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CIS–TRANS ISOMERIZATION OF THE (5-NITRO-2-FURYL)ACRYLAMIDE, AF-2, INITIATED BY ASCORBATE, GLUTATHIONE, Fe(II) and OH^-

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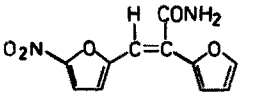
Abstract—The *cis–trans* isomerization of the (5-nitro-2-furyl)acrylamide, AF-2, has been investigated using some important biological reducing agents to initiate reaction. Physiological concentrations of L-ascorbic acid, glutathione and iron(II) all accomplish isomerization in a catalytic manner over a period of minutes. Base-catalysed isomerization has also been observed. In all cases, the presence of oxygen severely inhibits isomerization. It is proposed that the mechanism involves a free-radical chain process; AF-2 or analogues are thus extremely sensitive probes for the generation of nitro radicals in biochemical reducing systems because of the high efficiency of isomerization.

Isomerization of the 5-nitrofuran AF-2† from *cis* to *trans* forms (**I** to **II**, Fig. 1) is accomplished by "nitroreductase" enzymes and appropriate electron donors [1, 2]. Evidence that nitro radicals initiated isomerization [2] and electron spin resonance spectra [3] of these radicals produced in anaerobic microsomal incubations containing AF-2 proved that the isomerization occurred by a free-radical mechanism. Radiolytic generation of nitro radicals also provided evidence that the isomerization occurred via a chain reaction [2], and subsequent studies [4] clarifying the mechanism involved demonstrated that up to *ca* 500 molecules could be isomerized for each nitro radical produced, depending on the oxygen concentration and pH.

We now show that in addition to the several "nitroreductase" systems previously used as electron donors, some simple biochemical reducing agents can accomplish this isomerization under physiological conditions.



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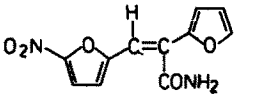

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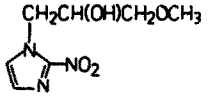

III

Fig. 1. Structural formulae of: **I**, *cis* AF-2 [*cis* 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide]; **II**, *trans* AF-2; **III**, misonidazole.

MATERIALS AND METHODS

Chemicals. AF-2 (*cis* and *trans*) was kindly donated by Prof. K. Tatsumi. Glutathione (Sigma) and all other chemicals (BDH, AnalaR) were used as received. Water was distilled and purified by Milli-Q treatment (Millipore Ltd.). N_2 (BOC Ltd.), oxygen-free grade, was purified by passage through Oxysorb (Messer Griesheim GmbH).

Spectrophotometry. A Pye-Unicam SP8-200 spectrophotometer was used with a 10 mm silica cell with a C10 socket and 8 mm "Suba Seal" rubber stopper in a thermostatted cell holder. The cell was stirred using a micro stirrer (Temtron Electronics Ltd.) and kept saturated with N_2 or air by bubbling. Reagents were added by microlitre syringe down a vent tube.

High performance liquid chromatography (HPLC). The eluant was 1 : 1 methanol–water, using Hypersil 5 ODS columns with a Waters 6000A pump, Cecil model Ce 2112 absorbance detector and Waters 730 integrating recorder.

RESULTS

Isomeric composition of the AF-2 samples and their spectral characteristics

Previous experiments [2] showed the *cis* and *trans* isomers to have equal extinction coefficients at 375 nm (cf. Fig. 3); HPLC analysis using absorbance

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† Abbreviations: AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; ε, decadic molar extinction coefficient; GSH, reduced glutathione; $FMNH_2$, reduced flavin mononucleotide; cis^-, *cis* AF-2 nitro radical anion; $trans^-$, *trans* AF-2 nitro radical anion; E, one-electron reduction potential vs NHE, the normal hydrogen electrode.

detection at this wavelength showed the "*cis*" sample to have 13% *trans* isomer present and the "*trans*" sample to have 1% *cis* isomer present. All the work reported below for "*cis*" AF-2 refers to this initial composition.

After making a correction for the isomeric composition, values of the extinction coefficient, ε, at 418 nm were calculated as: *cis*, 10,400 ± 500; *trans*, 21,700 ± 600 dm^3/mol per cm ($\Delta\varepsilon$ = 11,300 dm^3/mol per cm).

Isomerization of cis *AF-2 by ascorbate*

Figure 2, spectrum (a) shows the absorbance of *cis* AF-2 (83 μmole/dm^3) in phosphate buffer (2 mmole/dm^3, pH 7.0) containing EDTA (0.1 mmole/dm^3). Spontaneous spectral changes characteristic of isomerization occurred at higher pH (see below) but at pH 7.0 in anaerobic solution these did not exceed 5% isomerization per hr. Aerated stock solutions of *cis* AF-2 were stable for weeks at 4° in the dark.

On the addition of L-ascorbic acid (0.1 mmole/dm^3) to an anaerobic solution of *cis* AF-2 at pH 7.0, spectral changes occurred with a maximum at 415 nm and isosbestic points at 370 and 310 nm, but the absorption at 262 nm characteristic of ascorbate remained unchanged (Fig. 2). These spectral changes are consistent with *cis* to *trans* isomerization (cf. previously reported [2] isosbestic points at 372, 314 and 270 nm). The isomerization was 50% complete after ~18 min. An identical experiment using aerated solutions showed very small spectral changes over ~1 hr indicating ≥90% inhibition of the reaction by O_2 (*ca* 0.27 mmole/dm^3). (*Ca* 4% oxidation of the ascorbate was also observed over this period but this was clearly insufficient to lead to inhibition of the reaction.)

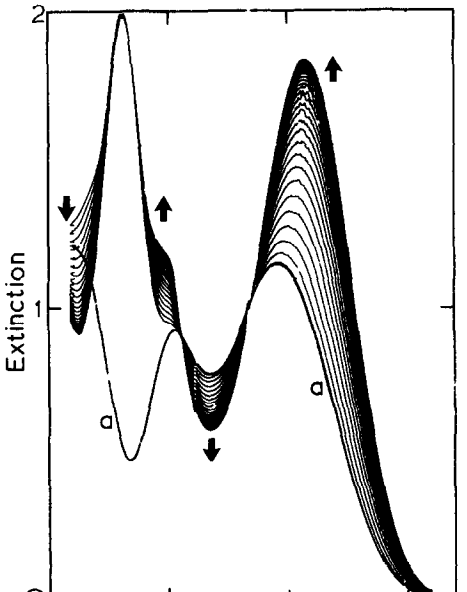

Fig. 2. Absorption spectra (path length = 1 cm) obtained during *cis–trans* isomerization of AF-2 initiated by ascorbate. Spectrum (a) shows *cis* AF-2 (83 μmole/dm^3) in phosphate buffer (2 mmole/dm^3, pH 7.0) with EDTA (0.1 mmole/dm^3) at 25°. Successive scans (at 200 sec intervals) show spectral changes on addition of L-ascorbate acid (0.1 mmole/dm^3). The vertical arrows indicate the direction of the change of extinction (optical density) for the regions shown.

Previous experience of the effects of pH on the reaction of ascorbate with another nitroaromatic compound (outlined below) led us to examine the isomerization of *cis* AF-2 by ascorbate at higher pH. Misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxypropan-2-ol] (Fig. 1, **III**) is a less powerful oxidizing agent than *cis* AF-2 and the chain isomerization of the latter greatly "magnifies" reductive attack, e.g. by electron transfer from ascorbate (see Discussion). As expected, reduction of misonidazole by ascorbate in anoxia could not be detected at pH 7. However, ascorbate is a more powerful one-electron reductant at higher pH [5] and using both spectrophotometry and HPLC we measured ~25% reduction of misonidazole (0.1 mmole/dm^3) by ascorbate (10 mmole/dm^3) after 1 hr at pH 13.0 (NaOH) providing the solution was deaerated (no reaction in aerated solution). Attempts to measure the effect of pH on the ascorbate-initiated isomerization of *cis* AF-2 revealed an unexpected effect of base alone on the reaction (see below). Thus *cis* AF-2 (40 μmole/dm^3) was completely isomerized in *ca* 10 min at pH 9.9 (NaOH) in the absence of O_2 without ascorbate; ascorbate (40 μmole/dm^3) had little additional effect at this pH.

Isomerization of cis *AF-2 by glutathione and* Fe(II)

All the experiments reported below involved spectral changes qualitatively similar to those shown in Fig. 2; for convenience the rates of isomerization are presented as plots of the percentage *trans* isomer calculated from absorbances at 418 nm (Fig. 3). Solutions from selected experiments were analysed by HPLC, showing only *cis* and *trans* AF-2 as products absorbing at 375 nm in the relative amounts expected from the absorption spectra recorded.

Because of the known reactivity of Fe(II)/thiol complexes towards nitroaromatic compounds [6, 7], previous experience with metal catalysis of nitroreduction by $FMNH_2$ [8], the effects of metal impurities in commercial thiols in other biochemical studies [9] and the well-known effects of heavy metals on thiol autoxidation [10], we examined the isomerization of *cis* AF-2 by glutathione and the effects of added Fe(II) with or without EDTA.

Figure 3(a) shows that GSH (2 mmole/dm^3) at pH 7.1 initiated isomerization of *cis* AF-2 (*ca* 84 μmole/dm^3) in anaerobic solution so that *ca* 25% was isomerized in *ca* 10 min. At this time, Fe(II) (ferrous ammonium sulphate, 10 μmole/dm^3) was added and the isomerization was considerably accelerated, as shown in the figure. The presence of EDTA (10 mmole/dm^3) only partially inhibited isomerization in the absence of added Fe(II) (*ca* 25% conversion in 35 min), but the addition of Fe(II) (10 μmole/dm^3) still accelerated the reaction. Very slow isomerization was observed using GSH (2 mmole/dm^3) in aerobic solution.

Figure 3(b) shows that ferrous ammonium sul-

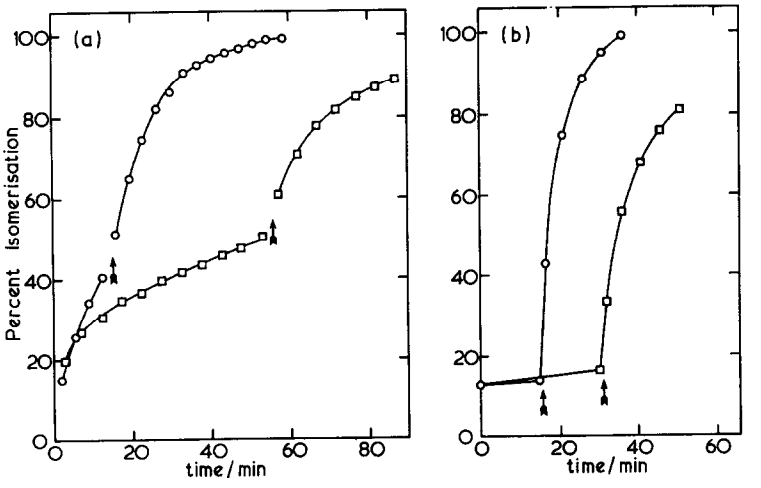

Fig. 3. Percentage *trans* isomer produced during the *cis–trans* isomerization of AF-2 by glutathione and Fe(II) in anaerobic solution at pH 7.1 (2 mmole/dm³ phosphate buffer) and 25°. (a) *cis* AF-2 (84 μmole/dm³) + GSH (2 mmole/dm³) in the absence of (○) and in the presence (□) of EDTA (10 mmole/dm³). Vertical arrows denote the time of addition of Fe(II) (10 μmole/dm³ ferrous ammonium sulphate). (b) *cis* AF-2 (85 μmole/dm³) in the absence (○) and presence (□) of EDTA (10 mmole/dm³). Arrows indicate the time of introduction of ferrous ammonium sulphate (10 μmole/dm³). All data calculated from changes in extinction at 418 nm.

phate (10 μmole/dm³) without GSH isomerized *cis* AF-2 (85 μmole/dm³) in a few minutes in the absence of O_2, and that EDTA (10 mmole/dm³) had little effect on this reaction. No isomerization was detectable with Fe(II) (10 μmole/dm³) in aerobic solution.

Isomerization of cis *AF-2 by* OH^-

Addition of NaOH (0.4 mmole/dm³, pH 10.1) to *cis* AF-2 (85 μmole/dm³) in deaerated water initiated the spectral changes shown in Fig. 4. The maxima at 416 and 300 nm, isosbestic points at 370, 312 and 270 nm, and HPLC analysis of a replicate run confirmed that the only reaction occurring was isomerization. HPLC analysis showed 4% *cis* isomer remained after spectral changes ceased to occur. Measurements of the rate of spectral change at several pH values showed 50% isomerization of *cis* AF-2 (85 μmole/dm³) occurred after *ca* 70, 20 and 2 min at pH 8.8, 10.1 and 11.2, respectively. At pH 12.0, *cis* AF-2 (40 μmole/dm³) was completely isomerized 2 min after the addition of base, but slow spectral changes subsequently occurred, the 416 nm absorption of *trans* AF-2 decreasing by *ca* 10% in 10 min.

Aerobic solutions of *cis* AF-2 (85 μmole/dm³) at pH 10.0 did not isomerize detectably over 30 min, but *ca* 15% isomerization was observed in an aerated solution at pH 11.1 over this period.

DISCUSSION

Chain isomerization of cis *AF-2 and its inhibition by oxygen*

Radical-initiated isomerization of *cis* AF-2 was previously reported [2] to be a chain reaction, as outlined in the following scheme [4]:

Initiation:	*cis* + e^-	$\rightarrow$ *cis*$^-$	(1)
Isomerization:	*cis*$^-$	$\rightleftharpoons$ *trans*$^-$	(2)
Propagation:	*cis* + *trans*$^-$	$\rightleftharpoons$ *cis*$^-$ + *trans*	(3)
Inhibition:	*cis*$^-$/*trans*$^-$ + O_2	$\rightleftharpoons$ *cis*/*trans* + O_2^-	(4)
Termination:	2 *cis*$^-$/*trans*$^-$	$\rightarrow$ products	(5)

Any electron donor capable of one-electron reduction of 5-nitrofurans will initiate isomerization. The chain propagation step results from the ~34 mV

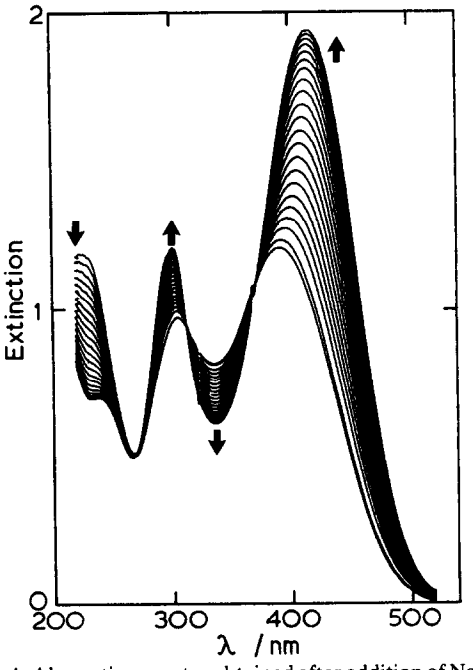

Fig. 4. Absorption spectra obtained after addition of NaOH (0.4 mmole/dm³, pH 10.1) to *cis* AF-2 (85 μmole/dm³) in deaerated water at 25°. Scans repeated at 210 sec intervals. Vertical arrows denote the direction of extinction change for each spectral region.

higher one-electron reduction potential for *cis* AF-2 than the *trans* isomer ($K_3 \sim 3.8$); estimates of $k_4 \simeq 6 \times 10^5$ or 2×10^6 dm^3/mol per sec for the *cis* and *trans* isomers, respectively, of AF-2 were reported [4]. In the absence of O_2, chain lengths of up to ~500 were measureable [4]. Such a chain reaction is consistent with the conclusion of Tatsumi *et al.* [2] that the isomerizing activity of several enzyme systems was much higher than their nitro-reducing activity.

Because of this chain reaction, isomerization of *cis* AF-2 is an extremely sensitive probe providing evidence for the generation of nitro radicals in systems where the steady-state concentration of the radicals would be too low to permit direct identification by, for example, electron spin resonance methods. Oxygen inhibits both isomerization and the formation of relatively stable reduction products [via reaction (5)] by the "futile metabolism" [11] well characterized for other nitroaromatic compounds, involving the electron transfer reaction (4) which has been observed directly [4, 12]. However, the efficiency of the chain isomerization step results in isomerization occurring to a readily detectable extent at oxygen concentrations where overall nitroreduction (net loss of AF-2) is so slow that it cannot be measured. Isosbestic points characteristic of *cis* to *trans* isomerization (but without net nitroreduction) are a feature not only of radiolytic radical-induced changes [2, 4] in AF-2 solutions, but were also observed upon treatment of *cis* AF-2 with other reductants as reported above; the likelihood of generating nitro radicals with these reductants must now be considered.

Thermodynamics of one-electron transfer between nitroaromatics and ascorbate

The equilibrium constant K_6

$$ArNO_2 + AH^- \rightleftharpoons ArNO_2^- + AH^\cdot \qquad (6)$$

where $ArNO_2$ = any nitroaromatic compound, AH^- = ascorbic acid and $AH^\cdot$ = the ascorbate radical can be calculated if the one-electron reduction potentials $E(ArNO_2/ArNO_2^-)$ and $E(AH^\cdot/AH^-)$ are known. The latter couple has been measured [5] at pH 13.5 and can be calculated at other pH values (e.g. 0.3 V at pH 7); for AF-2 the potentials for one-electron reduction of the *cis* and *trans* isomers are −0.242 and −0.276 V, respectively (vs NHE at pH 7; the values are unlikely to change at higher pH values) [4]. From these potentials we calculate $K_6 = 7 \times 10^{-10}$ and 2×10^{-10} for *cis* and *trans* AF-2, respectively. Initiation of isomerization by generation of nitro radical via reaction (6) would thus appear to be thermodynamically unfavourable.

However, if we assume the steady-state approximation:

$$d[ArNO_2^-]/dt = 0 = k_6[ArNO_2][AH^-] - k_6[ArNO_2^-][AH^\cdot]$$

where $K_6 = k_6/k_{-6}$, then for typical $[ArNO_2] = [AH^-] = 100\ \mu$mole/dm^3 we calculate a steady-state concentration of $ArNO_2^-$ radicals of *ca* 3 nmole/dm^3 when $ArNO_2$ = *cis* AF-2 [*cis*$^-$ depleted via reaction (2) will be immediately replenished by propagation step (3)]. The spectral changes in Fig. 2 correspond to an initial isomerization rate of *ca* 30 nmole/dm^3 per sec. Hence if reaction (6) was responsible for initiating isomerization via reactions (2) and (3), we might expect k_2 to be of the order of 10 sec^{-1} [equation (2) is well over to the right [4]). Independent experiments [4] generating *cis*$^-$ from *cis* AF-2 by radiolytic methods suggest an isomerization rate constant, k_2, in the range 5–40 sec^{-1}. Further, Kalyanaraman *et al.* [3] predicted k_2 to be 40 sec^{-1} from ESR observations, assuming $k_2 \simeq k_4[O_2]$ in a microsomal preparation and taking $k_4 = 2 \times 10^5$ dm^3/mole per sec. These observations provide independent support for the initiation of isomerization by reaction (6) in spite of the unfavourable thermodynamics involved. (Of course, this simple approach ignores other reactions such as disproportionation of $ArNO_2^-$ and $AH^\cdot$; detailed modelling of the reaction sequences, including these additional reactions, using numerical integration [4] confirms the conclusion that the concentration of $[ArNO_2^-]$ should be of the order derived above.)

For misonidazole, $E(ArNO_2/ArNO_2^-) = -0.389$ V at pH > 7 [13], so $K_6 = 2 \times 10^{-12}$ at pH 7 and 6×10^{-8} at pH 13. At the higher pH, we measured misonidazole loss at a rate of *ca* 7 nmole/dm^3 per sec with 100 μmole/dm^3 $ArNO_2$ and 10 mmole/dm^3 AH^-. Again making the steady-state approximation $d[ArNO_2^-]/dt = 0$ to calculate $[ArNO_2^-] \simeq 0.2\ \mu$mole/dm^3 we find that the reaction

$$ArNO_2^- + AH^- \rightarrow ArNO_2^{2-} + AH^\cdot \qquad (7)$$

would require $k_7 \simeq 3$ dm^3/mole per sec to explain the rate of loss of misonidazole. ($ArNO_2^{2-}$ would yield the nitroso intermediate ArNO by protonation and dehydration, and subsequent reduction steps are unlikely to be rate-determining.) Whilst reaction (7) has not been observed directly, the analogous one-electron oxidation of AH^- by O_2^- has a rate constant [14] of ~10^5 dm^3/mole per sec at pH ~8–9. It seems that, whilst nitro radicals may undergo many of the reducing reactions of O_2^- [e.g. reducing Cu(II) in superoxide dismutase (SOD) [15]], they are much less powerful oxidants than O_2^-: this would explain the failure [15] of misonidazole radicals to re-oxidize the Cu(I) centre in SOD to facilitate catalytic action as is thought to be the basis for the O_2^- dismutation. [Bigalow *et al.* [16] have studied the influence of nitroaromatic compounds on ascorbate oxidation by O_2. They also suggested generation of nitro radicals via equilibrium (6) but K_6 could not be calculated at that time because E ($AH^\cdot/AH^-$) was not reliably known.]

Isomerization of cis *AF-2 by glutathione and* Fe(II)

Conceivably, thiols such as glutathione (GSH) could produce nitro radicals in a reaction analogous to reaction (6):

$$ArNO_2 + RSH \rightleftharpoons ArNO_2^- + RS^\cdot + H^+ \qquad (8)$$

Again, net nitroreduction by thiols is normally undetectable under physiological conditions (the rate constant for reaction of misonidazole with GSH [17]

at pH 7.4 is $\leqslant 10^{-5}$ dm^3/mole per sec) and only the chain isomerization of AF-2 provides evidence for any reaction of the type described by equation (8). However, nitro radicals have been postulated as intermediates in the reduction of metronidazole, misonidazole, etc., by Fe(II)/thiol complexes [6, 7] and it is possible that the isomerization of *cis* AF-2 by GSH reported above is initiated not via reaction (8) but is catalysed by Fe(II) impurity in the GSH. Analysis of the GSH sample used in the present work (Sigma lot No. 81F-0304) by atomic absorption spectroscopy (kindly performed by Mr. R. H. Smith, Brunel University) showed 4 ppm Fe as impurity. Using 2 mmole dm^3 GSH [Fig. 3(a)] we would have 8 nmole/dm^3 Fe present.

Initiation of isomerization by Fe(II) alone [Fig. 3(b)] is explicable via reaction (9):

$$ArNO_2 + Fe(II) \rightleftharpoons ArNO_2^{\cdot-} + Fe(III) \qquad (9)$$

but K_9 cannot be calculated since the effective potential of the couple Fe(III)/Fe(II) is unknown at pH 7. It is likely that Fe(II)(EDTA) initiates isomerization efficiently since the standard reduction potential of Fe(III)(EDTA) is 0.117 V [18], i.e. significantly lower than that of the $AH^{\cdot}/AH^{-}$ couple at pH 7. The role of EDTA in inhibiting the GSH-initiated isomerization is not clear; other catalytic metal impurities (e.g. Cu [7]) may be a factor.

Isomerization of AF-2 is much slower in aerated solutions [regardless of whether the reaction is initiated by ascorbate, GSH or Fe(II)] consistent with radical intermediates and inhibition by reaction (4).

Base-catalysed isomerization of cis *AF-2*

Spontaneous or photolytic generation of nitroaromatic radical anions in strongly basic solution is well known [19, 20]; electron transfer from the base to the nitro compound or its excited state seems the likely initial step [21] and obvious analogy can be made with the formation of viologen radical cations on reaction of, for example, paraquat with base in the absence of oxygen [22]. Isomerization of *cis* AF-2 in deaerated basic solution occurs at much lower pH values than are required to generate sufficient radical anions for ESR observation of, for example, nitrobenzene radicals, because of the chain mechanism. In contrast, misonidazole was relatively stable (< 1% loss in 1 hr) at pH 13.

Conclusion

The isomerization of AF-2 and other (5-nitro-2-furyl)acrylamides occur with such high efficiency, especially in the absence of oxygen, that it is a very sensitive probe for the production of nitrofuran radicals. Reductants include not only the well-known 'nitroreductase' enzymes with their cofactors, but also non-enzymic electron donors. Ascorbate and glutathione, at physiological pHs and concentrations, are important examples. It should be stressed that the net rate of isomerization observed in all these systems depends critically on oxygen concentration: isomerization of *cis* AF-2 reflects the balance between reducing activity — *in its widest sense* — and the oxygen tension.

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