The Mechanism of the Free-radical-induced Chain Isomerisation of 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide

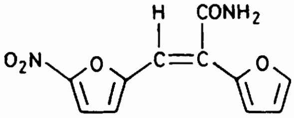
Eric D. Clarke, Peter Wardman,* and Ian Wilson
Cancer Research Campaign Gray Laboratory, Mount Vernon Hospital, Northwood, Middlesex HA6 2RN
Kiyoshi Tatsumi
Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Minami-ku, Hiroshima 734, Japan

The *cis–trans* isomerisation of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) has been studied by pulse and steady-state radiolysis techniques. A free-radical chain-isomerisation mechanism is proposed. Initiation involves production of nitro radical-anions from *cis*-AF-2 with subsequent isomerisation to give the *trans*-AF-2 radical. Propagation of the chain reaction occurs *via* electron transfer between the isomers and their radical-anions, disproportionation of the radicals representing the main chain-terminating step. Oxygen acts as an efficient inhibitor of isomerisation by electron-transfer reaction with the nitro radical-anions. Computer simulations using the Gear numerical integration algorithm show this mechanism to match the observed results closely.

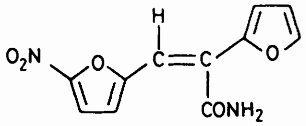
Nitroaryl compounds have found extensive use in medicine[1] and offer potential as adjuncts to radiation or chemotherapy in the treatment of cancer.[2,3] Reduction of nitroaryl compounds to nitro free-radicals by electron-donating enzymes (nitroreductases) is thought to be the basis of the selective reduction of these compounds in anaerobic biochemical reducing systems,[4] since the nitro radicals react rapidly with oxygen.[5] Radical-anions of several medically important nitroheterocyclic compounds have been observed by e.s.r. spectroscopy of suspensions containing nitroaryl compounds and *e.g.* hepatic microsomal preparations as electron donor.[6]

The 5-nitrofuran AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, exists in *cis* (**I**) or *trans* (**II**) forms which are readily distinguishable by their absorption spectra. Tatsumi *et al.*[7] found that xanthine oxidase (xanthine–oxygen oxidoreductase, E.C. 1.2.3.2) together with an electron donor catalysed the *cis–trans* isomerisation of AF-2 and suggested that the nitro free-radical was an intermediate in this isomerisation. This suggestion was supported by observation of efficient isomerisation of aqueous solutions of *cis*-AF-2 initiated by radiolytically produced nitro radicals,[8] and by direct observation of steady-state concentrations of the radicals by e.s.r. in microsomal preparations which also catalysed this isomerisation.[9]

The radiolytic generation of nitro free-radicals was of particular interest because the rate of isomerisation was observed to exceed the rate of production of nitro radicals (readily calculated) by at least an order of magnitude, *i.e.* the isomerisation was a chain reaction.[8] Radiolytic production of radicals is useful because radiolysis at low dose rates *e.g.* from a ^{60}Co γ-source generates radicals in a relatively slow, zero-order manner, simulating electron donation from cellular enzymes, whilst pulse radiolysis techniques[10] allow generation of micromolar concentrations of radicals in microseconds and direct observation by kinetic spectrophotometry of their subsequent reactions. We now report the application of these radiation-chemical techniques to a study of the mechanism of the *cis–trans* isomerisation of AF-2.



(I) *cis*



(II) *trans*

Experimental

Materials.—*cis*- and *trans*-AF-2 were kindly donated by Ueno Pharmaceutical Co. Misonidazole was kindly donated by Dr. C. E. Smithen (Roche Products Ltd.). Benzyl viologen (B.D.H.) was precipitated from methanol by acetone and dried. Sodium formate (Merck; zur Analyse), propan-2-ol (B.D.H.; AristaR), ammonium iron(II) sulphate, sodium chloride, sodium dihydrogenorthophosphate, disodium hydrogenorthophosphate, potassium thiocyanate, and sulphuric acid (B.D.H.; AnalaR) were used as received. Superoxide dismutase (E.C. 1.15.1.1; Miles 36-500; stated activity 3 000 units mg^{-1}) was used as supplied. Direct observation of its reaction with $O_2^{\cdot -}$ by pulse radiolysis[11] gave 24% of the expected activity by weight. Water was distilled and purified by Milli-Q treatment (Millipore Ltd.). Oxygen, nitrogen (<5 p.p.m. O_2), nitrous oxide (<10 p.p.m. O_2), and nitrogen–oxygen mixtures (99.5:0.5 and 98.85:0.15) were supplied by B.O.C. Ltd. Nitrogen was purified by passage through Oxysorb (Messr. Griesheim Gmbh).

γ-*Radiolysis.*—Solutions in a spectrophotometric cell or Drechsel bottle were irradiated by a ^{60}Co source. When deoxygenated conditions were required, the solution (2.5 ml) in a 1 cm silica spectrophotometric cell fused to a B10 cone was saturated with N_2 or N_2O by bubbling (*ca.* 15 min) *via* a needle passed through the bore of a stop-cock fused to a B10 socket into which the cell was inserted. The needle was withdrawn and the stop-cock turned to seal the cell. For irradiation of solutions containing N_2–O_2 mixtures a continuous bubbling technique was required to avoid radiolytic depletion of O_2. Solutions (50 ml) in a Drechsel bottle (125 ml) with sintered bubbling head (Quickfit), fitted with a stoppered side-arm for sample removal, were vigorously bubbled with the appropriate pre-washed N_2–

O_2 mixture. Dose rates of *ca.* 0.24—0.86 Gy min^{-1} were used, as measured by Fricke dosimetry,[12] assuming $\varepsilon = 2\,200$ dm^3 mol^{-1} cm^{-1} at 304 nm, 298 K, $d\varepsilon/dT = 0.7\%$ K^{-1}, and G (Fe^{3+}) = 1.61 μmol J^{-1} (G = radiation-chemical yield; 1 Gy = 1 J kg^{-1}). Pye-Unicam SP 8000, SP8-200, and Zeiss PMQ II spectrophotometers were used.

Pulse Radiolysis.—Solutions were saturated with the appropriate gas in syringes[13] and irradiated in 2 or 4 cm spectrophotometric cells in a flow system which facilitated solution changing. Either a 1.8 MeV linear accelerator[14] or a 4 MeV Van de Graaff generator was used to produce electron pulses of 0.2—2 μs duration. Spectrophotometric detection and photographic recording or transient digitisation followed the principles described earlier[10,14,15] using Philips and Varian xenon arc lamps, Hilger-Watts and SPEX (single pass) monochromators, a Hammamatsu R777 photomultiplier or RCA C30839 p-i-n photodiode and Tektronix 7612 digitiser.[16] A PDP11/34 computer was used for data analysis. Dosimetry was performed using 10 mmol dm^{-3} KSCN in aerated water[14] assuming the product $(SCN)_2^{\cdot-}$ absorbing at 480 nm had Gε = 2.13 × 10^{-3} dm^3 J^{-1} cm^{-1}.

H.p.l.c.—Equipment used was a Waters 6000A pump, WISP sample injector, Ce2112 u.v. detector, Waters 730 data module, and Hypersil ODS column with 1 : 1 methanol-water as eluant.

Computer Simulation of Reaction Kinetics.—The Gear numerical integration algorithm[17,18] formed the basis of a FORTRAN program using a PDP11/34 computer and Sigma 5000 graphics display.

Results

Spectral Characteristics and Isomeric Composition of cis- *and* trans-*AF-2.*—The absorption spectra of the *cis-* and *trans-*isomers of AF-2 are distinguishable in the 200—500 nm region,[7,8] with absorption maxima at 395 and 418 nm, respectively, and isosbestic points at 270, 313, and 375 nm. Analysis of the samples of '*cis*'- and '*trans*'-AF-2 using h.p.l.c. with spectrophotometric analysis at 375 nm indicated some spontaneous isomerisation of the solid samples occurred over the period of this work (4 years). Initially the '*cis*'-sample contained 4% *trans*-impurity; the '*trans*'-sample contained 1% *cis*-isomer [later experiments using significantly less pure '*cis*'-isomer (up to 11% *trans*) are noted as appropriate below]. Extinction coefficients ε, after correcting for the isomeric impurity, were calculated as: *cis* (1.26 ± 0.03) × 10^4 and *trans* (1.81 ± 0.06) × 10^4 dm^3 mol^{-1} cm^{-1} at 395 nm; *cis* (1.04 ± 0.05) × 10^4 and *trans* (2.17 ± 0.06) × 10^4 dm^3 mol^{-1} cm^{-1} at 418 nm.

Radiolytic Generation of Nitroaryl Radical-anions.—The radicals produced when deaerated water is irradiated include e_{aq}^-, H$^\cdot$, and OH$^\cdot$. In the presence of sodium formate, H$^\cdot$ and OH$^\cdot$ are converted into $CO_2^{\cdot-}$ by hydrogen abstraction; e_{aq}^- and $CO_2^{\cdot-}$ react with nitroaryl compounds to produce nitro radical-anions.[10] Values[19] of k_1 for nitrobenzenes, nitro-

$$ArNO_2 + e_{eq}^- \longrightarrow ArNO_2^{\cdot-} \qquad (1)$$

$$ArNO_2 + CO_2^{\cdot-} \longrightarrow ArNO_2^{\cdot-} \qquad (2)$$

imidazoles, and nitrofurans are generally *ca.* 3 × 10^{10} dm^3 mol^{-1} s^{-1}. If solutions containing sodium formate (50 mmol dm^{-3}) are saturated with nitrous oxide (*ca.* 25 mmol dm^{-3}), reaction of e_{eq}^- with N_2O generates OH$^\cdot$ and, in turn, $CO_2^{\cdot-}$. The radical-anions, $ArNO_2^{\cdot-}$ will therefore be produced solely by reaction (2). Observations of this reaction at 680 nm (the absorption maximum of $ArNO_2^{\cdot-}$, see below) in formate–N_2O solutions containing 23 or 45 μmol dm^{-3} AF-2 after a 0.2 μs, *ca.* 15 Gy pulse of 1.8 MeV electrons enabled estimates of k_2 = (3.0 ± 0.4) × 10^9 and (2.0 ± 0.5) × 10^9 dm^3 mol^{-1} s^{-1} for *cis-* and *trans-*AF-2 respectively. A dose of 1 Gy = 1 J kg^{-1} produces *ca.* 0.7 μmol dm^{-3} $CO_2^{\cdot-}$;[12] the low ε of the radicals necessitated a dose of 15 Gy, producing *ca.* 10 μmol dm^{-3} $ArNO_2^{\cdot-}$. The concentration of AF-2 was thus only 3—5 times higher than that of $CO_2^{\cdot-}$, resulting in non-exponential kinetics. The appropriate second-order algorithm[20] was used for kinetic analysis.

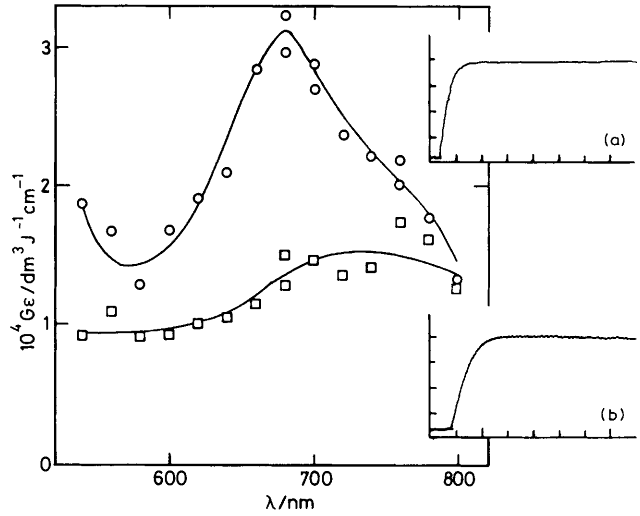


Figure 1. Absorption spectra of the nitro radical-anions produced by reaction of $CO_2^{\cdot-}$ with *cis*-AF-2 (○) and *trans*-AF-2 (□). Solutions contained AF-2 (0.2 mmol dm^{-3}), sodium formate (50 mmol dm^{-3}), and sodium phosphate (2 mmol dm^{-3}), pH 7.4, saturated with N_2O. Spectra were recorded 10 μs after a 14 Gy electron pulse. Insert: Oscillograms illustrating the growth of absorption at 680 nm following the pulse for (a), *cis*-AF-2, vertical sensitivity 0.6% absorption div.$^{-1}$, horizontal timescale 10 μs div.$^{-1}$; (b) *trans*-AF-2, 0.3% div.$^{-1}$, 10 μs div.$^{-1}$

Spectral Characteristics of the Radical-anions of cis- *and* trans-*AF-2.*—Pulse radiolysis of N_2O-saturated solutions containing AF-2 (0.2 mmol dm^{-3}), sodium formate (50 mmol dm^{-3}), and sodium phosphate (2 mmol dm^{-3}) at pH 7.4 generated $ArNO_2^{\cdot-}$ *via* reaction (2), the reaction being complete *ca.* 10 μs after the 0.2 μs, 14 Gy pulse. The absorptions of $ArNO_2^{\cdot-}$ measured at this time are shown in Figure 1, expressed as the product of ε and the radiation-chemical yield, G, in dm^3 J^{-1} cm^{-1}. Using[12] G ($ArNO_2^{\cdot-}$) = 0.7 μmol J^{-1} we estimate ε *ca.* 440 and 190 dm^3 mol^{-1} cm^{-1} for the *cis-* and *trans-*radicals respectively at 680 nm. The oscillograms show the radicals do not decay over *ca.* 100 μs; similar experiments observing over much longer timescales (see below) showed the different absorptions arising from *cis-* and *trans-*radicals persisted for much longer times.

One-electron Reduction Potentials of cis- *and* trans-*AF-2.*—One-electron transfer equilibria between $ArNO_2/ArNO_2^{\cdot-}$ and a redox indicator couple such as $BV^{2+}/BV^{\cdot+}$ (BV^{2+} = benzyl viologen, 1,1′-dibenzyl-4,4′-bipyridylium dichloride) can be generated and measured by pulse radiolysis in a few microseconds.[10,21] The use of propan-2-ol as OH$^\cdot$ scavenger [generating the reducing radical $(CH_3)_2\dot{C}OH$] as an alternative to formate[10] permits low ionic strengths. Nitrogen-saturated solutions containing propan-2-ol (0.2 mol dm^{-3}), *cis*- or *trans*-AF-2 (20—40 μmol dm^{-3}), BV^{2+} (200—1 200 μmol dm^{-3}), and sodium phosphate (4 mmol dm^{-3}) were pulse irradiated (*ca.* 2 Gy). The absorptions measured after *ca.* 100 μs corresponded to the position of the equilibrium (3) from which

$$ArNO_2^{\cdot -} + BV^{2+} \rightleftharpoons ArNO_2 + BV^{\cdot +} \quad (3)$$

K_3 was calculated.[21] Clear evidence for the rapid approach to equilibrium from the initial non-equilibrium condition was observed, very similar to that previously reported for the analogous redox equilibrium involving BV^{2+} and duroquinone.[21]

From these experiments we estimated $K_3 = (8.4 \pm 1.0) \times 10^{-3}$ and $(3.3 \pm 0.2) \times 10^{-2}$ for *cis*- and *trans*-AF-2 respectively at ionic strength $I = 0.008$—0.013. After applying corrections for ionic strength, we calculate[21] $\Delta E_3 = -112 \pm 3$ mV for *cis*- and -78 ± 2 mV for *trans*-AF-2. As $E(BV^{2+}/BV^{\cdot +}) = -354 \pm 6$ mV,[22] we have $E(ArNO_2/ArNO_2^{\cdot -}) = -242 \pm 7$ and -276 ± 6 mV for *cis*- and *trans*-AF-2 respectively at pH 7.1 ± 0.1, where E are values of the thermodynamically reversible one-electron reduction potentials of the stated couples *versus* the normal hydrogen electrode.

Rates of Electron Transfer between the Nitro Radicals and Oxygen.—Previous measurements[5] of the rates of reaction (4)

$$ArNO_2^{\cdot -} + O_2 \longrightarrow ArNO_2 + O_2^{\cdot -} \quad (4)$$

of nitro radicals with oxygen gave k_4 *ca.* 2×10^5 dm^3 mol^{-1} s^{-1} for 5-nitrofurans, *i.e.* half-lives of *ca.* 3 ms in O_2-saturated aqueous solutions. In deaerated solutions at pH 7.4, the natural life-times of the *cis*- and *trans*-AF-2 radicals (produced by pulse radiolysis of formate–AF-2 solutions as described above) were found to be > 50 ms (see below), suggesting that estimation of k_4 for AF-2 should be possible.

Solutions of *cis*- or *trans*-AF-2 (0.1—0.3 mmol dm^{-3}), sodium formate (50 mmol dm^{-3}), and sodium phosphate (2 mmol dm^{-3}) at pH 7.4, saturated with N_2, air, or O_2 were pulse irradiated (35—75 Gy) and the decay of $ArNO_2^{\cdot -}$ monitored at 680 nm. Whilst in nitrogen-saturated solutions little decay of *cis*- and *trans*-AF-2 radical-anions was observed in 10—20 ms (see below), in aerated or oxygenated solutions exponential decay was observed with first-order dependence on oxygen concentration (for the *cis*-radical-anion a half-life of *ca.* 5 ms was measured in aerated solution, and *ca.* 1 ms in O_2-saturated solution). Typical transients for *cis*- and *trans*-radical-anion decay in air are shown in Figure 2(a) and (b). The initial absorptions of $ArNO_2^{\cdot -}$ were reduced in the presence of air or O_2 because of the competing reactions (5) and (6) as expected

$$e_{aq}^{-} + O_2 \longrightarrow O_2^{\cdot -} \quad (5)$$

$$CO_2^{\cdot -} + O_2 \longrightarrow O_2^{\cdot -} + CO_2 \quad (6)$$

($k_5 = 1.9 \times 10^{10}$ dm^3 mol^{-1} s^{-1}, and $k_6 = 2.4 \times 10^9$ dm^3 mol^{-1} s^{-1}).[19,23] This effect, together with the low extinction coefficients of the radical-anions, made detailed kinetic analysis difficult. However, we estimate k_4 for *cis*-AF-2 radical + O_2 to be in the range 4—8 × 10^5 dm^3 mol^{-1} s^{-1}, and k_4 for *trans*-radical + O_2 as 1—3 × 10^6 dm^3 mol^{-1} s^{-1}.

Stabilities of the Nitro Radical-anions in the Absence of Oxygen.—In N_2-saturated aqueous solutions several nitroaryl radical-anions have been shown[5,24] to decay by disproportionation with second-order rate constants typically < 1 × 10^5 dm^3 mol^{-1} s^{-1} at pH 7 (*i.e.* half-lives ⩾0.1 s in typical pulse-radiolysis experiments). For AF-2 solutions the low ε of the radical-anions, together with the instability of the analysing light source, limits any observation of absorption change at these longer times. Nevertheless, pulse radiolysis (*ca.* 15 Gy) of nitrogen-saturated solutions of *trans*-AF-2 (0.3 mmol dm^{-3}), sodium formate (50 mmol dm^{-3}), and sodium phosphate (2 mmol dm^{-3}), pH 7.3 was possible giving reasonable measurements for the *trans*-radical absorption at 680 nm over 1 s. Figure 2(c) [transient (i)] shows little, if any, decay of the *trans*-radical-anion over 1 s. In contrast, the absorption produced by pulse radiolysis of a similar solution of *cis*-AF-2 {Figure 2(c) [transient (ii)]} changed with an apparent half-life of 0.25 s assuming decay to the *trans*-radical absorption. Increasing the dose to *ca.* 80 Gy reduced the *cis*-radical half-life to *ca.* 70 ms with no change in the *trans*-radical stability. Whether this observation represents *cis–trans* isomerisation or *cis*-radical disproportionation cannot be readily distinguished (see later).

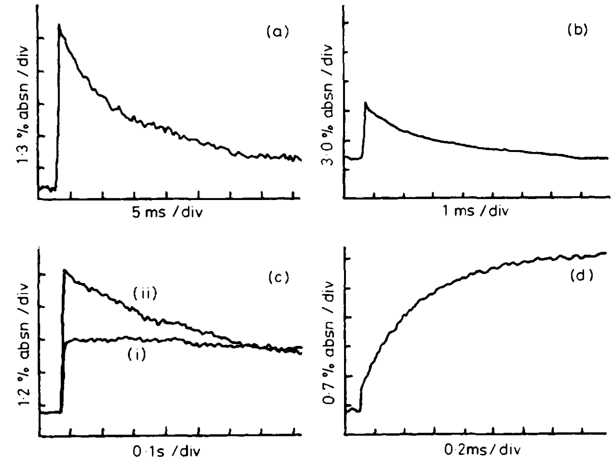


Figure 2. Oscillograms illustrating transient absorptions of AF-2 radical-anions at 680 nm following pulse radiolysis of solutions containing sodium formate, sodium phosphate, pH 7.4, and (a) *cis*-AF-2, air-saturated; (b) *trans*-AF-2, air-saturated; (c) (i), *trans*-AF-2, N_2-saturated, (ii), *cis*-AF-2, N_2-saturated; (d), *cis*-AF-2 and misonidazole, N_2-saturated

Rate of Electron Transfer from a Nitroimidazole Radical-anion to AF-2.—The relatively high one-electron reduction potentials of *cis*- and *trans*-AF-2 (−242 and −276 mV respectively, see above) compared with the 2-nitroimidazole misonidazole [1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol] which has $E(ArNO_2/ArNO_2^{\cdot -}) = -389$ mV[21] at pH 7 suggested that the electron-transfer reaction (7) between the

$$(ArNO_2)_a^{\cdot -} + (ArNO_2)_b \rightleftharpoons (ArNO_2)_a + (ArNO_2)_b^{\cdot -} \quad (7)$$

latter and AF-2 should be observable where $(ArNO_2)_a$ is misonidazole and $(ArNO_2)_b$ is *cis*- or *trans*-AF-2.

Pulse radiolysis (*ca.* 25 Gy) of nitrogen-saturated solutions containing *cis*- or *trans*-AF-2 (0.1 mmol dm^{-3}), misonidazole (2 mmol dm^{-3}), sodium formate (0.2 mol dm^{-3}), and sodium phosphate (4 mmol dm^{-3}), pH 7.4 produced absorption changes at 680 nm as illustrated in Figure 2(d), for *cis*-AF-2. As reactions (1) and (2) for misonidazole with e_{eq}^{-} and $CO_2^{\cdot -}$ have rate constants[19,24] $k_1 = 3.0 \times 10^{10}$ dm^3 mol^{-1} s^{-1} and $k_2 = 1.2 \times 10^9$ dm^3 mol^{-1} s^{-1} and as [misonidazole]:[AF-2] = 20:1, the misonidazole radical-anion forms 92% of the nitro-radical yield a few microseconds after the radiation pulse. The subsequent build-up of absorption at 680 nm is attributable to AF-2 radical formation *via* reaction (7) (misonidazole

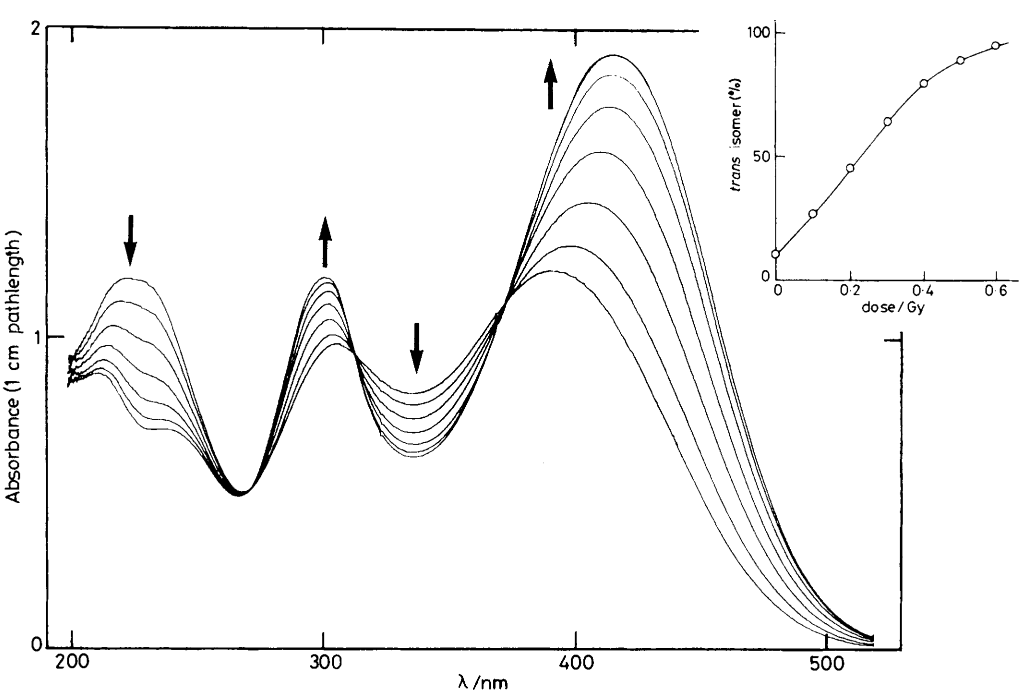


Figure 3. Absorption spectra obtained during isomerisation of *cis*-AF-2 (90 μmol dm^{-3}) to its *trans*-isomer on γ-radiolysis (0.24 Gy min^{-1}) in N_2-saturated sodium formate solution, pH 7.4, at 25 °C. Scans recorded for 0.1 Gy dose increments. Vertical arrows denote the direction of absorption changes for each spectral region. Insert: Plot of % *trans*-isomer *versus* radiation dose calculated from absorption changes at 418 nm

radical-anion does not absorb at this wavelength). The kinetics of the reaction are described by equation (8) where k_{obs} is the

$$k_{obs} = k_7[\text{AF-2}] + k_{-7}[\text{misonidazole}] \qquad (8)$$

observed first-order rate constant and k_7 and k_{-7} are the respective forward and reverse second-order rate constants for equilibrium (7). From their known one-electron reduction potentials we calculate K_7 $(=k_7/k_{-7}) = 310$ and 82 for misonidazole with *cis*- and *trans*-AF-2 respectively. Measuring $k_{obs} = (2.6 \pm 0.1) \times 10^3$ s^{-1} for misonidazole-*cis*-AF-2, we estimate $k_7 = (2.4 \pm 0.1) \times 10^7$ dm^3 mol^{-1} s^{-1}; for misonidazole-*trans*-AF-2 we have $k_{obs} = (9.8 \pm 0.3) \times 10^2$ s^{-1} and $k_7 = (7.9 \pm 0.3) \times 10^6$ dm^3 mol^{-1} s^{-1}.

Isomerisation of cis-AF-2 *initiated by γ-Radiolysis.*—γ-Radiolysis (0.24—0.86 Gy min^{-1}) of deaerated solutions containing *cis*-AF-2 (90 μmol dm^{-3}; actually 80 μmol dm^{-3} *cis*, 10 μmol dm^{-3} *trans* by h.p.l.c. analysis), sodium formate (25 mmol dm^{-3}), and sodium phosphate (2 mmol dm^{-3}) at pH 7.4 generates *cis*-AF-2 radical-anion in a zero-order manner (rate *ca.* 3—10 nmol dm^{-3} s^{-1}), as described above. An earlier report[8] described results from such experiments utilising total doses of *ca.* 70 Gy, where the spectral changes included effects from reactions such as (9), *i.e.* net loss of $ArNO_2$ to yield higher

$$2\,ArNO_2^{\cdot-} \longrightarrow \text{products} \qquad (9)$$

reduction products. In the present work it was realised that isomerisation was measurable after much lower doses.

Figure 3 shows the spectral changes observed when the above N_2-saturated *cis* AF-2 solution was γ-irradiated (dose rate 0.24 Gy min^{-1}), using dose increments of 0.1 Gy. After a total dose of 0.6 Gy spectral changes attributable to isomerisation ceased to occur, h.p.l.c. analysis showing that the solution contained 4 μmol dm^{-3} *cis*- and 86 μmol dm^{-3} *trans*-AF-2. Hence total isomerisation (to an equilibrium ratio, largely favouring the *trans*-isomer) of 76 μmol dm^{-3} *cis*-AF-2 required a dose of 0.6 Gy, corresponding to the generation of 0.4 μmol dm^{-3} *cis*-radical-anion. In this experiment, therefore, one nitro radical initiates the isomerisation of *ca.* 200 molecules, with a maximum value (Figure 3, insert) at 50% isomerisation (*i.e.* a 50:50 *cis–trans* mixture) of 240 molecules. Repeated experiments indicated considerable variability in the chain lengths (molecules isomerised/radicals produced) measured, *e.g.* at pH 7.4 and dose rate 0.24 Gy min^{-1} values in the range 190—320 were obtained. However, in spite of these variations, an increase in chain length from *ca.* 60 at pH 3.8 to *ca.* 450 at pH 8.4 was apparent from replicate runs at several pH values.

It is possible that the variation in chain length at a given pH arose from incomplete deoxygenation. Continuous vigorous bubbling of *cis*-AF-2 solutions with N_2 containing varying amounts of O_2 indicated that O_2 severely inhibited the isomerisation compared with that seen in N_2. Thus γ-irradiation of AF-2 (90 μmol dm^{-3}), sodium formate (25 mmol dm^{-3}), and sodium phosphate (2 mmol dm^{-3}) at pH 7.4 containing 0.15% O_2 (*ca.* 2 μmol dm^{-3}) gave chain lengths of 45 for dose rate 0.24 Gy min^{-1} and 22 at 0.86 Gy min^{-1}. 0.5% O_2 (*ca.* 6.5 μmol dm^{-3}) further inhibited isomerisation, reducing chain lengths to 14 (0.24 Gy min^{-1}) and 4 (0.86 Gy min^{-1}).

Since $O_2^{\cdot-}$ is a product of reaction (4) when AF-2 solutions containing O_2 are irradiated, the effect of the enzyme superoxide dismutase (SOD; E.C. 1.15.1.1) on the chain isomerisation was investigated. For *cis*-AF-2 continuously bubbled with 0.15% O_2 under the above conditions the addition of 0.4 mg ml^{-1} SOD completely prevented isomerisation even after a total irradiation dose of 10 Gy was delivered at 0.24 Gy min^{-1}. SOD is known to react with some nitro radical-anions[11] [reaction (10)]. Pulse radiolysis (20 Gy) of a N_2O-saturated

$$ArNO_2^{\cdot-} + \text{SOD} \longrightarrow \text{products} \qquad (10)$$

solution containing *trans*-AF-2 (60 μmol dm^{-3}), sodium formate (0.2 mol dm^{-3}), sodium phosphate (4 mmol dm^{-3}) at pH 7.3 and SOD (0.5 mg ml^{-1} of 24% active enzyme; molecular weight 32 500) showed the *trans*-radical-anion absorption at 680 nm to decay with a half-life of *ca.* 10 ms from which we estimate k_{10} *ca.* 2×10^7 dm^3 mol^{-1} s^{-1}. As found previously,[11] the product of reaction (10) was an inactive form of the enzyme; thus replicate pulses of the same solution gave increasingly longer life-times of the *trans*-radical.

Discussion

Radical-induced cis–trans *Isomerisation of AF-2.*—γ-Radiolytic reduction of *cis*-AF-2 yields the corresponding radical-anion, with eventual isomerisation to the *trans*-compound. This is in agreement with the earlier results of Tatsumi *et al.*[8] providing clear evidence that the *cis–trans* isomerisation of AF-2 is a radical-initiated process. Important proof of the free-radical mechanism has been shown by direct observations of the AF-2 radical-anions by e.s.r.[9] during isomerisation in anaerobic microsomal preparations. It has been estimated, from MO calculations,[25] that the barrier to rotation about the carbon–carbon double bond of AF-2 is reduced from 290 to 20—40 kJ mol^{-1} upon formation of the radical anion.

The Efficiency of Isomerisation.—The high yields of *trans*-AF-2 produced following low-dose γ-radiolysis clearly reflects a chain mechanism for isomerisation, leading to an equilibrium mixture largely favouring the *trans*-isomer. H.p.l.c. analysis of samples on completion of reaction showed an equilibrium mixture of the two isomers of 96% *trans*- and 4% *cis*-AF-2 to be present. This ratio of isomers was also obtained when solutions of *trans*-AF-2 (isomeric purity: 99% *trans*, 1% *cis*) were irradiated. An equilibrium ratio of 88% *trans*: 12% *cis*-isomers has been reported for enzymic isomerisation of AF-2.[8]

The propagation step for chain isomerisation is most likely to be electron transfer involving *cis*- and *trans*-AF-2 as outlined in reaction (11) where $cis^{\cdot -}$ and $trans^{\cdot -}$ refer to the appropriate

$$trans^{\cdot -} + cis \rightleftharpoons trans + cis^{\cdot -} \qquad (11)$$

radical-anions. Electron-transfer reactions analogous to (3), (4), (7), and (11) involving nitroaryl compounds with *e.g.* quinones[26] or oxygen[5] are well documented. Estimates of rate constants for *ca.* 30 reactions of the general form (7) have recently been measured[27] for a series of nitroaryl compounds and show a good correlation with free energy of reaction according to the Marcus theory of electron transfer.[28] The rate constants for AF-2 reaction with misonidazole radical-anion are in agreement with these findings. Reaction (11) can be calculated to have an equilibrium constant $K = 3.8$ from the difference in one-electron reduction potentials of 34 mV for the AF-2 isomers. Interpolation from the electron-transfer rate constants–free energy relationships of other nitroaryl compounds[27] provides estimates of $k_{11} = 2 \times 10^6$ dm^3 mol^{-1} s^{-1} and $k_{11} = 5 \times 10^5$ dm^3 mol^{-1} s^{-1} for the forward and reverse reactions of equation (11).

In the absence of oxygen, the chain termination step seems likely to be the disproportionation reaction (9). For several nitroaryl compounds at pH 7, this rate has been estimated[5,24] as typically $< 10^5$ dm^3 mol^{-1} s^{-1}. Indeed, our pulse radiolysis studies indicate the *trans*-AF-2 radical-anion to be stable for many seconds, *i.e.* estimating a rate constant ($2\,k_9$) for disproportionation $< 3 \times 10^4$ dm^3 mol^{-1} s^{-1}. Assuming steady-state conditions exist during γ-radiolysis, *i.e.* the rate of production of radicals equals rate of loss by disproportionation, we can estimate that for a dose rate of 0.24 Gy min^{-1} and disproportionation rate constant of 3×10^4 dm^3 mol^{-1} s^{-1}, steady-state concentrations of *ca.* 0.3 μmol dm^{-3} AF-2 radicals will be present. Thus the half-life of the radicals with respect to disproportionation reaction (9) {half-life = $(2\,k_9\,[\mathrm{ArNO_2}^{\cdot -}])^{-1}$} is *ca.* 100 s, very much slower than the chain-propagation step (11), which would have $t_{1/2}$ *ca.* 6 ms at *ca.* 45 μmol dm^{-3} *cis*- + 45 μmol dm^{-3} *trans*-AF-2 since $k_{obs} = k_{11}[cis] + k_{-11}[trans]$. Very long chain lengths are thus readily conceivable, although the potential susceptibility to reaction of AF-2 radicals with impurities, including oxygen, is obvious with such long natural radical lifetimes.

Chain termination by reaction (9) would be expected to be more efficient at lower pH values, since k_9 decreases steadily with increasing pH[5,24] (*cf.* $O_2^{\cdot -}$ decay[29]). The decreased chain lengths observed at lower pH values in the absence of oxygen supports the assignment of reaction (9) as the chain termination step. The rate of reaction (9) should increase with increasing dose rate and limited experiments at higher dose rates gave lower isomerisation yields, further supporting this assignment.

Inhibition of Isomerisation by Oxygen.—With *e.g.* 0.5% O_2 (6.5 μmol dm^{-3}) present during γ-radiolysis, we see that effective half-lives for reaction (4) are *ca.* 0.2 s and 50 ms for *cis*- and *trans*-AF-2, respectively, illustrating the efficient removal of AF-2 radicals by low concentrations of oxygen relative to the natural disproportionation process (9).

The Rate of cis–trans *Isomerisation.*—The major uncertainty in this work is the rate of reaction (12). Direct measurement of

$$cis^{\cdot -} \rightleftharpoons trans^{\cdot -} \qquad (12)$$

this reaction is difficult because of the complexity of the chain isomerisation process and possibly competing disproportionation. Micromolar quantities of *cis*-AF-2 radical-anion produced by pulse radiolysis will undergo isomerisation to the *trans*-radical, but the rapid propagation step (11) will maintain a relatively high *cis*-radical concentration with accumulation of *trans*-AF-2 ground state. It is possible that isomerisation is sufficiently rapid that the absorption change in Figure 2(c) represents the change in the $cis^{\cdot -}:trans^{\cdot -}$ radical ratio as isomerisation proceeds through to the final equilibrium mixture of ground-state isomers. Alternatively, Figure 2(c) can be explained if the *cis*-radical-anion disproportionates an order of magnitude faster than the *trans*-radical. We believe this to be unlikely. The kinetics observed cannot therefore represent simply reaction (12) alone.

Since most of the likely reactions occurring in γ-irradiated solutions have been individually characterised in the present work, or can be reliably estimated, it seemed appropriate to simulate a comprehensive reaction scheme using numerical integration techniques. The reaction steps to be incorporated into the scheme are outlined in the Table, and include all the initiation, isomerisation, propagation, termination, and inhibition rates discussed above. In addition, modelling of all γ-radiolysis experiments required the zero order input of e_{eq}^{-} and $CO_2^{\cdot -}$. Rate constants of 1.1×10^{-9} and 1.5×10^{-9} mol dm^{-3} s^{-1} for e_{eq}^{-} and $CO_2^{\cdot -}$ production respectively were used, corresponding to a dose rate of 0.24 Gy min^{-1}. From the Table it can be seen that the form of the differential equations to be used during simulaton are *stiff*,[18] *i.e.* many of the rate constants differ by up to six orders of magnitude, whilst some of the species involved will be maintained at almost constant concentration. The Gear algorithm[17,18] enables the integration of these stiff differential equations using an implicit, multistep predictor–corrector method in which the step-size is automatically varied after each integration step to allow maximum efficiency and accuracy.

As the rate constants for isomerisation remain uncertain, repeated computer simulations of the reaction scheme were

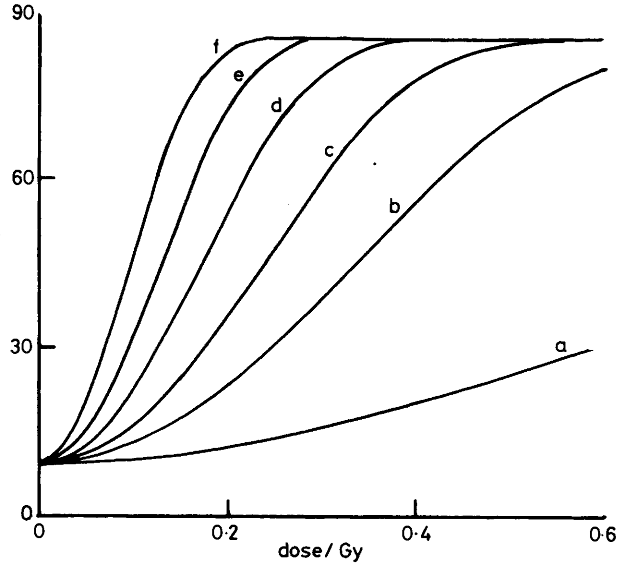


Figure 4. Computer simulations of the *cis–trans* isomerisation of AF-2. Plot depicts *trans*-AF-2 formation during γ-radiolysis (0.24 Gy min^{-1}) using rate constants as quoted in the Table with k_{12} and k_{-12} fixed at (a) 1.0 and 0.15 s^{-1}; (b) 5.0 and 0.75 s^{-1}; (c) 10 and 1.5 s^{-1}; (d) 20 and 3.0 s^{-1}; (e) 40 and 6.0 s^{-1}; and (f) 100 and 15 s^{-1}, respectively

carried out with k_{12} and k_{-12} adjusted to obtain the closest fit to experimental observations. With all other rate constants fixed at the values quoted in the Table, a ratio of $k_{12}:k_{-12}$ = 6.67:1 was found to be necessary to duplicate the observed final ground-state equilibrium of 96% *trans*-:4% *cis*-AF-2. This model also calculates the presence of sub-micromolar steady-state nitro radical-anion concentrations of 87% *trans*-radical and 13% *cis*-radical at equilibrium. (A similar steady-state ratio has also been derived[25] from direct e.s.r. measurements of the radicals in equilibrium mixtures of AF-2 in hepatic microsomal preparations, *viz.* 77%:23% *trans*:*cis*-radicals.) Maintaining the 6.67:1 constant ratio for k_{12}/k_{-12}, Figure 4 shows the results obtained for computer simulations of the isomerisation scheme with a series of k_{12} values between 1 and 100 s^{-1}. The conditions represented in the model are those of a typical γ-radiolysis experiment using 90 μmol dm^{-3} *cis*-AF-2 in nitrogen-saturated formate solution. Comparison of Figure 4 with the experimental observations of Figure 3 indicates that the closest approximation is obtained when k_{12} is in the range 5—40 s^{-1} (and consequently k_{-12} 0.75—6 s^{-1}). With k_{12} set at 10 s^{-1}, the model reveals that 50% isomerisation (also the maximum rate in the model) requires 0.23 Gy radiation, equivalent to a chain length of 230 molecules isomerised/radicals generated. Kalyanaraman *et al.*[9] have suggested k_{12} to be of the order of 40 s^{-1} from their e.s.r. observations assuming $k_{12} = k_4\,[O_2]$ and taking $k_4 = 2 \times 10^5$ dm^3 mol^{-1} s^{-1} in their microsomal system, in agreement with the estimates above. This relatively high isomerisation rate constant is in contrast to those determined for other free-radical-initiated chain-isomerisation processes, *e.g.* for *cis*-stilbene values of $k_{12} + k_{-12}$ = 1.5×10^{-3} s^{-1} have been estimated.[30] Indeed, with such a fast rate of isomerisation, we now see that the spectral changes observed on pulse radiolysis of *cis*-AF-2 solutions can be ascribed to rapid total isomerisation of the compound. Modelling of the system upon pulse radiolytic generation of *cis*-AF-2 radicals showed isomerisation to be complete in less than 1 s, with the rate of isomerisation being dose dependent. The best fit to experimental data was obtained for k_{12} values approaching 40 s^{-1}.

Table. Proposed reaction scheme for *cis–trans* isomerisation of AF-2

Reaction	k_{for}/dm^3 mol^{-1} s^{-1}	k_{rev}/dm^3 mol^{-1} s^{-1}	Ref.
Initiation			19
$cis + e_{aq}^- \longrightarrow cis^{\cdot -}$	3×10^{10}		19
$trans + e_{aq}^- \longrightarrow trans^{\cdot -}$	3×10^{10}		*a*
$cis + CO_2^{\cdot -} \longrightarrow cis^{\cdot -}$	3×10^9		*a*
$trans + CO_2^{\cdot -} \longrightarrow trans^{\cdot -}$	2×10^9		
Isomerisation			
$cis^{\cdot -} \rightleftharpoons trans^{\cdot -}$	5—40 s^{-1}	0.75—6 s^{-1}	*b*
Propagation			
$cis + trans^{\cdot -} \rightleftharpoons cis^{\cdot -} + trans$	2×10^6	5×10^5	*b*
Termination			
$2\,cis^{\cdot -} \longrightarrow cis +$ reduced products	3×10^4		*b*
$2\,trans^{\cdot -} \longrightarrow trans +$ reduced products	3×10^4		*b*
Inhibition by O_2			
$cis^{\cdot -} + O_2 \rightleftharpoons cis + O_2^{\cdot -}$	6×10^5	2×10^4	*a*
$trans^{\cdot -} + O_2 \rightleftharpoons trans + O_2^{\cdot -}$	2×10^6	1.8×10^4	*a*
$O_2 + e_{aq}^- \longrightarrow O_2^{\cdot -}$	1.9×10^{10}		19
$O_2 + CO_2^{\cdot -} \longrightarrow O_2^{\cdot -}$	2.4×10^9		23
$2\,O_2^{\cdot -} \longrightarrow O_2 + H_2O_2$	4×10^5		29

[a] Measured in this work. [b] Estimated in this work.

Computer simulations of the γ-irradiated system in the presence of O_2 reflected the inhibitory effect of O_2 on *cis–trans* isomerisation. In all cases, however, the model predicted less efficient inhibition than was actually observed. Thus, incorporation of 2 μmol dm^{-3} O_2 into the reaction scheme with k_{12} = 10 s^{-1} gave calculated chain lengths (for 50% isomerisation) of 130 molecules isomerised/radicals generated, whilst 6.5 μmol dm^{-3} O_2 was calculated to reduce this to *ca.* 60 molecules (*cf.* observed values of 45 and 14 molecules, respectively). Although depletion of nitroaryl radicals by reaction with oxygen (4) represents the prime inhibition step, the reverse step involving interaction of ground-state AF-2 with $O_2^{\cdot -}$ cannot be ignored. This will effectively reduce the efficiency of inhibition by O_2 making isomerisation extremely sensitive to the natural lifetime of $O_2^{\cdot -}$. The complete prevention of isomerisation by the addition of SOD to the system is most likely to be due to the dismutation of $O_2^{\cdot -}$ by the enzyme. However, it is also possible that inhibition by SOD could be due to direct reaction with AF-2 radicals as in reaction (10). Computer simulations incorporating these steps show either process to yield very efficient inhibition of isomerisation.

The reaction scheme in the Table omits reaction (13) which

$$cis^{\cdot -}/trans^{\cdot -} + O_2^{\cdot -} \longrightarrow \text{products} \qquad (13)$$

could also decrease the isomerisation efficiency in the presence of O_2. A suggestion[5] that nitrofuran radical-anions react with $O_2^{\cdot -}$ with *k ca.* 10^7 dm^3 mol^{-1} s^{-1} has been made; addition of reaction (13) to the computer model with $k_{13} = 5 \times 10^6$ dm^{-3} mol^{-1} s^{-1} gave a reasonable fit to the experimental data.

The free-radical-initiated chain-isomerisation mechanism described above allows *cis*-AF-2 to be used as a probe for nitro-radical generation. This is of particular importance in physiologically relevant reducing systems,[31,32] although the possible interaction of AF-2 radicals with other species capable of electron-transfer reactions (such as other nitroaryl compounds and/or O_2) should also be considered. For example, Raleigh *et al.*[31] have reported that the yield of *trans*-AF-2 formed by electron transfer to *cis*-AF-2 from *e.g.* aerobic xanthine oxidase with xanthine as electron-donating substrate, is reduced in the presence of other nitroaryl compounds *via* redox-related competition for electrons from the enzyme. Raleigh *et al.*[31] found that misonidazole (1.6 mmol dm^{-3}) reduced the isomerisation efficiency of *cis*-AF-2 (30 μmol dm^{-3}) by 50%. Using these concentrations together with the equilibrium data for reaction (7), we calculate a *cis*-radical to misonidazole radical equilibrium ratio of 6:1. This ratio would be independent of electron-transfer rate to the nitroaryl compounds from the enzyme, indeed electron transfer to misonidazole from the enzyme need not occur. The rate of loss of misonidazole radical by reaction with oxygen[5] ($k_4 = 3.8 \times 10^6$ dm^3 mol^{-1} s^{-1}) is *ca.* 6 times faster than reaction (4) for *cis*-AF-2. Consequently the overall rate of loss of both *cis*-AF-2 and misonidazole radicals in aerated solutions would be the same, resulting in a 50% reduction of *cis*-AF-2 radicals under the conditions used. The net reduction in isomerisation efficiency of aerobic solutions of *cis*-AF-2 caused by the addition of other nitroaryl compounds reflects reactions (4) and (7) rather than direct competition for electrons donated by the reducing system, since $(k_{12} + k_{-12}) \ll k_4[O_2]$ or $(k_7[ArNO_2]_a + k_{-7}[ArNO_2]_b)$. Whilst electron transfer from xanthine–xanthine oxidase to nitroaryl compounds is indeed redox related,[33] the contributions from the redox-controlled[5,27] reactions (4) and (7) to the experiments reported by Raleigh *et al.*[31] cannot be ignored.

Acknowledgements

This work was supported by the Cancer Research Campaign. We thank Dr. M. R. L. Stratford for performing h.p.l.c. analysis.

References

1 E. Grunberg and E. H. Titsworth, *Annu. Rev. Microbiol.*, 1973, **27**, 317.
2 P. Wardman, *Curr. Top. Radiat. Res. Q.*, 1977, **11**, 347.
3 'Chemical Modification: Radiation and Cytotoxic Drugs,' ed. R. M. Sutherland, Pergamon Press, New York, 1982; *Int. J. Radiat. Oncol. Biol. Phys.*, 1982, **8**, 323.
4 R. P. Mason and J. L. Holtzman, *Biochem. Biophys. Res. Commun.*, 1975, **67**, 1267.
5 P. Wardman and E. D. Clarke, *Biochem. Biophys. Res. Commun.*, 1976, **69**, 942; 'Techniques and Applications of Fast Reactions in Solution,' eds. W. J. Gettins and E. Wyn-Jones, Reidel, Dordrecht, 1979, p. 535.
6 R. P. Mason, in 'Free Radicals in Biology,' ed. W. A. Pryor, Academic Press, New York, 1982, vol. 5, ch. 6.
7 K. Tatsumi, S. Kitamura, N. Koga, H. Yoshimura, and Y. Kato, *Biochem. Biophys. Res. Commun.*, 1976, **73**, 947.
8 K. Tatsumi, N. Koga, S. Kitamura, H. Yoshimura, P. Wardman, and Y. Kato, *Biochim. Biophys. Acta*, 1979, **567**, 75.
9 B. Kalayanarman, E. Perez-Reyes, R. P. Mason, F. J. Peterson, and J. L. Holtzman, *Mol. Pharmacol.*, 1979, **16**, 1059.
10 P. Wardman, *Rep. Prog. Phys.*, 1978, **41**, 259.
11 P. Wardman, in 'Radiation Biology and Chemistry: Research Developments,' eds. H. E. Edwards, S. Navaratnam, B. J. Parsons, and G. O. Phillips, Elsevier, Amsterdam, 1979, 189.
12 'The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis,' eds. J. H. Baxendale and F. Busi, Reidel, Dordrecht, 1982, pp. 49—62, 241—266.
13 R. L. Willson, *Int. J. Radiat. Biol.*, 1970, **17**, 349.
14 G. E. Adams, J. W. Boag, and B. D. Michael, *Trans. Faraday Soc.*, 1965, **61**, 492.
15 D. S. Sehmi, *Meas. Control*, 1978, **11**, 3.
16 B. Vojnovic, Ph.D. Thesis, University of London, 1983.
17 R. J. Field, Technical Report, NDRL-2161, 1980, University of Notre Dame, Indiana.
18 C. W. Gear, 'Numerical Initial Value Problems in Ordinary Differential Equations,' Prentice-Hall, New Jersey, 1972.
19 M. Anbar, M. Bambenek, and A. B. Ross, 'Selected Specific Rates of Reactions of Transients from Water in Aqueous Solution. 1. Hydrated Electron,' Nat. Stand. Ref. Data. Ser., National Bureau of Standards, 1973.
20 J. W. Moore and R. G. Pearson, 'Kinetics and Mechanism,' Wiley, New York, 1981, 3rd edn.
21 P. Wardman and E. D. Clarke, *J. Chem. Soc., Faraday Trans. 1*, 1976, **72**, 1377.
22 E. Steckham and T. Kuwana, *Ber. Bunsenges. Phys. Chem.*, 1974, **78**, 253.
23 G. E. Adams and R. L. Willson, *Trans. Faraday Soc.*, 1969, **65**, 2981.
24 P. Wardman, unpublished work; *Int. J. Radiat. Biol.*, 1975, **28**, 585.
25 B. Kalyanaraman, R. P. Mason, R. Rowlett, and L. D. Kispert, *Biochim. Biophys. Acta*, 1981, **660**, 102.
26 D. Meisel, *Chem. Phys. Lett.*, 1975, **34**, 263.
27 I. Wilson, P. Wardman, and E. D. Clarke, to be published.
28 R. A. Marcus, *Annu. Rev. Phys. Chem.*, 1964, **15**, 155.
29 B. H. Bielski, *Photochem. Photobiol.*, 1978, **28**, 645.
30 S. Sorensen, G. Levin, and M. Szwarc, *J. Am. Chem. Soc.*, 1975, **97**, 2341.
31 J. A. Raleigh, F. Y. Shum, D. R. Koziol, and W. M. Saunders, *Cancer Clin. Trials*, 1980, **3**, 55; J. A. Raleigh, F. Y. Shum, and S. F. Liu, *Biochem. Pharmacol.*, 1981, **30**, 2921.
32 E. D. Clarke, P. Wardman, and I. Wilson, *Biochem. Pharmacol.*, 1984. **33**, 83.
33 E. D. Clarke, K. H. Goulding, and P. Wardman, *Biochem. Pharmacol.*, 1982, **31**, 3237.

Received 28*th July* 1983; *Paper* 3/1313