlinde (1982). Having assured ourselves that we were able to reproduce the key features of the earlier work, we proceeded with measurements designed to determine the chemical repair rates for a series of thiols of varying net charge.

Two thiols having a net charge of zero were selected for study. One, 2-mercaptoethanol, is similar to DTT in that it possesses no ionized groups at pH 7; however, being a monothiol, it would not be expected to exhibit the special characteristics sometimes found for dithiols. The other, cysteine, has a net charge of zero because the charges of the ionized amino and carboxyl groups cancel each other while the thiol group remains largely unionized at this pH. As seen in Figures 1(b) and 1(c), these thiols decrease markedly the conductivity yield relative to that seen with poly(U) alone, and at the same time increase substantially the value of  $k_1^{\text{obs}}$ . In the case of Cys it was found that a sharp increase in the conductivity often preceded the slower exponential increase. The former, but not the latter, change was seen with Cys alone, and could be attributed to conductivity changes associated with oxidation of the thiol. This portion of the curve was therefore ignored in fitting a first-order exponential curve to the results. In each determination of  $k_1^{\text{obs}}$  at least two doses were employed, and it was established that the observed rate was dose-independent. The value of  $k_1^{\text{obs}}$  was measured as a function of thiol concentration (Figures 2(a) and 2(b)), the results showing a reasonably linear dependence. Values of the second-order repair rate calculated from the slopes were nearly identical for the two thiols.

We sought next to measure the rate of cysteamine, which has a net charge of +1 at pH 7, but it became immediately apparent that this would be a much more difficult task. Concentrations of CyA above several micromolar completely eliminated the conductivity change by an apparently very rapid repair of the poly(U) radicals. The repair could be slowed by utilizing low CyA concentrations, but there was then the necessity of restricting the dose so that the thiol would not be converted to the disulphide via the thiyl radical. By optimizing the sensitivity of the measuring system it was eventually possible to obtain measurements with CyA

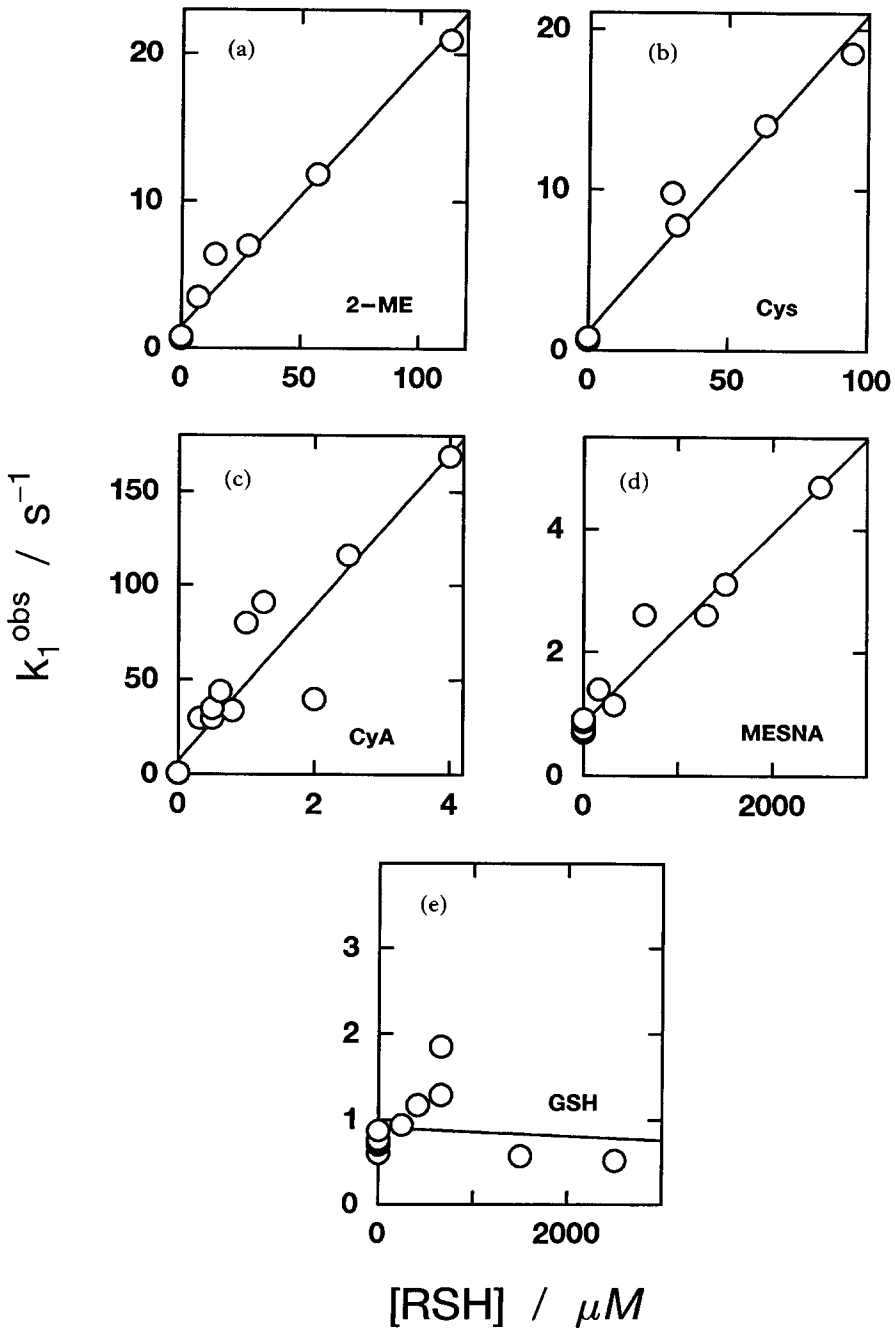


Figure 2. Variation of  $k_1^{obs}$  with concentration of thiol at pH 7. The solid lines represent the unweighted least-squares fits of the data and the slopes were utilized to calculate the values for  $k_2$ .

(Figure 1(d)) and to show that the rates were dose-independent. The variation in  $k_1^{\text{obs}}$  with the concentration of CyA (Figure 2(c)) yielded a value of  $k_2$  which was more than two orders of magnitude greater than that found for 2-ME and Cys.

Although autoxidation of the thiol was unlikely, since oxygen was rigorously excluded from the system by prolonged gassing with  $\text{N}_2\text{O}$ , we felt it important to establish that CyA actually remained present as the thiol during the pulse experiment at the low concentrations employed. We sought to accomplish this by derivatizing the sample with monobromobimane and analysing for the CyA derivative by HPLC with fluorescence detection (Fahey and Newton 1987). In order to protect the HPLC column it was necessary to remove the poly(U) prior to the chromatography step, and this was accomplished by filtering the sample through a molecular-weight selective membrane in a Centricon-10 microconcentrator. The precision of the analysis was limited by the fact that recovery of standards through the procedure was unreliable below  $0.3 \mu\text{M}$  CyA and was only 40–80% in the concentration range  $0.3$ – $2.5 \mu\text{M}$  CyA, lowest recoveries occurring at the lowest concentration, due apparently to absorption losses during the filtration step. When samples of poly(U) plus cysteamine were prepared, loaded in the pulse conductivity cell in the usual manner, collected upon exit from the cell and derivatized with monobromobimane, subsequent analysis revealed no major loss (<20%) of thiol during gassing, loading and manipulation of the sample when corrected for losses during filtration. Thus, the actual level of CyA present when the pulse is administered is not substantially less than the stated levels. When poly(U) samples containing  $1 \mu\text{M}$  CyA received a 3 Gy pulse, or one containing  $0.5 \mu\text{M}$  CyA received a 1.6 Gy pulse, the CyA levels declined measurably but by no more than 25%, confirming the conclusion based on the independence of  $k_1^{\text{obs}}$  on dose that radiolytic thiol depletion under the conditions of measurement is not large enough to influence the results appreciably.

Although marked losses of cysteamine were ruled out as a complicating factor, we were also concerned that the observed repair phenomenon might result from the presence of traces of cystamine in the cysteamine solutions. Since this disulphide bears a net charge of +2 it should be more efficiently concentrated near poly(U) than cysteamine with its net +1 charge. However, when the value of  $k_1^{\text{obs}}$  was measured for a poly(U) solution containing  $1 \mu\text{M}$  cystamine, it was found to be only one-fourth that found for  $1 \mu\text{M}$  CyA so that the rates observed for CyA cannot result from reactions involving contaminating cystamine.

Two thiols bearing a net charge of  $-1$  at pH 7, sodium 2-mercaptoethanesulphonate (MESNA) and GSH, were also examined. In both cases the presence of thiol at concentrations approaching millimolar gave transients yielding values of  $k_1^{\text{obs}}$  which were not significantly different from those obtained with poly(U) alone. Moreover, with poly(U) at  $60 \text{ mg l}^{-1}$  and concentrations of MESNA or GSH in the millimolar range, the fast increase in conductivity due to direct reaction of thiol with hydroxyl radical became very large, and the slow increase in conductivity due to poly(U) cleavage could not be observed. To overcome this difficulty the poly(U) concentration was increased to  $360 \text{ mg l}^{-1}$  to allow poly(U) to compete more effectively with thiol for hydroxyl radicals. Although the initial fast conductivity increase was not eliminated at the higher poly(U) concentration, the slower increase ascribed to poly(U) strand breakage could be observed (Figures 1(e) and 1(f)). The observed dependence of  $k_1^{\text{obs}}$  upon [MESNA] yielded a value of  $k_2 = 1.5 \times 10^3$



(Figure 2(d)), more than two orders of magnitude slower than the corresponding rates for 2-ME and Cys. However, with GSH no increase in  $k_1^{\text{obs}}$  could be detected at even the highest GSH concentration (Figure 2(e)) and only an upper limit could be established for  $k_2$ .

The failure to observe measurable effects of GSH upon  $k_1^{\text{obs}}$  was consistent with conclusions drawn from the observed effect of GSH upon the conductivity yield. In the presence of GSH the yield was expected to decrease owing to the competition between GSH reacting with hydroxyl radical,  $k_2 = 1.5 \times 10^{10}$  (Quintiliani *et al.* 1977), and poly(U) reacting with hydroxyl radical,  $k_2 = 1.5 \times 10^9$  (Bothe and Schulte-Frohlinde 1982). At 1.5 mM GSH and 1.1 mM poly(U) one can calculate from the known rates that GSH should reduce the yield by a factor of 0.09 relative to the yield in the absence of GSH. The observed factor was 0.14, showing that GSH does not reduce the yield to even the extent predicted theoretically for hydroxyl radical scavenging, much less providing any further reduction that might be attributed to repair reactions of poly(U). Although the rate for reaction of MESNA with hydroxyl radicals has not been measured, it can be expected to have essentially the same diffusion-controlled rate as measured for GSH. When measured on the same day and under the same conditions as for GSH, the presence of 1.5 mM MESNA caused a reduction in yield that was 5–7-fold greater than observed with 1.5 mM GSH. This further decrease over that expected for hydroxyl radical scavenging is in acceptable accord with the measured rate for repair by MESNA (Figure 2(d)) which predicts an approximately 4-fold reduction in yield as a result of repair. Thus, the measured conductivity yields support the conclusion that repair by GSH is not measurable at low millimolar concentrations, whereas repair by MESNA is just measurable under these conditions.

We wished to determine  $k_2$  for the radioprotective thiol WR-1065 which has a net charge of approximately +2 at pH 7, and was therefore expected to be even more effective than CyA in repairing poly(U) radicals. However, preliminary experiments indicated that measurements at pH 7 with WR-1065 were likely to be extremely difficult because the high repair rate requires that very low thiol concentrations be used in order to allow some non-repaired radical to form and produce a measurable signal. However, at very low thiol concentrations one must also use very low doses to preclude radiolytic depletion of the thiol. This also reduces the radical yield, the net result being that the window where meaningful measurements can be made becomes vanishingly small for WR-1065 at pH 7. However, measurements with WR-1065 were possible at pH 4. Bothe and Schulte-Frohlinde (1982) had found that  $k_1^{\text{obs}}$  for poly(U) increases with decreasing pH, the value at pH 4 being some two orders of magnitude greater than the value at pH 7, and we were able to confirm this result (Figure 3(a)). The larger value of  $k_1^{\text{obs}}$  in the absence of thiol greatly facilitates the measurements with reactive thiols, permitting rates to be determined for WR-1065 as well as the CyA and 2ME (Figures 3 and 4). This improvement occurs mainly because at pH 4 and a given thiol concentration a larger fraction of the poly(U) radicals produce strand breaks, and the resulting conductivity signal is therefore greater. Another advantage of measurement at pH 4 was that there was no significant concern over loss of thiol at low micromolar concentrations, since thiols are less reactive in acidic solutions. Table 1 lists the values of  $k_2$  obtained at pH 4, along with those measured at pH 7, and the relative rates computed from the results at both pH values.

#### 4. Discussion

The observed variation in the rate of repair of poly(U) radicals with thiol structure of nearly six orders of magnitude (Table 1) is difficult to understand in terms of conventional structure–reactivity concepts. All of the thiols studied have the general structure  $\text{XYCHCH}_2\text{SH}$ , where X and Y are variable groups. The presence of two saturated carbons between the SH and the variable groups precludes resonance effects and since all of the thiols have primary structures, steric effects are not likely to be large. This leaves inductive effects but these are non-existent for H-atom abstraction from a carbon atom two carbons removed from the substituent (Tedder 1982) and therefore would not be expected to be important for the corresponding abstraction from sulphur. Moreover, the measured rates for H-

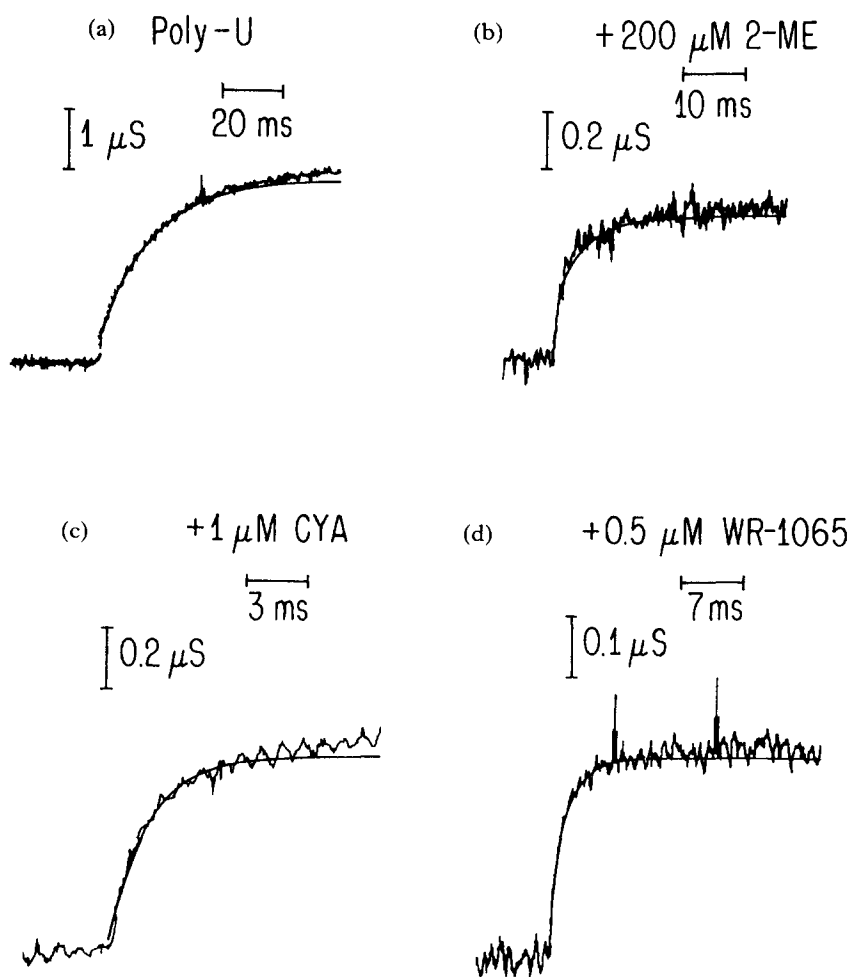


Figure 3. Representative traces of conductance vs time obtained as in Figure 1 but at pH 4. (a) Poly(U) alone, 2.5 Gy, single measurement,  $k_1^{\text{obs}} = 46 \text{ s}^{-1}$ . (b) Poly(U) plus 2-ME, 8 Gy, single measurement,  $k_1^{\text{obs}} = 179 \text{ s}^{-1}$ . (c) Poly(U) plus CyA, 1.3 Gy, average of 10 measurements,  $k_1^{\text{obs}} = 400 \text{ s}^{-1}$ . (d) Poly(U) plus WR-1065, 0.7 Gy, average of five measurements,  $k_1^{\text{obs}} = 560 \text{ s}^{-1}$ .

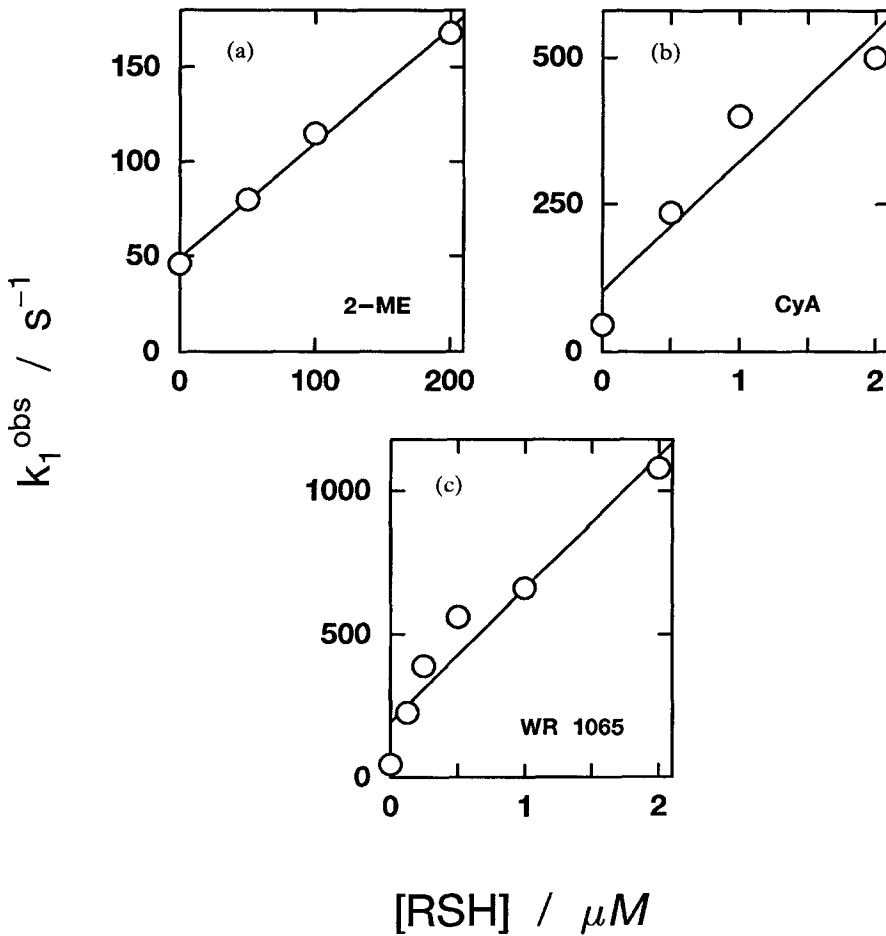


Figure 4. Variation of  $k_1^{obs}$  with concentration of thiol at pH 4. The solid lines represent the unweighted least-squares fits of the data and the values of  $k_2$  were calculated from the slopes of these lines.

Table 1. Rates for repair of poly(U) radicals by thiols.

Thiol	Net charge at pH 7	pH 7		pH 4	
		$k_2(M^{-1} s^{-1})$	Rel. rate	$k_2(M^{-1} s^{-1})$	Rel. rate
GSH	-1	< 500	< 0.003	—	—
MESNA	-1	$1.5 \pm 0.1 \times 10^3$	0.009	—	—
2-ME	0	$1.8 \pm 0.1 \times 10^5$	1.0	$6.1 \pm 0.3 \times 10^5$	1.0
Cys	0	$2.0 \pm 0.2 \times 10^5$	1.1	—	—
CyA	+1	$4.1 \pm 0.4 \times 10^7$	230	$2.2 \pm 0.5 \times 10^8$	360
WR-1065	+2	—	—	$4.6 \pm 0.7 \times 10^8$	760

Errors shown are  $\pm 1$  standard error of regression.

Int J Radiat Biol Downloaded from informahealthcare.com by The Keeper of Scientific Books on 04/26/10 For personal use only.

atom transfer to alcohol radicals from GSH, Cys, and CyA differ by no more than a factor of two (Willson 1983, Nucifora *et al.* 1972) so that the present marked rate differences reflect a special property of the polyanion poly(U) in reactions with charged molecules. The observed variation of  $k_2$  values with net charge is plausibly explained by the counter-ion condensation and co-ion depletion properties of polyanions, and no alternative explanation is immediately apparent. In the accompanying paper (Fahey *et al.* 1991), we show that for DNA similar, although reduced, dependences of repair rate upon thiol net charge arise at higher ionic strength. This also supports the view that the effects are due to electrostatic interactions.

GSH is less effective in repairing poly(U) than is MESNA (Table 1) and therefore would appear to be depleted near poly(U) to a greater extent than MESNA, even though both thiols bear a net charge of  $-1$ . A possible basis for this difference derives from the fact that GSH is significantly larger than MESNA. The Monte-Carlo calculations of Le Bret and Zimm (1984) revealed that smaller cations are condensed at DNA more favourably than larger cations and, although calculations were not done to explore the role of size with anions, it seems likely that the approach of large anions to DNA or other polyanions would be similarly disfavoured on the basis of the larger volume excluded to occupancy by cations.

If the foregoing analysis is correct, then all of the thiols in Table 1 should have essentially the same inherent reactivity as 2-ME and Cys, and the apparent differences in the values for  $k_2$  shown in Table 1 derive from a breakdown of the assumption that the thiols are homogeneously distributed in solution as a consequence of counter-ion condensation or of co-ion depletion. On this basis we can estimate from the results that the effective concentration of CyA near poly(U) is  $\sim 100$ -fold greater than its average concentration, whereas the effective concentration of MESNA near poly(U) is  $\sim 100$ -fold less than its average concentration. Such differences appear to be of the same magnitude as those calculated on theoretical grounds for DNA (Le Bret and Zimm 1984).

It seemed surprising at first that WR-1065, with its net charge of  $+2$ , was only slightly more reactive than CyA when the values of  $k_2$  were measured at pH 4, but a consideration of the stoichiometry involved shows that this is in accord with the counter-ion condensation phenomenon. Thus, Bothe and Schulte-Frohlinde (1982) estimate that poly(U) is 71% dissociated under these conditions, which means that 29% ( $53 \mu\text{M}$ ) of the  $180 \mu\text{M K}^+$  ions present at  $60 \text{ mg l}^{-1}$  poly(U) are condensed. Assuming that all monovalent cations are equivalent with respect to their ability to be condensed, then at  $1 \mu\text{M}$  thiol concentration the CyA represents 0.55% of the condensed ions which corresponds to  $0.29 \mu\text{M}$  or 29% of the total CyA present. If we now consider WR-1065 it is apparent that although the net  $+2$  charge may enhance condensation, the maximum possible is 100%, resulting in only about a 3-fold increase in the amount of condensed thiol. Other factors, such as the assessment somewhat, but it is clear that with a major fraction of the cationic thiol being condensed under these conditions the observed somewhat more than 2-fold greater reactivity of WR-1065 than that of CyA is in accord with the theory. Larger differences between WR-1065 and CyA were seen in their binding to DNA (Smoluk *et al.* 1988) and in their protection against radiation-induced base release from DNA (Zheng *et al.* 1988) but the stoichiometry in those cases was such that very little of the thiol was bound. This analysis suggests that a more significant difference between the values of  $k_2$  for CyA and WR-1065 might have been seen

had the measurements been made at higher ionic strength where a smaller fraction of the thiol is bound; however, the conductivity method is restricted to low ionic strength solutions and this precludes such studies.

It is possible to detail more specifically the nature of some of the repair processes measured here. Hydroxyl radical attacks poly(U) primarily through forming an adduct at uracil (Deeble *et al.* 1986); this adduct has been directly measured by ESR (Hildenbrand and Schulte-Frohlinde 1989) and its rate of decay accords with the rate of strand breakage measured here and earlier (Bothe and Schulte-Frohlinde 1982) by pulse radiolysis with conductivity detection. The values of  $k_2$  reported in Table 1 at pH 7 therefore can be taken to represent H-atom transfer rates to the C(5) hydroxyl radical adduct. At pH  $\leq 4$  this radical is not observed by ESR and the spectrum found has been ascribed to a sugar radical produced by elimination of the 3'-phosphate group (Hildenbrand and Schulte-Frohlinde 1989). This observation, together with the observed pH dependence of strand break formation (Bothe and Schulte-Frohlinde 1982), were interpreted as resulting from acid-catalysed dehydration of the C(5) adduct to yield the radical cation of uracil which directly or indirectly produces a sugar radical precursor to strand breakage at a faster rate than does the C(5) adduct itself. Our finding that the values of  $k_2$  for 2-ME and CyA at pH 4 are 3–6 times greater than are observed at pH 7 indicate either that a different and more reactive poly(U) radical is involved in the repair process at low pH or that the reactivity of the C(5) adduct is somehow increased by environmental effects at low pH.

The results of the present study demonstrate that with a single-stranded polynucleotide at low ionic strength counter-ion condensation and co-ion depletion can play a dramatic role in determining rates of chemical repair of polyanion radicals by thiols, and it seems likely that such effects are also important with double-stranded DNA, even at physiological ionic strength. Such effects were seen in the protection of calf thymus DNA measured in 0.1 M sodium perchlorate (Zheng *et al.* 1988), and it seems probable that a significant portion of the protection measured resulted from repair processes. Although repair rates for thiols cannot be determined at physiological ionic strength by the present methodology, they can be measured using the gas explosion technique (Michael *et al.* 1979, Held *et al.* 1984b). We report in the accompanying paper (Fahey *et al.* 1991) studies using this approach which show that the effects of counter-ion condensation and co-ion depletion upon the repair rates of DNA radicals by thiols at physiological ionic strength, while not as large as the effects shown here, are nevertheless significant.

### Acknowledgements

The authors thank Brian Hall and Keith Allen for operating the Van de Graaff accelerator, Michael Stratford and Rodeina Watfa for performing the HPLC analyses of the bimeane derivatives, Rosalind Orchard for construction of electronics equipment used in these studies, Eberhard Bothe for helpful advice on technical procedures, and Dorothy Brown for preparation of the manuscript. This work was supported by the Cancer Research Campaign, by a Cancer Scholar Grant from the American Cancer Society to R.C.F., and by Grant CA-39582 from the National Institute of Health, US Public Health Service.

### References

- ALEXANDER, P. and CHARLESBY, A., 1955, Physico-chemical methods of protection against ionizing radiations. *Radiobiology Symposium, Liège, 1954*, edited by Z. M. Bacq and P. Alexander (Butterworth, London), pp. 49–60.

- ALPER, T. and HOWARD-FLANDERS, P., 1956, Role of oxygen in modifying the radiosensitivity of *E. coli* B. *Nature*, **178**, 978–979.
- BOTHE, E. and SCHULTE-FROHLINDE, D., 1982, Release of  $K^+$  and  $H^+$  from poly(U) in aqueous solution upon  $\gamma$  and electron irradiation. Rate of strand break formation in poly(U). *Zeitschrift für Naturforschung*, **37c**, 1191–1204.
- DEEBLE, D. J., SCHULTZ, D. and VON SONNTAG, C., 1986, Reactions of OH radicals with poly(U) in deoxygenated solutions: sites of OH radical attack and the kinetics of base release. *International Journal of Radiation Biology*, **49**, 915–926.
- ELLMAN, G. L., 1959, Tissue sulphhydryl groups. *Archives of Biochemistry and Biophysics*, **82**, 70–77.
- FAHEY, R. C. and NEWTON, G. L., 1987, Determination of low-molecular-weight thiols using monobromobimane fluorescent labelling and high-performance liquid chromatography. *Methods in Enzymology*, **143**, 85–96.
- FAHEY, R. C., PRISE, K. M., STRATFORD, M. R. L., WATFA, R. R. and MICHAEL, B. D., 1991, Rates for repair of pBR 322 DNA radicals by thiols as measured by the gas explosion technique: evidence that counter-ion condensation and co-ion depletion are significant at physiological ionic strength. *International Journal of Radiation Biology*, **59**, 901–917.
- HELD, K. D., HARROP, H. A. and MICHAEL, B. D., 1981, Effects of oxygen and sulphhydryl-containing compounds on irradiated transforming DNA. I. Actions of dithiothreitol. *International Journal of Radiation Biology*, **45**, 613–622.
- HELD, K. D., HARROP, H. A. and MICHAEL, B. D., 1984a, Effects of oxygen and sulphhydryl-containing compounds on irradiated transforming DNA. II. Glutathione, cysteine and cysteamine. *International Journal of Radiation Biology*, **45**, 615–626.
- HELD, K. D., HARROP, H. A. and MICHAEL, B. D., 1984b, Effects of oxygen and sulphhydryl-containing compounds on irradiated transforming DNA. III. Reaction rates. *International Journal of Radiation Biology*, **45**, 627–636.
- HILDENBRAND, K. and SCHULTE-FROHLINDE, D., 1989, E.s.r. studies on the mechanism of hydroxyl radical-induced strand breakage of polyuridylic acid. *International Journal of Radiation Biology*, **55**, 725–738.
- KLAYMAN, D. L. and COPELAND, E. S., 1982, Radioprotective agents. *Kirk-Othmer: Encyclopedia of Chemical Technology*, 3rd edn, edited by M. Grayson (John Wiley & Sons, New York), vol. 19, pp. 901–832.
- LANDOLT-BÖRNSTEIN, 1960, Leitfähigkeit von Ionen in wässrigen Lösungen. *Zahlenwerte und Funktionen*, 6 Aufl., **II/7**, 258.
- LE BRET, M. and ZIMM, B. H., 1984, Distribution of counter-ions around a cylindrical polyelectrolyte and Manning's condensation theory. *Biopolymers*, **23**, 287–312.
- LILIE, J. and FESSENDEN, R. W., 1973, An alternating current conductivity method for studies of pulse radiolysis in aqueous solutions. Determination of the state of ionization of several  $e_{aq}^-$  adducts. *Journal of Physical Chemistry*, **77**, 674–677.
- MANNING, G. S., 1978, The molecular theory of polyelectrolyte solutions with applications to the electrostatic properties of polynucleotides. *Quarterly Reviews in Biophysics*, **11**, 179–246.
- MICHAEL, B. D., HARROP, H. A. and MAUGHAN, R. L., 1979, Fast response methods in the radiation chemistry of lethal damage in intact cells. *Radiation Research*, edited by S. Okada, M. Imamura, T. Terashima, and H. Yamaguchi (Japanese Association for Radiation Research, Tokyo), pp. 288–296.
- MINCHINTON, A. I., 1984, Measurements of glutathione and other thiols in cells and tissues: a simplified procedure based on the HPLC separation of monobromobimane derivatives of thiols. *International Journal of Radiation Oncology, Biology and Physics*, **10**, 1503–1506.
- NUCIFORA, G., SMALLER, B., REMKO, R. and AVERY, E. C., 1972, Transient radicals of DNA bases by pulse radiolysis. Effects of cysteine and cysteamine as radioprotectors. *Radiation Research*, **49**, 96–111.
- QUINTILIANI, M., BADIELLO, R., TAMBA, M., ESFANDI, A. and GORIN, G., 1977, Pulse radiolysis of glutathione. *International Journal of Radiation Biology*, **32**, 195–204.
- SMOLUK, G. D., FAHEY, R. C. and WARD, J. F., 1986, Equilibrium dialysis studies of the binding of radioprotector compounds to DNA. *Radiation Research*, **107**, 194–204.

- SMOLUK, G. D., FAHEY, R. C. and WARD, J. F., 1988, Interaction of glutathione and other low molecular weight thiols with DNA: Evidence for counter-ion condensation and co-ion depletion near DNA. *Radiation Research*, **114**, 3–10.
- TEDDER, J. M., 1982, The importance of polarity, bond strength and steric effects in determining the site of attack and the rate of free radical substitution in aliphatic compounds. *Tetrahedron*, **38**, 313–329.
- VOJNOVIC, B., 1985, A sensitive single-pulse beam charge monitor for use with charged particle accelerators. *Radiation Physics and Chemistry*, **24**, 517–522.
- WILLSON, R. L., 1983, Free radical repair mechanisms and the interactions of glutathione and vitamins C and E. *Radioprotectors and Anticarcinogens*, edited by O. F. Nygaard and M. G. Simic (Academic Press, New York), pp. 1–22.
- ZHENG, S., NEWTON, G. L., GONICK, G., FAHEY, R. C. and WARD, J. F., 1988, Radioprotection of DNA by thiols: Relationship between the net charge on a thiol and its ability to protect DNA. *Radiation Research*, **114**, 11–27.