E.S.R. Observation of Enamine Radical Cations from Thiamine (Vitamin B_1) and Related Compounds

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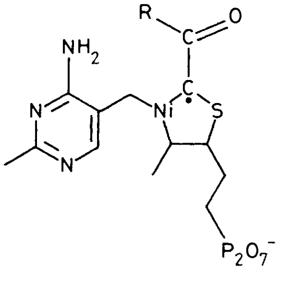
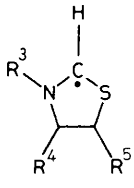
Reactions of thiamine and thiazolium salts with hydroxyalkyl radicals yield enamine radical cations by radical and/or formal hydrogen addition at the =CH– position of the thiazolium ring. Their high resolution e.s.r. data lead to reinterpretations of previous spectroscopic observations on thiamine radicals and suggest a structure for a radical observed during the enzymic action of pyruvate:ferredoxin oxidoreductases from archaebacteria and anaerobic eubacteria.

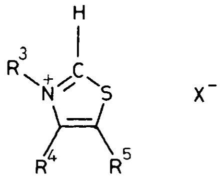
In recent work Oesterhelt *et al.* demonstrated[1–3] the involvement of a free radical intermediate in the thiamine-diphosphate dependent decarboxylation of 2-oxoacids by 2-oxoacid:ferredoxin oxidoreductases obtained from archaebacteria and anaerobic eubacteria. E.s.r. spectra of frozen solutions exhibited $g = 2.006$, a total width of *ca.* 5 mT, only slightly anisotropic features, and indications of hyperfine couplings of *ca.* 0.5 mT to several nuclei which depended on the 2-oxoacid employed. For the radical a vinyloxy-type structure was suggested[2–4] which should better be represented by its alkyl form ($R = CH_3$ for pyruvic acid) and a mechanism for its role in the catalytic cycle was given. Unfortunately, there are practically no e.s.r. data available for related radicals[5] which would lend support for the proposed structure. Furthermore, the variation of the spectral features with group R is somewhat in conflict with the assignment since the spin population at the carbonyl carbon atom is expected to be low. To confirm or rule out structure (**1**) we therefore attempted to generate radicals of the general structure (**2**) through reduction of thiamine and related thiazolium salts (**3**) by photochemically generated α-hydroxyalkyl radicals $(CH_3)_2\dot{C}OH$ and $\dot{C}H_2OH$ in liquid solutions. These radicals have reduction potentials of −1.50 and −0.95 V, respectively,[6] and should transfer one electron to thiamine (−0.5 V[7]) and the model compounds.[8] Interestingly, instead of the expected neutral radicals, the reactions led to enamine radical cations by formal addition of either alkyl radicals or hydrogen atoms at the C-2 carbon of the thiazolium ring, *i.e.* radicals of the type:



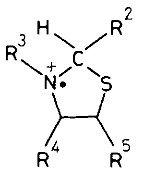
(**1**)



(**2**)



(**3**)



(**4**)

R^2 = H or hydroxyalkyl

Since such species have apparently not been studied before we report their e.s.r. data here. This also leads to reinterpretations of earlier work on thiamine-derived radicals[8,9] and to a new suggestion for the structure of Oesterhelt's enzyme radical.

Experimental

All chemicals were used in the purest available form (Fluka or EGA-Chemie). The thiazolium salts 3-methylthiazolium iodide (**3a**), 3,4-dimethylthiazolium iodide (**3b**) and 3,4-dimethyl-5-(2-hydroxyethyl)thiazolium iodide (**3c**; $R^5 = CH_2CH_2OH$) were prepared from the parent thiazoles and methyl iodide, and 3-benzyl-4-methyl-5-(2-hydroxyethyl)thiazolium chloride (**3d**) from the thiazole and benzyl chloride following standard procedures.[10] They were purified by recrystallization from ethanol–ether mixtures. Thiamine was used as the chloride salt.

Oxygen-free solutions of either 0.5–2.5 mol dm^{-3} acetone and 0.5–15.0 mol dm^{-3} propan-2-ol in water or 0.2 mol dm^{-3} di-t-butylperoxide and 5–8 mol dm^{-3} water in propan-2-ol or methanol were photolysed ($305\ nm \leqslant \lambda \leqslant 330\ nm$) during a slow flow within the cavity of an e.s.r. spectrometer at (303 ± 1) K. They showed the formation of $(CH_3)_2\dot{C}OH$ by photoreduction of acetone or $(CH_3)_2\dot{C}OH$ and $^{\bullet}CH_2OH$ *via* reaction of t-butoxyradicals with the alcohols. Upon addition of 10–30 mmol dm^{-3} of the thiazolium compounds the spectra of the primary radicals were completely replaced by those described below. The *g*-factors of the thiazolium-derived radicals were measured relative to those of $(CH_3)_2\dot{C}OH$ [2.00314(2)[11]] or $^{\bullet}CH_2OH$ (2.00317[11]). Spectrum simulations were carried out with ESREXN.[12]

Results and Discussion

N-*Methyl Thiazolium Salts*.—Reaction of $(CH_3)_2\dot{C}OH$ from both methods of generation with 3-methylthiazolium iodide (**3a**) gave rise to the e.s.r. spectrum shown in Figure 1(*a*). The simulation in Figure 1(*b*) was obtained using coupling to one

Table 1. Hyperfine coupling constants of enamine radical cations/mT.

Radical	a_2	a_N	a_3	a_4	a_5	Other
(**4a**)[a]	2.930 (H)	0.734	0.804 (3 H)	0.177 (H)	1.010 (H)	
(**4b**)	2.571 (H)	0.655	0.756 (3 H)	0.243 (3 H)	1.082 (H)	
(**4c**)	2.385 (H)	0.676	0.779 (3 H)	0.263 (3 H)	0.849 (2 H)	
(**4d**)	2.048 (H)	0.868	0.560 (2 H)	0.297 (3 H)	0.826 (2 H)	
(**4e**)	2.155 (H)	0.625	0.652 (2 H)	0.343 (3 H)	0.831 (2 H)	0.053 (1 H)
(**5d**)[d]	2.494 (2 H)	0.698	0.777 (2 H)	0.259 (3 H)	0.835 (2 H)	
(**5e**), pH < 4.8	3.025 (2 H)	0.662	0.556 (2 H)	0.314 (3 H)	0.797 (2 H)	
(**5e**), pH > 4.8	3.055 (2 H)	0.675	0.673 (2 H)	0.274 (3 H)	0.805 (2 H)	

[a] R adducts, (**4e**): R = CH_2OH, others: R = $(CH_3)_2COH$. [b] H-adducts.

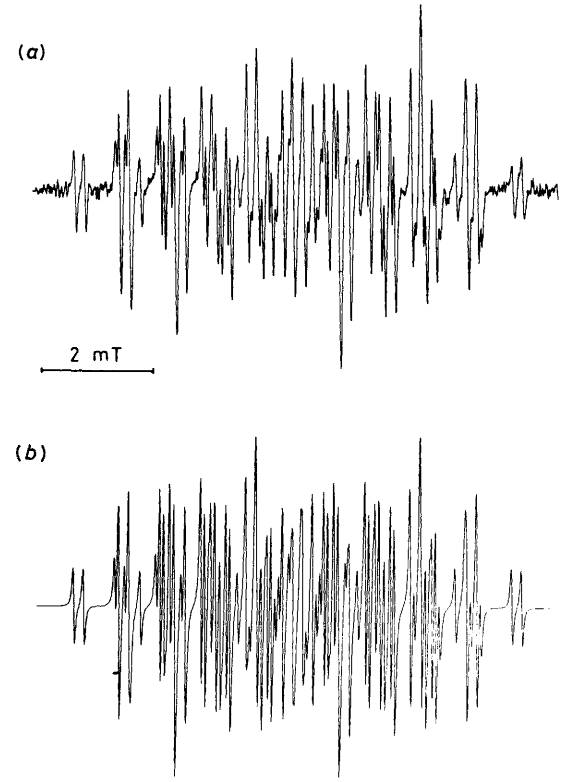
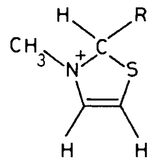


Figure 1. E.s.r. spectrum of the $(CH_3)_2\dot{C}OH$ adduct to *N*-methylthiazolium iodide (**4a**), (*a*), and simulation (*b*).

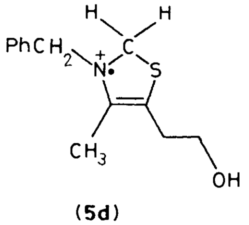
nitrogen nucleus (0.734 mT), three equivalent protons (0.804 mT), and three protons with coupling constants of 2.930, 1.010, and 0.177 mT, respectively. The total spectral width is *ca.* 8.0 mT, and $g \approx 2.006$. From the distribution of the coupling constants and comparisons with those of amine radical cations[13] and cyclopentenyl radicals[14] the assignment of the radical as the enamine radical cation (**4a**) is immediately evident:



(**4a**)

$a(CH_3)$, a(N) and two of the remaining H-couplings are as expected for a 1-aza-allylic system and the large proton coupling is typical for a β-proton in substituted cyclopentenyl radicals. Furthermore, the bonding configuration of the nitrogen must be planar[13] and the five-membered ring must be fairly rigid. The nature of group R can not be deduced from the spectrum but is very likely to be $(CH_3)_2COH$, *i.e.* the radical is formed by addition of the primary hydroxyalkyl radical to (**3a**) at C-2. The two other *N*-methyl compounds (**3b**; $R^4 = CH_3$) and (**3c**; $R^4 = CH_3$, $R^5 = CH_2CH_2OH$) led to e.s.r. spectra which confirmed the above assignment. For (**3b**) the small coupling of 4-H is replaced by a small coupling to three equivalent protons of a CH_3 group. The same is true for (**3c**) which in addition shows a coupling to two protons of the CH_2CH_2OH group at C-5. The coupling constants are collected in Table 1. Variation of pH from 1.0 to 8.5 did not change the spectra. Further, it is noteworthy that no adduct of type (**4a**) could be obtained if the primary species was $^{\bullet}CH_2OH$ instead of $(CH_3)_2\dot{C}OH$.

N-*Benzylthiazolium Salt* (**3d**).—Reaction of this compound with $(CH_3)_2\dot{C}OH$ in solutions containing a large excess of propan-2-ol over water led to the $(CH_3)_2COH$ adduct (**4d**) with coupling constants quite analogous to those of (**4c**) (Table 1) However, this compound behaved somewhat differently from (**3a–c**). Firstly, reaction of $^{\bullet}CH_2OH$ also led to a corresponding adduct radical. It also exhibited a small splitting of one of the CH_2OH protons (0.05 mT). Secondly, reaction of $(CH_3)_2\dot{C}OH$ in solutions with reduced propan-2-ol content yielded, in addition to (**4d**), a second radical with somewhat sharper lines and a much larger total width. Figure 2(*a*) shows the observed spectrum. The stronger lines in the centre are due to (**4d**) and the weak lines of the additional radical are visible in the wings. Figure 2(*b*) shows these lines amplified and Figure 2(*c*) is the wing part of a simulation based on parameters similar to those of (**4d**), with the difference of two large β-proton couplings instead of one. Based on the coupling pattern, the second radical is assigned as (**5d**), *i.e.* a formal adduct of hydrogen at C-2. Unfortunately, (**5d**) could not be observed alone. Variation of pH from 2 to 7.5 did not change the spectral features of (**4d**) and (**5d**), and $g \approx 2.006$ as for the other species.



(**5d**)

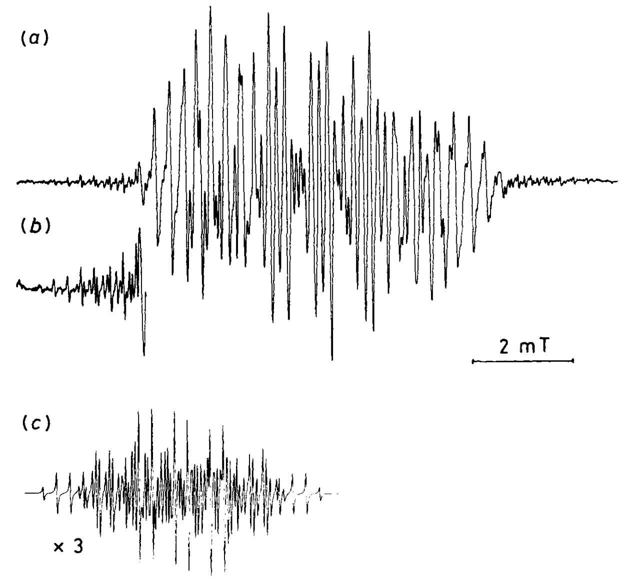


Figure 2. (*a*) E.s.r. spectra of the $(CH_3)_2\dot{C}OH$ adduct (inner lines) and the H adduct [wings, (*b*)] to the *N*-benzylthiazolium salt; (*c*) partial simulation for the H adduct.

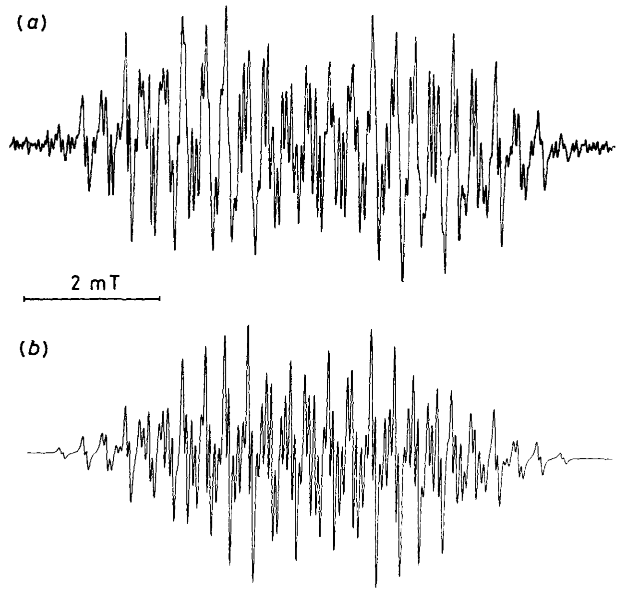


Figure 3. E.s.r. spectrum of the $\dot{C}H_2OH$ adduct to thiamine (*a*) and simulation (*b*).

Thiamine (**3e**).—$^{\bullet}CH_2OH$ reacted with thiamine to form the adduct (**4e**), the e.s.r. spectrum of which exhibited a small coupling to one of the CH_2OH protons (Figure 3). The adduct of $(CH_3)_2\dot{C}OH$ was observed in solutions with high propan-2-ol content. However, it was completely replaced by the addition product of H at C-2 for aqueous solutions containing only a little propan-2-ol. Figure 4 shows the spectrum and simulation of this radical for pH 7. In experiments with D_2O, a spectrum was obtained which was compatible with a D adduct, *i.e.* (**5e**) with one of the CH_2 protons substituted by a deuteron. Lowering of the pH led, for pH < 5.5, to the appearance of a third radical which gradually replaced the second species. It had very similar coupling constants to the latter, the major difference being the coupling to the protons of the CH_2-group linking the thiazolium and pyrimidine rings. This indicates that the third radical is (**5e**) with the pyrimidine moiety at a different stage of protonation. The pK_a was found to be 4.8 ± 0.2. This agrees perfectly well with the pK_a reported for protonation of the amino pyrimidine group of thiamine;[15–17] this is not expected to be altered in the H adduct since radical formation does occur at a fairly remote position. The *g*-factors of the CH_2OH adduct and the H adduct are 2.00604(8) and 2.00620(8), respectively.

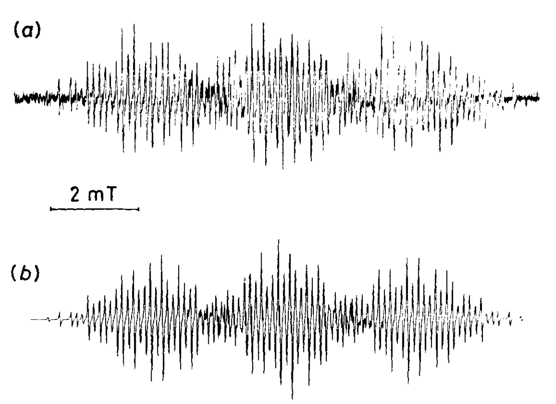


Figure 4. E.s.r. spectrum of the $H^{\bullet}$ adduct to thiamine (pH = 7) (*a*) and simulation (*b*).

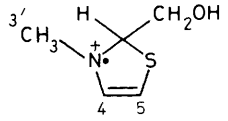
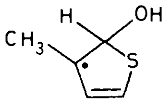
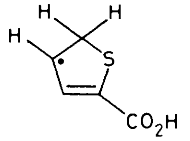
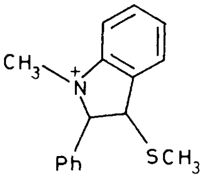
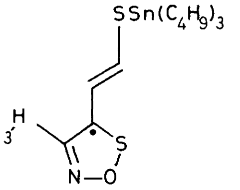
In previous work Damerau *et al.*[9] treated $^{\bullet}CH_2OH$, $CH_3\dot{C}HOH$, $CH_3CH_2\dot{C}HOH$, and $(CH_3)_2\dot{C}OH$ radicals with thiamine at pH 1 and observed the e.s.r. spectra which are very similar to those found here for the hydroxyalkyl adducts, though somewhat less resolved. They suggested radical formation by addition to the pyrimidine ring. This must now be revised since from our studies on the model compounds it is clear that addition occurs to the thiazolium residue.

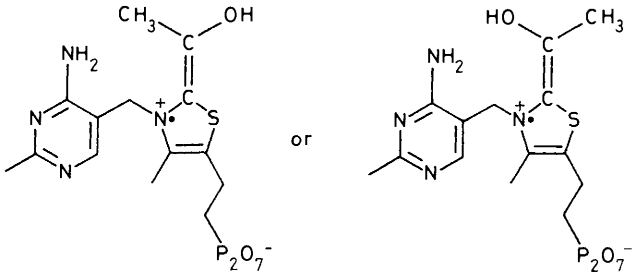
Also related to this work are the u.v. spectra of Hayon *et al.*,[8] observed during reactions of $(CH_3)_2\dot{C}OH$ with thiamine in aqueous solutions. The transients showed spectra with two broad maxima which shifted in the pH range 0.5–3. The authors attributed the spectrum to the neutral radical (**2e**) with a pK_a for protonation of the thiazolium nitrogen of 1.6 ± 0.3. We found that in the pH range studied the relative rates of formation of $(CH_3)_2\dot{C}OH$ and H adducts changed in favour of the former, and therefore suggest that Hayon *et al.* also observed different enamine radical cations.

Reaction Mechanism.—It was shown that the three *N*-methylthiazolium cations (**3a–c**) react with $(CH_3)_2\dot{C}OH$ by addition but not with $\dot{C}H_2OH$, whereas the *N*-benzyl compound (**3d**) and thiamine (**3e**) are able to add to both radicals. The latter two compounds also gave formal H adducts by reaction with $(CH_3)_2\dot{C}OH$ but not with $^{\bullet}CH_2OH$, and the rate of formation of the H adduct increased with the water content of the solvent. Both hydroxyalkyl radicals have very low reduction potentials and are strongly nucleophilic with $(CH_3)_2\dot{C}OH$ being the stronger nucleophile. Furthermore, it is known from Breslow's classic studies on vitamin B_1 and model compounds that the reactivity towards nucleophiles at C-2 increases in the order *N*-methylthiazolium < *N*-benzylthiazoliumion < thiamine.[18] Thus, the apparent order of ease of addition is in accord with, and explained by, the increasing electron-withdrawing effect of the *N*-substituents. This explanation is also supported by the addition behaviour of other nucleophilic radicals to substituted alkenes.[19]

Of particular interest is the formation of the H adduct radicals by reaction of the *N*-benzyl compound and thiamine with $(CH_3)_2\dot{C}OH$. We believe that this is caused by an initial electron transfer to give the anticipated neutral thiazole radicals (**2d**) and (**2e**) plus $(CH_3)_2CO^+H$, followed by a rapid protonation at C-2 (or deuteriation in D_2O). In fact, the

Table 2. E.s.r. parameters of enamine radical cations and related species (*a*/ mT).

Radical	g	a_2	a_3	$a_{3'}$	a_4	a_5	Ref.
	2.0060	2.930 (1 H)	0.734 (N)	0.804 (3 H)	0.177 (1 H)	1.010 (1 H)	
	2.0039	1.78 (1 H)	—	1.255 (3 H)	0.26 (1 H)	1.36 (1 H)	23
	2.0061	3.3 (2 H)	—	1.3 (1 H)	—	—	24
	—	—	0.415 (N)	0.845 (3 H)	—	—	25
	2.0065	—	—	0.186 (1 H)	0.497 (N)	—	26



(6)

electron transfer from $(CH_3)_2\dot{C}OH$ to various compounds is a well known reaction,[20] and in the present case a rapid protonation of an intermediate neutral thiazole radical with a pyramidal configuration at the nitrogen atom will be strongly favoured because of the gain in delocalization energy.[21,22] The simultaneous observation of addition and electron transfer and its solvent-dependent ratio may even point to a common intermediate or highly polar transition state for both pathways.

Related Radicals.—Table 2 shows the e.s.r. parameters of one of the enamine radical cations and of some related radicals. They support the arguments used in the above assignments, and show that g *ca.* 2.006 is quite characteristic for radicals with aza-allyl or azapentadienyl groups flanking an S-atom. Furthermore, in combination with the pathways for radical formation they lead to a suggestion for the structure of Oesterhelt's radical. We have shown that enamine radical cations are easily formed and that any neutral precursor will rapidly be protonated at C-2. Therefore, we believe that the originally proposed neutral species (**1**) is very unlikely. From $g \approx 2.006$ a relatively large spin population is indicated at C-5, but the total spread of the spectrum of only 5 mT rules out an aza-allylic system with a β-proton at C-2. Thus, we arrive at an aza pentadienylic structure and suggest (**6**) for the case of pyruvic acid. With an average 30% reduction[14] of coupling constants on the transition from the allylic to the pentadienyl system the data of Table 1 lead to the predictions of a(N) *ca.* 0.45 mT, a_3(2 H) *ca.* 0.45 mT, a_5(2 H) *ca.* 0.55 mT, a_4(3 H) *ca.* 0.20 mT, and $a(CH_3)$ *ca.* 0.50 mT for this species. This yields a total width of *ca.* 5 mT, as observed. Furthermore, the proposed structure agrees with the observation of only low hyperfine anisotropy and with the dependence of the spectrum on the 2-oxoacid employed in the enzymatic reaction. Of course, the newly suggested structure is only a protonated form of the original version (**1**). However, the difference is more subtle since even the species deprotonated at —OH would rather adopt a zwitterionic form with a planar positively charged nitrogen than the bent geometry of structure (**1**).

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References

1 L. Kerscher and D. Oesterhelt, *Eur. J. Biochem.*, 1981, **116**, 587.
2 R. Cammack, L. Kerscher, and D. Oesterhelt, *FEBS Lett.*, 1980, **118**, 271.
3 L. Kerscher and D. Oesterhelt, *Trends Biochem. Sci.*, 1982, **7**, 371.
4 D. Oesterhelt, personal communication.
5 Landolt–Börnstein, New Series, vols. 1, 9, 17, Springer Verlag, Berlin, 1965–1989.
6 J. Butler and A. Henglein, *Radiat. Phys. Chem.*, 1980, **15**, 603.
7 P. N. Moorthy, K. N. Rao, and K. Kishore, *Radiat. Eff. Lett.*, 1979, **43**, 1.
8 P. N. Moorthy and E. Hayon, *J. Org. Chem.*, 1977, **42**, 879.
9 W. Damerau, D. Schwarz, G. Lassmann, G. Hübner, and A. Schellenberger, *Z. Chem.*, 1973, **13**, 179.
10 'Organikum,' VEB Verlag Deutscher Wissenschaften, Berlin, 1977.
11 G. P. Laroff and R. W. Fessenden, *J. Phys. Chem.*, 1973, **77**, 1283.
12 J. Heinzer, *Mol. Phys.*, 1971, **22**, 167.
13 S. F. Nelsen in 'Magnetic Properties of Free Radicals,' Landolt-Börnstein, New Series, vol. II/9d2, Springer Verlag, Berlin, 1980.
14 A. Berndt in ref. 13, vol. 11/9b, Springer Verlag, Berlin, 1977.
15 R. F. W. Hopmann and G. P. Brugnoni, *Nature (London), New Biol.*, 1973, **246**, 157.
16 A. H. Cain, G. R. Sullivan, and J. D. Roberts, *J. Am. Chem. Soc.*, 1977, **99**, 6423.
17 J. Suchy, J. J. Mieyal, G. Bantle, and H. Z. Sable, *J. Biol. Chem.*, 1972, **247**, 5905.
18 R. Breslow and E. McNelis, *J. Am. Chem. Soc.*, 1959, **81**, 3080.
19 H. Fischer and H. Paul, *Acc. Chem. Res.*, 1987, **20**, 200.
20 K.-D. Asmus and M. Bonifačić in 'Radical Reaction Rates in Liquids,' Landolt-Börnstein, New Series, vol. II/13b, Springer Verlag, Berlin, 1984.
21 R. Houriet, J. Vogt, and E. Haselbach, *Chimia*, 1980, **34**, 277.
22 B. Albrecht, M. Allan, E. Haselbach, L. Neuhaus, and P.-A. Carrupt, *Helv. Chim. Acta*, 1984, **67**, 220.
23 B. C. Gilbert, R. O. C. Norman, and P. S. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1981, 207.
24 B. Eda, R. J. Cook, and D. H. Whiffen, *Trans. Faraday Soc.*, 1964, **60**, 1497.
25 M. Colonna, L. Greci, and M. Poloni, *J. Chem. Soc., Perkin Trans. 2*, 1982, 455.
26 K. U. Ingold, D. H. Reid, and J. C. Walton, *J. Chem. Soc., Perkin Trans. 2*, 1982, 431.

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