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Isothiocarbamido Radicals from Thiourea: Electron Spin Resonance Spectroscopy of *N*-Benzylidene-*t*-butylamine-*N*-oxide and 5,5-Dimethyl-1-pyrroline-*N*-oxide Spin Adducts

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Amidinothio radicals, $NH_2(=NH)CS\cdot$, formed in the reaction of thiourea and hydrogen peroxide and trapped in aqueous solution by *N*-benzylidene-*t*-butylamine-*N*-oxide (PBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) spin traps are reported for the first time. Stability of the radical is highly dependent on the pH as well as the concentration of thiourea. ESR spectral lines give a^{N} and a_{β}^{H} values as 1.53 mT and 0.24 mT respectively for PBN and 1.46 mT and 2.08 mT respectively for DMPO spin adducts. Preliminary kinetic experiments on the spin trapping reaction are also reported.

Thiourea is often used as a reducing agent in redox initiated free radical polymerization reactions.[1] Amidinothio radicals are believed to be the primary radicals formed in situ.[2] In the case of redox systems involving peroxide, $\cdot OH$ radicals may also be formed. In the latter case, generation of free radicals has been shown to be due to a one-electron transfer with concomitant cleavage of the –O–O– bond. In these reactions, the effect of thiourea at high concentration is, however, rather confusing. While the presence of thiourea increases the rate of polymerization of vinyl compounds by H_2O_2 dramatically, high concentration of thiourea affects neither the rate nor the degree of polymerization. In some cases, the percentage of graft polymerization yield is even found to decrease with an increasing concentration of thiourea.[2] The scavenging effect of thiourea at high concentration is probably due to the formation of 1,1′-dithiobis(formamidine) more readily, thereby decreasing the concentration of the amidinothio radicals in the system. Thiourea is also used in biochemical reactions as an effective $\cdot OH$ scavenger. It inhibits NADPH dependent mi crosomal oxidation of ethanol and 1-butanol, which is more effective in the presence of catalase-inhibiting azide.[3] Of all $\cdot OH$ scavengers, thiourea has been shown to be the most effective one. The highly reactive $\cdot OH$ radicals which may be the true damaging species in vivo, are produced via superoxide anions (O_2^-) by the iron-catalyzed Haber–Weiss reaction and are most effectively inhibited by thiourea.[4] Recently, 1-methyl-2-mercaptoimidazole, which has a isothiourea moiety has been found to directly interact with $\cdot OH$ and protect a sensitive enzyme against inactivation.[5] It has also been shown that ergothioneine, the only mercaptoimidazole derivative in nature, is a more effective radio protector than cystein and cysteamine.[6] A thiyl type radical (amidinothio) is probably an intermediate in the above scavenging reactions (which readily forms disulfides).[7] Detailed chemical and spectroscopic aspects of this radical have not been studied because of the failure of attempts to detect it in solution by ESR spectroscopy. Radiolysis of thiourea in a solid matrix, however, is reported to generate the radical but poor resolution of the resultant spectra restricts proper analysis.[8] Spin trapping of a few thiyl radicals is known but, no such study with thiourea has been reported so far. In the present communication, results of spin trapping experiments and ESR spectroscopy of PBN and DMPO adducts of amidinothio radicals are reported.

Experimental

Reagents: 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and *N*-benzylidene-*t*-butylamine-*N*-oxide (PBN) were supplied by Aldrich Chemical Co., U.K. Control experiments did not show any ESR signal from impurities in the samples of both the spin traps. Thiourea was recrystallized twice from hot distilled water before use. All other chemicals were either from B.D.H., U.K. or Aldrich/Sigma Chemical Co., U.K. and were of analytical grade. Hydrogen peroxide solution or $FeCl_3$ (in 0.1 M HCl, 1 M=1 mol dm^{-3}) was mixed with citrate buffer and was degassed for 20 min under a blanket of oxygen-free nitrogen. Similarly, a mixture of thiourea and the spin trap was also degassed before mixing finally with the former solutions separately in a thermostatic bath at 25±0.1 °C.

ESR Spectroscopy: An aliquot of the reaction mixture was taken up and transferred into a flat aqueous cell and ESR spectra were recorded in a Varian V4502 spectrometer with a field modulation frequency of 100 kHz. The ESR spectra recording was started from 5 min after mixing. The microwave power level was maintained at 10 mW or lower to avoid saturation. A typical spectrometer setting was as follows: Modulation amplitude 0.2 mT, signal level 2.0×10^2, sweep time 5 min, response 1 s, and microwave frequency 9.53 MHz (measured by a cavity wavemeter). The magnetic field sweep was calibrated using a purpose-built marginal oscillator to measure the corresponding ^{1}H NMR frequency, read by a digital frequency meter.

Kinetics of Spin Trapping: Appropriate amounts of reaction components were mixed in a thermostatic bath at required temperature under a blanket of pure nitrogen. The

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solution was then drawn through the aqueous ESR cell placed in the ESR cavity by a peristaltic pump at a flow rate of 3 ml per min. The appropriate magnetic field was set to monitor a particular line of the spectrum and the intensity was measured at the required interval of time. The pump was turned off temporarily during recording.

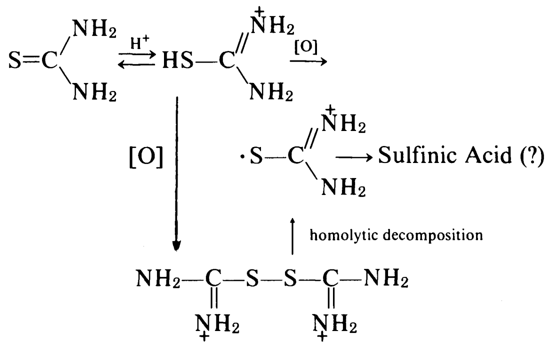
Results and Discussion

Although aminoiminiomethanesulfinate, $\genfrac{}{}{0pt}{}{HN^+}{H_2N}{>}C{-}SO_2^-$, is known to be one of the variety of products formed during oxidation of thiourea in a medium of low acidity, generation of amidinothio radical by H_2O_2 as an intermediate is clearly possible at the moderate pH of ca. 3.0.[9] The removal of reactive hydrogen atom attached to the sulfur of isothiourea, HS–C(=NH)–NH_2, which exists in tautomeric equilibrium with thiourea, could lead to formation of the radical. Because of the low symmetry of molecular structure, the ground state of this radical is probably not purely degenerate. However, even a 'quasi-degenerate' ground state of this thiyl radical leads to considerable spin orbit coupling of the unpaired electron's angular momentum, which in turn results in high *g* anisotropy and, perhaps, very short spin relaxation times. This makes the ESR lines too broad to be detected. On the other hand, since the stability of the radical is an acute function of its concentration and the solution pH, spin trapping of amidinothio radical is tricky. The alternative fate of the radical is rapid formation of 1,1′-dithiobis(formamidine) probably by the following reactions:[7]

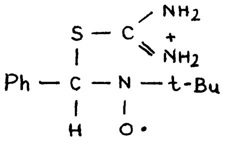
$$2\,NH_2(=NH)CS\cdot \rightarrow NH_2(=NH)CSSC(=NH)NH_2 \quad (1)$$

$$NH_2(=NH)CS\cdot + NH_2(=NH)CSH \rightarrow [NH_2(=NH)CSSC(=NH)NH_2]^- + H^+ \quad (2)$$

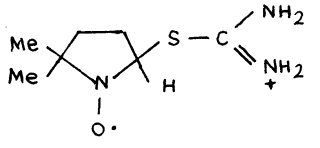
However, possible pathways of amidinothio radicals formation could be identified as follows:



ESR Spectra of PBN and DMPO Spin Adducts: When H_2O_2 (0.6%) was added to the system of thiourea (0.05 M) containing PBN (0.007 M) in citrate buffer (pH 2.8), signals were detected by the ESR spectrometer at room temperature. The characteristic six line signal was observed 5 min after the addition of H_2O_2. The *g* value of the spectrum was 2.0064, a^N=1.53 mT, a_β^H=0.24 mT, (Fig. 1a), which could be assigned to the adduct **A**. A drop in pH, which was observed during



(A)



(B)

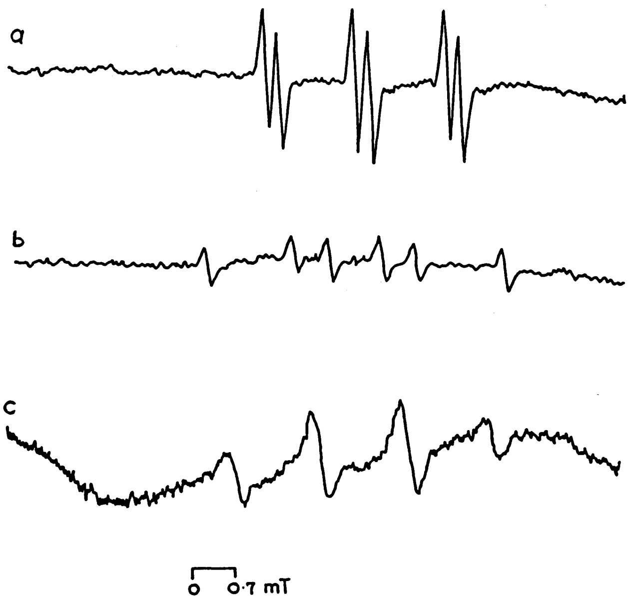
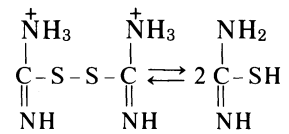


Fig. 1. ESR spectra of amidinothio radical spin adduct in citrate buffer (a,b). Concentration of: thiourea=58 mM, H_2O_2=0.56%, pH 3.0. a, PBN (7.0 mM) spin adduct. b, DMPO (8.0 mM) spin adduct. c, ESR spectrum of an unidentified radical spin adduct formed in PBN (7.0 mM)/$FeCl_3$ (100 mM) system. The broad background line is one of the six ESR lines of Mn (II), present as trace impurity in the sample of $FeCl_3$.

the redox reaction at neutral pH and in absence of any buffer, may be partly due to the formation of disulfides by reaction (2) and, partly due to sulfinate formation. Figure 1b shows the spectral lines arising from the DMPO adduct under similar experimental conditions. The spectrum shows six characteristic lines with a *g* value of 2.006. The corresponding a^N=1.46 mT and a_β^H=2.08 mT, which could be assigned to trapping of NH_2 (=NH) CS· radical forming adduct **B**. As with PBN, DMPO could not trap the radical above pH 3.5, but, even at optimum pH, DMPO adduct gives lower intensity of the ESR lines. This spectrum is completely different from that of the well-known DMPO·OH adduct (a^N=1.49 mT, a_β^H=1.49 mT).[10] This demonstrates that although ·OH could be formed in the reaction conditions, trapping of this radical did not occur under the present experimental conditions. This view is substantiated by observing the effect of thiourea and H_2O_2 concentrations on ESR intensities (discussed in the latter part of the paper). The pH of the experimental medium is, however, unfavorable from the point of view of the stability of spin traps. Nitrones are prone to hydrolysis in aqueous solutions, to form an aldehyde and hydroxylamine, hydrolysis being more rapid in low pH, occassionally forming a nitroxide.[11] Janzen et al., for example, have shown, the addition of water across the double bond of 4-POBN (*N*-(4-pyridylmethylene)-*t*-butylamine-*N,N'*-dioxide), followed by H_2O_2 oxidation, produced 4-POBN·OH.[12] For the sake of checking of the reliability of present observations, each set of experiments was replicated for control, in order to examine the possibility of nitroxide formation either by such hydrolysis or by each of the species present in the reaction mixture in presence of another by any unknown side reaction at the experimental pH. Despite careful and intensive observation by ESR spectrocopy no such reaction was identified. Moreover, the possibility that the observed radicals might be arising from buffer (citrate) derived species is discounted because the same signal was found in control experiment (at pH 3.0) in absence of any buffer but adjusting pH with carefully added dil HCl. Further, the only report of the radical generation in Ti (III)-citrate+H_2O_2 system, which gives a a_β^H value of 2.28 mT. However, this value is considerably higher than that observed in the present study.[13]

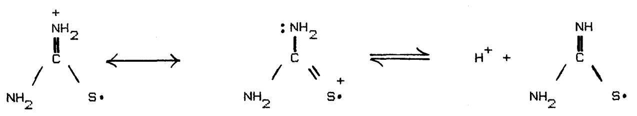
Attempts to trap the radicals by PBN, generated in thiourea/$FeCl_3$ redox couple fails, because $FeCl_3$ (in 0.1 M HCl) itself leads to a weak 1:2:2:1 spectrum in presence of PBN (Fig. 1c). Analysis of the spectrum gives a *g* value of 2.002, a^N=1.42 mT and a_β^H=1.35 mT. Although the radical could not be identified, similar ESR lines from PBN have been reported earlier.[13]

Influence of PH: Preisler and Berger[14] did not observe any dependence of electrode potential on pH for the reaction,



$$\mathrm{HN{=}C(\overset{+}{N}H_3){-}S{-}S{-}C(\overset{+}{N}H_3){=}NH} \rightleftarrows 2\,\mathrm{HN{=}C(NH_2){-}SH}$$

since, according to these authors there is no involvement of H^+ in the above process. They observed a decomposition of disulfide above pH 3.0. The present spin trapping experiment, however, shows a maximum ESR spectral intensity in the pH region of 2.5—3.0, above and below of which the intensity decreases rapidly (Fig. 2). At low pH the tautomeric equilibrium of thiourea in aqueous solution is shifted towards isothiourea form facilitating the formation of the radical. Moreover, the protonated form of the amidinothio radical at low pH is surely more stable than the radical itself and consequently the dimerization process is less favorable.[15]



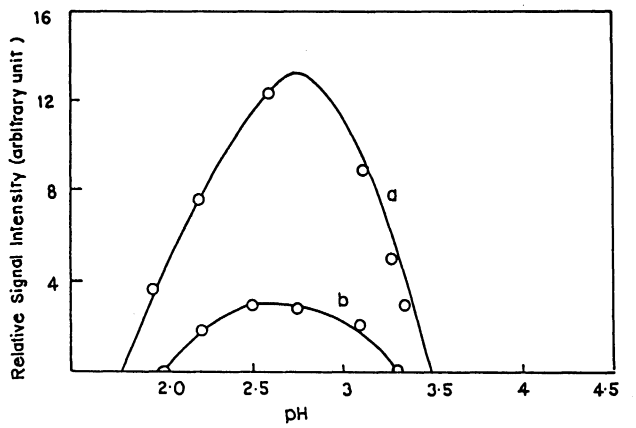


Fig. 2. ESR signal intensity of amidinothio radical spin adduct in citrate buffer, as a function of pH. Concentration of: thiourea=58 mM, H_2O_2=0.56%. a, PBN (7.0 mM) spin adduct. b, DMPO (8.0 mM) spin adduct.

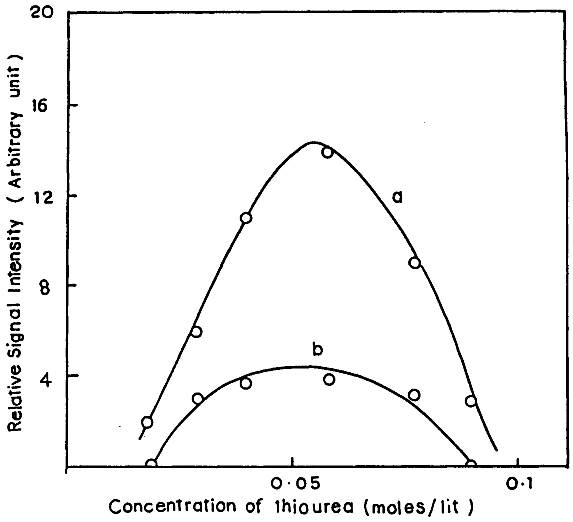


Fig. 3. ESR signal intensity of amidinothio radical spin adduct in citrate buffer, as a function of thiourea concentrations. Concentration of H_2O_2=0.56%, pH 3.1. a, PBN (7.0 mM) spin adduct. b, DMPO (8.0 mM) spin adduct.

At the pH level below 2.0, the spin traps themselves are probably no longer stable.

Influence of Thiourea, H_2O_2 and PBN Concentrations: ESR signals from both PBN and DMPO adducts could be detected only within a short range of thiourea concentrations (0.025—0.1 M) with the maximum intensities at 0.05 M (Fig. 3a, b). At thiourea concentrations lower than 0.025 M, spin concentration of the generated radicals is too low to be trapped. On the other hand, above 0.1 M, the rate of the dimerization process is so high that spin trapping process is unfavorable. This situation makes the detection of the radical difficult unless proper concentration conditions are maintained, but nevertheless, the observation indi-

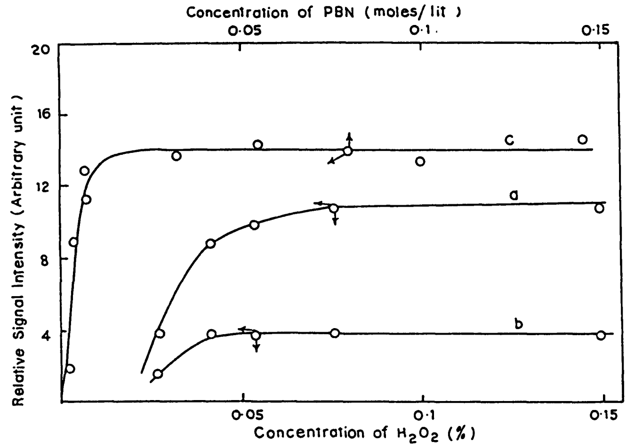


Fig. 4. ESR signal intensity of amidinothio radical spin adduct in citrate buffer, as a function of concentrations of H_2O_2 (a,b) and PBN (c). a, PBN (7.0 mM) spin adduct b, DMPO (8.0 mM) spin adduct, Concentration of thiourea=58 mM. c, PBN spin adduct, Concentration of: thiourea=58 mM, H_2O_2=0.56%.

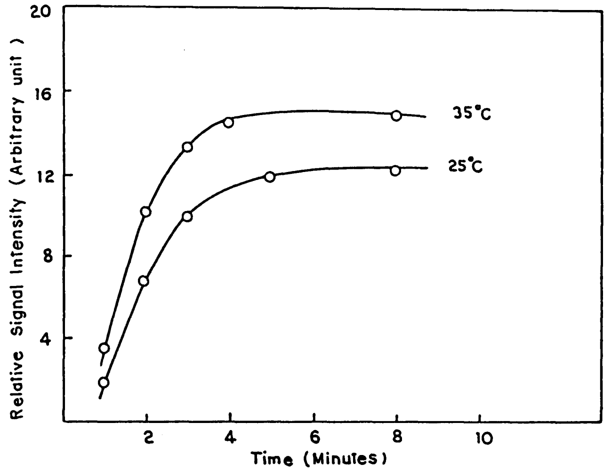


Fig. 5. Kinetics of formation of PBN-amidinothio radical spin adduct in citrate buffer. Concentration of: thiourea=58 mM, H_2O_2=0.56%, PBN=7.0 mM, pH 3.1.

rectly substantiates the proposition that not ·OH, but the amidinothio radical is trapped by PBN. On the other hand, with increase in H_2O_2 concentrations, signal intensities gradually increase attaining a plateau on the intensity vs. concentration curve (Fig. 4). Dependence of ESR intensities on PBN concentration is shown in Fig. 4c. Ten fold increase in PBN concentration (0.01 to 0.1 M) did not result in any appreciable increase in the intensity. This shows that the steady state concentration of the radical is low. Moreover, the release of enthalpy on the formation of the C–R bond (R being the radical) in the spin adduct is probably not high. The weak C–R bond might be due to severe steric strain for a bulky addition. Comparatively less spin concentrations of DMPO spin adduct in the experimental pH range restricts further experiment with DMPO.

Kinetics of Spin Trapping: A preliminary study on the kinetics of spin trapping by PBN of the amidinothio radical generated by the reaction of thiourea and H_2O_2 in aqueous solution at 25 °C and 35 °C shows that the adducts are formed after a short time lag, and reach a maximum concentration within 5 min time (Fig. 5). Steady state concentration of the adduct as observed from the plateau of the intensity vs. time curves, is a function of temperature. Unlike the DMPO spin adduct, which is stable for several hours, PBN adducts start decaying after 15 min, and ESR signals vanish completely within 30 min time.

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