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Chapter 1

History of the Use of Nitroxides (Aminoxyl Radicals) in Biochemistry: Past, Present and Future of Spin Label and Probe Method

Lawrence J. Berliner

Additional information is available at the end of the chapter

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1. Introduction

The perspective of this chapter is very much historical. The author was fortunate enough to have begun his graduate studies at the very inception of the technique of spin labeling. Mind you, the topic of this book is nitroxides a.k.a. aminoxyl radicals which in fact preceded the spin labeling method and its inception. Hence the chapter will cover a history of the synthetic developments with nitroxides, the history of the development of spin labels, and the use of nitroxides and will provide an overview to the future of its applications. The intent is to cover the very beginning and then discuss some of the key areas (always dominated by synthetic organic chemistry) that allowed this technique to blossom more and more. Needless to say, while the definition of spin labeling is the incorporation of a stable, free radical into a macromolecular system of choice, we have yet to find anything (aside from the trityl radicals) that will fulfill this purpose. And the trityl radicals, which may be covered briefly in this chapter, give no dynamic or structural information whatsoever as they yield a single narrow line spectrum. The author feels confident to discuss these areas since he was deeply involved in the synthetic organic chemistry of these compounds, frequently repeating the syntheses of the basic starting compounds that were either commercially unavailable or quite expensive at the time. This includes the synthesis of phorone/triacetonamine from ammonia, acetone and calcium, in a 1898 synthesis[1], which was the basis for the nitroxide TEMPONE(2,2,6,6-tetramethylpiperidinone 1-oxyl).

Perhaps one of the earliest papers describing 'nitroxides' was from the American Cyanamid Company laboratories about the reaction of t-nitrobutane with metallic sodium[2]. They found a g value of 2.0065 and a single line linewidth of 8.5G (probably because they observed the compound in neat form where exchange and dipolar broadening were predominant). A follow-up publication produced a plethora of compounds derived from

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phenyl derivatives. They were able to measure a hyperfine coupling constant for DTBN of 15.25G [3]. We should recall that the earliest example of these radicals was the famous Fremy's salt, used to calibrate EPR machines to this day. This long-lived free radical, shown below, was discovered in 1845 by Edmond Frémy [4].

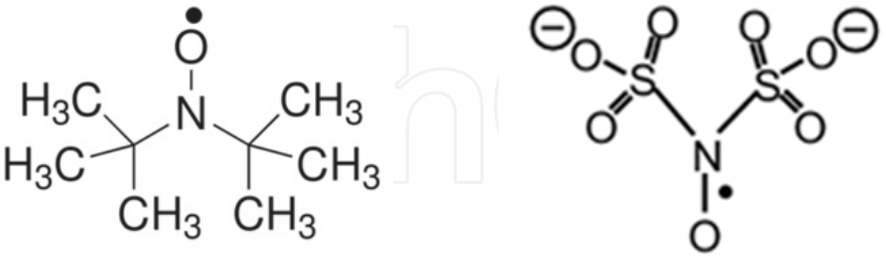


Figure 1. (left) di-t- butylnitroxide[2];(right) Fremy's salt [1]

In the 1960s, stable paramagnetic compounds were developed extensively in the USSR Academy of Sciences, Institute of Chemical Physics, that contained aminoxyl (or iminoxyl or nitroxyl or nitroxide) 'reporter' groups. Until these compounds became commercially available, one was obligated to prepare them homemade, but their syntheses were fairly straightforward (starting with the either phorone or triacetoneamine) [1]. The Russian group was led by organic chemists M.B. Neiman and E.G. Rozantsev and the group expanded these syntheses into a broad range of compounds, some of which could be applied as protein modification reagents [5-6].

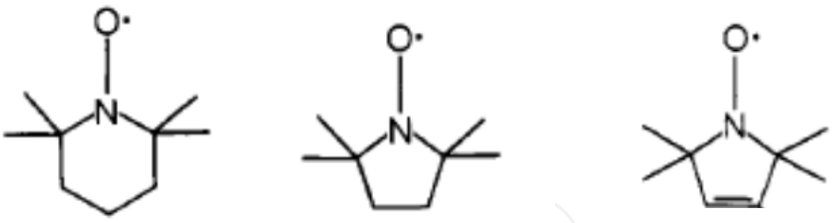


Figure 2. Piperidine, pyrrolidine and pyrroline nitroxides.

Let us not overlook the tremendous advantages of nitroxides that contribute to their versatility in the study of (biological) macromolecules i.e., they are very stable in most solvents over a wide range of pH values. The paramagnetic N-O bond moiety is quite tolerant to various synthetic conditions, specifically those in the tetramethyl flanked piperidine, pyrroline or pyrrolidine rings. Freezing, thawing, distilling or boiling usually impart no adverse affects on their stability, ie, paramagnetism is retained. Since EPR does not require optical transparency, and is not sensitive to magnetic susceptibility effects (which plagues NMR), one can work in opaque solutions, solids or mixtures. And the EPR sensitivity is 600- 700 times higher per spin compared with a proton in NMR. Thus, with very narrow linewidth spectra, one could detect nitroxide spectra in solution down to

nanomolar levels with high sensitivity cavities. The EPR spectral lineshape reflects nitroxide tumbling motion, hence one can distinguish freely tumbling, 'unattached' or unreacted label in a sample with other bound species. The only real drawback of spin labels is their susceptibility to reduction to the corresponding diamagnetic hydroxylamine in the presence of organic or biological reducing agents, which will be addressed under in-vivo studies. Yet, where some synthetic recipes may utilize e.g., $NaBH_4$ which reduces the N-O moiety, the radical is easily regenerated in mild H_2O_2 or exposure to O_2.



Figure 3. First meeting of L. J. Berliner with E. G. Rozantsev, USSR Institute of Chemical Physics, 1979

2. Horrible, terrible nomenclature: IUPAC versus 'common usage'

Spin labels are commonly called 'nitroxides,' also the title of this book. In addition the terms iminoxyl or nitroxyl have been used as well as the occasional use of the term aminoxyl. Yet IUPAC RNRI Rule RC-81.2.4.D defines compounds with the structure $R_2NO\cdot$ as 'radicals derived from hydroxylamines by removal of the hydrogen atom from the hydroxy group, and they are in many cases isolable.' While Chemical Abstracts Service uses nitroxide as the parent name for $H_2N–O\cdot$, e.g., $(ClCH_2)_2N–O\cdot$ or bis(chloromethyl) nitroxide, the IUPAC name is bis(chloromethyl) aminoxyl. It is correct to state that nitroxide should not be used as a name of a class of compounds that are specifically and correctly (a la IUPAC) aminoxyl radicals. As for the use of iminooxy or iminoxyl radicals, this has been used incorrectly for alkylideneaminoxyl radicals (also called iminoxyl radicals, $R_2C=N–O\cdot$. Its use is strongly discouraged. In the Sigma/Aldrich catalog, the spin probe TEMPO is listed as 2,2,6,6-tetramethylpiperidine 1-oxyl.

Hence, the most inappropriate term for these radicals, nitroxides, has been used most widely and, as of 2009, has been cited about 115,000 times, nitroxyl about 29,500 times, iminoxyl (initiated by E.G. Rozantsev and coworkers) about 4,150 times. Aminoxyl, the most correct, has been cited 3,910 times. Obviously, the term nitroxyl is way out of line, pushed only bnefly by the late Andre Rassat, but is not relevant to this class of radicals (although I have two colleagues who continue to propogate this misuse!) I recall a friendly conversation with my long time colleague, Jim Hyde, who emphasized that if it becomes common usage, it's here so stay and to just give up on the issue. However when we academics teach organic chemistry to our young students, we try to imbue them with the correct terminology. Furthermore, standard states and nomenclature were designed so that scientists in the world can understand one another. It is clear that the correct nomenclature that the spin-label community should be using is aminoxyl radicals. It would be great if, from this point in our history moving forward, we might correct this error in the future and abide by the IUPAC rules.

3. Early applications to studying subtle aspects of protein/enzyme structure

The spin label method is a reporter group technique, a concept in the 1960s [7], as depicted in Fig. 4.

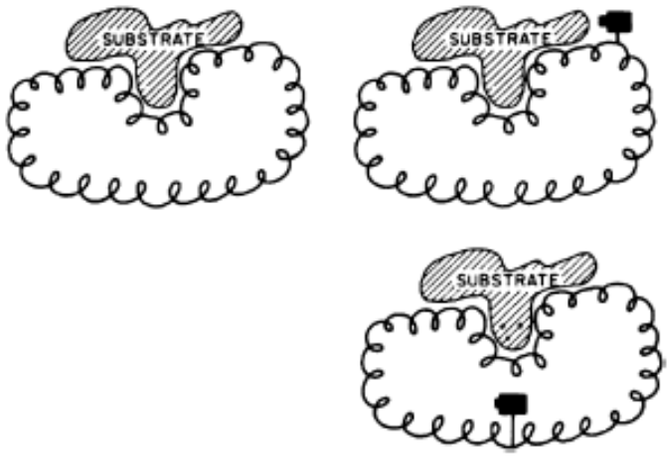


Figure 4. Schematic representation of an enzyme-substrate complex in native protein (top left), protein containing reporter group (solid black area) adjacent to substrate binding area (top right), and reporter group distant from substrate binding area (bottom right). From [7] with permission

The revolutionary developments in organic synthesis of nitroxide spin labels helped us overcome a major challenge for biochemical studies, where the plan was to attempt to fit the spin label to the biological system in as subtle a manner as possible, that is to "fool" the system into thinking it was binding to, or interacting with, a real, natural substrate or cofactor. One of the early attempts in the McConnell laboratory was the synthesis, a nitrophenylester of 1-carboxyl-2,2,5,5-tetramethylpyrollidine, depicted in Figure 5 (left), so that one could take advantage of the esterase activity of the enzyme α–chymotrypsin and

virtually 'hook' the enzyme during its action on the compound. The approach was to isolate the active acyl-enzyme intermediate, covalently attached at serine 195, which is where the intermediate in the enzyme catalyzed hydrolysis resides. Indeed, the spin labeled acyl-enzyme intermediate reflected a tightly bound (possibly rigid, uniquely oriented) spin label at the active site [8]. However, it became much more difficult when one wanted details as to how the enzyme handled this spin labeled substrate analog and business area to do single crystal studies of the spin labeled chymotrypsin order to derive information about the precise orientation of the label. This could be gleaned from knowledge of the anisotropic hyperfine constants and anisotropic g-factors. One could determine orientation of the N-O bond and work backwards to find the orientation of the nitroxide five-membered ring on the protein. With knowledge of the x-ray structure of α-chymotrypsin and its reactive intermediate structures already known, the process was straightforward, Bauer and Berliner were able to obtain individual binding orientation of the R-and S-enantiomers of this particular house substrate panel and from that understand why the more slowly released enantiomer substrate acyl group was mis-oriented at the active site making hydrolysis by an activated water molecule quite difficult [9].

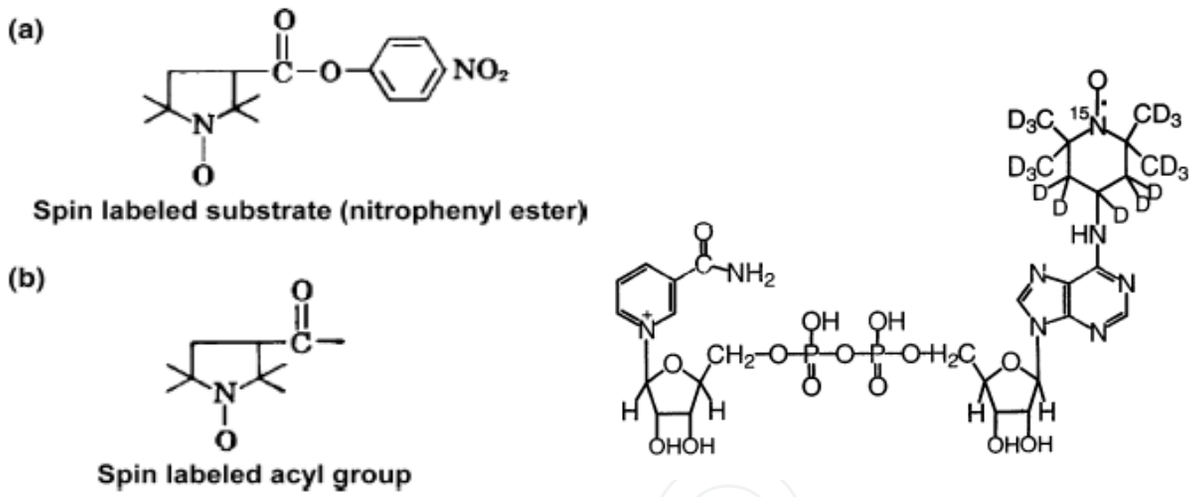


Figure 5. (left). Spin-labeled substrate (R,S)-2,2,5,5-tetramethyl-3-carboxy-pyrrolidine-p-nitrophenyl ester (a). The acyl-nitroxide group that is covalently linked to Ser 195 of α-chymotrypsin (b). It was later found that the "specificity" for a spin-labeled acyl-chymotrypsin was the S- enantiomer, although both enantiometers can be isolated as acyl enzymes. (right) Chemical structure of SL-NAD$^+$,nicotinamide N^6-([^{15}N,$^2H_{17}$]2,2,6,6-tetramethylpiperidine-4-yl-l-oxyl)adenine nucleotide [10]

The real sophistication came in studies of enzymes that bound nucleotide analogues or, in fact, DNA and nucleotide complexes. In some beautiful work Trommer and colleagues synthesized NAD analog, SL-NAD$^{+,}$ where the nitroxide ring was fused onto the nicotinamide, the structure, shown above in Figure 5b (right) [10]. The enzyme bound very tightly to this NAD analog and its precise orientation could be determined. What was interesting was that in the example glyceraldehyde phosphate dehydrogenase, a tetrameric enzyme that binds one NAD per subunit in each tetramer, the distance between two NAD

spin labeled analogs could be determined from the electron-electron dipole interaction. This was the first example of distance measurements involving two spin labels within a protein structure and, due to the fortuitous situation of a perfectly, rigidly bound spin label, distances could be determined precisely [10]. This study still remains the gold standard of distance measurements by electron-electron dipolar interactions.

4. Lipid spin probes (oxazolidinyl or doxyl, proxyl)

The development of spin labeling and spin probes expanded to lipids and membranes. In order to probe these biological structures, one needs a label that mimics or looks like a lipid and can be incorporated into a phospholipid membrane structure. As late as the late 1960s, one could only prepare an ester of a fatty acid with one of piperidine or pyrrolidine nitroxides, but one could not incorporate a probe somewhere in the middle of the lipid chain in order to probe various depths of a membrane. It was not until John Keana demonstrated that one can incorporate an oxazolidine ring at specifically placed ketone (keto) groups in a lipid, resulting in a rigid five membered ring fused to the lipid chain that was easily oxidized to the radical nitroxide (doxyl) [11]. This virtually led to a revolution in our ability to probe membrane structure and dynamics with structural and dynamic accuracy. Several of these compounds are shown below. It took a while before these were commercially available, however, the synthesis was reasonably straightforward and scientists in the area were willing to share their compounds with one another. This was a clear departure from the relatively straightforward chemistry of the piperidine, pyrroline and pyrrolidine based aminoxyl radicals that had been developed by the Russian groups up to that point. The synthesis is relatively straightforward: take a lipid of interest, which can be purchased as a halo-derivative or occasionally as the desired keto derivative. Then the oxazolidine ring is formed at this position on the chain, then oxidized to the radical. Spin labeled lipid probes became available with aminoxyl radical group at the 5-, 12-, or 16 position in the lipid chain, and later at other positions. The resulting biochemistry, i.e., to incorporate these lipid nitroxides at either the 2- or 3- position of a phospholipids, was fairly straightforward as the fatty acid interchange or ester interchange chemistry was already well known. The synthetic schemed and some example probes are shown below, with a phospholipid analog in Figure 6.

Some years later, the problem of the oxazolidine ring being essentially reversible, (i.e. hydrolyzable), a newer development involved the incorporation directly of a five-membered ring (proxyl) into the structure of a lipid molecule at a strategically placed double bond. The chemistry again was somewhat sophisticated but straightforward; the synthetic route (Figure 7) leads to a side-chain-substituted 2,2,5,5-tetramethylpyrrolidine-N-oxy1 (proxyl) nitroxide lipid spin probes from a commercially available nitrone is treated with an organometallic reagent, which after Cu^{2+}-air oxidation gives a new intermediate nitrone, followed by a second selected organometallic reagent, which after Cu^{2+}-air oxidation yields the proxyl spin probe [11]. The advantage of proxyl chemistry over oxazolidine chemistry in order to make lipid spin probes was that one could tailor the orientation of the N-O group with respect to the lipid axis. This became important since the

hyperfine coupling constant along the z-axis of the label (i.e. directly above and perpendicular to the N-O plane) yielded a large splitting, upwards of 32G, that allowed a quite accurate estimate of the orientation, order parameter and dynamics of this portion of the lipid spin probe within the membrane.

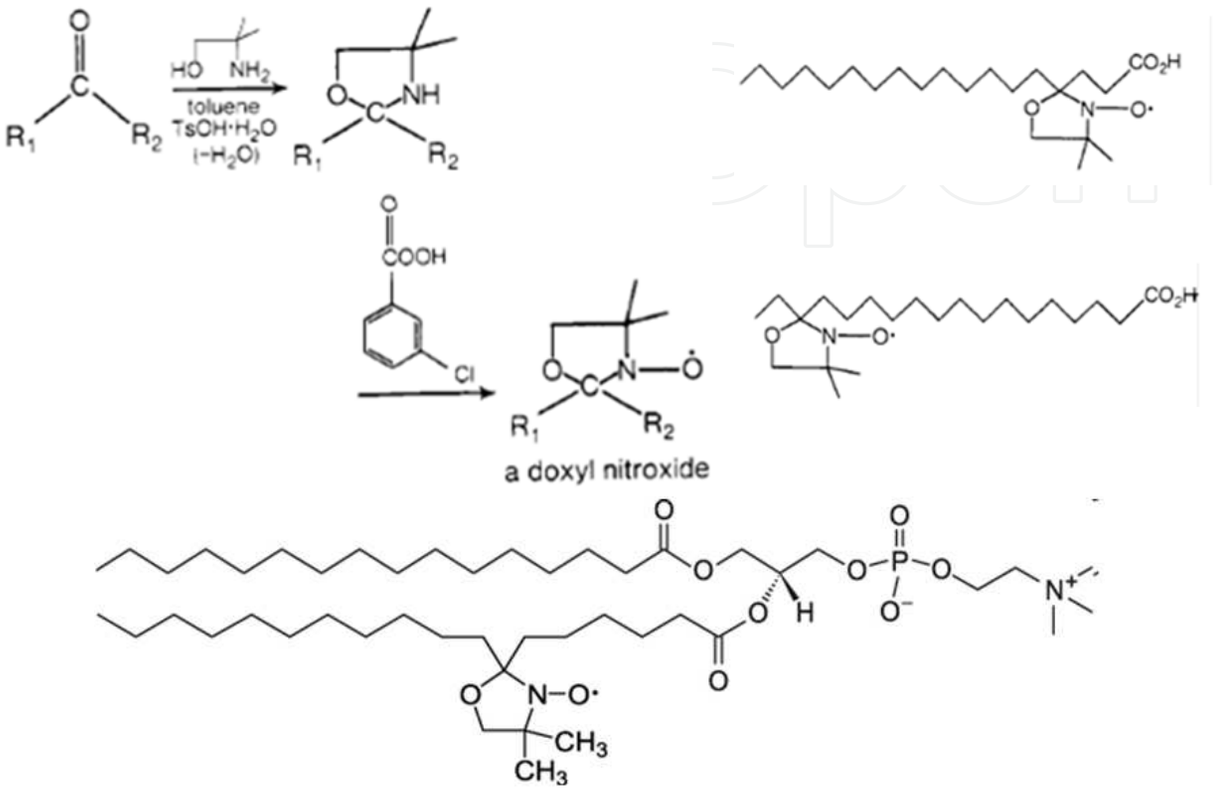


Figure 6. 16:0-7 Doxyl PC 1-palmitoyl-2-stearoyl-(7-doxyl)-sn-glycero-3-phosphocholine.

Even more rigid lipid probes were possible with the advent of racemic azethoxyl lipid probes nitroxides (called minimum steric perturbation spin labels). In the azethoxyl the nitrogen atom is actually embedded in the hydrocarbon chain. Cis-trans isomerism is possible and modeling suggests that the trans isomer should resemble a saturated lipid, whereas the cis isomer introduces a bend in the chain which approximates that observed with a cis carbon-carbon double bond.

The general synthetic route to the azethoxyl nitroxide spin labels is similar to that of the proxyl nitroxides, except that a different nitrone is used in the beginning (Figure8), where in this specific example, the trans isomer predominates [11].

Synthetic development was also carried out by several chemists in Ljubljana, Slovenia, as well as other synthetic organic labs, all of which were principally in Eastern Europe. In the U.S.A., the plight of an organic chemist attempting to obtain tenure in an academic department required the synthesis of complex natural products for the development of new synthetic reactions. Frequently the synthetic procedures for preparing these aminoxyl radicals, spin labels or spin probes were albeit modern but not new and novel; the organic chemist simply adapted the new, clever synthetic procedures to obtain the required label. It

wasn't until the late 1990s, or perhaps the new millennium, where chemistry departments accepted applied chemistry as a valid academic area of new ideas and novel techniques. Certainly, it was the synthetic organic chemist who solved this problem and, for that matter, most biophysical studies involving probes depend on clever synthetic abilities. EPR had a great advantage in membrane and cell studies and cell membranes since the technique did not require optical transparency, did not have the magnetic susceptibility problems encountered in NMR, and required a fairly low level of spin label doping of the biological system in order to obtain a strong, highly sensitive spectrum. Indeed, it is fair to say that EPR added a tremendous amount of knowledge to our understanding of lipid, membrane and related polymeric systems, which was a great complement to that learned from NMR, solid-state NMR and microscopic methods The real leadership in the implications of these problems started, again, in the McConnell lab at Stanford University and with people like Joe Seelig, Wayne Hubbell and others who followed. Nobel laureate Roger Kornberg was also a graduate student in this laboratory, and his work also was involved in studies of lipids and membranes through the use of spin labels and spin probes [12].

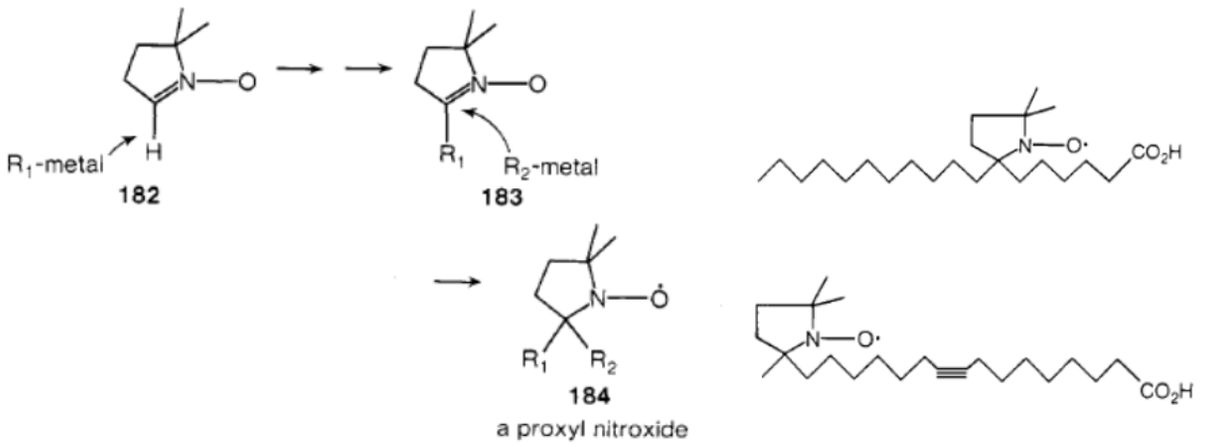


Figure 7. Synthesis of a proxyl nitroxide

5. Nucleic acid analogues

The Bobst laboratory at the University of Cincinnati synthesized some very novel nucleotide analogues where the label was covalently tethered to various purine and pyrimidine rings in such a manner that the tether did not distort the DNA structure and was rigid enough to not create ambiguities in an interpretation of the backbone or sidechain motion of the polynucleotide where the label was incorporated [13]. A series of these novel, unique structures are shown in Figure 9. This work was then copied and extended by other groups, particularly the Seattle group (University of Washington) that also designed nucleotide analogues for probing DNA [14]. In all cases the syntheses were truly challenging, could only be carried out by very proficient organic chemists, and support the view of this author that synthetic organic chemistry is the rate-limiting step in many of these biophysical probe experiments.

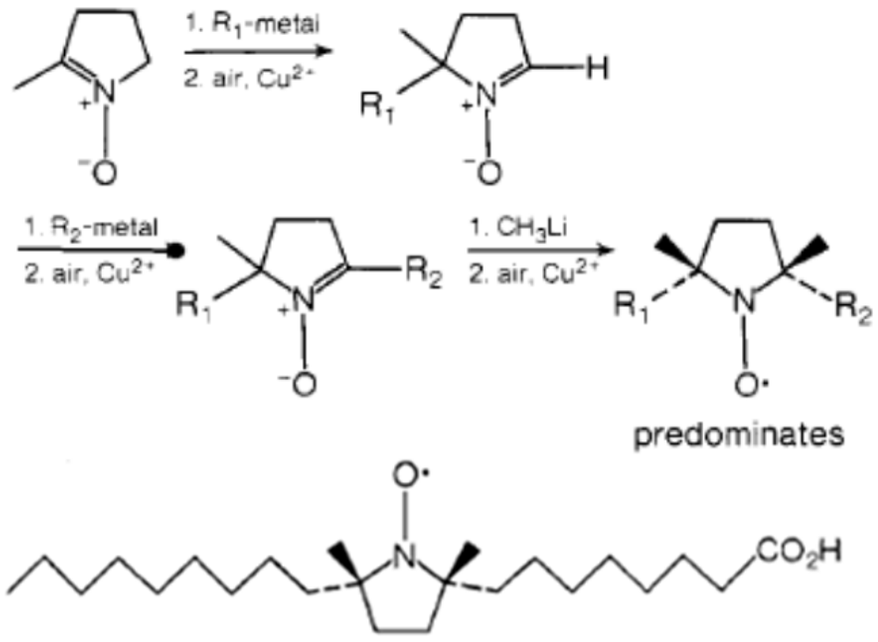


Figure 8. Synthesis of an azethoxyl nitroxide

6. Specificity in protein labeling: Thiol groups

The ideal goal with spin labeling is a universal method to label any tailored site with high specificity. Let's face it; spin labeling of proteins is a protein chemical modification methodology. That aside, it is the chemistry of the functional groups utilized in order to label a protein. If one examines the 20 common amino acids, one finds that the advantageous modification chemistry is both quite limited, ambiguous, and is very much dependent on pKa values where charged sidechains are targeted. This leaves us only with the cysteine thiol as the best candidate for any sort of specific modification. If one looks at the standard array of protein modification functional groups, at least what existed in the 1960s, 1970s and 1980s, we were limited to the maleimde, the alpha halo-acetamide groups and a few disulfide-based reagents, all of which had limitations, particularly the former two. Reagents such as iodoacetamide or N-ethylmaleimide (NEM) will react with thiol groups, amino groups (alpha- amino groups, lysine) and occasionally with hydroxyl groups of a nucleophilic serine or threonine or a tyrosyl side chain. Secondly, the preponderance of these sidechains is usually multifold in proteins, while thiol groups are usually small in number, or occasionally nonexistent. In a quest for highly specific reversible thiol reagents, Berliner and Hideg capitalized on the chemistry pioneered by George Kenyon with methylthiomethane sulfonate, a reagent that undergoes disulfide interchange with a cysteine eliminating the methylsulfonate leaving group [15]. This was definitely advantageous over dithiol reagents, where one loses half of the label in the exercise and it also created other problems involving thiol interchange that could eventually negate of the advantages. Hence the label shown in Figure 10 (bottom), affectionately known as MTSSL or MTSL, was synthesized and was shown to be highly reactive, uniquely specific for cysteine

thiol groups and could be easily released with a small concentration of mercaptoethanol or dithiothreitol, allowing one to recover the protein and also allowing for a second labeling stoichiometry quantitation based on the released label [16]. Berliner and Hideg showed eloquently how this works with the reactive protease papain, which contains a cysteine SH at the active site analogous to the serine OH in chymotrypsin [16]. Initially this label wasn't used much by other research groups, but the advent of molecular biology and the power of site-specific mutation triggered a revolution in this area, pioneered by Wayne Hubbell. The technique, named site directed spin labeling, has really been the method of choice since the 1990s and has created a renaissance in spin labeling [17].

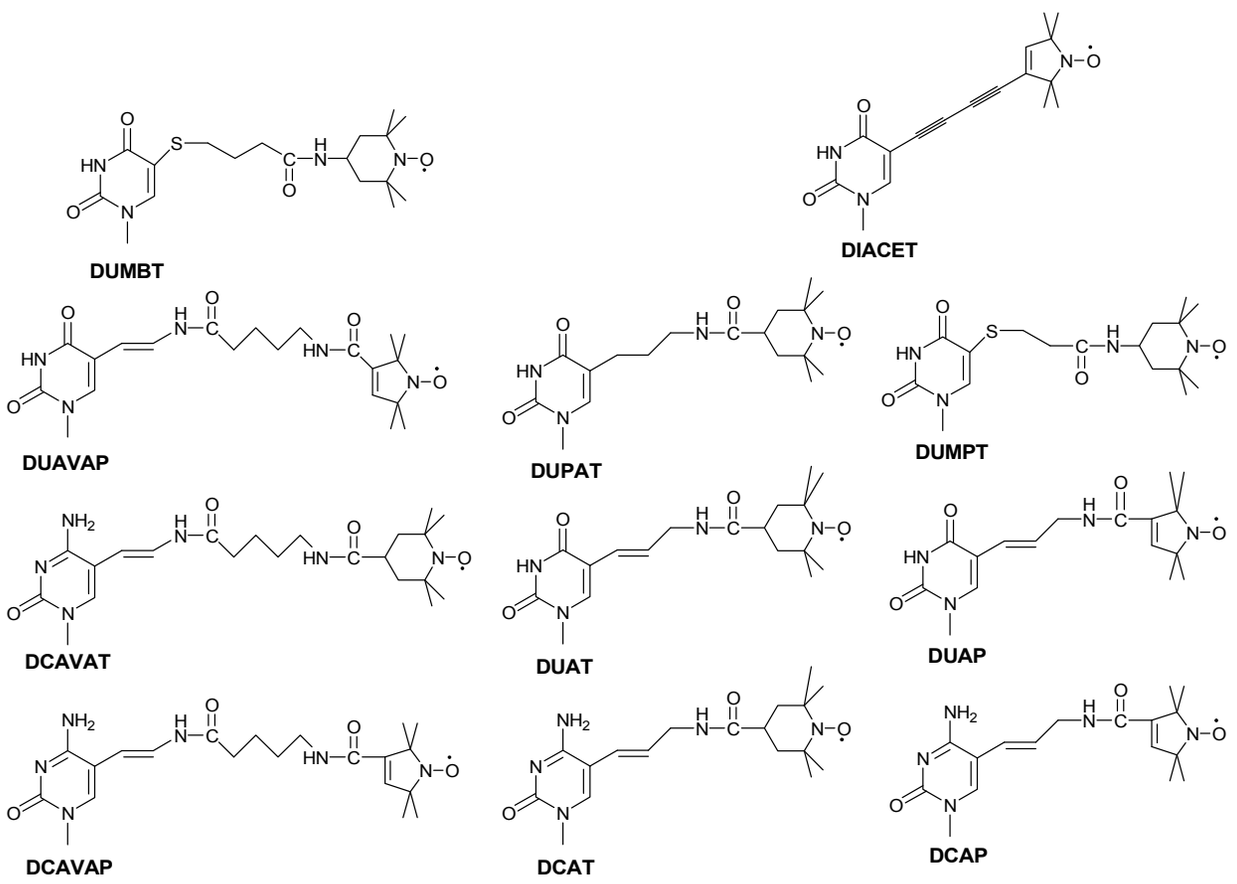


Figure 9. Representative spin labeled pyrimidine bases that can be incorporated into nucleic acid structures. Adapted from [13] with permission.

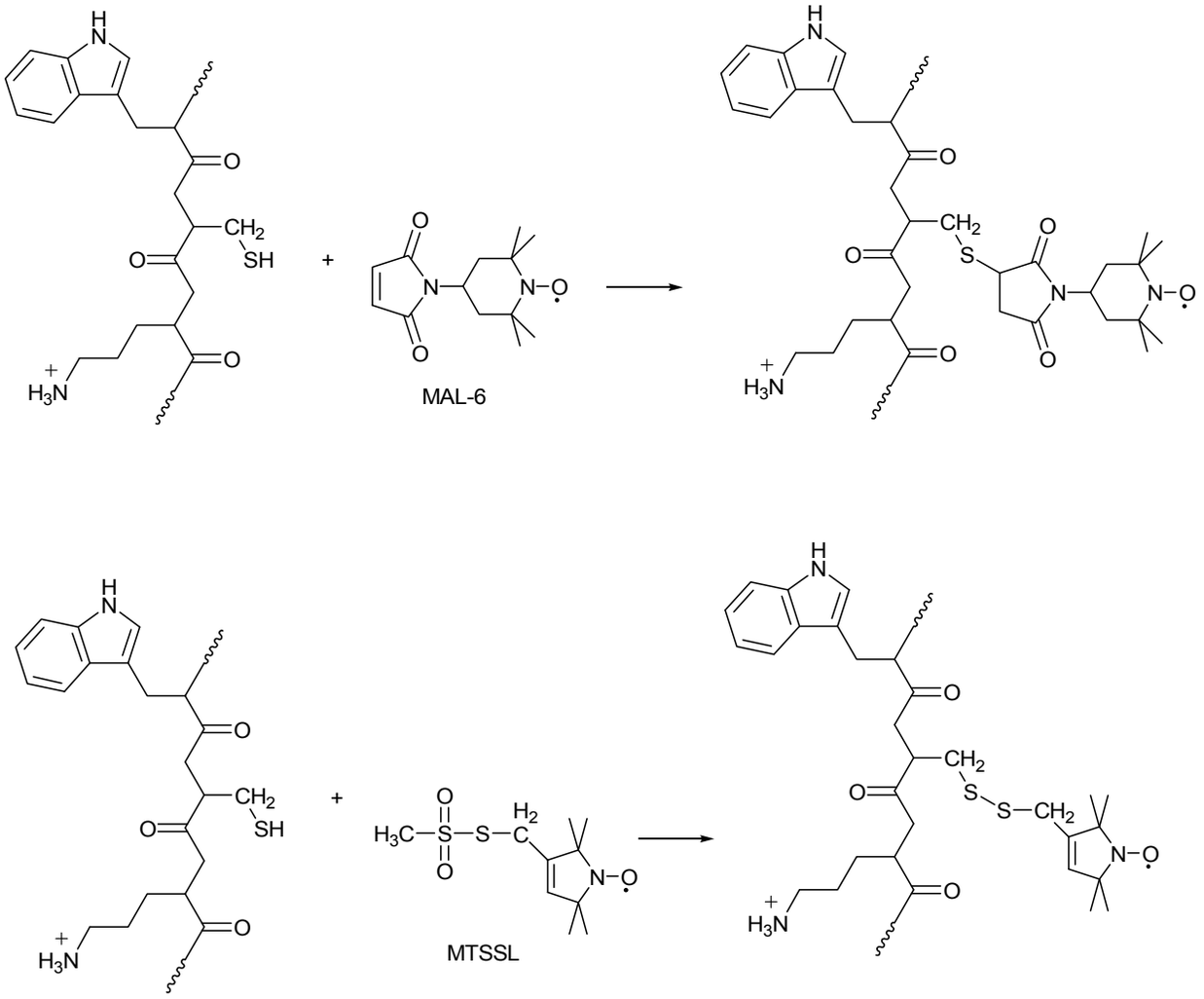


Figure 10. Comparison of thiol labeling reagents. The top reaction utilizes the NEM analog, which reacts irreversibly with the protein thiol group, but can also partially react with other nucleophiles. On the other hand, MTSSL reacts specifically with SH groups and can be reversed in the presence of another thiol reagent, such as mercaptoethanol or DTT. Adapted from [18] with permission

Wayne Hubbell's important contribution was to realize that one could incorporate thiol groups into protein sequences with ease, almost at choice. If there was an example where the disulfide bridge or a few free thiol group caused a major perturbation in the structure or the folding of the protein, it was usually pretty obvious by some functional or conformational (e.g. CD, ORD) analysis. Hubbell attacked the most pressing problems in protein science, which is membrane proteins, which are neither soluble nor amenable to x-ray crystallography or NMR. He started first in collaboration with Nobel Laureate H. G. Khorana on bacteriorhodopsin, a protein whose structure and function had eluded us up to this point, particularly with respect to the light induced conformational changes that occur [19]. The technique, in concert with the well known molecular oxygen Heisenberg exchange relaxation (broadening) of the N-O, allowed assessment of secondary structure characteristics, particularly that of bundled helical structures, which are typical of membrane spanning proteins. If one mutates every residue in a helical protein to a Cys, in

each case labels the protein, and then assesses accessibility under increased oxygen, a periodicity of about 3 -4 would be expected in the oscillation of oxygen exposure (since O_2 has a higher solubility in the interior vs the solvent environment). For β-sheet structures, the periodicity would be 2 since every other residue is exposed to the solvent and vice-versa. The two figures below depict the theoretical behavior for a β-sheet and α-helical domain, respectively. Further confirmation occurs when using aqueous paramagnetic reagents such as chromium oxalate or potassium ferricyanide, which selectively relax (broaden) spin labels on the exterior of the protein pointing into the solvent [17]. Hence these accessibility parameters could be quantified and used as sensitive probes of secondary and super-secondary structure. Theoretical plots are shown below in Figure 11. In a study on lac permease, an SDSL 'scan' was done and the results are shown in Figure 12 below [21].

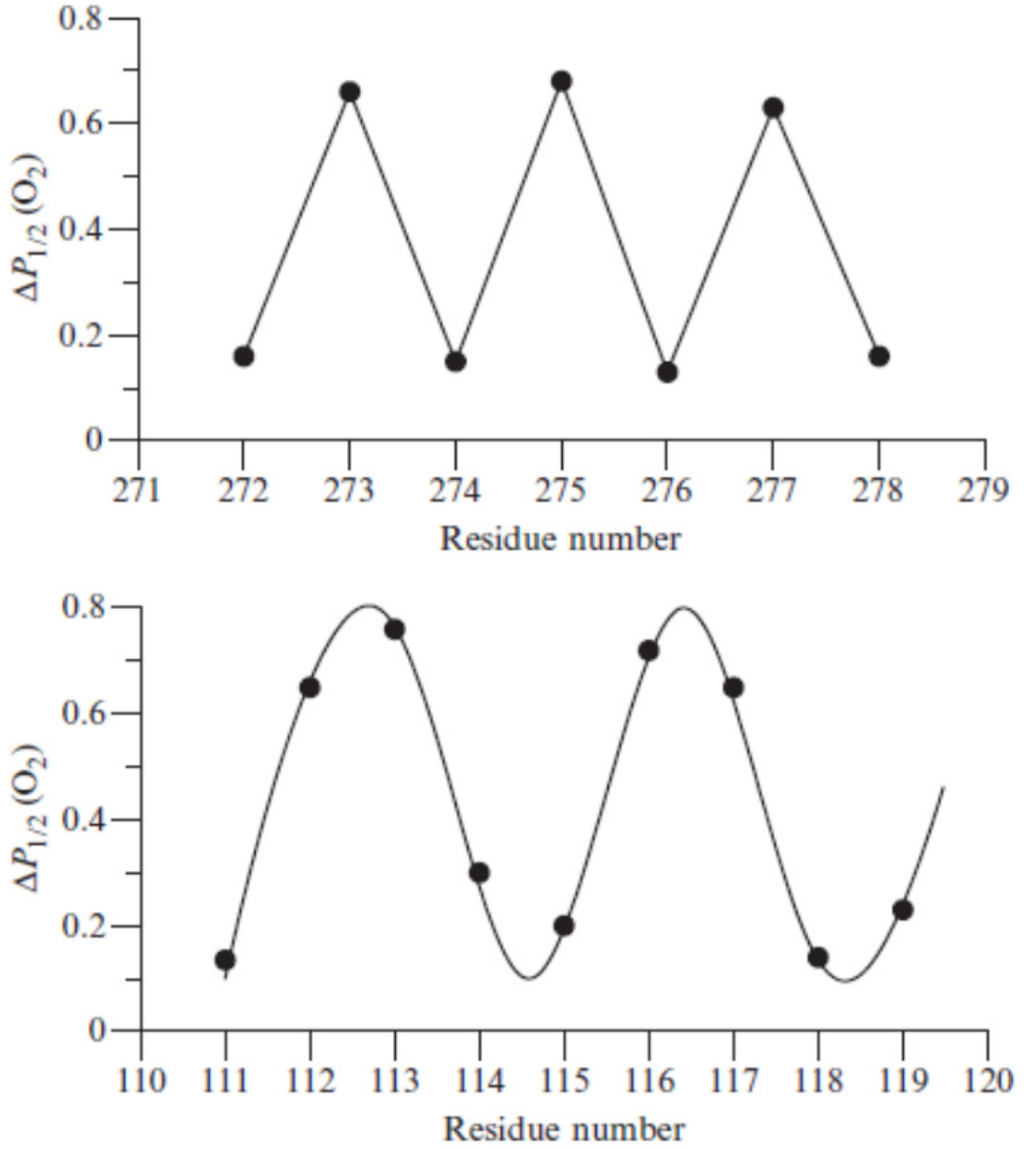


Figure 11. Idealized accessibility data plots indicating β-strand and α-helical secondary structure. From [20] with permission

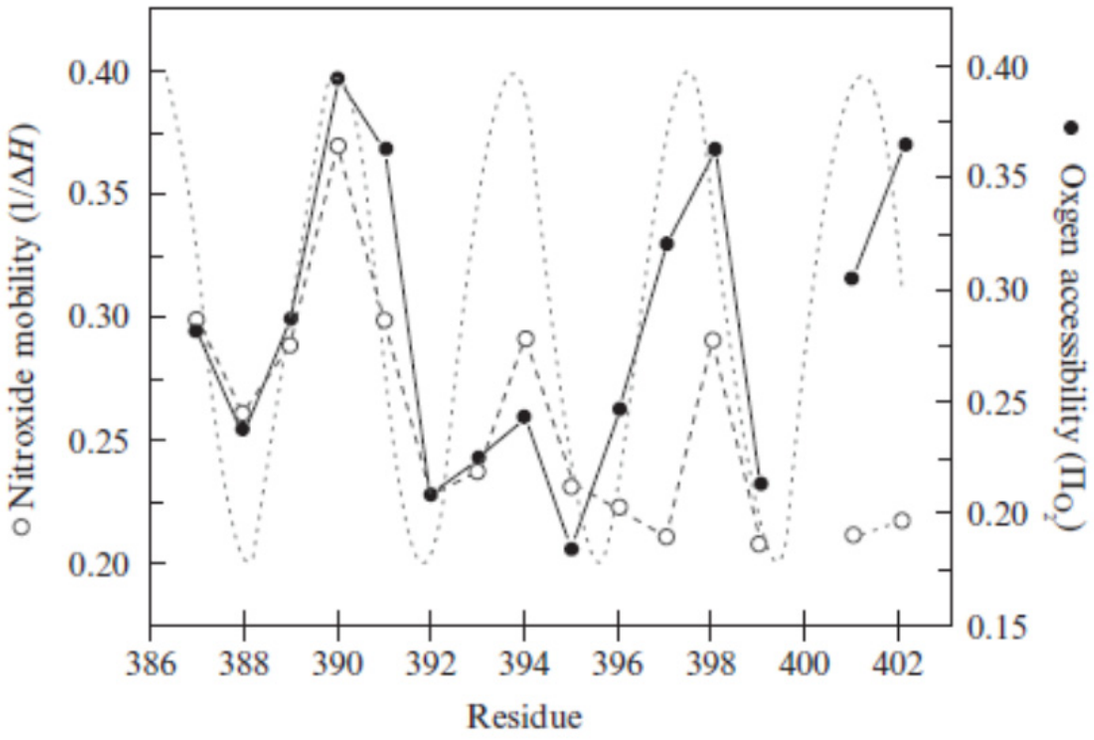


Figure 12. Π(O_2) (solid line) and 1/ΔH (broken line) versus sequence position for the nitroxide-labeled single-Cys residues at positions 387-402 in lac permease. The dotted curve is that for a function of period 3.6, and comparison with the Π(O_2) and 1/ΔH functions confirms that the data are consistent with an α-helical structure. Ada[ted from [21] with permission.

The site directed spin labeling (SDSL) method blossomed by the early to mid-1990s, with dedicated sessions at meetings on the use of SDSL and EPR in protein structure. Of course, the MTSL label had some disadvantages: it still had some conformational flexibility, it could perturb the protein structure and lastly, in order to obtain an unambiguous assessment of protein structure and function, the use of additional spin labels would be desirable. Hence the Hideg lab propagated several more labels and analogues [22]. Recent work has involved distance measurements within proteins, i.e., the incorporation of two cysteines at selected positions in protein with the idea of mapping the structure by distance triangulation. This is a major effort since one obtains only the distance between the electrons on the two labels, respectively, and each spin label must be correlated back to the protein backbone with inferences from amino acid side chain structure and the aspects of motion of the label in multiple orientations. Consequently, one pair of incorporated cysteines yields just one distance. Figure 13 shows the dilemma of attaining very accurate distance measurements from a double labeling experiment. Nonetheless, the technique has still been valuable and people have developed sophisticated motional simulations in order to localize the label in the protein structure. One looks at motion around a cone and workers have attempted to come up with distances within 15 to 20% accuracy. Needless to say, the x-ray crystallographers have a major advantage (assuming that the protein can be crystallized) but the NMR spectroscopists have a bigger advantage because they can incorporate one single,

relatively rigid, spin label and obtain more than 100 distances from electron-proton paramagnetic relaxation enhancements over distance ranges of 5 to 15 Å. In fact, the EPR double label method was quite limited on distances, about that of the electron-proton distance limits. Remembering that we only obtain one distance for each labelled pair, it wasn't until Jack Freed and coworkers incorporated DHQC methodology enabling one to assess distances upwards of 50 to 60 Å [23].

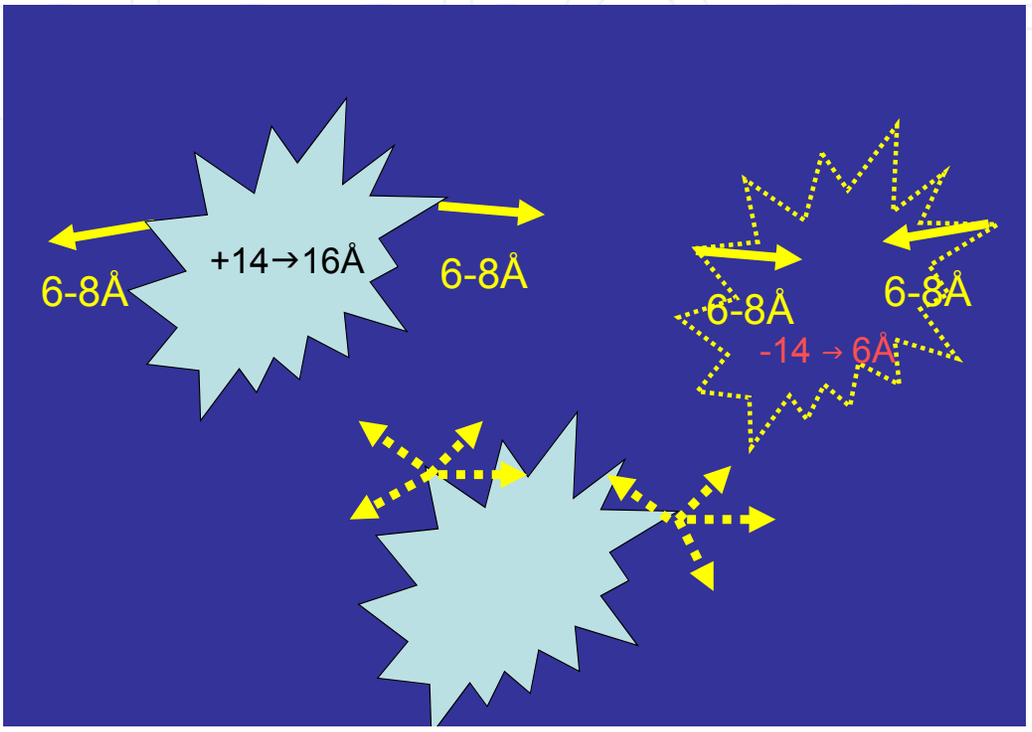


Figure 13. Uncertainty about spin label motion and orientation(s). The spin label ring can orient in several directions, depending on the flexibility of the tether. Ideally, one would like a rigid unique orientation, but the likelihood is not high.

Some possible approaches are to anchor the label in two points on the protein. The label shown below in Figure 14 would require two mutated cysteines at proper spacing in order to meet that requirement.

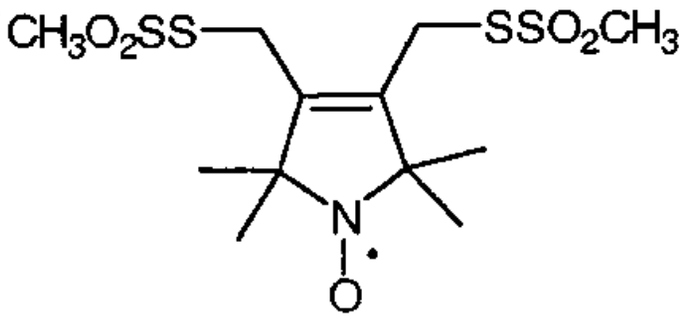


Figure 14. Rigid two site attachment spin label. bis-MTSSL [pyrrolinyl] 2,5-Dihydro-3,4-bis(methanesulfonylthiomethyl)-2,2,5,5-tetramethyl-1H-pyrrol-1-yloxyl radical

7. Nitrones and spin traps: The adducts form nitroxides

These compounds are actually a class of chemical functional groups that had been known quite early since one of the synthetic methods of producing of nitroxides is by a controlled, specific oxidation of a nitrone compound. However, these have found tremendous use in the characterization of free radicals in solution, particularly in the biological field where a plethora of potential radicals are possible. In fact, the reaction of a nitrone with carbon or oxygen-based radicals yields a nitroxide adduct with the spectrum that is characteristic of the chemistry of that particular initial radical (with some caveats that are discussed below). The use of radical-addition reactions to detect short-lived radicals was first proposed by E. G. Janzen in 1965 [24]. The early pioneers in this field studied two classes/types of spin traps which were commercially available at the time and are still on the market today: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide, and PBN, alpha-phenyl N-tertiary-butyl nitrone. These and other second generation spin traps are shown in Figure 15 below.

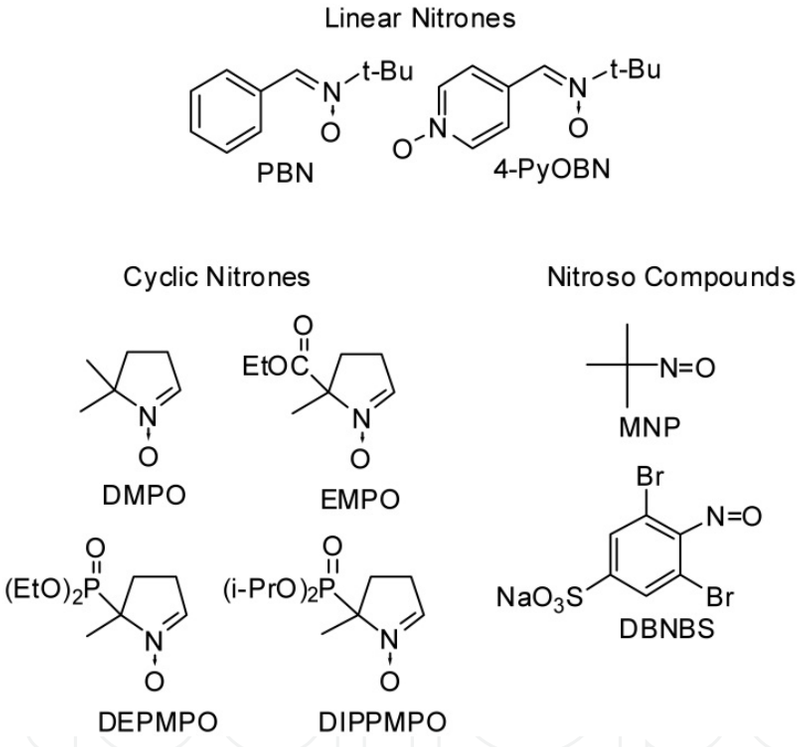
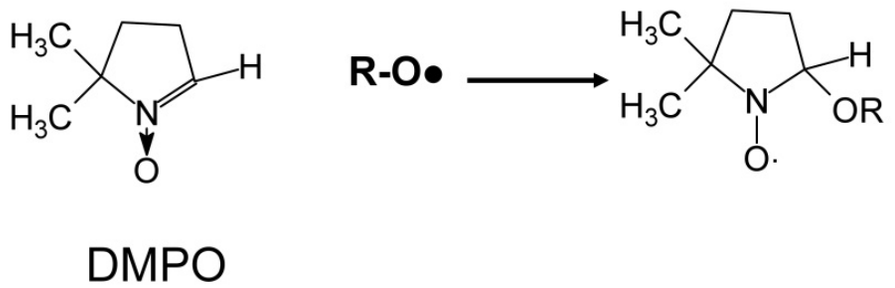


Figure 15. Structures of various spin trap types including second generation nitrones. Structure abbreviations: PBN α-phenyl N-tert-butyl nitrone, 4PyOBN; DMPO 5,5-dimethylpyrroline N-oxide, EMPO 5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide, DEPMPO 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide, DIPPMPO 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide, AMPO 5-carbamoyl-5-methyl-1-pyrroline N-oxide, MNP 2-methyl-2-nitroso propane, DBNBS 3,5-dibromo-4-nitrosobenzenesulfonate, CPCOMPO 7-oxa-1-azaspiro[4.4]non-1-en-6-one 1-oxide

DMPO could cross the cell membrane and was in fact sensitive to radicals that were present in both the aqueous media as well as the lipid medium. Some derivatives of DMPO with selected substitutions at the 1- or 4- position allow one to affect its partitioning properties between aqueous and lipid environments. Janzen successfully showed that DMPO could

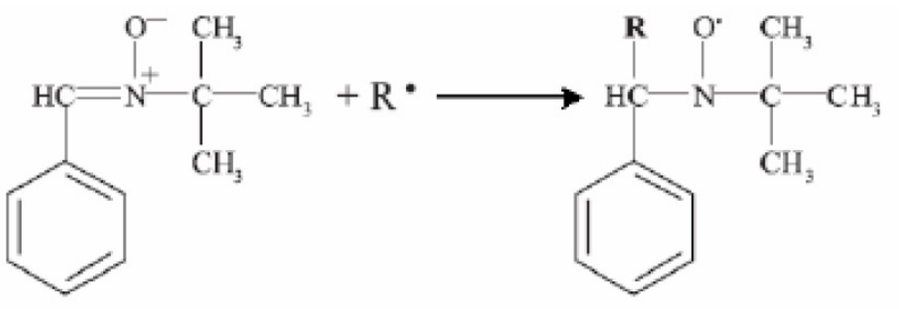
trap the important reactive oxygen species: superoxide and hydroxyl radical [25]. He clearly demonstrated the differences in their EPR spectra, allowing characterization of these radicals in vivo. However it wasn't until a few years later that Rosen and colleagues found that the superoxide radical adduct of DMPO could decompose to the hydroxyl adduct by a mechanism which, to date, is still not totally clear. Hence one has to take special care, e.g., including SOD in an experiment in order to include or exclude superoxide [26].



DMPO

Figure 16. Reaction of DMPO with an oxyradical.

The PBN spin trap was, by virtue of its non-polar lipophilic behavior, quite valuable for trapping lipid radicals and those in a membrane milieu. If PBN reacted with oxygen radicals, such as hydroxyl radical, it decomposed without any radical adduct remaining. On the other hand, the PBN-lipid adducts, if partitioned into the membrane, were stable for long periods of time and one could e.g., isolate lipid-radical adducts of PBN in erythrocytes, then extract and concentrating them later. In fact, some lipid biologically produced lipid radicals are stable in lipid media and erythrocytes could be 'post labeled' with PBN after some oxidative stress event [26].



PBN PBN spin aduct

Figure 17. Reaction of PBN with a carbon based radical.

These two spin traps remained with us for almost 20 years and, along with the particular annoying side reaction noted with DMPO and superoxide, the DMPO adducts tended to be fairly unstable. In the late 1980s, Tordo's group prepared a DMPO analog that contained a phosphoester-type group in one of the positions of the flanking methyl groups [28]. One of these compounds, DEPMPO, was quite successful in that the half life of the radical adduct was much longer than that known for DMPO. It is important to point out at this juncture

that the reaction kinetics for all of these radical traps were quite poor, involving the necessity of having 50 – 100 mM concentrations of spin trap in the solutions. Further development of other analogs by Tordo's group and also some quality control by the commercial laboratory that were selling DEPMPO was quite an advance for the community.

However the real, major effort in our understanding and designing nitrone spin traps, based on the DMPO skeleton, was a result of an intense effort at Ohio State University by Villamena, who did synthetic, kinetic and computational design studies of these traps as well as their aminoxyl radical adducts. Hence Villamena studied both their reactivity and the stability of the adducts [29]. Overall, spin traps are powerful reagents, albeit more limited for in vivo studies due to their low sensitivity and kinetics and the concentration limits of reactive radicals in vivo. The hope was to accumulate radicals in vivo up to levels where the trapped adducts exceeded the normal in vivo level. Suffice it to say we are 'part way' there. But we still suffer from the breakdown of the radical adducts and have not yet attained optimal kinetics. Some future concepts for applications of these type of compounds would be to prepare 'spin trap labels' that could be incorporated at specifically targeted organ sites in vivo which would then would convert to the radical adduct at the time and place of radical generation. There have also been some efforts to attach fluorescent moieties onto these labels, whereby the fluorescence is quenched upon formation of the nitroxide radical adduct [30-31]. This area has great promise and some examples are diagrammed below under future developments (Section 8).

8. In-vivo EPR using aminoxyl radicals: History and fate

The last frontier of applications of aminoxyl (a.k.a. nitroxide) radicals is in their applications to in-vivo studies. Since the aminoxyl (nitroxide) radicals were the first, and for a long time the only radicals that were stable and detectable in aqueous solution, such as cell and other components, it was straightforward and logical to try to examine the fate and behavior of these radicals in living systems. Early on, attempts were made to mix development of spin labeling that one attempted to mix aminoxyl radicals and living systems. In fact, in an undocumented experiment in the McConnell laboratory the toxicity of a nitroxide was tested on a goldfish. A beaker-full of the radical, t-butylnitroxide, was emptied into a Bell jar containing a goldfish. While the concentration was not accurately estimated, it was certainly in the tens to hundreds of millimolar; needless to say the fish lived, and as we learned later these labels are actually life-sustaining compounds). But someone accidentally left the hot water slowly dripping into the Bell jar and eventually the fish expired. One postdoc in the lab actually monitored his urine for the ingestion of these compounds as several of them are volatile and there was an extensive amount of synthesis and gas chromatography ongoing them lab with these volatile compounds. Needless to say, no adverse effects were found.

So what happens if you mix a nitroxide with e.g., a cell or tissue suspension? If it is the six-membered ring species, i.e., the piperidinyl nitroxides, and easily those that can cross the cell membrane into the cytoplasm, they are rapidly reduced, i.e., 'neutralized,' within a few minutes, since a plethora of intracellular biological reducing agents ready to take on their antioxidant role and convert the nitroxide to its corresponding hydroxylamine. For example,

TEMPONE, or for that matter TEMPOL, are rapidly converted to the hydroxylamine with an immediate loss of the paramagnetism. This occurs within a few minutes. The five-membered ring species, however, have much longer half-life, i.e. of the order of 15 to 30 min., allowing one to study some aspects of the metabolism and perhaps the ability to image this paramagnetic material in a living species. The first experiments were done by the Brasch group where they were evaluating nitroxides as MRI contrast agents[33-34]. This was followed by a plethora of studies on animals, tissue samples, blood samples, etc. where we obtained a wealth of pharmacokinetic data (although no totally clear understanding of the mechanism and detailed rate constants) [35-36]

Suffice it to say, imaging by EPR methods is challenging, if not hopelessly low resolution, since most nitroxide labels have linewidths of, at best, 0.3-0.5G for a compound that is deuterium and N-15 enriched. It has only been with the trityl radicals mentioned early where any hope of imaging was possible. However, if one takes advantage of the power and high resolution of magnetic resonance imaging (MRI) and the fact that the contrast agents in this methodology are paramagnet, then organic radicals have a place. Therefore nitroxide spin label/spin probe analogs have been tested as MRI contras agents and have met with some success. One must overcome the problem of biological reduction, also a problem with the radical adducts of nitrone spin traps since the cellular/tissue milieu contain many reducing agents such as NADH, ascorbic acid, and mitochondrial reduction sources [33]. The real quest here is to produce a well protected, aminoxyl radical that is highly resistant to biological reduction yet can be incorporate into the tissue system of choice. A few examples have been reported to date, particularly where the tetramethyl groups that flank the N-O group are replaced by long aliphatic chains such as lipids or tertiary butyl chains or cages.

9. Conclusions/prognosis/summary/future developments

This author has frequently concluded, about once per decade, that spin labeling has met its limits and should go into the category of the 'on the shelf' routine technique given all of its limitations. However, we have found one or two cases of a renaissance in the use of nitroxides, particularly the inception of the SDSL technique using MTSL labels which have given it a major rebirth. The future should involve marrying various techniques that can utilize paramagnetic materials, some of which that have already been mentioned earlier: NMR, fluorescence, dynamic nuclear polarization (DNP) and other technologies yet to be developed or discovered. Some examples are shown below.

Optical probes, e.g., absorb in the visible or are fluorescent, when coupled to a paramagnetic moiety, experience shifts or lifetime relaxation from a nearby free electron. One example below (Figure 18), is a nitroxide fluorophore, which exhibits significantly quenched fluorescence emission. Some applications are cartooned in Figures 19 and 20.

This nitrone spin trap depicted in Fig. 21 is one of several synthesized and tested by David Becker [32] at Florida International University. Upon reaction with reactive oxygen species, the absorption properties of the nitrone shifts are depicted below.

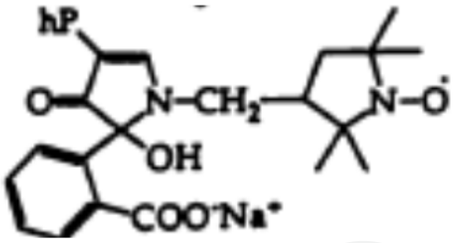


Figure 18. ((2-Carboxy)phenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one sodium salt [30]

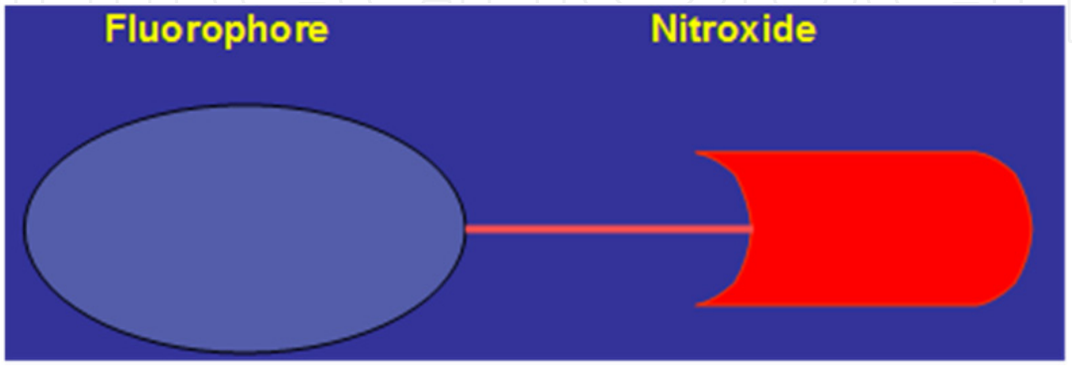


Figure 19. Schematic of the spin label in Figure 18 , where the red nitroxide depicts the paramagnetic N-O group, while the weak fluorescence reflects quenching by the paramagnet.

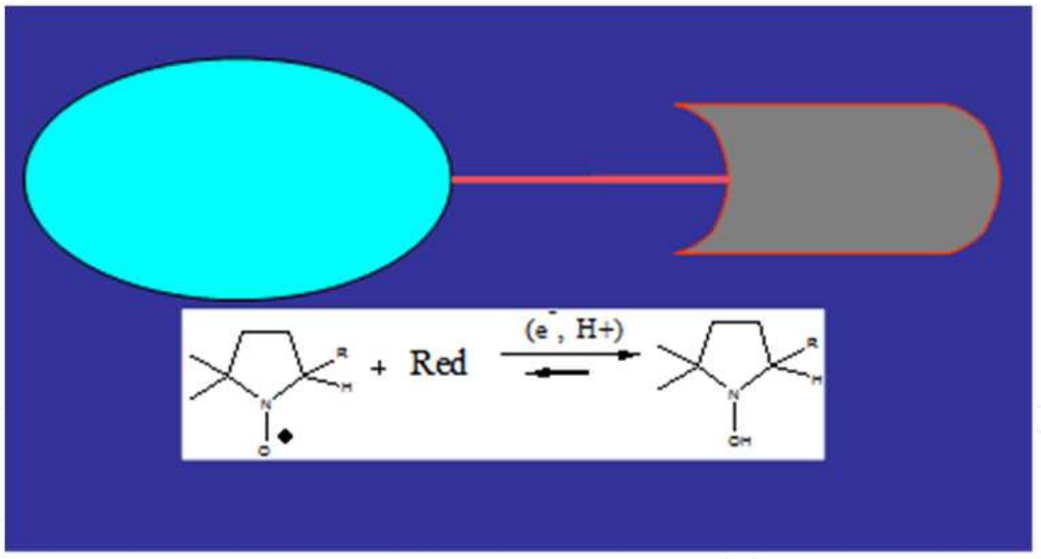


Figure 20. Upon reduction of the spin label the corresponding hydroxylamine, e.g., in a biological system by NADH or ascorbic acid, the fluorescence emission is strong and the EPR spectrum from the spin label has disappeared.

One can take advantage of NMR/MRI by spin labeling a cell surface with multiple nitroxide labels. The highly labeled surface now acts as an excellent paramagnetic relaxation enhancement site for exchanging water molecules, enhancing contrast in MRI [33-34] and being uniquely sensitive to changes in conformation, permeability and flexibility of the cell membrane surface as depicted below.

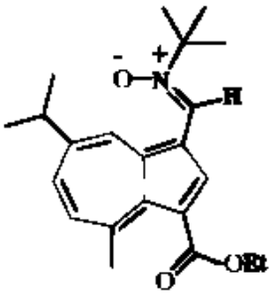


Figure 21. A colorimetric nitrone spin trap.

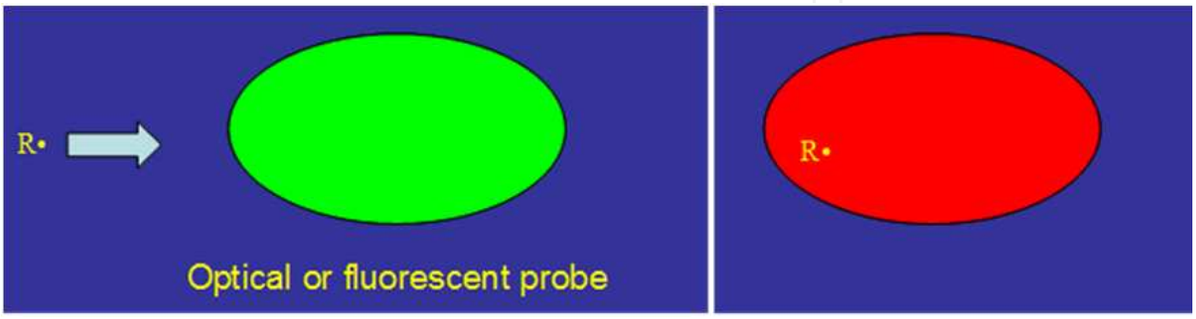


Figure 22. Schematic of the reaction of a colorimetric or fluorescent nitrone spin trap with a radical

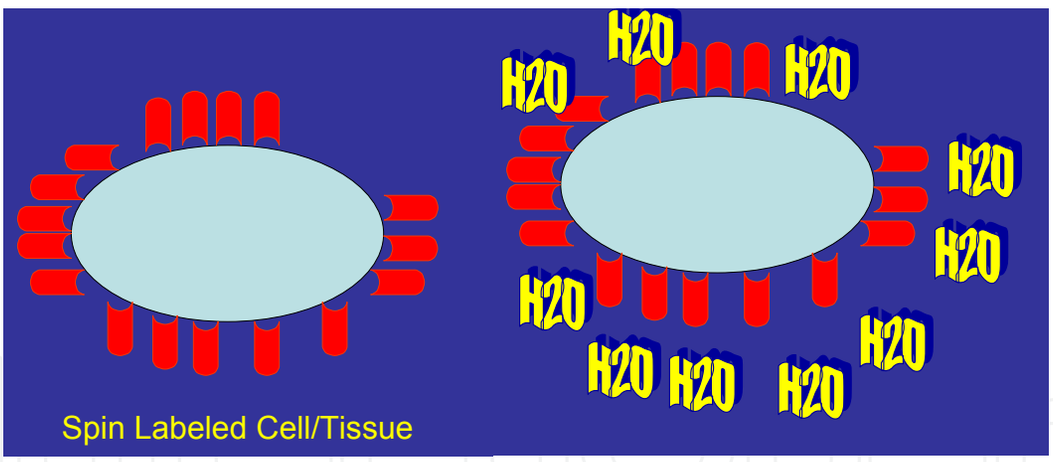


Figure 23. Schematic of a proton relaxation enhancement (PRE) spin labeled cell. Multiple paramagnetic labels are affixed to the cell surface by specific binding or covalent attachment. This results in a significantly enhanced PRE, which is detectable in MRI.

Hence future developments in several of these areas should show great promise for the future.

Author details

Lawrence J. Berliner
Dept. of Chemistry and Biochemistry, University of Denver, USA

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