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Invited Review
A Water Soluble C-nitroso-aromatic Spin-trap – 3,5-Dibromo-4-nitrosobenzenesulphonic Acid. 'The Perkins Spin-Trap'

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INTRODUCTION

It is now widely accepted that Reactive Oxygen (ROS) and Reactive Nitrogen (RNS) Species (including free radicals) are involved in the pathogenesis of several disease states (reviewed in Ref. 1). The only technique which can detect low concentrations of radicals directly is Electron Spin Resonance spectroscopy (ESR). Although the technique is highly sensitive (thresholds of 10^{-7}–10^{-6}M spins), it is not directly applicable to the study of biological oxidations or indeed to the majority of radical chemistry. A more successful technique, permitting ESR investigation of short lived reactive free radicals by transforming them into more persistent species, is the so-called 'spin trapping' method.

SPIN TRAPPING

In this approach, a radical scavenger molecule (better known as a 'spin trap') is used to detect the radicals by trapping them to give radicals of greater stability, whose concentrations will rise to readily detectable levels (Ca. 10^{-6}–10^{-7}M or greater). The general reaction is represented by scheme [1].

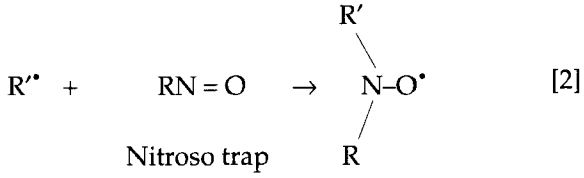
The spin adducts are then detected by conventional ESR methods and in many cases the identity of the short-lived radical can be ascertained from its ESR spectrum. The method was termed 'spin trapping' by Janzen and Blackburn[2] to avoid confusion with the already established technique of spin labelling.[3] However, the origins of this novel procedure were first reported by de Boer *et al.*[4] as discussed later in this review.

$R^{\bullet}$	+	ST	→	(St-$R^{\bullet}$)	[1]
Reactive Radical		Spin trap		'spin adduct'	

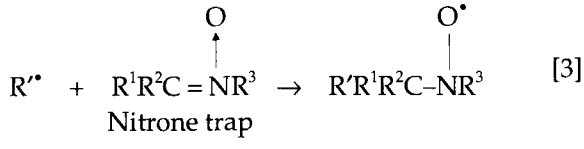
The spin-trapping technique gained great importance in the late 1960's as a result of reports by several independent groups, particularly Perkins *et al.*,[5,6] Janzen and Blackburn[2] and Lagercrantz and Forshult.[7]

THE MOST COMMONLY USED SPIN TRAPS

The most common spin traps in general use are nitrones and C-nitroso-compounds, schemes [2] and [3]. In both cases trapping of a radical by these traps leads to a more stable nitroxide ($R_2NO\bullet$);

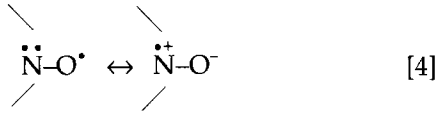


[2]



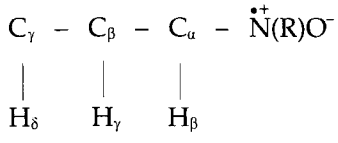
[3]

These nitroxides are of sufficient stability and persistence at concentrations high enough to afford well defined ESR spectra. The nitroxide function can be represented as a hybrid of the two resonance structures (a) and (b), scheme [4]



[4]

The unusually high stability of nitroxides is associated with this delocalisation of the electron. The balance between the non-polar (a) and dipolar (b) extremes is perturbed by the polarity of the immediate surroundings. The measured ESR parameters may change by as much as 20% in studying a nitroxide in an organic solvent as well as in water. The ESR spectra of the nitroxides invariably exhibit a main triplet (1:1:1) splitting, a_N, due to the interaction of the unpaired electron with the ^{14}N nucleus of the nitroxide group. Additionally, secondary splittings $a_{another}$, may arise from magnetic nuclei in the trapped radical and in some instances from other magnetic nuclei in the spin trap. The α, β, γ and δ positions of the nuclei in the nitroxide are defined with respect to the unpaired electron of the nitrogen atom (cf [4]), scheme [5]



[5]

The magnitude of the nitrogen splitting, a_N, measured in units of magnetic flux (gauss or millitesla; 10 G = 1mT) is found in the range of 4 to 30 G, but is commonly closer to 15 G. The precise value of a_N will depend critically upon the nature of substituents on nitrogen and upon the medium in which the nitroxide is dissolved. Hence, the ideal spin trap would be one in which the hyperfine coupling in the nitroxide would be due purely to the nuclear spins of the radical trapped. The splitting pattern, the value of the nitrogen and other nuclear hyperfine splitting constants, the *g*-value* and the line widths of individual lines are all important factors which contribute to an assignment of structure of the nitroxide. Although *g*- values can provide useful information on the distribution of spin in a free radical, little emphasis has been placed on their utilisation in discussions of spin adduct spectra.

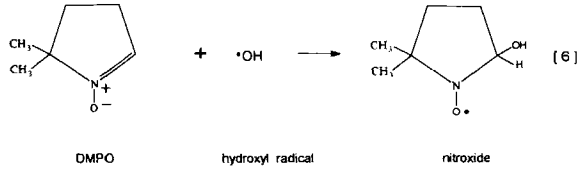
The utility of a spin trap depends on the properties of the trapping agent itself on the one hand, and those of the spin adduct derived from it on the other. The spin trap should be fairly soluble and inert to chemical and photochemical reactions, other than the radical trapping reaction (scheme [I]) under study. It is also desirable that the spin-trapping reaction, should be very rapid,

*The *g*-value determines the position of the centre of the spectrum on the display.

so that low concentrations of trap are sufficient i.e. the spin trap should have a particularly high affinity for radicals.

The pre-eminent advantage of C-nitroso-compounds as spin traps over nitrones is that in the spin adduct the scavenged radical is directly attached to the nitroxide nitrogen, scheme [2]. Consequently, the ESR spectrum of the nitroxide will often reveal splittings from magnetic nuclei in the trapped radical, and these will greatly facilitate its identification.

In contrast, a major drawback with the nitrone traps is that the information regarding the nature and structure of the trapped radical is not immediately available from the spectrum of the derived nitroxide. This occurs because the radical trapped is quite far from the nitroxide centre and hence often contributes little or nothing to the ESR splitting pattern. For example, the ESR spectrum of the nitroxide from the most widely used nitrone trap C-phenyl N-*t*-butyl nitrone (PBN), always exists as a triplet of doublets due to the nitrogen and β-hydrogen coupling. Thus identification of nitrone spin adducts requires a great deal of expertise, especially when a mixture of adducts is formed. This can only be achieved after comparison of the splittings achieved with a reference nitroxide recorded under strictly comparable conditions. Ambiguities may still remain, however, and a better method involves the use of isotopically labelled compounds, where additional splittings may assist with structure elucidation. Despite these difficulties, nitrones have one distinct advantage over nitroso-compounds in biological studies, in that only the former yield reasonably persistent spin adducts with oxygen-centred radicals. They have therefore been used quite extensively to investigate the participation of oxidising radicals in biological systems.



Specifically, the much utilised nitrone trap 5,5-dimethylpyrroline N-oxide (DMPO), has been employed to trap the hydroxyl radical, scheme [6].

A point worthy of mention here is that the spin trapping method will reveal the free radicals which react with the spin trap with a sufficiently large rate constant to produce a nitroxide whose life-time is then suitable for detection. The absence of an expected nitroxide does not by itself prove that the expected radical has not been produced in the system.

When designing a spin-trapping experiment or interpreting the results of an investigation using this method the following questions were set as a guide by Perkins[6] to assist in formulating conclusions.

a) Can the chosen spin-trap participate in reactions other than with reactive radicals generated in the experiment? Can these alternative reactions yield nitroxides which will hamper spectrum interpretation?
b) How readily can the identity of the trapped radical be discerned from the spectrum achieved, keeping a) in mind.
c) How fast is the trapping reaction and how persistent are the spin adducts formed?
d) Does the detection of a spin adduct signify a major reaction pathway, or could it be a minor side reaction? Here one may also consider the possible metabolism of spin adducts *in vivo* e.g. reduction of spin adduct to ESR-silent species by ascorbate or enzymes?

To these questions Mottley and Mason[8] have added another:

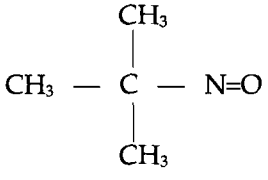
e) Has evidence of structure been achieved using isotopically labelled (^{13}C, ^{2}H, ^{17}O, ^{15}N etc.) compounds? If not then an independent synthesis of the spin adduct should be undertaken.

Several reviews have addressed the application of spin-trapping to chemical[6,9,10] and biological[11–15] systems, the effects of ionising[16,17] and UV irradiation[18] in molecules of biologicalsignificance and also in the sonochemistry of aqueous

solution reactions illustrated by ultrasound.[19] There are also two spin-trapping data bases, one by Li *et al.*[20] and the other by Du Bose *et al.*[21] which includes mass spectral data to help take full advantage of this very useful technique.

NITROSO SPIN TRAPS

The first reported use of C-nitroso compounds as radical scavengers was by de Boer *et al.*[4] where they confirmed the trapping of alkyl radicals to form nitroxides. They pioneered the use of now the most widely used aliphatic C-nitroso spin trap which is 2-methyl-2-nitrosopropane (*t*-nitrosobutane; MNP) and also emphasised the importance of ESR spectroscopy.



2-methyl-2-nitrosopropane (MNP)

Experimental evidence for the exclusive involvement of monomeric C-nitroso-compounds in the reactions leading to a nitroxide was also presented by them. However, they did not remark upon the possibility that radical scavenging by C-nitroso-compounds might be of more general utility.

The merits of C-nitroso-compounds as spin traps over nitrones have been mentioned earlier in this review. The major drawbacks of these compounds are that they are thermally and photochemically labile and that spin adducts with oxygen-centred radicals are rather unstable. Their solubility is also reduced due to their tendency to dimerise. In solution at room temperature only a fraction of the compound (monomer) may be available for trapping the radicals i.e. approximately 90% for MNP in organic solvents.

Nitroxides in general may disproportionate as the radical is bonded to the nitroxide function: Monosubstituted nitroxides, $RN(H)O^{\bullet}$, disproportionate rapidly to nitroso-compound and hydroxylamine, scheme [7]

$$2RNHO^{\bullet} \rightarrow RN=O + RNHOH \qquad [7]$$

Nitroxides may also be reduced to diamagnetic products in the presence of hydrazines, hydroxylamines, thiols and ascorbic acid. The presence of any of these may result in rapid loss of the nitroxide signals. This is a very serious problem in biological studies as we shall see later.

The most widely used nitroso spin trap, MNP, exemplifies drawbacks of C-nitroso compounds in that it decomposes under the influence of red light and on thermal warming (Ca. 50°C in dark) to give di-*t*-butyl nitroxide (DTBN), scheme [8]

$$Bu^tN=O \xrightarrow{h\gamma} NO^{\bullet} + Bu^{t\bullet} \xrightarrow{MNP} \underset{DTBN}{Bu^t{}_2NO^{\bullet}} \qquad [8]$$

The ESR spectrum of DTBN is a broad triplet (due to the unresolved splitting by the *t*-butyl protons) which often overlaps the radical adducts of interest. This shortcoming can be lessened by using fully deuterated nitrosobutane[22] (MNP-d_9), when the spectra of the spin adducts are sharper and better resolved. Caution must be exercised when reporting an ESR spectrum resulting from the use of MNP; a simple 3 line spectrum with a_N Ca. 15G will most probably be due to unwanted DTBN. Makino *et al.*[23] have found that formation of DTBN cannot be suppressed even if the experiment is carried out at low temperature. In an effort to overcome this the best compromise has been achieved by preparing the aqueous solutions of MNP by stirring in the dark for less than 2 hrs. at 45°C. Another trick for reducing the signal from DTBN is to adjust the pH of the reaction system to around 4. This has been reported to decay the DTBN signal before signals from other adduct species.[24]

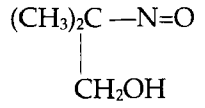
BIOLOGICAL STUDIES USING MNP WHICH LED TO THE DESIGN AND SYNTHESIS OF DBNBS

Ingall *et al.*[25] examined the metabolic activation by liver microsomes of carbon tetrachloride (CCl_4) using MNP and assigned the spectrum achieved to the trapping of a secondary peroxyl radical ($CCl_3O_2^{\bullet}$) or a secondary lipid peroxyl radical rather than the $^{\bullet}CCl_3$ radical. However, this was shown to be incorrect by Kalyanaraman *et al.*[25] who repeated the investigation, and, in conjunction with further experiments, were able to assign the spectrum to a lipid diethyl radical adduct and a second species; this species was identified as *t*-butyl hydronitroxide ($Bu^tNHO^{\bullet}$) resulting from the reduction of MNP by liver microsomes.

Lai and Piette[27] were first to attempt trapping of the •OH produced in the Fenton (Fe^{2+}/H_2O_2) reaction using MNP as the spin trap. They reported the production of *t*-butyl hydroxy nitroxide, but Kalyanaraman *et al.*[28] showed that the splitting parameters corresponded exactly with those of $Bu^tNHO^{\bullet}$. Therefore, MNP probably cannot trap •OH in a biological system and the only reported success in trapping of •OH by MNP has been in radiolysis experiments.[29]

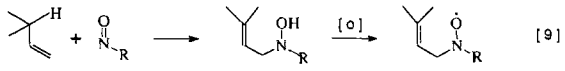
Fruitful use of MNP has been accomplished when the experiment is carefully designed to minimise the formation of DTBN e.g. in liposomes where the radical versus nonradical mechanisms of spin adduct formation were distinguished using radical scavengers.[30]

Modifications of MNP have been synthesised with a view to applications in aqueous systems. De Groot *et al.*,[31] in a pioneering investigation of an enzyme-catalysed reaction system, examined catalysis of the oxidation of linoleic acid by soybean lipoxygenase using the little used trap 2-methyl-2-nitrosopropanol ('hydroxy-MNP'; this trap has the hydroxyl group incorporated to enhance water solubility but shares the disadvantages of MNP). It was demonstrated, by deuterium labelling experiments, that the spin adduct is derived from a C-9 radical and the 9-hydroperoxide is



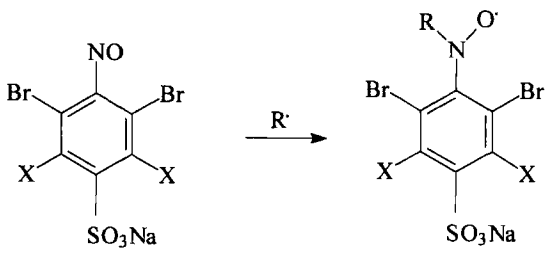
2-methyl-2-nitrosopropanol

formed in the absence of the trap. However, in their report De Groot *et al.* failed to consider the ene reaction which aliphatic nitroso compounds undergo with olefins resulting in allylic hydroxylamines.[32] These are readily oxidised to nitroxides, scheme [9].



[9]

Spin trapping of the superoxide radical anion, hydroperoxyl and hydroxyl radicals have been the main goals attempted utilising this technique in biological studies. In this context, nitrone traps have found greater application, since, as already pointed out, they form relatively stable adducts with oxygen-centred radicals, although attempts to use *C*-nitroso traps to achieve conclusive results are continuing. In particular, trapping of •OH in biological studies by C-nitroso-compounds would have been the most important application of the spin-trapping technique. It became quite apparent in the late 70's that these traps were undergoing reduction in the biological environment. Although, the nitrone traps are successful at trapping •OH, in many applications the concentration of trap required to achieve useful results can be fairly toxic; e.g. PBN (10 mM) caused 50% inhibition of the rate-pressure product (an index of cardiac function) as observed in a controlled Langedorff-type heart system.[33] Here, an alternative method using naturally occurring traps and utilising high pressure liquid chromatography (HPLC) to detect the hydroxyl-radical adducts formed is 'aromatic hydroxylation'.[34]



A ; X = H
B ; X = D

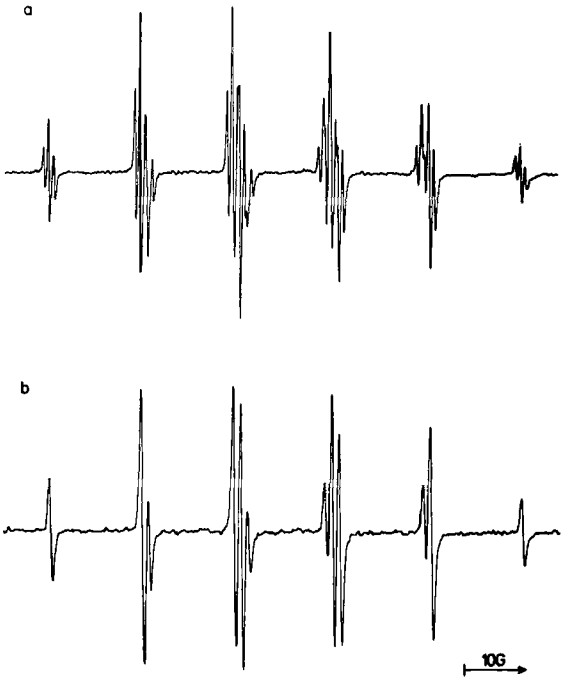


FIGURE 1 E.S.R. spectra of methyl spin adducts (a) of sodium salt of 3,5-dibromo-4-nitrosobenzenesulphonic acid [A], and (b) of sodium 3,5-dibromo-4-nitrosobenzenesulphonate-d_2 [B], obtained as described in text. The hyperfine splitting parameters obtained with [B] were $a_N = 14.5$ G and $a_H = 13.5$ (3H) G. Adapted from H. Kaur, PhD Thesis[36].

Sodium Salt of 3,5-Dibromo-4-Nitrosobenzenesulphonic Acid (DBNBS)

Hindered nitrosobenzenes, investigated as spin traps by Konaka *et al.*,[45] have proven to be especially useful in chemical studies. It seemed of interest to develop a water-soluble analogue of these compounds, which might overcome many of the problems associated with lipophilic nitroso compounds, especially the aliphatic ones such as MNP. Hence, DBNBS (A) and its dideutero-derivative (B) were successfully synthesised.[36†] Although DBNBS dimerises, there is sufficient monomer, even at low concentration of the trap, to give spin adducts. It does not give nitroxides on exposure to visible light or on variations in temperature, though DBNBS-oxygen-centered radical adducts are unstable.[36] Saturated solutions of DBNBS are pale green and a more pronounced blue-green colour develops only on warming (the compounds have a solubility of *Ca.* 100 g L^{-1} in water at 20°C, though remaining substantially dimeric). The spin traps do not yield observable spin-adducts with hydroxyl radicals, but the inclusion of dimethyl sulphoxide (DMSO) in the aqueous H_2O_2 system (H_2O_2/H_2O/DMSO/hγ at 20°C) gives intense spectra of the adducts of methyl radicals which arise via hydroxyl radical attack on DMSO; this provides an indirect indication of hydroxyl participation.[37] The ESR spectra of methyl adducts of DBNBS and its dideutero derivative are illustrated in Figure 1. The two spectra illustrate extremely well the spectral simplification upon using the deuterated compound (B). This is particularly advantageous in studies of complex systems where the absence of splittings from protons of the trap greatly facilitate spectral assignment.

†A solution of 3,5-dibromosulphanilic acid (10 mmol) in a mixture of glacial acetic acid (30 ml), 30% aqueous hydrogen peroxide solution (70 mmol), and anhydrous sodium acetate (10 mmol) was warmed gently to bring the solids into solution. The solution was allowed to stand at room temperature for 14 days, when the straw-coloured blades which had formed were separated and washed with acetic acid (5 ml) and three times with dried ether (50 ml). Acetic acid was removed by crystallisation from ethanol to give analytically pure sodium 3,5-dibromo-4-nitrosobenzene-sulphonate as a pale yellow powder, m.p. >300°C. The yield was 34%.

APPLICATIONS

DBNBS was designed with a view to overcoming some of the drawbacks of MNP, with the added advantage that this will be soluble in water and more amenable to use in biological systems. Its expected hydrophilic character might give information regarding the location of free radical formation, i.e. intracellular radicals might differ from extracellular ones.

Although this trap is water-soluble, stable, and apparently insensitive to light, Samuni *et al.*[38] demonstrated that within several minutes of incubation in a cellular system only a small amount of DBNBS is able to penetrate the cell membrane. They also reported an as yet unassigned triplet characterised by $a_N = 12.4$ G with no further hyperfine coupling, arising from leaving the trap in solution at room temperature for a few days.

SUPEROXIDE ($O_2^{\bullet -}$)

The general conclusion reached by us and other researchers is that DBNBS does not produce stable adducts with $^{\bullet}OH$. However, a great deal of interest was stirred when a very persistent adduct was attributed to trapping of the superoxide radical, $O_2^{\bullet -}$. In this first report by Ozawa and Hanaki[39] the $O_2^{\bullet -}$ was generated from xanthine-xanthine oxidase in aqueous solution or in alkaline DMSO. Stolze and Mason[40] questioned the utility of this reagent especially under the latter condition, and reported the formation of a number of radicals in this system. The radical which forms the adduct with DBNBS in alkaline DMSO has been identified as sulphur trioxide anion radical ($\bullet SO_3^-$) rather than $O_2^{\bullet -}$. This extremely stable sulphur trioxide adduct was also observed in a model system where cyanide radicals were expected to be formed[40,41]. The radical species produced from DMSO at high pH are methyl, superoxide and $\bullet SO_3^-$, hence extreme caution is needed in spectral identification when this common organic solvent is used.

Mani and Crouch[42] repeated the original study[39] and found that addition of superoxide dismutase or catalase did not attenuate the ESR signal but actually increased it. These authors did attempt to identify the adduct, but concluded that it was not the superoxide anion. They also pointed out the need for caution in spectral interpretation in the absence of relevant controls.

Samuni *et al.*[38] independently reached the conclusion that xanthine-xanthine oxidase system did not result in an observable ESR signal with DBNBS but the $O_2^{\bullet -}$ adduct was detected with the nitrone trap, DMPO. This lack of signal with DBNBS was attributed to the absence of DMSO in their experiment.

The final evidence to settle this controversy was obtained in a pulse radiolysis experiment showing that DBNBS reacts rapidly with $O_2^{\bullet -}$.[43] The DBNBS-$O_2^{\bullet -}$ adduct is reported to be unstable and not detected by ESR spectroscopy. Furthermore, Nazhat *et al.*[43] in this study showed that the adduct observed by some researchers, but not others, when DBNBS is exposed to the $O_2^{\bullet -}$ generating xanthine/xanthine oxidase system, is produced by a peroxidatic oxidation using hydrogen peroxide formed by the dismutation of $O_2^{\bullet -}$. The formation of this radical depends on the presence of peroxidase activity in the batch of xanthine oxidase used. The spectrum of this artefact appears as a triplet of triplets with splitting parameters $a_N = 12.8$ G and $a_H = 0.7$ G (2H); the latter is the ill-resolved hyperfine splitting from the two *meta*-hydrogens.

This supposed trapping of $O_2^{\bullet -}$ by DBNBS is a prime example of how an unexpected secondary reaction gives rise to an adduct which attracted the efforts of many experts for four years.

NITRIC OXIDE (NO•)

DBNBS has also been applied in another field of major interest, namely the attempted trapping of nitric oxide (NO•) – the endothelial derived relaxing factor.[44] A nitrone trap (DMPO) and the

nitroso spin traps (DBNBS and MNP) were tested for their ability to trap gaseous $NO^{\bullet}$.[45]. The resulting spectra indicate that the nitrone traps are hydrolysed while the nitroso traps, in particular DBNBS, gave spectra perhaps compatible with the trapping of a nitrogen centered radical. However, in experiments carried out with human platelets[46] and in mouse neuroblastoma cells[47] when $^{15}NO^{\bullet}$ was substituted for $^{14}NO^{\bullet}$, the spectra did not produce the expected change in nitrogen splitting. Four spin adducts of DBNBS were reported[47] which were later assigned as NO-related, S-centred and two C-centred radicals derived from human platelets.[48] Ichimori *et al.*[48] further explained that, the adduct assigned as NO-related is not DBNBS-$NO^{\bullet}$, but a dimerised secondary product, since the monomeric product is unstable. Hence, the unambiguous trapping of $NO^{\bullet}$ or NO-derived radicals in a biological system remains to be demonstrated. Once again extreme caution must be exercised in reporting any spectrum purporting to result from the trapping of $NO^{\bullet}$ directly.

Recently Rosen *et al.*[49] attempted trapping $NO^{\bullet}$ generated directly from L-arganine by the enzyme nitric oxide synthase (NOS) with various nitrones and DBNBS dissolved in buffer. The e.s.r. spectra obtained were similar to those reported by them earlier.[44] Further investigations by them using $^{15}NO^{\bullet}$ and hydroxylamine (a by product of L-arganine metabolism by NOS and known to generate $O_2^{\bullet-}$ at physiological pH) indicated the spectra to be artefactual. Hence they[49] concluded that currently available spin traps are not capable of trapping $NO^{\bullet}$ generated from NOS.

LOW DENSITY LIPOPROTEIN – LDL

DBNBS has continued to be used to study carbon-centred, sulphur-centred and oxygen-centred radicals. Studies by Kalyanaraman *et al.*[50] have shown that the reaction of DBNBS with low density lipoprotein (LDL) gives spin adducts associated with both lipid and protein radicals. The mechanism proposed is that DBNBS forms a covalent bond with apoprotein B_{100} and lipids of LDL by a lysine-independent process resulting in increased recognition and degradation by macrophages. Furthermore, DBNBS itself was found to cause rapid modifications of LDL without inducing peroxidation. A sulphonic acid analogue of PBN failed to modify LDL in a similar manner, suggesting that the presence of sulphonic acid alone does not ensure modification. The formation of DBNBS-LDL-lipid adduct has been attributed to the facile 'ene' reaction, scheme [9], between the nitroso group in DBNBS and the lipid.[51]

These results have been substantiated though not in the same detail by Davies and Rice-Evans.[52]

REPERFUSION INJURY – HEART

DBNBS is not particularly toxic to cells in culture,[38] although at the concentration [10 mM] used for ESR studies in isolated perfused rat heart, where the trap is solubilised in bicarbonate buffer, toxicity is a problem.[33]

PROCARBAZINE

The anti-cancer drug procarbazine is a hydrazine derivative used in chemotherapy. The mode of its action was postulated to produce methyl radicals, thus DBNBS has been utilised to trap the radicals. The methyl radicals have been identified during oxidation of procarbazine in rat liver microsomes and isolated hepatocytes *in vitro*, as well as in several organs following administration of this drug *in vivo*.[53]

GILVOCARCIN V

Gilvocarcin V (GV) is a naturally occurring antibiotic reported to produce high phototoxicity even at low doses in biological systems when compared to other phototoxic compounds i.e.

trioxsalen and 8-methoxypsoralen. In the presence of UV or visible radiation, GV becomes a DNA damaging agent in both bacteria and mammalian cells.[55] Photolysis of GV in argon saturated DMSO or 50% DMSO-water solutions in the presence of DBNBS-d_2 (B) generated the CH_3-DBNBS-d_2• spin adduct (b).[56] GV also produces singlet oxygen, $^1O_2$, hence it was concluded that GV photochemistry proceeds by both Type I (involves production of $O_2^{\bullet-}$ which disproportionates to H_2O_2 giving $^{\bullet}OH$ in the presence of trace metals) and Type II (generation of $^1O_2$) pathways which could explain its reported phototoxicity.[56]

ULTRASOUND

There is widespread use of diagnostic and therapeutic ultrasound in medicine (medical diagnosis and hyperthermic cancer therapy). There may be free radicals formed, hence this possibility no matter how small needs careful investigation and the risks involved documented. Riesz[19] has employed DBNBS most extensively to detect and identify the free radicals formed by continuous wave and pulsed ultrasound in aqueous solutions. In this application DBNBS has been proclaimed the spin trap of choice for aqueous sonolysis studies. The sulphonate group ensures non-volatility and several carbon-centred radical adducts have been identified.

BIOLOGICAL MACROMOLECULES

As presented in this review, spin trapping has been employed extensively and at times successfully to detect and identify low-molecular-mass reactive radicals in biological systems. In contrast, it is now being used to investigate high-molecular-mass species generated as a result of radical-induced damage to bio-logical macro-molecules, such as DNA, RNA, proteins, phospholipids and carbohydrates.[56] These macromolecules are either destroyed, altered or both in a large number of cellular injuries and diseases.

PROTEINS

Damage to proteins by free radicals causes alterations in primary, secondary and tertiary structure as a result of changes in amino acid composition, fragmentation and cross-linking. Davies *et al.*[57,58] have used DBNBS to trap the radicals formed in a relatively large protein such as bovine serum albumin (BSA), and a smaller protein, histone 2A, when attacked by $^{\bullet}OH$ (generated by simple Fenton reaction – Fe^{2+}/H_2O_2). The larger protein adduct is a highly immobilised species whereas the smaller protein adduct yields spectra which reflect the presence of both highly immobilised and relatively mobile (i.e. sharp-lined signals) species.[57,58] The results have been explained[57] in terms of random attack of the initiating radical on the surface of the protein and the trapping of the radicals formed. The difference in the proteins is due to the presence of both a globular core and (relatively mobile) N- and C-termini domains in the histone protein.

A similar process has been used to study the effect of denaturing agents on the motion of protein radicals produced on the exposure of photosensitisers (such as hematoporphyrin) bound to BSA, to visible light in the presence of the spin trap DBNBS.[59]

Use of DBNBS and the nitrone, DMPO, has allowed primary, secondary, and tertiary carbon-centred species to be detected and distinguished with a number of proteins and high-molecular-mass amino acid homopolymers.[57,58] Additionally the DBNBS-protein adduct is more stable than the DMPO-protein adduct, this may be due to a decreased rate of radical-radical termination and/or disproportionation because of the increased steric bulk of the adducts.

Kikugawa *et al.*[60] have recently reported the appearance of ESR signals in reactions of DBNBS with non-radical biological components such as amino acids, peptides and unsaturated fatty acids. DBNBS was also found to cause strand breaks in supercoiled DNA but no signals were seen with DNA, nucleosides and nucleobases. Hence, these

investigators emphasised caution when using DBNBS to detect free radicals in biological systems containing amino acids or other biological components after long incubation which may give misleading results.

CARBOHYDRATES

Carbohydrates undergo fragmentation, cross-linking and alteration of structure when attacked by $^{\bullet}OH$. High-molecular-mass polymers such as polygalacturonic acid, chitin, chondroitin sulphate A and hyaluronic acid have been studied by Davies *et al.*[57] to document the pattern of spectra obtained resulting from fragmentation.

Horseradish peroxidase has been found to catalyse the oxidation of the deoxyribose sugars, 2-deoxyribose and 2-deoxyribose-5-phosphate resulting in carbon-centered radicals which were trapped with DBNBS.[61] In the absence of the sugar a three line spectrum obtained was reported to result from a one electron oxidation of the spin trap i.e. giving the $DBNBS^{+\bullet}$ radical cation.

THE DBNBS RADICAL CATION

DBNBS may be used to detect oxidising species capable of forming its radical cation $DBNBS^{+\bullet}$ and Blake *et al.*[62] have utilised this to demonstrate the generation of ROS within the inflamed human synovium following an *ex vivo* hypoxia-reoxygenation cycle. They observed that the microvascular endothelial based enzyme xanthine oxidase is the predominant source of ESR detectable oxidising species in inflamed synovial specimens exposed to hypoxia-reoxygenation.

Blake *et al.*[63] have also detected unidentified oxidants in plasma of subjects with severe renal disease capable of oxidising DBNBS. They extracted an oxidant from the plasma with an upper molecular weight limit of about 3,000 Daltons and it has been reported to be stable for months. Furthermore, physiological plasma concentrations of Vitamin C, Vitamin E or glutathione were not found to inhibit the oxidising capacity of uremic plasma.

CONCLUSION

DBNBS is a trap tailored for use in aqueous or hydrophilic environments. In particular, it is valuable where carbon-centred radicals are produced, though it has serious shortcomings in other circumstances.

New spin traps continue to be reported, but Perkins' goal[6] of an ideal spin trap 'the Philosophers stone among diamagnetic scavengers' – seems unattainable.

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