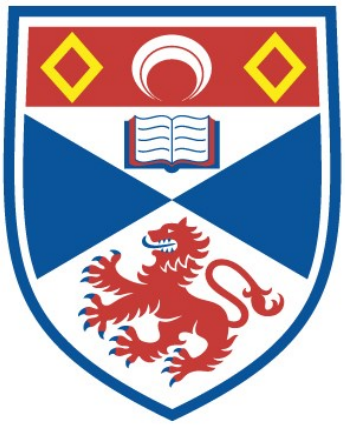
AN INVESTIGATION OF THE REACTIONS OF NITRIC OXIDE WITH SELECTED ORGANIC COMPOUNDS

Jonathan S. B. Clark

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1996

AN INVESTIGATION OF THE REACTIONS OF NITRIC OXIDE WITH SELECTED ORGANIC COMPOUNDS.

A thesis presented by Jonathan S. B. Park, B.Sc., to the University of St. Andrews in application for the degree of Doctor of Philosophy.

September 1995.

Declarations

I, Jonathan S.B. Park, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed _____ _____ Date 5/10/95

I was admitted to the School of Chemistry of the University of St. Andrews under Ordinance General No. 12 on the 1st October 1992 and as a candidate for the degree of Doctor of Philosophy on 1st October 1993.

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List of abbreviations

ACh	Acetylcholine
ACVA	4,4'-Azobis-4-cyanovaleric acid
ADP	Adenosine diphosphate
AIBN	Azobisisobutyronitrile
AIVN	2,2'-Azobis-2,4-dimethylvaleronitrile
BOOB	Di-t-butyl peroxide
cAMP	Cyclic adenosine monophosphate
CBC	Carbamyl choline
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
DBNBS	3,5-Dibromo-4-nitrosobenzene sulphonate
DETC	Diethylthiocarbamate
DIW	Deionised water
DMPO	5,5-Dimethylpyrroline-N-oxide
DMSO	Dimethylsulphoxide
DPPH	Diphenylpicrylhydrazyl
EDRF	Endothelium derived relaxing factor
EPR	Electron paramagnetic resonance
EtOH	Ethanol
FAD	Flavine adenine dinucleotide
Fe-PPIX	Iron protoporphyrin IX
FMN	Flavine mononucleotide
FT-IR	Fourier Transform infra red
GC	Guanylate cyclase
GC-MS	Gas chromatography-mass spectrometry
GTP	Guanosine triphosphate
GTP-CH	Guanosine triphosphate cyclohydrase I

H_4B	Tetrahydrobiopterin
hfs	Hyperfine splitting
HPLC	High performance liquid chromatography
L-NHMA	N-hydroxy-N-methyl-L-arginine
L-NMMA	N-monomethyl-L-arginine
MeCN	Acetonitrile
MeOH	Methanol
MetHb	Methaemoglobin
MGD	N-methyl-D-glucamine dithiocarbamate
MNP	2-Methyl-2-nitrosopropane
MS	Mass spectrometry
N-Arg	Nitroarginine
NADPH	Reduced nicotinamide adenine dinucleotide
NANC	Non-adrenergic non-cholinergic
NHA	N-hydroxyarginine
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NOCT	Nitric oxide cheletropic trap
NOS	Nitric oxide synthase
OxyHb	Oxyhaemoglobin
PBN	α-Phenyl-N-t-butyl nitrone
$PhBu^t$	t-Butylbenzene
PK	Protein kinase
RBS	Roussin's Black Salt
SDS	Sodium dodecyl sulphate
SIN-1	3-Morpholinosydnonimine
SNAP	S-nitroso-N-acetyl penicillamine
SNP	Sodium nitroprusside
SOD	Superoxide dismutase

TBDMS	t-Butyldimethylsilyl
THF	Tetrahydrofuran
UV	Ultra violet

Abstract

The reaction of nitric oxide (NO) with several organic and model biological compounds was studied to determine the ultimate fate of NO in a biological milieu, and to devise novel methods of trapping and analysing NO.

A series of potential spin traps including anthracene, *o*-quinone and 2-acetylcyclohexanone was investigated by bubbling NO through a solution of the above compounds. The resulting mixture was analysed by EPR spectroscopy and GC-MS. The EPR spectra obtained showed no radicals were produced and GC-MS analysis revealed only unreacted starting material.

Dienes were investigated because of their willingness to react with radical species. *Trans, trans*-1,4-diphenyl-1,3-butadiene did not form a nitroxide when reacted with NO, but in the presence of molecular oxygen, 2,5-dimethyl-2,4-hexadiene gave a mixture of a di-*t*-alkyl nitroxide and a *t-s*-dialkyl nitroxide.

Traps related to nitromethane anion were investigated in basic solution to generate the *aci* form, but the only compound to give a spin adduct was nitroethane, and this did not give the expected EPR spectrum.

2-Diazocycloheptanone was synthesised from cycloheptanone and reacted with NO. This reaction produced E and Z iminoxyl radicals and a carbonyl nitroxide. When the solvent was changed from *t*-butyl benzene to water, no reaction was observed. A known NO donor, S-nitroso-N-acetyl penicillamine (SNAP), was mixed with the spin trap and photolysed, but this did not produce a spin adduct. A second diazo compound, 5-diazouracil, was investigated, and this produced a radical species with complex but weak EPR spectra.

Nitric oxide was reacted with stable free radicals to provide a quantitative spectrophotometric test for NO. Tetraphenylhydrazine was prepared which is in equilibrium with the diphenylaminyl radical in solution. The diphenylaminyl radical trapped NO and was detected by UV/vis spectroscopy, reaching a λ_{max} after about one hour. When the solvent was changed from *t*-butyl benzene to acetonitrile, the reaction

took place much more rapidly. From these results it appeared that NO could be detected at concentrations beteween 10^{-4} and 10^{-7} M. Nitric oxide did not react with the parent compound, diphenylamine. When mixed with NO_2, diphenylamine formed nitro addition products. Another stable radical, galvinoxyl, was investigated. This did not rapidly scavenge NO to give an identifiable adduct.

Nitric oxide was reacted with several alkenes with electron-releasing groups and alkenes with electron-withdrawing groups. The reaction between *n*-butylvinyl ether and NO was investigated at room temperature. Many oxidation products were identified from the mass spectra obtained. Oxidation products were also identified from the reaction between NO and 1-cyclohexenyloxytrimethylsilane. Analysis by EPR spectroscopy revealed the presence of two radicals, likely to be dialkyl nitroxides. No products were observed in the reaction of NO and methyl linoleate.

EPR spectroscopy of a solution containing mesityl oxide and NO revealed the presence of three radicals, E and Z conformers of the iminoxyl radical, and a carbonyl nitroxide. 1-Acetylcyclohexene reacted with NO to give a di-*t*-alkyl nitroxide. Synthesis and subsequent photolysis of 1-acetylcyclohexenyl oxime confirmed this result. Another acceptor compound studied was tetracyanoethene; this did not react with NO.

Cyclopropylacetyl peroxide was investigated, as decomposition gives the cyclopropyl methyl radical. Analysis of the resulting solution showed that bicyclohexyl had been formed, but there were no traces of any nitrogen-containing products.

Nitric oxide did not react with either glutathione or dibenzyl disulphide, but NO_2 reacted with L-tryptophan forming a radical adduct. Analysis by EPR spectroscopy yielded a strong three-line signal, characteristic of a nitroxide.

The abstraction of hydrogen from, and the rearrangement of selected silylamines was investigated. The amines were photolysed in the presence of di-*t*-butyl peroxide in the cavity of the EPR spectrometer at temperatures ranging from

160K to 250K. The spectra obtained showed the unrearranged and rearranged radicals. No nitrogen-centred radicals were observed by EPR.

CHAPTER 1.

Background to nitric oxide research

1.1. Nitric oxide in chemistry and biology.

1.1.1. Biology of Nitric oxide.

Nitric oxide (NO) has recently become one of the most prominent molecules to be investigated by the scientific community. It has long been known that it is present as a pollutant in the atmosphere from exhaust emissions from flue gases and engines, but little was known about its biological properties. It came as a surprise to discover that such a transient and toxic molecule has what are now known to be vital roles in the mediation of several physiological processes.

In 1980, Furchgott and Zawadzki[1] had shown that the relaxation of blood vessels in response to acetylcholine (ACh), a neurotransmitter, and other substances, required the endothelium or inner lining of the vessel to be intact. Without this, vasodilation would not occur. It was found that the endothelium released a labile substance which diffused into the surrounding smooth muscle causing relaxation. This substance, originally termed the 'endothelium derived relaxing factor' (EDRF), was eventually identified as NO[2,3]. Prior to this, NO had been implicated as the active metabolite of nitro-glycerine, a known vasodilator, and other organic nitrates in the dilatation of blood vessels. The dilatation effected by NO occurs via the activation of guanylate cyclase (GC), and the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP)[4,5]. Nitric oxide was first implicated in the brain when cerebral cultures stimulated by excitatory amino acids released a substance which possessed the properties of NO[6-8].

1.1.1.1. *NO Formation.*

With the identity of NO firmly established as the EDRF, the question of production arises. How can such a molecule be endogenous? Work done by the Wellcome group of Ashton, Palmer and Moncada[9] gave evidence that it is a product of the L-arginine pathway, and is synthesised *in vivo* by the action of nitric oxide synthase (NOS) on L-arginine. Further work has since been carried out[10-12] which supports

both the identity of EDRF and confirms the requirement of L-arginine for function. This is summarised in figure 1.0.

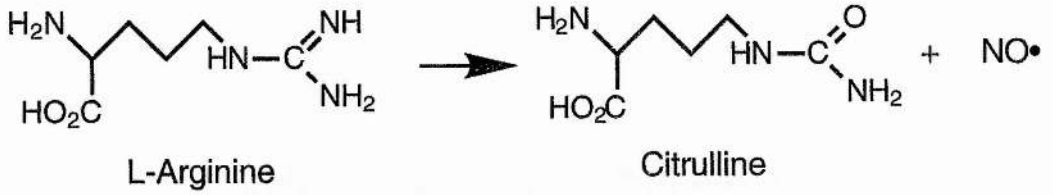


Fig. 1.0 The production of NO from L-arginine.

The synthesis of NO involves some unusual oxidative chemistry, which is mediated by two isoforms of NOS. The first of these is a constitutive isoform which is present constantly in the cell. This enzyme is regulated by calmodulin, a Ca^{2+}-dependent regulatory protein, and Ca^{2+}. The second isoform is of an inducible nature, and is found in macrophages. Isolation and purification of the constitutive form from rat and porcine cerebellum showed cytosolic dimeric proteins of M_r=150,000-160,000Da[13-15]. The inducible form from rat macrophage is also a dimeric protein Mr=130,000Da[16,17]. A membrane-bound endothelial isoform has also been isolated from bovine aorta, and this was reported to have M_r=135,000Da[18]. This too is an inducible isoform. Thus, there are no absolute criteria for the structure of these proteins, and as more cell types are discovered that produce NO, it may follow that there are different types of NOS for each cell type. Nitric oxide synthases oxidise the guanidine group of L-arginine in a process that involves the transfer of five electrons and the formation of NO and L-citrulline. Inhibition can be brought about by the use of simple alkyl arginine analogues for reversible inhibition of constitutive NOS, and irreversible inhibition for inducible NOS can be brought about with N-monomethyl-L-arginine (L-NMMA), or N-hydroxy-N-methyl-L-arginine (L-NHMA). The use of inhibitors is of clinical importance, and will probably need to be isoform specific, as a reduction in, for example, endothelial NOS would be detrimental combined with a reduction in brain NOS.

An important factor for elucidation of the mechanism of NOS action is the discovery of the amino acid sequence. This provides clues as to binding sites.

Structural similarities between the isoforms can be studied, and this has led to the suggestion that a single distinct gene is responsible for the production of the isoform[19]. Sequencing has been carried out on brain NOS[20], endothelial NOS[19,21] and macrophage NOS[22-24], and it has been shown that there is much regional conservation between each isoform, and also when compared to cytochrome P_{450} reductase. There are two domains, the reductase domain at the C-terminus end which contains binding sites for NADPH, FAD and FMN, and the haem domain which, in the NOS, is at the N-terminus end, unlike the P_{450} enzyme. These binding sites are involved in electron transport and thus it can be assumed that NOS acts in a similar fashion to P_{450}.

1.1.1.2. *Cofactors required for NOS.*

Like many enzymes in biological systems, NOS need cofactors to function. There are five cofactors used in the NOS redox system. As previously stated, the sequencing of NOS has proved the homology with cytochrome P_{450} reductase, including binding sites for FAD, FMN and NADPH. It could therefore be assumed that the electron flow will be in the same direction, such that NADPH reduces FAD, which in turn reduces FMN. This will occur in the reductase domain which is present on both NOS and P_{450} reductase[20]. Another link between P_{450} and NOS is that it has been shown that inducible NOS incorporates a prosthetic group, iron protoporphyrin IX (Fe-PPIX)[25], which is also provided by P_{450}. As further proof of this requirement, NOS is inhibited by CO[26] as is the conversion of N-hydroxyarginine (NHA) to L-citrulline.

The fifth cofactor required by NOS is tetrahydrobiopterin (H_4B). It has been reported that macrophage NOS is completely dependent on this cofactor[27,28], but purified brain NOS retains activity in the absence of H_4B[13,15]. This can be explained by the purification of brain NOS; H_4B is so tightly bound to the NOS that it copurifies, almost as an enzyme subunit[29]. It is still unclear what the exact role of H_4B is in the redox system. Initially it was thought that it was involved in hydroxylation of L-arginine, but this was challenged by Giovanelli *et al.*[30], who proposed that it had an allosteric effector role, or stabilised the NOS in some way. This latter suggestion was

supported by Bassenbacker and co-workers[31] who used pterin analogues to probe the NOS reaction; 6(RS)-methyltetrahydropterin which stimulates the initial rate of inducible NOS, and 6(RS)-methyl-5-deazatetrahydopterin, which inhibits citrulline formation. Marletta *et al.* [32] also confirmed this hypothesis. The domains can be represented thus (figure 1.1):

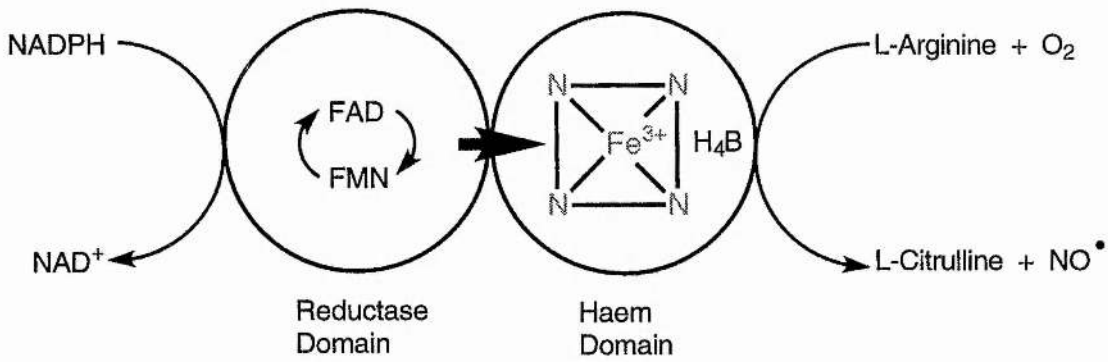
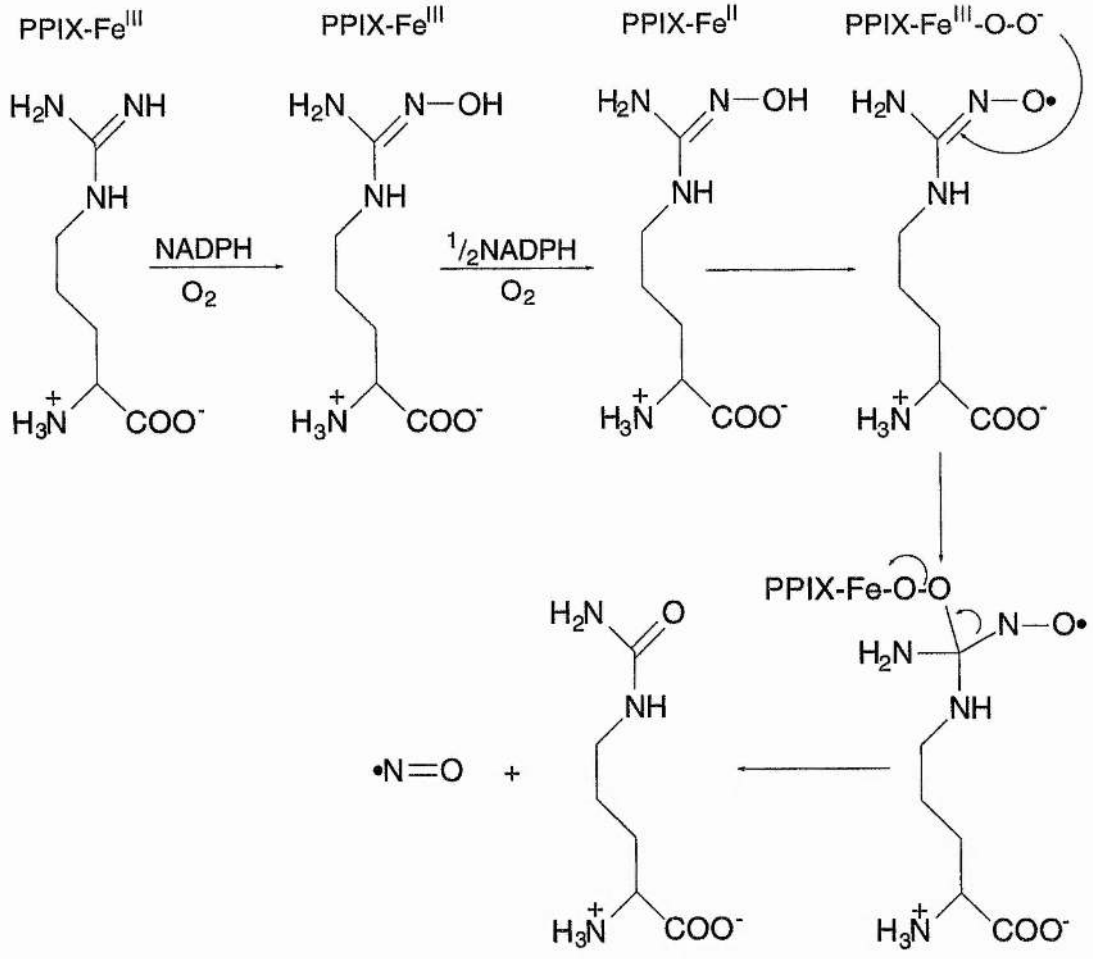


Fig. 1.1 The domains of NOS

1.1.1.3. *Mechanism of NOS action.*

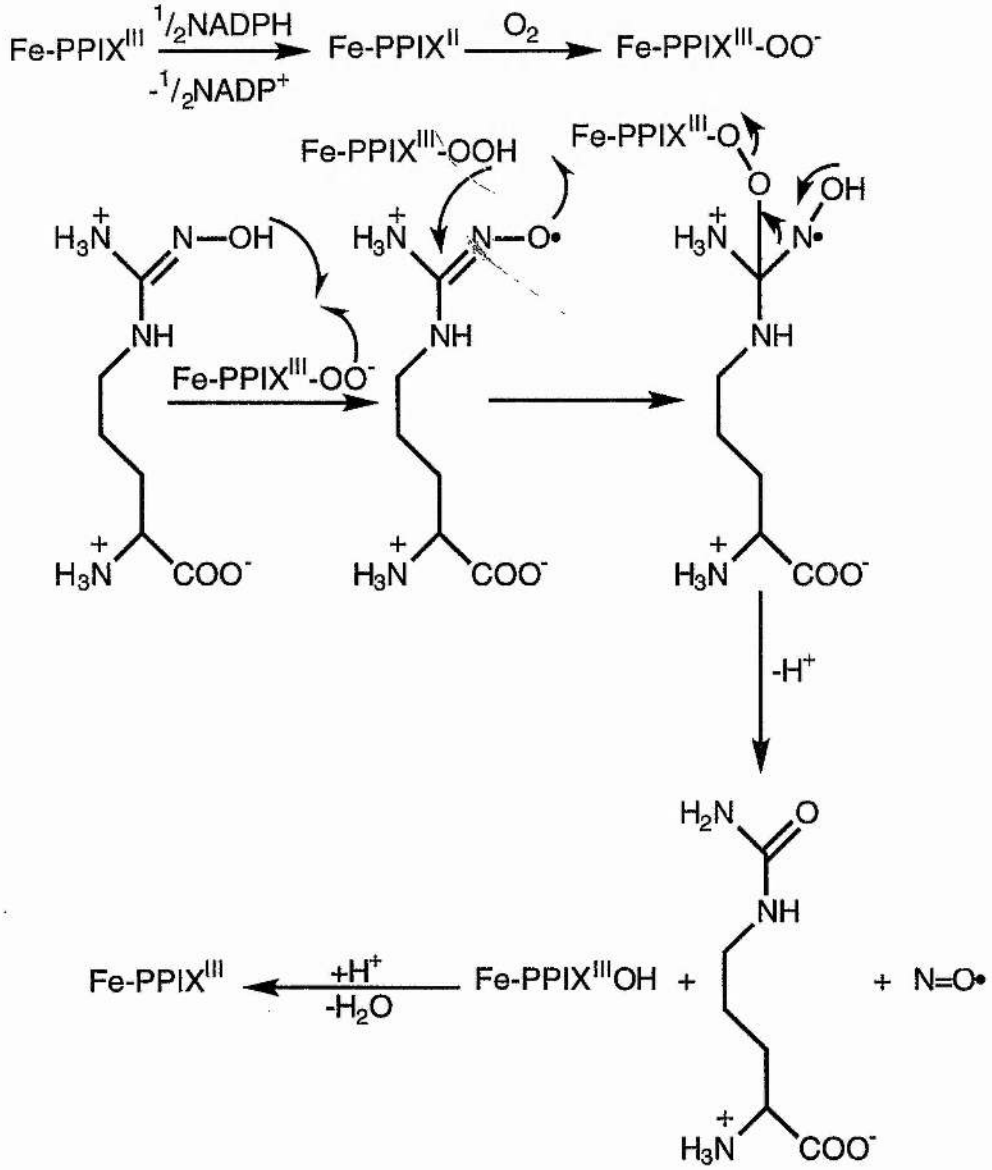
As previously mentioned, CO inhibits the formation of NO and L-citrulline, and the reaction requires Ca^{2+} and calmodulin as activators. The proposed mechanism for this reaction is analogous to that of the P_{450} enzyme aromatase. Aromatase catalyses three successive chemical steps all at the same active site. The first two steps involve formation of an Fe^{III} peroxide[33] which attacks the aldehyde functionality. The analogous mechanism for the action of NOS was first thought to be that shown below in figure 3[26]. The peroxide attacks the imine which decomposes to give L-citrulline and NO. This is represented in figure 1.2.

Fig.1.2 Proposed mechanism of NOS



A more recent proposal[34] aimed to clarify the second step of the mechanism proposed by Marletta, i.e. that of the conversion of NHA to the iron-arginine complex. NADPH is required for the initial conversion of Fe-PPIX^{III} to Fe-PPIX^{II}, which then picks up molecular oxygen to form the FePPIX^{III}OO• adduct. However, another electron must be provided to form the haem-peroxo complex Fe-PPIX^{III}OO$^-$, and this must come from the substrate. Korth and co-workers[34] assumed an intermediate is formed, the protonated oxime. A hydrogen is then abstracted from this by the Fe-PPIX^{III}OO• peroxyl radical. This is a more kinetically favourable reaction. The second electron is therefore transferred as a hydrogen atom. Consequently, it will be the peroxy species Fe-PPIX^{III}OOH which will act as a nucleophile toward NHA. The release of NO could then be from an aminyl radical, rather than the nitroxide radical. The Fe-PPIX^{II} is regenerated via the Fe-PPIXOH species. This is summarised in figure 1.3:

Fig. 1.3 Formation of haem-peroxo complex
and regeneration of iron complex



This would explain the production of NO and the renewal of the haem complex very neatly. The synthase would use both its haem and reductase domains for successive processes at a common active site, requiring NADPH and molecular oxygen for the oxidation of the arginine, and more oxygen for oxidation of the haem group.

1.1.1.4. *Regulation of NO synthase.*

There are several possible mechanisms of regulation *in vivo*. For example, cerebral NOS is activated by glutamate-stimulating N-methyl-D-aspartate (NMDA)

receptors which open calcium channels causing an influx of Ca^{2+}. This binds to calmodulin which activates NOS. Thus there is a rapid generation of NO in the brain. In vascular tissue, ACh will activate the phosphoinositide cycle to generate Ca^{2+}, also stimulating NOS. Again this is a rapid process. This is backed up by the use of calmodulin antagonists which inhibit cerebellar and endothelial enzymes[13]. The inducible macrophage NOS is resistant to Ca^{2+} activation due to the close binding of calmodulin to the enzyme[35].

Macrophages do not display any NOS protein under normal circumstances. If they are subjected to an external inflammatory stimulus, such as interferon-γ, then NOS protein will be synthesised over a period of time, mediating the cellular response.

It has been found that many cell types other than macrophages are capable of producing inducible NOS in response to the appropriate stimulus[36,37]. These cells include hepatocytes, endothelial cells, pancreatic islets and fibroblasts. The main agents of stimuli are microbes, their toxic products and inflammatory cytokines. Not all, however, elicit the same responses in each cell type[38].

Phosphorylation has a regulatory effect on NOS. Amino acid sequences for phosphorylation by cAMP-dependant protein kinase (PK) are evident in brain and endothelial NOS, but are not as apparent in macrophage NOS[39]. Studies carried out, however, indicate that neuronal NOS can be phosphorylated by cAMP-dependant PK, PK-C, cGMP-dependant PK, and Ca^{2+}/calmodulin dependant PK[40-42]. Phosphorylation by all of these lowers catalytic activity[40]. These methods of activation/inhibition are thus linked and could be loosely termed self-regulatory.

In addition to these regulators of NOS as an enzyme, there also arises the question of substrate and cofactor availability. For instance, arginine is the starting molecule for NO production, and thus the availability of this amino acid must regulate the quantity of NO produced. It has also been shown[43-45] that uptake and synthesis of arginine are increased when there is induced NOS stimulation. Arginine is actively transported into the cell via protein carriers when there is a stimulus such as endotoxin or cytokine invasion. Increased arginine transport could not maintain high levels of

NO at certain sites, for example at a wound, so increased synthesis must account for extra NO production. The major sites of arginine synthesis are the liver, where it is formed and rapidly degraded in the urea cycle, and the kidney, where citrulline is extracted from the plasma and converted to arginine. This is subsequently released into the vascular system[46]. Recently it was discovered that induction of NOS and increased activity of argininosuccinate synthase occur in tandem[47,48]. Argininosuccinate synthase is a key enzyme in the conversion of citrulline to arginine, in conjuction with argininosuccinate lyase. Thus, citrulline can be used to provide a supply of arginine, and consequently NO production is raised.

The level of H_4B present in the cell will also affect the rate at which NO is produced. This cofactor is synthesised from GTP[49], and the main enzyme in the pathway is GTP cyclohydrase-I (GTP-CH). It has been reported that GTP-CH levels are lower in unstimulated rat endothelial tissue than in stimulated[50], indicating a coinduction of NOS and H_4B. This latter will maintain the activity of the induced NOS.

1.1.2. Nitric oxide in the body.

The production of NO has been discussed, but what exactly is the role of NO when it is released? The versatility of this small molecule is quite spectacular, but it is still a dangerous species if unchecked.

1.1.2.1. *Vascular NO.*

Nitric oxide produced by the endothelial cells lining the blood vessels can have wide ranging effects. As mentioned above, it causes relaxation of the surrounding smooth muscle leading to reduced blood pressure[2,3], and it is also involved in a variety of other regulatory processes. Endothelial NO affects the aggregation of platelets in the vascular system to prevent clotting. This is not advantageous in a wound situation, but it does have therapeutic value, i.e. to prevent infarcts in heart disease, for example[52]. The actual mechanism responsible for inhibition of platelet aggregation is not fully understood, but could include effects on guanylate cyclase, phospholipase C[53], or adenosine diphosphate (ADP) ribosylation[54-56].

Nitric oxide also inhibits leukocyte adhesion and degranulation of mast cells[57], and is thought to be antiatherosclerotic[58]. It has regulatory effects on the lungs contributing to bronchodilation and ventilation-perfusion matching, whereby the blood flow is balanced with the oxygen uptake.

Nitric oxide has an important role in both the gut and the immune system. When stimulated by bacteria or other foreign substance, there will be induction of NOS in the macrophages leading to the production of NO. This destroys the invasive bacterium, and has recently been shown to be tumouricidal[36]. Recent studies have revealed that NO has antiviral action[59]. Despite all the benefits of NO, excessive production can lead to hypotension, which is a killing factor in septic shock, inflammation, microvascular leakage and tissue damage. This can be dealt with at a pharmacological level by the administration of NOS inhibitors.

1.1.2.2. *Nitric oxide and the CNS.*

In certain cell types NO is not always present. It is synthesised on demand and is not stored, and has intracellular targets rather than specific receptors. This fits the description of both a hormone and a neurotransmitter. Thus NO could be expected to have roles in nervous transmission, interpretation of stimuli, sensory perception and any other process involved with cerebral input/output.

In the peripheral non-adrenergic non-cholinergic (NANC) nerves, NO is used as a mediator to decrease muscle contractility, free Ca^{2+} and promote bronchodilation[57]. It is also responsible for gut peristalsis, and NOS inhibitors block this process[60,61], indicating that NO is the neurotransmitter. Nitric oxide is present in neurons in the cerebral cortex and the cerebellum. Here it acts at NMDA receptors to mediate the response to glutamate, an excitatory neurotransmitter[6]. Glutamate release is presynaptic, and causes an influx of Ca^{2+} into the cell. This will induce NOS to act on arginine and produce NO. This is a useful process, as neural damage resulting from stroke is attributed to overstimulation of glutamate receptors[62]. Nitric oxide has also been implicated in memory and learning[63], and also in sensory processing. The application of NOS inhibitors interferes with the acquisition and retention of

behavioural tasks based on spatial learning, and also with olfactory social memory. Pre-acquired memory does not appear to influenced by NO.

The visual, olfactory and pain pathways also appear to be linked to NO. In the eye rod photoreceptors, two cGMP systems are regulated by opposite changes in free Ca^{2+}. In one, GC is Ca^{2+} inhibited, while in the other, a Ca^{2+}-activated NOS is coupled to a soluble GC[64]. Breer and Shepherd[65], studying the olfactory system of mammals and insects, found that increases in cGMP response to pheromones or high odourant concentrations modulate synaptic transmission. There could feasibly be recruitment of neurons dependent on the stimulus, if there was an increase in both types of stimuli.

Even in the area of reproduction NO plays a part. NOS neurons are prominent in penile tissue. Electrical stimulation of the cavernous nerve in rats gives penile erection, which is blocked by low doses of intravenous NOS inhibitors[66]. Nitric oxide must therefore be the transmitter.

As mentioned above, an excess of NO is in no way beneficial, especially concerning the CNS. In addition to stroke, glutamate stimulation may also contribute to neurodegenerative diseases, such as Alzheimer's and Huntingdon's Chorea. Experiments carried out by Dawson *et al.* [67] showed that NO mediates glutamate neurotoxicity. Addition of nitroarginine (N-Arg) and L-NMMA, potent NOS inhibitors, reduce cell death in a concentration-dependant fashion.

Nitric oxide can also act as a radical scavenger. The superoxide free radical anion, $O_2^{\bullet-}$ can be produced in the body and cause extensive tissue damage. It was generated from polymorphonuclear leukocytes[68], and when analysed spectrophotometrically it was found that NO had combined with the superoxide. This is beneficial in that superoxide is a major cause of cell injury. However, the combination of $NO^{\bullet}$ and $O_2^{\bullet-}$ leads to the formation of the peroxynitrite anion:

$$NO^{\bullet} + O_2^{\bullet-} \longrightarrow ONOO^-$$

At physiological pH this anion becomes protonated, but is extremely unstable, having a half life of ~ 1.9s at pH 7.4[69]. This decomposes homolytically to give:

The production of the hydroxyl radical, OH•, is important, because this is far more reactive than O_2^{-}• or NO•. Superoxide dismutase (SOD) will often reduce tissue injury, indicating that O_2^{-}• plays a significant part in the cytotoxicity of NO *in vivo*. However, other work has suggested that NO acts as a scavenger for $O_2^{\bullet-}$ and has a cytoprotective role[70], therefore it could also be effective against OH•.

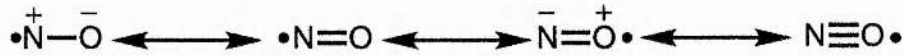
1.1.3. Summary.

From the above, it can be seen that NO is one of the most important molecules to have been 'rediscovered' in recent times. It is small, simple, reactive and present in many types of cell. It has an essential role as a bodily mediator, but can also be detrimental. An apt description came from the BBC in 1992- A Vital Poison.

1.2. Chemistry of nitric oxide.

1.2.1. Introduction.

Nitric oxide is a simple diatomic molecule which exists as a colourless gas at room temperature and is highly toxic. It possesses a lone pair of electrons together with an unpaired electron which imparts free radical character. It can best be represented by the following canonical structures:



It does not dimerise readily, and is paramagnetic, and although no EPR spectra have been recorded in the gas phase, EPR spectra have been obtained when NO was trapped in a matrix. Its reactions with other free radicals have been widely studied.

1.2.2. Preparation of NO.

Nitric oxide has been produced in a variety of ways. The direct combination of nitrogen and oxygen at room temperature is thermodynamically unfavourable[71], and is only achieved at elevated temperatures, such as in engines. Escape of this into the environment leads to formation of NO_x pollutants from the atmospheric oxidation of

NO. On an industrial scale, NO is produced from the oxidation of ammonia over a platinum catalyst:

$$4NH_3 + 5O_2 \xrightarrow{Pt} 4NO + 6H_2O$$

In the laboratory, NO can be generated from the reduction of nitric acid with copper:

$$3Cu + 8HNO_3 \longrightarrow 3Cu^{2+} + 6NO_3^{2-} + 4H_2O + 2NO$$

or by treating hydrogen iodide with nitrous acid:

$$NO_2^- + H^+ \longrightarrow HONO$$

$$I^- + H^+ \longrightarrow HI$$

$$2HI + 2HONO \longrightarrow I_2 + 2H_2O + 2NO$$

It is likely that all these methods of production also generate impurities in the form of NO_2. One method that gave high quality NO was the reaction of sodium nitrite with ascorbic acid, as described in section 2.4.1.1.

1.2.3. Properties.

One of the most studied reactions of nitric oxide is its oxidation to NO_2, a reaction that could feasibly occur under physiological conditions. There is still speculation about the mechanism of this reaction, but the accepted explanation is that a dimer is formed (with negative ΔH^o value), which then reacts with oxygen in the rate limiting step to form NO_2. Kinetic studies of this oxidation in aqueous solution have revealed that no nitrate is formed[72], but the products are nitrous acid and nitrite, depending on the pH. Awad and Stanbury[73], investigated the kinetics of the reaction in aqueous solution and found by ion chromatography that the sole product of reaction between NO and O_2 was NO_2^-. The overall kinetics of the aqueous reaction were summarised into three steps:

$$NO + O_2 \longrightarrow NO_2 \qquad k$$

$$NO_2 + NO \longrightarrow N_2O_3 \qquad \text{fast}$$

$$N_2O_3 + H_2O \longrightarrow 2NO_2^- + 2H^+ \qquad \text{fast}$$

The results also showed that the reaction was pH independent and worked in excess of NO or O_2. Recent work on the oxidation of NO and HNO_2 in aqueous solution has confirmed these observations[74]. In the early 1960's, NO was investigated as a potential electron pair acceptor[75], as it was known that NO reacted with the sulphite ion. It was proposed that NO reacted with amines acting as Lewis bases to generate nitrosamines. Interest in nitrosamines was stimulated when it was suggested that they were carcinogenic[76]. Much work was carried out in the area, but results became somewhat muddled, as groups reported findings that are now realised to be erroneous. The most common mistake was having a non-airtight system, so the entry of oxygen facilitates the formation of NO_2. Once complete removal of oxygen was carried out, it was found that pure NO only reacted slowly with secondary amines[77]. If oxygen was allowed access to the reaction, rapid nitrosation was effected. Direct nitrosation involving carbon-centred radical intermediates has been reported[71] and this leads to the formation of the oxime. Nitric oxide reacted with dialkylmercury compounds on U.V. irradiation or heating to give nitroso compounds via radical intermediates[78]. Other displacement reactions include the nitrosation of alkyl Grignard reagents to give alkyl nitroso compounds from intermediate N-nitrosohydroxylamines.

One particular group of compounds that seem to be immune to nitrosation by NO alone are thiols. It was first found in 1962[79] that the reaction of NO with thiols gave rise to symmetrical disulphides. Later, reaction of several thiols with NO and NO_2 was studied[80], and it was found that although the disulphide was the major product in each reaction, only those reacted with NO_2 yielded the nitrosothiol as an intermediate reaction product. A radical mechanism was proposed for this pathway. There has been much recent interest in the actions and reactions of nitrosothiols[81].

Related to NO in their action as nitrosating agents are dinitrogen trioxide, N_2O_3, and dinitrogen dioxide, N_2O_4. Gaseous N_2O_3 breaks down into NO and NO_2, and in aqueous solution the accepted convention is that nitrous acid exists with N_2O_3 in equilibrium:

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

At elevated nitrous acid concentrations, the solution may appear blue due to the quantity of N_2O_3 present. N_2O_4 exists in equilibrium with NO_2 as:

$$2NO_2 \rightleftharpoons N_2O_4$$

Both N_2O_3 and N_2O_4 are effective nitrosating agents in the gas phase or in the liquid phase. In organic solvents, gaseous N_2O_3 reacts with secondary amines to give N-nitroso compounds[77,82] and N_2O_4 reacts with secondary amines to produce N-nitroso compounds and N-nitro compounds[83]. The formation of nitroso compounds has been of paricular interest in the study of cigarette smoke and atmospheric chemistry. Other studies have revealed that a radical process is responsible for the formation of the nitroso compound from the photodissociation of HNO_2 and its subsequent reaction with NO. This was thought to occur in the atmosphere which aids in the production of nitroso pollutants:

$$HONO \xrightarrow{h\upsilon} OH\bullet + NO\bullet \xrightarrow{R_2NH} H_2O + R_2N\bullet \xrightarrow{NO} R_2NNO$$

In aqueous media, nitrosation was expected to occur either slowly or not at all, because it was thought that there would be hydrolysis to NO_2^-:

$$N_2O_3 + 2OH^- \longrightarrow NO_2^- + H_2O$$

$$N_2O_4 + 2OH^- \longrightarrow NO_2^- + NO_3^- + H_2O$$

Hydrolysis does occur, but is slower than the nitrosation of some amines. It has been suggested that N_2O_3 and N_2O_4 exist as tautomers, each with a more reactive nitrite form:

$$O{=}N{-}NO_2 \rightleftharpoons O{=}N{-}ONO$$

$$O{=}N{-}O{-}NO_2 \rightleftharpoons O_2N{-}NO_2$$

It has been shown that reactions in aqueous media are inhibited by acids, sodium azide, alcohols and primary amines. However, they are catalysed by the presence of nucleophilic anions, 1,2-alkanolamines, and 1,2-dihydroxy compounds. Nitrosation in aqueous media is dependent on the basicity of that medium and also on the basicity of the amine to be nitrosated.

In the absence of acid, simple alcohols retard nitrosation of amines because they react with the nitrosyl gases formed to give a nitrite ester, where Y= any halide group:

$$ROH + YNO \xrightarrow{OH^-} RONO + Y^- + H_2O$$

Alcohols that possess an electron-withdrawing group in the position β to the hydroxy usually increase the yield of nitrosamine. A mechanism for this reaction involves the formation of the nitrite ester and then upon addition of the secondary amine, this undergoes a transfer of the nitroso group to regenerate the alcohol. Activation by the β-substituent renders the alcohol reactive toward the amine under nonacidic conditions. Experimental evidence has shown that the reactivity of simple alkylnitrite esters toward secondary amines increases with the electron-withdrawing ability of the β-substituent. The extent of nitrosation is also dependent on the reactivity of the amine.

From this it can be seen that N_2O_3 and N_2O_4 are particularly effective nitrosating agents in organic and aqueous media.

Nitric oxide has been known to act as a ligand in transition metal complexes for many years. Possibly the most well known of these are the nitroprusside anion, $[Fe(CN)_5NO]^{2-}$, and Roussin's Black Salt (RBS), $[Fe_4(NO)_7]^-$. These are known as nitrosyl complexes and many have been synthesised for a large number of the transition metals. Synthesis of these complexes can take place by a variety of methods. Direct use of NO has only a few precedents[84], with ruthenium and osmium. The problem with using NO in this way is that the nitrogen donates three electrons. If, for example, a carbonyl ligand is replaced, the third excess electron can cleave the metal-metal bond to promote decomposition[85]. More usual methods involve the nitrosonium cation, NO^+, or nitrite ion to introduce the NO subunit. The structures of these complexes were elucidated by ^{14}N NMR, EPR, vibrational and photoelectron spectroscopy, and X-ray crystallography. Those ligands which contain the NO unit are capable of releasing that unit when decomposed thermally or photolytically.

Much work has been carried out on the reaction of NO with haem and porphyrin systems[86] which have been analysed by EPR spectroscopy. Kinetic studies have also been carried out on the binding of NO to metal nitrosyls. It was found that

phthalocyaninatoiron (II) bound NO in a 1:1 mole ratio via *pseudo* first order kinetics[87]. Many more studies on the action of NO as a ligand have been undertaken, and there are numerous papers on the subject[88]. A number of nitrosyl complexes can act as nitrosating agents by direct nucleophilic attack. The most studied of these is sodium nitroprusside (SNP) anion[77], and its reaction with amines. It was determined that a complex between the anion and the amine was produced, rapidly but reversibly, which was then decomposed by the reaction between another molecule of amine or water.

1.2.4. Summary.

From its reactions, we can see that the chemistry of nitric oxide is far from fully understood, and the opportunities for further work are vast. Nitric oxide is surprisingly reluctant to take part in reactions as a radical, and does so usually in the presence of its gaseous relative, NO_2. It is only willing to form complexes under selected conditions.

CHAPTER 2.

Nitric oxide and spin traps

2.1. Development of spin traps for nitric oxide.

2.1.1. Detection of nitric oxide and its metabolites.

Nitric oxide is too unstable in biological milieu to be studied at length as a discrete molecule. The study of NO by the spin trap method is becoming increasingly important as a research tool. It eliminates the need for the large quantities of material that are used in continuous flow experiments, and in systems which are too complex or large to monitor continually *in vivo*.

Spin trapping may become the analytical method of choice, but there are other methods available for the detection of NO. For instance, the development of a recent method of analysis utilises the spectrophotometrically monitored, NO induced conversion of oxyhaemoglobin (oxyHb) to methaemoglobin (MetHb)[89], the reaction being detected via its vasodilatory activity.

$$HbO_2 + NO\bullet \longrightarrow MetHb + NO_3^-$$

In the gas phase, the chemiluminescence of NO_2 from the reaction of NO with ozone [90] was utilised.

$$O_3 + NO\bullet \longrightarrow O_2 + \bullet NO_2 + h\upsilon$$

Linked with NO are the anions nitrite (NO_2^-) and nitrate (NO_3^-). These two groups are secondary products of the oxidation of NO. Spectrophotometric assay can be used therefore to detect the presence of NO via the Greiss reaction[91], involving diazo coupling. In the case cited, biological solutions such as urine, saliva and deproteinised plasma were investigated. The nitrate in a sample was reduced to nitrite which reacted with a Greiss reagent to form a purple azo dye. Reduction of nitrate is facilitated by passing NO_3^- down a cadmium column. Nitrite will react with the Greiss reagent anyway, so if this is present, the column can be bypassed. Detection is via a UV/vis spectrophotometer. The reagent used comprised 1 part 1% naphthylethylenediamine in distilled water plus 1 part 1% sulphanilic acid in 5% concentrated H_3PO_4. For quantification of results, the acid-catalysed nitration of benzene by NO_2^+, and its subsequent detection by GC-MS was employed. To reduce

interference from other compounds, especially in urine, such as citrate and phosphate, High Performance Liquid Chromatography (HPLC) was used.

The detection of NO by the above methods is mainly suited to *in vitro* analyses. In biological systems, the quantity of NO generated is nanomolar, and decays usually in less than one second. An easily applicable *in vivo* tool is thus required for constant monitoring of NO production. Such apparatus is the NO-selective electrode. This was first applied to detect the presence of NO in brain tissue slices[92]. A platinum wire in a glass micropipette filled with KCl solution was used as the working electrode. A thin membrane sealed the tip, and was only permeable to small gaseous molecules such as NO. The current measurement was based on the following electrochemical equation:

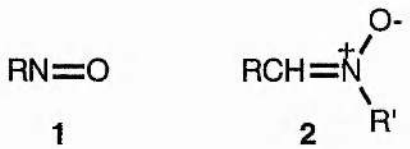
$$NO\bullet + 2H_2O \longrightarrow NO_3^- + 4H^+ + 3e^-$$

A positive voltage was applied to counter any current from contaminants such as O_2. The disadvantages of this first electrode were that it was too fragile, it was difficult to construct, and it could not penetrate more resistant tissue. An improved version using a stainless holder was developed[93], and another group[94] devised a more sensitive electrode with a nickel (II) porphyrin coating. The most recent innovation came from Ichimora *et al.*[95], utilising a Pt/Ir alloy electrode coated with a nitrocellulose film. This is more stress resistant than its predecessors. The electrode consists of three layers; an outer silicon membrane to reduce ionic effects and unwanted reactions. The middle membrane is the NO-detecting nitrocellulose polymer, and the inner layer is made up of KCl, deposited to prevent overvoltage in the NO discharge. The counter electrode is made of carbon fibre. Nitric oxide was generated by means of S-nitroso-N-acetyl penicillamine (SNAP) which decomposes either thermally or photochemically. Segments of rat aorta were also used to test the efficacy of the electrode biologically. The results showed that the electrode was very sensitive and able to detect nanomolar concentrations with a rapid response time. However, there was also a disadvantage, in that the electrode required calibration with an NO standard prior to use, because each electrode was different, and subject to time-dependent decay. Secondary products,

such as NO_2^- and NO_3^- do not interfere with the electrode's efficiency. It would appear that the selectivity is due to large molecules or charged species being unable to penetrate the membrane layers. Additionally, O_2 is not involved because the electrode operates at a range (0.4 to 0.8V) where O_2 is not electrolysed. The effects of various parameters were also tested; pH, temperature, and oxygen dependence. Negligible effects were recorded by a change in pH (range 6 to 8.5), but the current did rise with temperature. Increased oxygen concentration decreased the current, because O_2 scavenges NO. Thus, O_2 and temperature must be kept constant for the electrode to function with maximum effect. It appears then, that the development of a sensitive, NO-selective electrode is a major analytical method for *in vivo* detection of NO.

All the aforementioned types of detection have a limited range and/or a lack of specificity. Consequently, new methods are desirable.

It appears that the first systematic study of the production and trapping of free radicals was carried out by Lewis and co-researchers in the 1940's[96]. They discovered that irradiation of aromatic compounds by UV light caused dissociation either into two radicals, a positive and negative ion, or a positive ion and an electron. If a reaction known to produce radicals is carried out in the presence of a radical scavenger, this scavenger will then react with the radical produced and form a relatively long-lived, stable radical species. This can then be observed using electron paramagnetic resonance (EPR) spectroscopy. The two most commonly used types of trap are nitroso compounds **1**, and nitrones **2**.

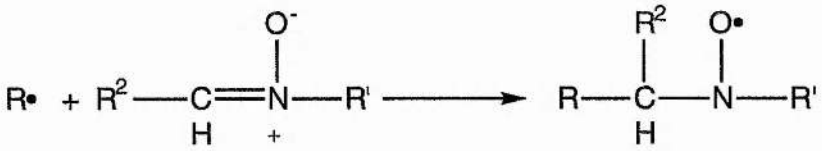


1 **2**

Both of these react to give stable nitroxides. The EPR spectra of nitroxides give a characteristic 3-line signal, in the ratio 1:1:1. This splitting is due to the interaction of the single electron with the ^{14}N nucleus. The triplet signal will also be split by magnetic nuclei of the R or R' groups coupling with the lone electron. Thus, the structure of the new adduct could feasibly be deduced in favourable cases by

interpretation of the spectrum. The trapping of NO is desirable because it allows the direct study of the sites of production of NO and possibly its ultimate fate, i.e. if the reaction of NO to form NO_2 for example occurred to 'mop up' excess NO after inhibition of NOS, then a spin trap could be introduced at a strategic point in a proposed pathway in order to determine where the NO finally reacted.

The trap employed would have to be selective for NO, as NO_2 is itself a radical so would also be detectable by EPR spectroscopy. Another advantage of spin trapping is that EPR spectroscopy is very sensitive, able to detect nanomolar quantities which is a sufficient concentration for biological systems. The use of nitroso compounds is favoured over nitrones, because, even with increased thermal and photochemical stability, the nitrone spectrum only shows a nitrogen triplet and a proton doublet, as the incoming radical will attack at the carbon β to the nitrogen:



Magnetic nuclei in the trapped radical are too far away to show significant coupling with the unpaired electron. Thus, it would be difficult for the structure of the radical R• to be determined[97].

2.1.2. Spin traps in use.

The technique of spin trapping has now been with us for some thirty years. Many reviews have been published and it would be impractical to review all of the work carried out. Some investigations will be described as they are pertinent to the general subject matter.

Nitric oxide has not been used extensively on its own as a trapping agent. It has been shown that it is possible[98] to trap biradicals produced by photocleavage of cycloalkanones. More recently, Tudos and coworkers[99] generated dialkylnitroxide radicals by the irradiation of solvents, then bubbling NO through the mixture. It was found that the radical formation occurred in two steps, the first being the formation of

the nitroso compound, then the pick-up of a further alkyl radical. If the solution was saturated with NO, a further reaction would occur:

$$R\bullet + NO \longrightarrow RNO$$

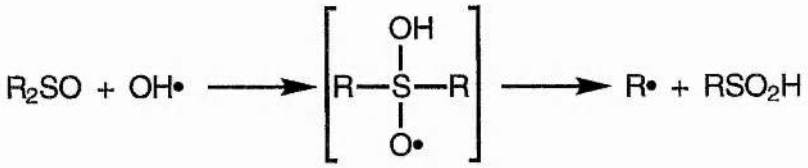
$$R\bullet + RNO \longrightarrow R_2NO\bullet$$

$$R_2NO\bullet + NO\bullet \longrightarrow R_2NONO$$

This reaction was also dependant on the temperature. If the temperature was lowered to -50^0C, then the lifetime of the dialkylnitroxide radical increased, even in NO saturated solution. On warming, the EPR signal disappeared.

It has been demonstrated that spin trapping is a good diagnostic technique for structure determination of radical adducts. If a radical scavenger, e.g. a nitroso compound RNO, is used, then it is crucial that the structure of the trap does not interfere with the structure of the newly formed adduct, i.e., if an asymmetric nitroxide of the form RR'NO• is generated, then R' must not obscure the secondary splittings on the EPR spectrum. Lagercrantz[97] generated stable nitroxide radicals by the use of t-alkylnitroso compounds as radical scavengers. Irradiation by UV light in the presence of an initiator, $Pb(OAc)_4$, provided evidence of oxidation and the formation of stable nitroxides. This led to the successful identification of the intermediates in the oxidative decarboxylation of acids, and the oxidation of alcohols. Hydrogen peroxide was also used as an initiator to abstract a hydrogen from aliphatic hydroxy and carbonyl compounds via the production of the hydroxyl radical (OH•). The spectra recorded showed that the trapped radicals had generally been produced by abstraction of the hydrogen α to the functional group, i.e., RC•HOH were produced from alcohols and RC•HCOOH were formed from acids. Cyclic ketones gave rise to radicals formed by ring-opening reactions. Radicals have also been produced from aryl iodides[100] and organometallic compounds[101] by photochemical fission, both types of which have been trapped as nitroxides. Symmetrical and asymmetrical sulphoxides produce radicals when photolysed in the presence of H_2O_2. These are of the same general nature as those produced by dialkyl compounds, i.e., R_2SO gives the radical R•, but $R^1S(O)R^2$ will furnish a mixture of radicals $R^1\bullet$ and $R^2\bullet$. Lagercrantz also studied the action of

H_2O_2 on sulphones. Generally, their behaviour is different to that of sulphoxides. No radicals were trapped from dimethyl- and diethylsulphone, but hydrogen abstraction from the methylene groups of di-n- propyl- and di-n-butylsulphones did give trappable free radicals. A mechanistic proposal for the formation of the radicals from sulphoxides can be generalised:



3

i.e. it proceeds via OH• attack. As the sulphone sulphur is already bonded to four atoms, it will not undergo this reaction. However, when a more synthetic approach was taken, involving alkylation of aromatic and heteroaromatic molecules, it was suggested that an adduct is formed first which subsequently fragments to give the radical complex **3** shown above. Methylated products were obtained when t-BuO• was used instead of OH•, but it was found that the CH_3• radical was derived from t-BuO•, and not the sulphoxide. Labelling experiments with deuterated DMSO confirmed this suggestion.

Investigations have also been carried out on N-halogen substituted compounds and phosphorous-centred radicals. Imides such as N-bromo- and N-chlorosuccinimide have been studied, and these gave very similar spectra when photolysed in the presence of t-nitrosobutane. Radicals are also formed from organophosphorus reactions as intermediates in the addition of primary and secondary phosphines to alkenes. When a solution of Na_2HPO_3 was photolysed with t-nitrosobutane, a 6-line EPR spectrum was observed. This was thought to be from the trapping of the radical anion $\bullet PO_3^{2-}$, formed by hydrogen abstraction from the parent anion, HPO_3^{2-}.

More recently, Lagercrantz[102] found that reactive alkyl radicals were formed during the photochemical decomposition of various azo compounds, including 2,2'-azobisisobutyronitrile (AIBN), 2,2'-azobis-2,4-dimethylvaleronitrile (AIVN), and

4,4'-azobis-4-cyanovaleric acid (ACVA). The interaction between the above, NO• and different solvents was studied, and this led to the following proposal for the reaction between EtOH, AIBN, and NO•. The initiator, AIBN, is photolytically cleaved into two 2-cyanopropyl radicals and nitrogen:

$$NCC(CH_3)_2{-}N{=}N{-}C(CH_3)_2CN \xrightarrow{h\upsilon} 2NC{-}\dot{C}(CH_3)_2 + N_2$$

The resultant propyl radical brings about homolytic abstraction of a methylene hydrogen from EtOH:

$$NC{-}\dot{C}(CH_3)_2 + CH_3CH_2OH \longrightarrow CH_3\dot{C}HOH + NCC(CH_3)_2H$$

A 2-cyanopropyl radical can also pick up NO• forming the 2-cyanopropylnitroso compound. This is then subject to attack by the carbon-centred hydroxyethyl radical which generates the dialkyl nitroxide:

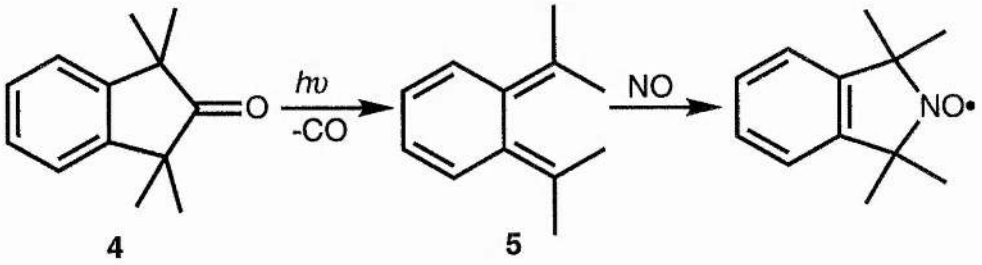
$$NC{-}\dot{C}(CH_3)_2 + NO\bullet \longrightarrow NCC(CH_3)_2NO$$

$$NCC(CH_3)_2NO + CH_3\dot{C}HOH \longrightarrow NCC(CH_3)_2N(O\bullet)CH(CH_3)OH$$

Similar results were obtained with methanol and propan-2-ol and with AIVN and ACVA. No radicals were detected in the absence of NO, so the 1:1:1 nitrogen splitting observed by EPR spectroscopy must be from the nitrogen of NO rather than that of AIBN. Isotope studies confirmed this. The yield of aminoxyls was lowered if O_2 was introduced. The solvent was changed to dimethyl sulphoxide (DMSO) but not degassed. Consequently, NO_2• was produced and this gave rise to a previously identified product. Nitric oxide concentration was estimated from the maximum amplitude of the peaks from the EtOH/ H_2O ACVA experiment. Thus it can be seen that the alkyl radicals take part in all aspects of the reaction, as hydrogen abstractors from the substrate and as nitroso precursors. Eventually, this technique could lead to an accurate quantitative method for analysing NO production *in vivo*.

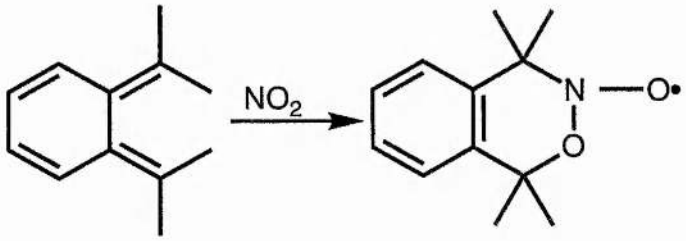
As NO is so hard to detect *in vivo*, it was suggested that custom made traps could be designed[103]. It was known that biradicals have been trapped by NO[98], so a biradical acting in a cheletropic manner should also trap NO. However, structurally

suitable biradicals are transient and react rapidly with oxygen. The synthesised trap would also have to be lipid and/or water soluble. Korth and coworkers photodecarbonylated 1,1,3,3-tetramethyl-2-indanone, **4**, to give 1,2-bis(*exo*-isopropylidene)cyclohexa-3,5-diene, **5**. This was found to trap NO successfully, giving the 1,1,3,3-tetramethylisoindolin-2-oxyl radical:



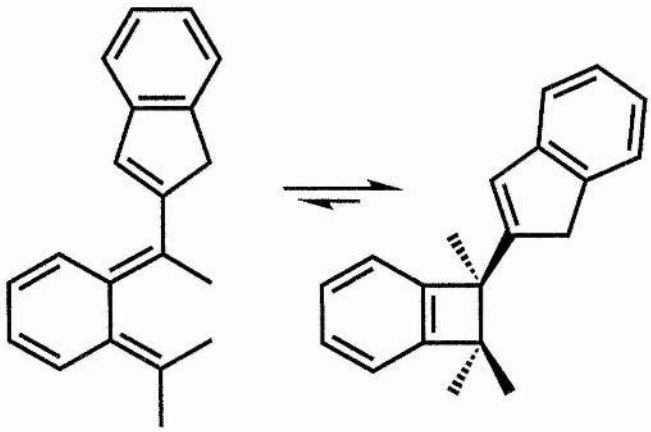
4 **5**

However, this trap is unstable and unselective; it can also trap NO_2•. It is negligibly soluble in water, and it is subject to 1,5-sigmatropic H shifts under photolysis at similar wavelengths used for decarbonylation.



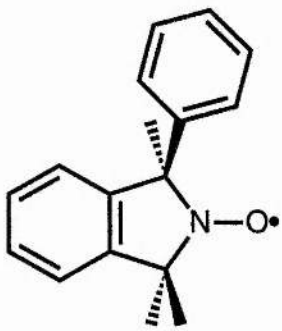
In a more recent investigation[104], other nitric oxide cheletropic traps (NOCTs) were synthesised. A similar starting point was taken, i.e. *o*-quinodimethanes, and various alkyl groups were attached. It was discovered that many of the NOCTs trapped NO_2• far more efficiently than NO•. In addition they were all still susceptible to the sigmatropic shift. To overcome this, the starting ketone dispiro-[cyclopropane-1,1'-indane-3',1''-cyclopropane]-2'-one was synthesised from indan-2-one, because it was thought that the sigmatropic shift could be retarded if the methyl groups were replaced by cyclopropyl groups. However, irradiation of the cyclopropyl compound did not cause significant decarbonylation, which was therefore ineffective as a trap. When a photolysed solution of the indanone precursor was left overnight, it gave a 3-line nitroxide signal when analysed by EPR spectroscopy. This could not be due to the original trap, (the *o*-quinodimethane), as its lifetime is only 140ms. It was eventually

realised that in the preparation of the original indanone, there was a significant amount of an impurity, subsequently identified as 1-(2-indenyl)-1,3,3-trimethyl-2-indanone. This was subsequently separated from the mother solution and photolysed. It was expected this would generate 7-(2-indenyl)-7,8,8-*o*-quinodimethane, **6**, but ^{1}H NMR did not give the expected *o*-quinodimethane signals. It was thought that these were masked by aromatic protons.



6 **7**

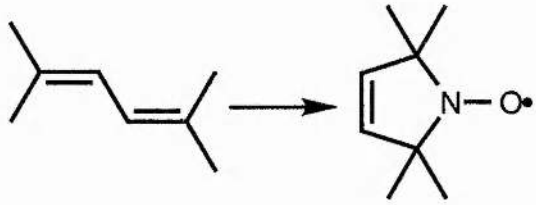
Even at low concentrations, the 7-(2-indenyl)-7,8,8-*o*-quinodimethane proved a very effective NOCT. This was thought to be due to its equilibrium with its cyclised isomer, 7-(2-indenyl)-7,7,8-trimethylbenzocyclobutene, **7**, with the equilibrium favouring the latter. Thus it was shown that this nitroxide was very persistent - after 20 days at 20°C the EPR signal had only reduced in intensity by about 20%. Substitution of a benzene ring instead of the indene gave a no less efficient, but slower acting trap:



To promote water solubility, various functional groups were introduced into the benzene ring. Firstly, oxidation of the 5-bromo- substituted tetramethylindanone to the

hydroxyl-substituted indanone, and secondly formation of the tetra-alkylammonium-substituted indanone were attempted. Neither proved to be a successful trap for NO. The best result arose from substitution by a carboxylic acid group. This gave increased water solubility and did not significantly alter the decay constant i.e. the 1,5-shift. Thus modification of the NOCTs by addition of a carboxylic acid group could prove to be an excellent biological trap.

In 1993, Symons and coworkers[105] postulated that NO reacted with 2,5-dimethyl-2,4-hexadiene to give a stable nitroxide radical via a ring closure reaction:



Rockenbauer and Korecz[106] did not agree with these findings, claiming that the formation of the nitroxide requires the presence of molecular oxygen, i.e. NO_2 must react with compound first to form a carbon-centred radical. The following mechanism was proposed:

$$R + \bullet NO_2 \longrightarrow O_2NR\bullet$$

$$O_2NR\bullet + NO\bullet \longrightarrow O_2NRNO$$

$$O_2NRNO + O_2NR\bullet \longrightarrow (O_2NR)_2NO\bullet$$

A different form of spin trapping is based on triplet trapping. In this case, the usual diamagnetic nitroso or nitrone scavenger, is replaced by a paramagnetic scavenger; itself a radical. Forrester and Sadd[107] carried out studies on carbene trapping with diazo compounds. A solution was degassed and saturated with NO. The sample was then irradiated with UV light in the cavity of an EPR spectrometer. Some of the molecules gave iminoxyls of structure R=N-O• without photolysis. Iminoxyl radicals have a characteristic ^{14}N triplet splitting of ~27-30G. Thus they can be distinguished from any other type of nitroxide radical, e.g. the carbonyl nitroxide [a(N) ~7G], or dialkyl nitroxide [a(N) ~14G], that may also have been formed. Other carbene sources tested were diazirenes, oxiranes, diphenylketone, phospholan,

phenyl(trichloromethyl)mercury, lithio-salts of tosylhydrazones and 3H-pyrazole. Iminoxyls were only generated from the diazirines and oxiranes. The remainder were either unreactive, (tosylhydrazones), or only produced nitroxides. Nitroxides were formed in all cases. Photolysis of nitrene precursors failed to produce nitroxides or iminoxyls, except for ethoxycarbonyl azide, which generated a nitroxide. From this it was concluded that diazo compounds provide the best triplet traps for NO and that nitrene precursors fail to scavenge NO.

More recently, Reszka *et al.*[108], noted that the *aci-* form of nitromethane acted as a spin trap. If nitromethane is treated with 0.5M NaOH, then it is deprotonated to form $CH_2{=}NO_2^-$. It was shown that if NO was bubbled through the solution, an EPR spectrum consisting of 18 lines could be observed. This was due to the formation of the radical dianion $^-ON{=}CHNO_2^{\bullet-}$, in turn produced from β-hydrogen abstraction from the primary spin adduct $O{=}NCH_2NO_2^{\bullet-}$. This undergoes a negative charge shift from the carbon to the oxygen, as illustrated:

$$CH_2{=}NO_2^- + {\bullet}NO \longrightarrow O{=}NCH_2NO_2^{\bullet-}$$

$$O{=}NCH_2NO_2^{\bullet-} \xrightarrow{-H^+} O{=}N\bar{C}HNO_2^{\bullet-}$$

$$O{=}N\bar{C}HNO_2^{\bullet-} \rightleftharpoons {}^-ON{=}CHNO_2^{\bullet-}$$

This radical was quite persistent, and addition of strong base (2M NaOH) caused intensification of the EPR spectrum. This approach too could be a useful analytical tool for the detection of NO in aqueous solutions. The only drawback to this is the high pH required for generation of the *aci* form.

As stated previously, spin traps have been available for many years[109] and the biological aspects of free radicals have been inferred for some time also. Many of the redox systems, such as cytochromes and some other enzymes act via radical intermediates. Although spin trapping has been used in biology for many years, now that NO is at the forefront of research this technique will be increasingly utilised. Early NO work by Arroyo and coworkers[110] proved that a radical was formed when cultured mouse neuroblastoma cells were stimulated by carbamylcholine, an acetylcholine analogue. The spin trap used for detection was 3,5-dibromo-4-nitrosobenzene

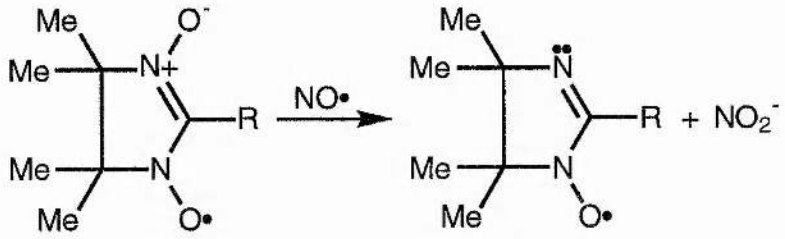
sulphonate (DBNBS). The cells were incubated with the trap and nutrients and then stimulated by CBC, via its action on muscarinic receptors. An EPR signal comprising six lines was observed. The signal was enhanced by superoxide dismutase (SOD) and a similar signal was seen upon incubation with L-arginine. Product assay by ion-exchange chromatography revealed that cGMP formation increased and also GC activity increased, two major effects of NO production. This would seem to verify the fact that NO is indeed the product. When L-NMMA was introduced, the formation of cGMP was inhibited in conjunction with the DBNBS adduct. This would indicate that the activation of GC and the EPR signal originated from a product of NOS. Labelling experiments revealed that the NO source was the guanidine nitrogen of L-arginine.

However, when NO was bubbled directly into DBNBS, a markedly different 3-line signal was observed. Thus it appeared that NO was not the trapped radical previously detected. The conclusions of this work were that a free radical was formed from L-arginine which is associated with an increase in Ca^{2+} levels, (L-arginine activation of GC is dependant on free Ca^{2+}), and may act as an intercellular transmitter for receptor activation.

Another type of spin trapping experiment was carried out by Mordvinctev *et al.*[111]. This involved the trapping of NO released from the decomposition of 3-morpholinosydnonimine (SIN-1) by an iron (II) complex, Fe^{2+}-diethyldithiocarbamate $[(DETC)_2]$ which was bound to the membrane of yeast cells. The resulting iron nitrosyl complex, NO-Fe^{2+}-$(DETC)_2$ was detected by EPR spectroscopy and gave the characteristic 3-line signal. It was found that an increase of oxygen led to nitrite formation, and consequently a decrease in the signal intensity. Exogenous nitrite had no effect on the trapping efficiency.

More biological trapping has been carried out on ischaemic rat brain. Diethyldithiocarbamate and Fe^{2+} (see above) were again used. This study provided evidence that the potentiation of NO production occurs in the ischaemic brain. Brains from control rats, i.e. those that had not been subjected to ischaemia or arterial occlusion, showed only normal physiological levels of NO.

Other types of trap have been used to study NO. Joseph *et al.*[112] used nitronyl nitroxides to study the uptake of NO. This type of trap possesses both nitronyl and nitroxide functionalities, and when NO is trapped an imino nitroxide **8** is formed:



8

The photolytic decomposition of SNP provided the supply of NO, and the investigators found that there was almost complete conversion of the trap into the imino nitroxide. The concentration of NO_2^- was determined by the Greiss reaction. It appears that this method of trapping is very successful as it produces quantitative results and many of the nitronyl nitroxide analogues are available in lipid and water soluble form. A new series of nitronyl nitroxides was studied by Woldman *et al.*[113]. These were of the same general structure, but with varying functional groups at the carbon atoms α and β to the nitroxide. In a similar set of experiments to those of Joseph, it was found that these compounds successfully trapped NO to give stable nitroxides. The only drawback to this was that the radicals produced were quickly reduced in biological solutions (homogenised rat cerebellum). To overcome this, a nitronyl nitroxide with a charged functional group was synthesised and was incorporated into the inner volume of large unilamellar phosphatidylcholine liposomes. This lowered the penetration capability of the nitroxide and therefore its reduction rate to a level that allowed more accurate assessment by EPR spectroscopy.

The application of spin traps to *in vitro* systems gives evidence that NO is present in cell cultures and homogenates, but what about living systems? Komarov *et al.*[114] reported an *in vivo* trapping technique to measure NO release in conscious mice. In this case, SNP was used as the NO donor, and a metal chelator complex consisting of Fe^{2+} and N-methyl-D-glucamine dithiocarbamate, (MGD). This forms the stable and water soluble NO-Fe^{2+}-$(MGD)_2$ complex, analogous to the NO-Fe^{2+}-$(DETC)_2$

system reported elsewhere. The high water solubility is advantageous in that it can be used under physiological conditions. A saline solution containing SNP and the Fe^{2+}-$(MGD)_2$ complex was injected into the tail vein of the mouse. The tail tip was examined by EPR spectroscopy and a 3-line signal was observed. This had identical splittings to a spectrum obtained by addition of Fe^{2+}-$(MGD)_2$ to a solution of SNP *in vitro*. Analysis of whole blood, i.e. that from the sacrificed animal, also revealed a similar spectrum. This indicated that the complex was present in the entire circulatory system, not just the tail. This was the first time that NO had been detected by EPR spectroscopy in a living system, and was therefore of great importance.

Spin trapping has also been used to detect thiyl radicals. These were generated by the decomposition of thionitrites, to give the thiyl radical, RS•, and NO•[115]. The spin trap 5,5-dimethyl-1-pyrroline-N-oxide, (DMPO), was used to trap the thiyl radical. This gave the most intense signal when compared with other traps tested (PBN or 2-methyl-2-nitrosopropane, MNP). This could be significant from a biological angle, as NO is implicated in the direct S-nitrosation of cysteine[116].

With the association of superoxide, $O_2^{\bullet-}$, with NO in the body, there is current interest in the spin trapping of the products of peroxynitrite decomposition. As stated previously, NO reacts with $O_2^{\bullet-}$ to form peroxynitrite, $ONOO^-$, which is protonated at physiological pH, and is unstable, decaying to OH• and NO_2•. However, it has been suggested[117], that homolysis of peroxynitrous acid is not a thermodynamically viable mechanism for decomposition, and oxidative damage does not arise from OH• production. Thus, an attempt was made to obtain evidence of OH• formation by spin trapping[118]. Stock supplies of $ONOO^-$ were mixed with DMPO and analysed by EPR spectroscopy. This gave rise to a 4-line signal, confirming the presence of the DMPO-OH• adduct. Yields of these adducts were low, and higher concentrations of $ONOO^-$ gave weaker spectra. This was thought to be due to reaction of DMPO-OH• with $ONOO^-$ or its other degradation product $NO_2^{\bullet-}$. When $ONOO^-$ was added to preformed DMPO-OH•, no EPR spectra were recorded. It is known that $ONOO^-$ and NO_2 react with thiols[119], so addition of a sulphur containing compound could facilitate

increased yields of DMPO-OH•, due to their scavenging of excess $ONOO^-$ and $NO_2^{\bullet-}$. Consequently, an increased DMPO-OH• yield was detected in the presence of reduced glutathione (GSH) and cysteine. When GSH was added, two other radicals were observed. An unidentified carbon-centred radical, and a glutathionyl-DMPO adduct. This latter is transient so it did not interfere with the DMPO-OH• spectra. If DMPO was added after GSH and $ONOO^-$, no DMPO-OH• adducts were formed, so they must therefore have been generated from the decomposition products of $ONOO^-$. Competition experiments, i.e. those carried out in the presence of EtOH and desferrioxamine, revealed that there must be an alternative route of DMPO-OH• production, as the corresponding competitor-DMPO adducts were detected, but the DMPO-OH• adduct appeared at a reduced concentration. The most studied route of extra production involves the decay of the DMPO-$O_2^{\bullet-}$ adduct. The production of $O_2^{\bullet-}$ from $ONOO^-$ may be possible via reaction of oxygen with the thiyl radical from $ONOO^-$ oxidation of GSH. Proof of this arose from the addition of SOD . When incubated with the reaction mixture, it was found that the yield of the DMPO-OH• adduct had fallen significantly. The presence of desferrioxamine inhibited the formation of the DMPO-OH• adduct and formate addition led to the complete disappearance of the DMPO-OH• signal. From this it can be deduced that the decay of the DMPO-$O_2^{\bullet-}$ adduct is not a major route of DMPO-OH• production in the presence of formate, but it is in the presence of EtOH, as the EPR spectrum showed evidence of DMPO-OH•.

Following on from these experiments, SIN-1 autoxidation was investigated. It has already been stated[111] that SIN-1 decays to give NO•, but it also yields $O_2^{\bullet-}$. Evidence that a OH• radical was produced was provided by EPR spectroscopy. The DMPO-OH• yield increased in the presence of GSH, and in the presence of DMSO the DMPO-Me adduct was observed. The presence of GSH also potentiated this radical adduct. Thus, from these experiments it can be seen that peroxynitrous acid decomposition can trigger free radical production in a metal-independent pathway in low pH or thiol-containing environments.

In summary, it can be seen that the technique of spin trapping now covers a wealth of subjects and is no longer as restricted to the study of free radical chemistry or the more physical sciences. With the discovery of more free radical systems in biology, this will be an invaluable method of analysis and must certainly be developed further.

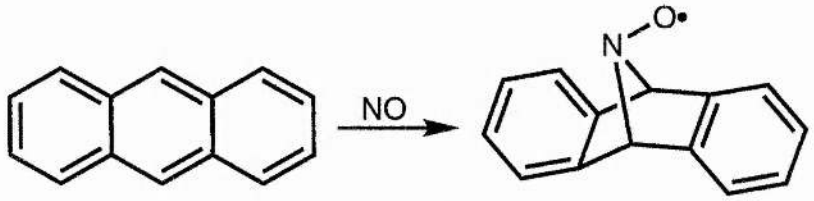
2.2. Results and discussion.

One of the aims of this project was to develop new types of spin trap to detect the presence of NO, and to build on previous experience in this field. Consequently, past literature was consulted and ideas expanded to suit our requirements. The main considerations for a biological spin trap are that it is stable and operational at physiological pH and is both water and lipid soluble. Thus, several types of compound were tested.

2.2.1. Traps related to NOCTs.

2.2.1.1. *Anthracene.*

By analogy with the reaction of NO with **5** we proposed that NO might react with anthracene as a spin trap (scheme 2.0) to give a bicyclic nitroxide which could be detected and analysed by EPR spectroscopy.



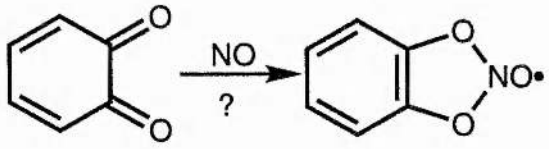
Scheme 2.0

It was anticipated that the retention of aromaticity in the two outer rings would facilitate this reaction. In fact when pure NO was bubbled into a solution containing anthracene no reaction was observed at room temperature. Examination of the solution by EPR revealed no nitroxides and GC-MS analysis showed no product, although unchanged anthracene was readily observed. Changing the solvent from *tert*-butylbenzene to cyclohexane to toluene to 1,2-dichlorobenzene had no effect. On raising the

temperature to 70°C and increasing the contact time to five hours, some minor reaction was observed. A small amount (<10%) of a product was detected and identified as benzophenone. Because of the lengthy contact time and the high temperature, we believe traces of oxygen may have leaked into the reaction chamber and so this product was formed by oxidative degradation possibly initiated by NO_2.

2.2.1.2. o-*Quinone.*

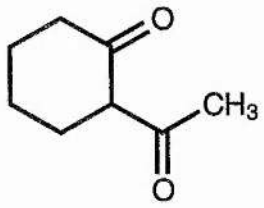
By analogy with **5** we also investigated o-quinone as a possible spin trap.



Scheme 2.1

o-Quinone was prepared by the oxidation of catechol using celite-supported silver carbonate[120]. Nitric oxide was bubbled into a solution of the o-quinone in cyclohexane. EPR spectroscopic investigation of the resulting solution showed a very weak three line nitroxide signal, which was almost indistinguishable from the background.

2.2.1.3. *2-Acetylcyclohexanone.*



Again it was thought that this compound might form a spin trap for NO, by analogy with **5**. Nitric oxide was bubbled through a solution of 2-acetylcyclohexanone in 20 cm^3 cyclohexane. A sample was taken after 3.5 hours and submitted for EPR spectroscopy. No paramagnetic species were present in the solution, or in any subsequent samples taken at 5, 23 and 25 hours.

2.2.2. The reaction of NO with dienes.

By way of comparison with the work of Gabr *et al.*, several other conjugated dienes were examined to see if they gave cyclic nitroxides when reacted with NO. In addition, 2,5-dimethyl-2,4-hexadiene was also studied to determine if molecular oxygen was required for the reaction.

2.2.2.1. Trans,trans-*1,4-diphenyl-1,3-butadiene.*

The reaction of NO with this compound was carried out in cyclohexane at 60°C. After nitric oxide was bubbled through, the solution turned from colourless to yellow, and yellow crystals were obtained. A sample of the crystals was analysed by mass spectrometry and gave a parent peak at 206, indicating the starting material was present. Analysis by EPR spectroscopy showed no paramagnetic species were present, i.e. nitroxide had not been formed. The experiment was repeated using *tert*-butylbenzene as solvent. In this case, no crystals were formed and the solution remained colourless. Analysis by EPR spectroscopy again showed no nitroxide had been formed.

2.2.2.2. *2,5-dimethyl-2,4-hexadiene.*

Nitric oxide was bubbled through a degassed solution of the hexadiene in $20cm^3$ t-butylbenzene. After two hours, a blue colour had developed, indicating the presence of a nitroso compound. A sample was taken which was not degassed prior to EPR analysis, and this revealed a 3-line triplet masking a triplet of doublets (figure 2.0):

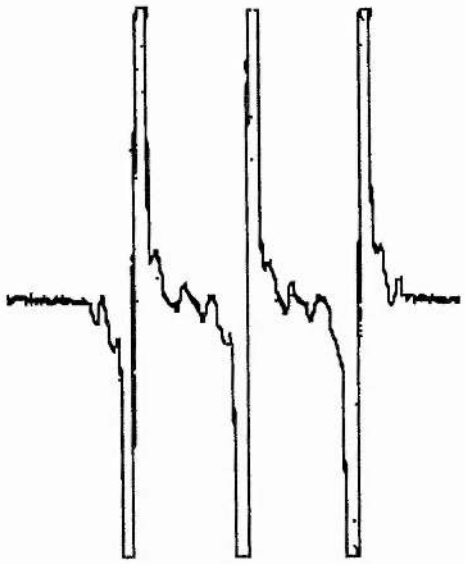
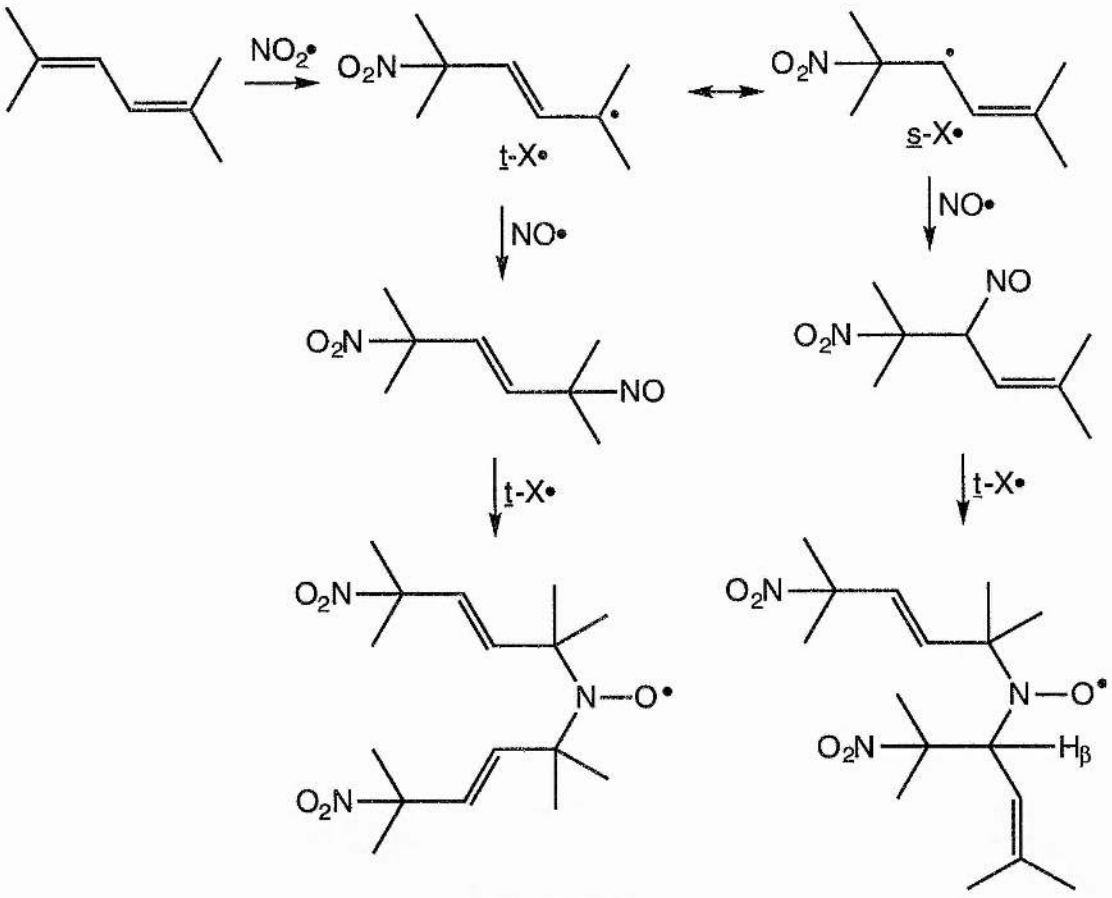


Figure 2.0

Splittings of a(N)= 15.5G for the major triplet, and a(N)= 14.6G, a(H)= 10.8G for the minor peaks were recorded. After exposure to O_2, the blue colour disappeared and a further sample was taken. The EPR spectrum observed in this case showed that the intensity of the triplet had increased, but that of the triplet of doublets had decreased. This appeared to contradict the findings of Rockenbauer[106], until it became apparent that there was a leak in the system, possibly via the N_2 line or when samples were taken. Subsequently, a different line and degassed syringes were used, and when air was excluded, no signals were observed. It must be, therefore, that NO_2 is required first and forms a carbon-centred radical which can then pick up NO. Scheme 2.2 explains the generation of both sets of radicals:



Scheme 2.2

The initial addition of NO_2 gives a delocalised allyl radical which can combine with NO at either end, as shown. From the spectrum it can be seen that the di-t-alkylnitroxide is much stronger than the t-s-di-alkylnitroxide, due to its increased stability. The hyperfine splitting from the β-hydrogen of the latter was measured at a(H)= 10.8G. No di-s-alkylnitroxide was observed because it will disproportionate too rapidly. This mechanism of formation is consistent with the proposal of Rockenbauer *et al.*

When analysed by GC-MS, no products were discernible. This is probably due to the decay of the radicals and their rapid degradation on the column.

2.2.3. Traps related to nitromethane anion.

As stated earlier, Reszka *et al.*[108] reported a novel spin trap for nitric oxide i.e. the anion generated on treatment of nitromethane (CH_3NO_2) with base in aqueous

solution. Compounds of types RCH_2NO_2 and $RR'CHNO_2$ were examined where R,R' are electron acceptor or alkyl groups.

2.2.3.1. *Nitromethane.*

The reaction of nitromethane with NO in basic conditions was investigated, and after several attempts using the same experimental conditions, the following spectrum was obtained, essentially the same as that of Reszka *et al.* This was satisfactorily simulated (figure 2.1) using the following parameters: a(N)= 11.56G (NO_2), a(N)= 6.5G (NO), and a(H)= 2.83G. When the sample was left overnight and re-examined by EPR spectroscopy the following morning, no signal was observed, even when the basicity was increased by addition of 2M NaOH.

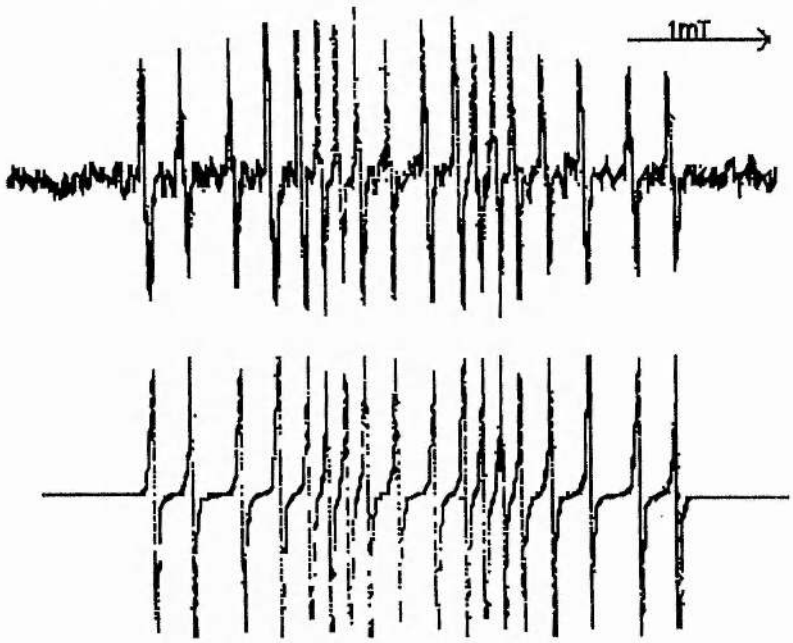
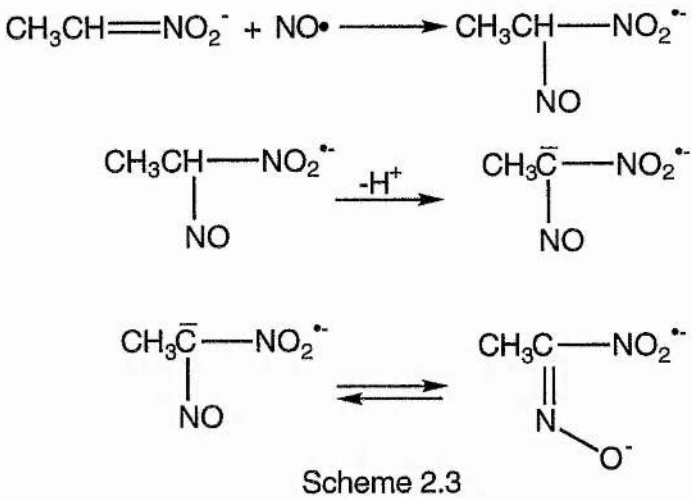


Fig. 2.1

2.2.3.2. *Nitroethane.*

Nitric oxide was bubbled through a 50mM solution of degassed nitroethane for a period of 24 hours. Samples were taken after 0.5, 3, and 20 hours. The only sample to show any sign of spin trap activity was that taken at 20h. A spectrum of greater than 10 lines was observed as shown. By analogy with nitromethane, the *aci* form of nitroethane is $CH_3CH{=}NO_2^-$, and thus the following, scheme 2.3, was proposed:



Scheme 2.3

Thus, from the above it can be seen that there would be two triplet splittings from the non-equivalent nitrogens, further split into quartets by the methyl hydrogens. As can be seen from the spectrum shown below, figure 2.2, this is not the case. Also shown is a simulation of the nitroethane spectrum, using the following values; a(1N)= 16.5G, a(1H)= 6.8G, and a(1H)= 4.2G. When compared to the literature, these splittings are consistent with those exhibited by a dialkyl nitroxide in an aqueous environment. The structure of this nitroxide has not yet been determined.

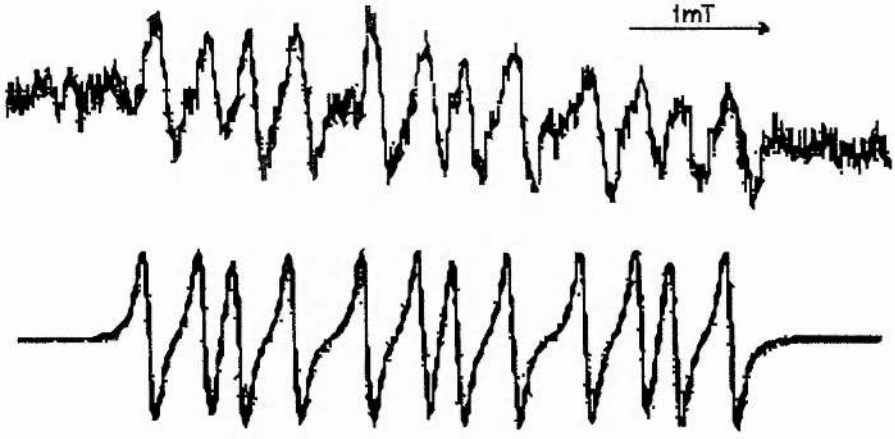
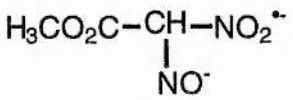


Fig. 2.2

The intensification of the signal on increasing the pH with 2M NaOH could be due to precursors formed at lower pH from the primary adduct $CH_3CH(NO)NO_2^{\bullet-}$ by addition of a second molecule of NO to the nitroso moiety, itself a spin trap. Analysis by GC-MS showed that no identifiable products were present.

2.2.3.3. *Methyl nitroacetate.*

A solution of methyl nitroacetate was prepared in the same concentration as that used for nitromethane, i.e. 50mM. The solvent was 0.5M NaOH. The solution was degassed and NO was bubbled through for 5 minutes. No EPR signal was observed. The pH was raised by addition of $5cm^3$ 2M NaOH but the expected spectrum was absent. Analysis by GC-MS did not reveal any trace of the expected spin adduct **9**,



9

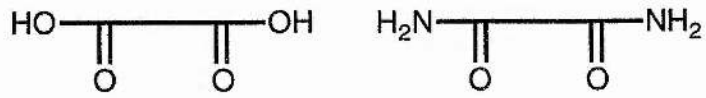
or its degradation products. The reason for this could be that there is some interference from the $-CO_2-$ group, but this is unlikely as this functionality should promote anion formation. Alternatively, it could be that the radical is too transient to be detected, or the time taken for observation was too great.

2.2.3.4. *Diethyl malonate.*

A 50mM solution of diethyl malonate ($C_2H_5O_2CCH_2CO_2C_2H_5$) in 0.5M NaOH was degassed for 1 hour. Nitric oxide was bubbled through for 1 hour and a sample taken for EPR. No paramagnetic species were present. Analysis by GC-MS indicated that no reaction had taken place.

2.2.3.5. *Malononitrile.*

A 50mM solution of malononitrile in 0.5M NaOH was degassed for 1 hour. Nitric oxide was bubbled through for 2 minutes, and a sample was taken. No EPR signal was observed . The basicity was increased by addition of 2M NaOH ($5cm^3$) and NO bubbled for a further 2 minutes. There was still no signal observed. After 3 hours of gassing, no EPR spectrum was obtained. On removal of the reaction vessel, white smoke was emitted and identified as ammonia. This must have been produced from the hydrolysis of the malononitrile. Nitriles can be hydrolysed to the acid or the amide, but in basic conditions acid formation is favoured. When samples were analysed by GC-MS, no products corresponding to the following were observed:



2.2.3.6. *2-Nitropropane.*

A 50mM solution of 2-nitropropane in 0.5M NaOH was degassed and NO bubbled through for 24 hours. There was no reaction during this period, and analysis by EPR spectroscopy revealed no formation of a radical adduct. 2-Nitropropane does not act as a trap for NO.

2.2.3.7. *Ethyl nitroacetate.*

In line with $MeNO_2$, a 50mM solution of ethyl nitroacetate was prepared in 0.5M NaOH. The solution was degassed and NO bubbled through. No EPR spectra were recorded after 0.5, 1.5, and 4 hours. There was a colour change from colourless to pale yellow, but it was concluded that ethyl nitroacetate does not act as a spin trap for NO.

2.2.3.8. *General conclusions.*

From the above investigations it can be seen that only the nitromethane anion will successfully form an adduct with NO. The trapping of NO by nitroalkanes is obviously highly specific. It appears that a high pH is required to promote the *aci* form of the compound, and this will be difficult to adapt for trapping in a physiological medium. The complexity of the splitting pattern may cause some problems for interpretation, but may also be beneficial in that they will be easily identified as characteristic nitric oxide adducts.

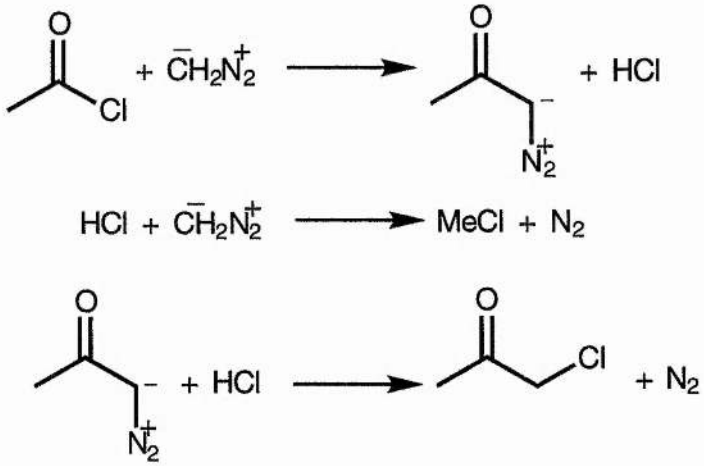
2.2.4. Diazo spin traps.

Nitric oxide was found to react with carbenes to give iminoxyl radicals, and most significantly, certain carbene precursors were themselves observed to react directly with NO, in the absence of irradiation[107]. It appeared to us that this could form the basis of a useful and feasible method for spin trapping NO. We attempted to trap NO using two diazo compounds which act as carbene precursors. The first compound, 2-diazocycloheptanone was synthesised from cycloheptanone, and the

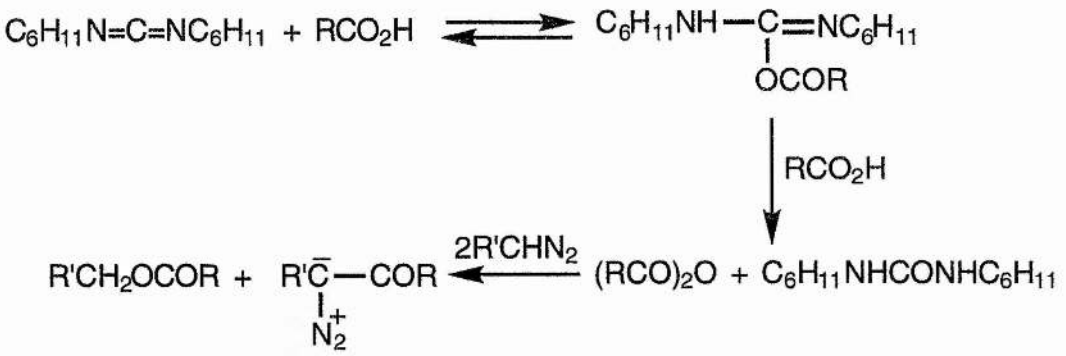
second compound investigated was 5-diazouracil, the syntheses of which are reported in the experimental section.

2.2.4.1. *Synthesis of diazo ketones.*

Diazo ketones have been known since the turn of the century, but it was only a fortuitous discovery of their rearrangement properties that led to their being investigated thoroughly[121]. Several methods have been developed for the synthesis of these compounds, the most common and earliest being the Arndt-Eistert procedure. This results in the formation of an α-diazoketone from the reaction between a primary diazoalkane, usually diazomethane, and an acid chloride. Sometimes, acid anhydride is used, but this reagent is less favoured. The classical Arndt-Eistert synthesis involves the production, in quantitative yield, of the diazoketone by the slow addition of the acid chloride to an ice-cold ethereal solution of diazomethane. In the reaction, there are two equivalents of diazomethane used to one acid chloride. The HCl produced by the action of the first equivalent of diazomethane on the acid chloride reacts with the second equivalent to give methyl chloride and N_2. Formation of the α-chloroketone occurs if there is no second equivalent of diazomethane. This can be summarised thus:

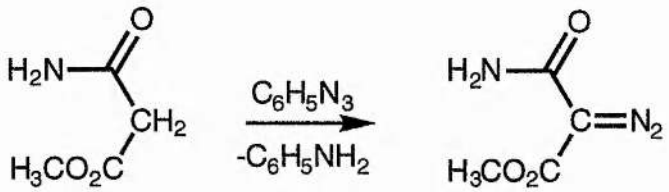


Diazoketones can also be prepared from the addition of a mixture of a carboxylic acid and dicyclohexylcarbodiimide in ether to ice-cold ethereal diazomethane (or diazoethane). This reaction gives the corresponding diazoketone via acid anhydride intermediates[122]:

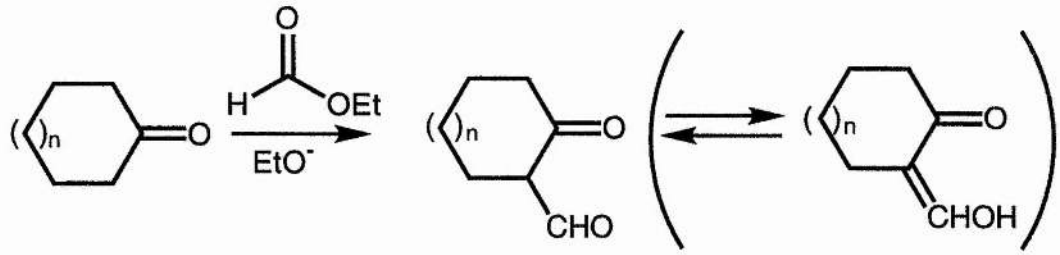


Limitations to the Arndt-Eistert procedure are that other functional groups present in the acid choride will react with the diazoketone unless suitable protection is afforded.

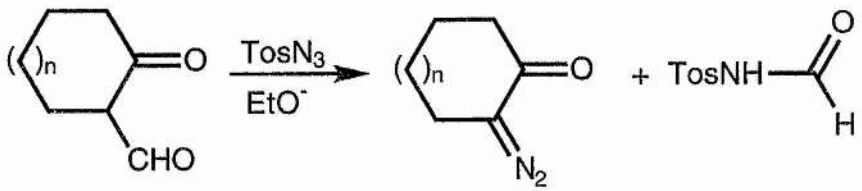
Extensive studies have been carried out on the transfer of a diazo group from an organic azide to a substrate containing an active methylene group. The basic reaction was discovered by Dimroth in 1910[123], and is shown below.



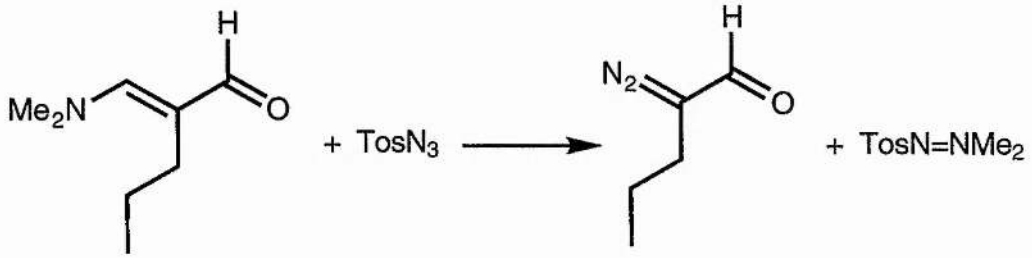
ρ-Toluenesulphonyl azide (tosyl azide) is now used as the transfer agent as it is readily prepared and its coproducts are easily removed. Basic conditions are required as it is the enolate anion which reacts with the tosyl azide. The strength of the base used is dependent on the pK_a of the methylene group. The presence of two activating groups is preferable, i.e. start with a diketone. If only one carbonyl group is present, as in simple cyclic ketones, a Claisen reaction can be carried out to introduce a temporary activating group into the ring:



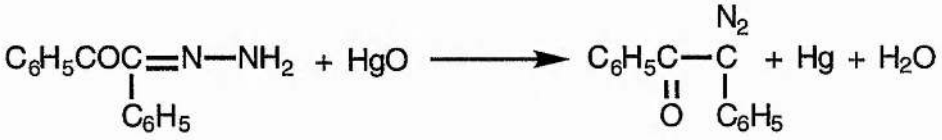
This is then treated with the tosyl azide in base and the diazoketone is formed via a cyclic intermediate.



Diazoketones can also be produced by azide group transfer to enamines. This method is used mainly for synthesis of diazoaldehydes, an example of which is described below[124]. Although this method of diazoketone production is effective, the other routes are generally superior.



Various other methods of diazoketone preparation are available. These include the oxidation of monohydrazones of α-aldehydoketones and α-diketones. This was initially carried out by Curtius[125] who obtained benzoylphenyldiazomethane from the oxidation of benzilhydrazone with mercury (II) oxide.

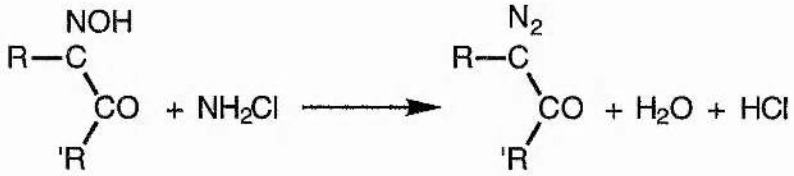


Other metal oxides, such as silver[126] and manganese (IV)[127] have been used, and also nickel peroxide[128]. The oxidation of hydrazones is used mainly for the preparation of acyclic monodiazoketones and aryl-substituted diazomethanes.

Diazotisation has been utilised in the preparation of diazoketones. Diazoacetophenone was produced from aminoacetophenone in the late nineteenth century[129].

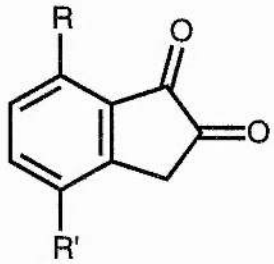
This procedure was used to synthesise cyclic diazoketones, and diazotisation of an amino group between two carbonyl groups gives the corresponding diazoketone[130].

Diazotisation using nitrosyl sulphuric acid was carried out by Mosby and Silva[131] to produce 2-diazo-1,3,4-trioxotetralin from 2-amino-3-chloro-1,4-naphthoquinone. Diazotisation can also be used to generate diazoketones from ketoximes. Much work was done in this area by Forster[132]. Treatment of the monoxime of a diketone with chloroamine in a cooled alkaline medium yielded the diazoketone.

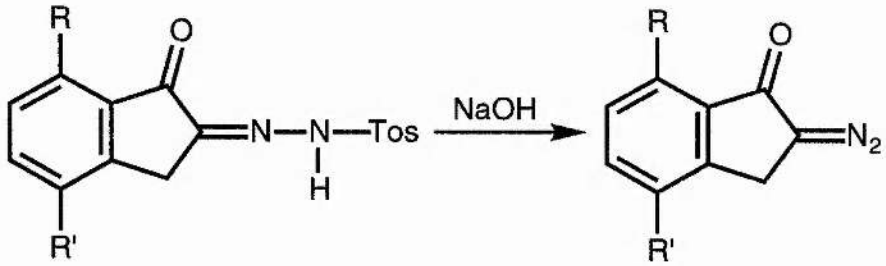


This method has been used to synthesise a variety of cyclic diazoketones and polycyclic bisdiazoketones[130].

Alkaline cleavage has also been used to produce diazoketones from tosylhydrazones of α-carbonyl compounds. The first attempted synthesis was of a series of substituted indanones. These displayed the general structure:

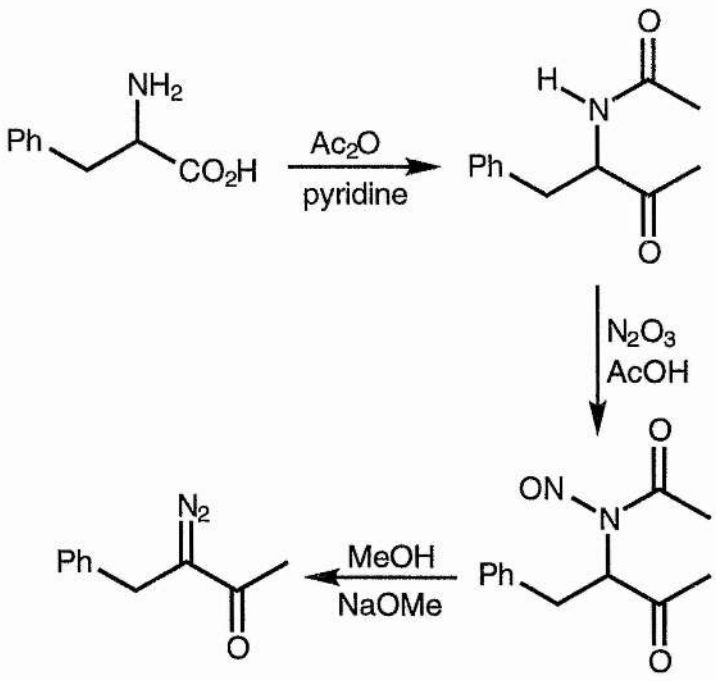


They reacted readily with ρ-toluene sulphonylhydrazine to give the tosylated adducts. Upon treatment with base, (NaOH), the tosyl group was removed and the diazoketone was isolated.



Another form of alkaline cleavage exploits the Dakin-West[133] production of N-acyl-α-aminoketones. Franzen[134] took these α-aminoketones and reacted them with nitrous fumes (N_2O_3) to generate the nitroso derivative. This derivative was then decomposed

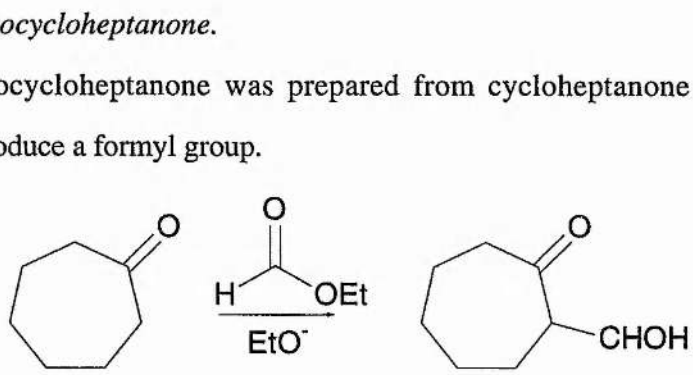
under basic conditions to give the diazoketone. This method was useful for producing secondary diazoketones.



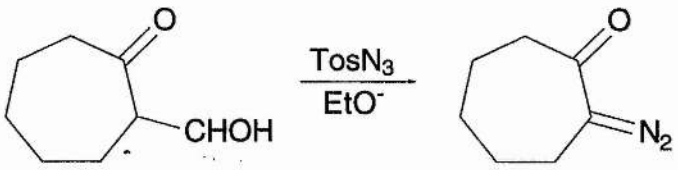
It can be seen that there is a great variety of syntheses for diazoketones. It still appears that the synthesis methods of choice are the Arndt-Eistert synthesis and diazo transfers, as these are the simplest and give good yields.

2.2.4.2. *2-Diazocycloheptanone.*

2-Diazocycloheptanone was prepared from cycloheptanone via a Claisen reaction to introduce a formyl group.

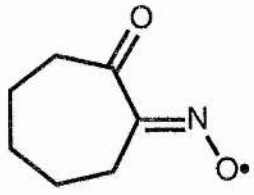


The 2-formylcycloheptanone was then treated with tosyl azide in base to generate the diazoketone.

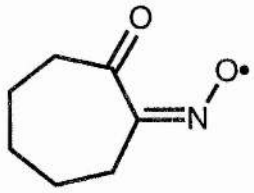


In the first set of experiments, the freshly synthesised diazo compound was dissolved in 0.5cm^3 t-butylbenzene and degassed for 20 minutes. Nitric oxide was bubbled through the solution and no reaction took place at 300ΔT (300ΔT corresponds to about 300K). On increasing the temperature to 330K, a weak triplet was seen. On lowering the temperature and photolysing the solution, a radical was observed with a(N)= 14G. This was similar to that observed by Forrester[107]. In another experiment, 2-diazocycloheptanone was dissolved in 0.6cm^3 t-butylbenzene and degassed for 20 minutes. A control tube was analysed by EPR spectroscopy at room temperature, 325 and 260K. No spectra were observed. Upon photolysis at 325K, still no spectra were observed, but at 260K, a weak spectrum started to form. Nitric oxide was then bubbled through the solution for 5 minutes at 275K. This gave rise to a weak signal. Upon further exposure to NO at 260K, two signals appeared, the major radical being a triplet of multiplets a(1N)=26.5G, and the minor also being a triplet of multiplets with a(1N)= 33.5G. Second derivative studies resolved the complex splitting pattern for each set of peaks. On raising the temperature to 330K, the signals became much weaker. The major radical was persistent for about 24 hours, after which it was undetectable. When re-photolysed after this period, the wide iminoxyl signal vanished to be replaced by a 5-line signal similar to that obtained in the earlier investigation. Because of the excellent resolution of both radicals, simulations giving all of the hfs were eventually successful. Parameters obtained for the major radical were a(1N)= 26.5G, a(1H)= 2.1G, a(1H)= 1.54G, and a(2H)= 0.58G, and for the minor radical were a(1N)= 33.5G, a(1H)= 0.23G, a(1H)= 0.49G, a(1H)= 0.44G, a(1H)= 1.65G, and a(2H)= 0.9G. This can be attributed to the other conformer of the iminoxyl. Figure 2.3 shows the complete experimental spectrum. The good correspondence with the simulated spectrum is shown (figure 2.4) for the low field multiplet under high resolution with second derivative presentation. These hfs are reasonably consistent with literature data for structurally related iminoxyls.

The two conformers are known as the E and Z forms, and are of the following structures:



10 Major E conformer

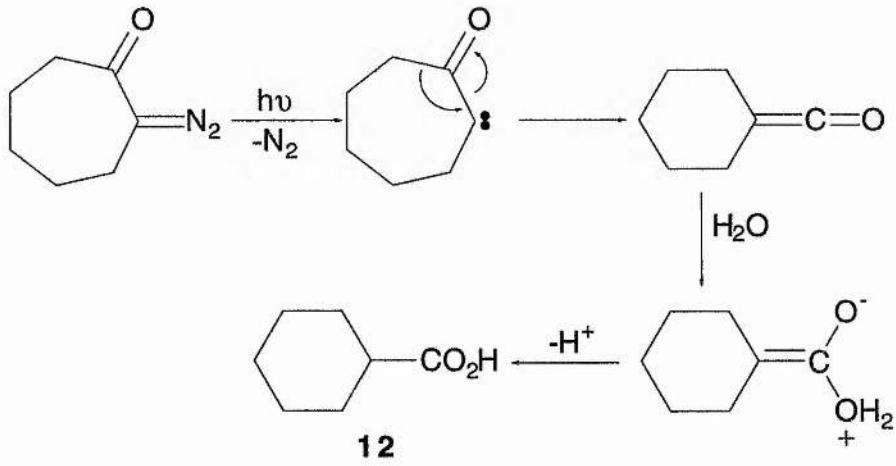


11 Minor Z conformer

Iminoxyl radicals can undergo long-range coupling, and the carbonyl functionality reduces the extent of spin density across the molecule[135]. For our conformers, by analogy with literature iminoxyls, the greatest couplings occur when hydrogens are *syn* to the oxygen of the iminoxyl group, and the nitrogen hfs will be largest. Thus we can assign the splittings of a(1N)=33.5G, a(1H)= 2.1G, a(1H)= 1.54G, and a(2H)= 0.58G to **11**, the Z-conformer, as the E-conformer, **10**, will be able to couple to a greater number of hydrogens. The nitroxide formed has a smaller splitting of a(N)= 14G. All the values obtained are consistent with the literature.

A third set of experiments revealed similar results, i.e. the presence of the iminoxyl and the nitroxide. The peak heights were measured and this revealed a half life of about 2.5 hours for the major triplet (iminoxyl). Analysis by GC-MS showed one major product, cyclohexane carboxylic acid, **12**. This is probably formed via a Wolff rearrangement of the diazo compound when the carbene is formed (scheme 2.4):

Scheme 2.4



12

It is thought that the hydrolysis of the ketene proceeds via the zwitterionic form, as shown above[136].

Figure 2.3. EPR spectrum obtained from the reaction between diazocycloheptanone and NO.

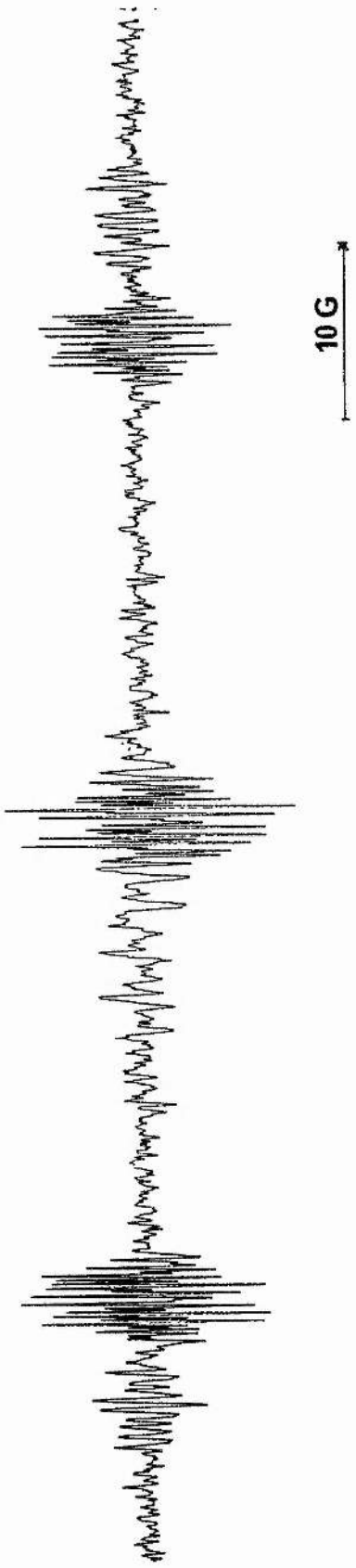
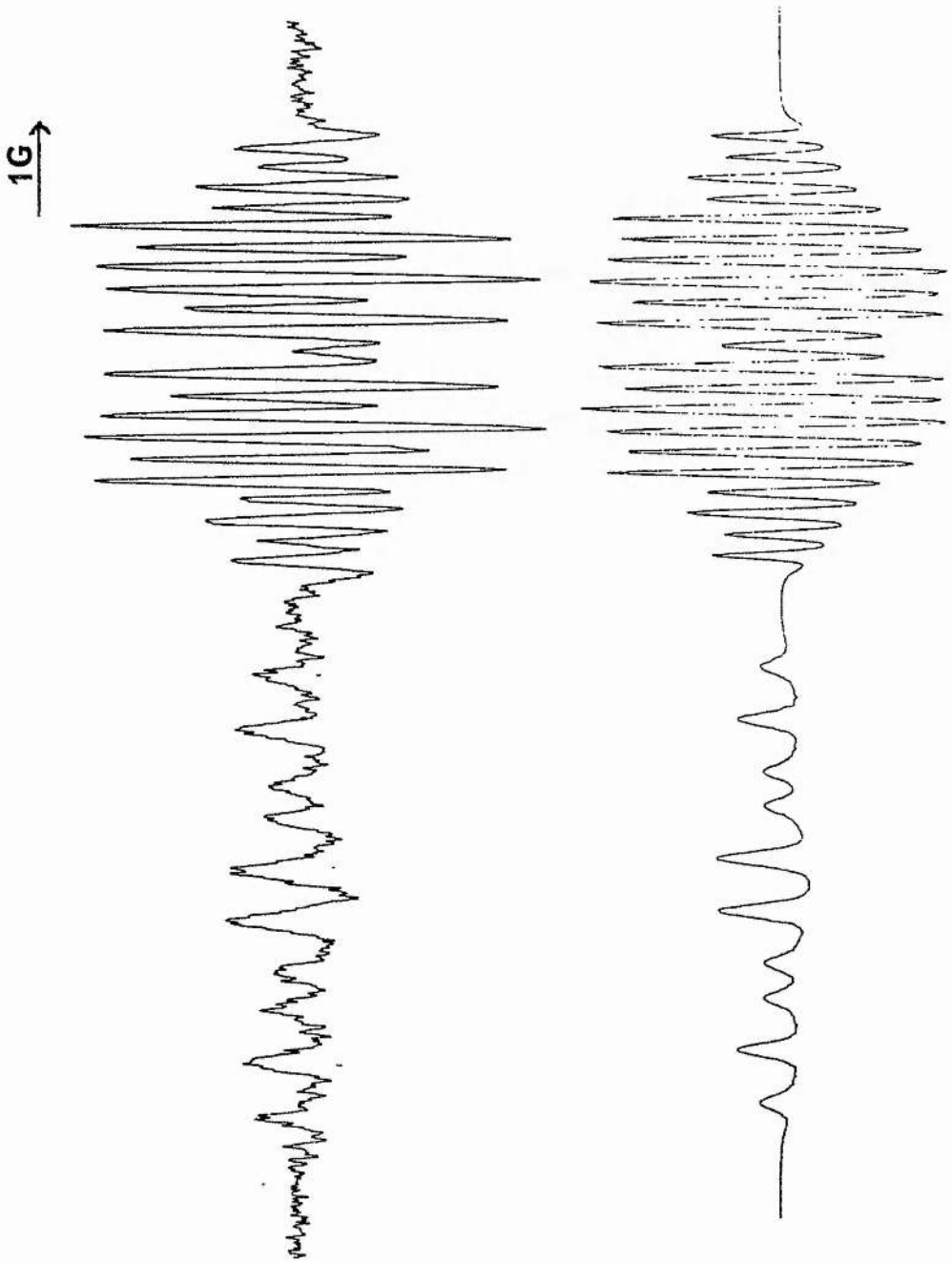


Figure 2.4. Second derivative presentation of the EPR spectrum of the low field multiplet obtained from the reaction between diazocycloheptanone and NO.



The GC-MS revealed several minor products, together with isopropyl-t-butylbenzene (m/z 177), which was present as an impurity in the solvent (t-butylbenzene). Also identified was the ethenyl ester of cyclohexanecarboxylic acid (m/z 154), triphenylphosphine oxide (m/z 278) which was most likely to be a contaminant on the column, and bis-(2-ethylhexyl)-phthalate (m/z 390), a plasticiser present in the tubing. The radical adducts were not, of course, seen on the chromatograms.

Further work revealed the presence of a third radical, the ubiquitous carbonyl nitroxide. This, however, was only observed at high concentrations of the diazocycloheptanone. The relative excess of NO ensures complete conversion to the iminoxyl and nitroxide. As the amount of iminoxyl adduct increased, there was formation of the carbonyl nitroxide. This had a(N) ~8G. The concentration of NO played an important part in which radical was formed. From our work it was shown that at low concentrations of NO, the reaction time was very long. At low NO concentration, the iminoxyl pair was formed preferentially and this was not as transient as the nitroxide. When the NO concentration was raised, more of the dialkyl nitroxide was generated. An interesting difference that was observed was that when the flasks containing the diazo spin trap and the NO solution were kept dark, only the nitroxide [a(N)= 14G] was formed. This means that light plays an important role in the generation of the iminoxyls, a fact that was hinted at earlier. On changing the solvent to MeCN, the main difference detected was that the reaction exclusively forms the carbonyl nitroxide and not the iminoxyl. This could be due to solvent effects if NO is more soluble in MeCN than in t-butylbenzene.

From these experiments it is shown that there are three species generated, the E and Z conformers of the iminoxyl radical, a dialkyl nitroxide, and a carbonyl nitroxide. The structures of these nitroxides have not yet been elucidated. The following data show the relative peak heights for the radicals as a function of time and NO concentration. The peak height shown is an average value from the three peaks present in each spectrum.

Time (min.)	Average peak height (mm)	
	Major iminoxyl	Dialkyl nitroxide
0.00	0.00	0.00
14.5	67.0	6.75
54.5	75.0	11.5
94.5	36.0	11.0
131.5	21.0	10.0
164.5	17.0	10.0

Figure 2.4 shows the development of the peak height with time for the iminoxyl and the dialkyl nitroxide generated. It can be seen that the iminoxyl radical begins to decay after 60 minutes. The dialkyl nitroxide, although weaker, appears to be more persistent.

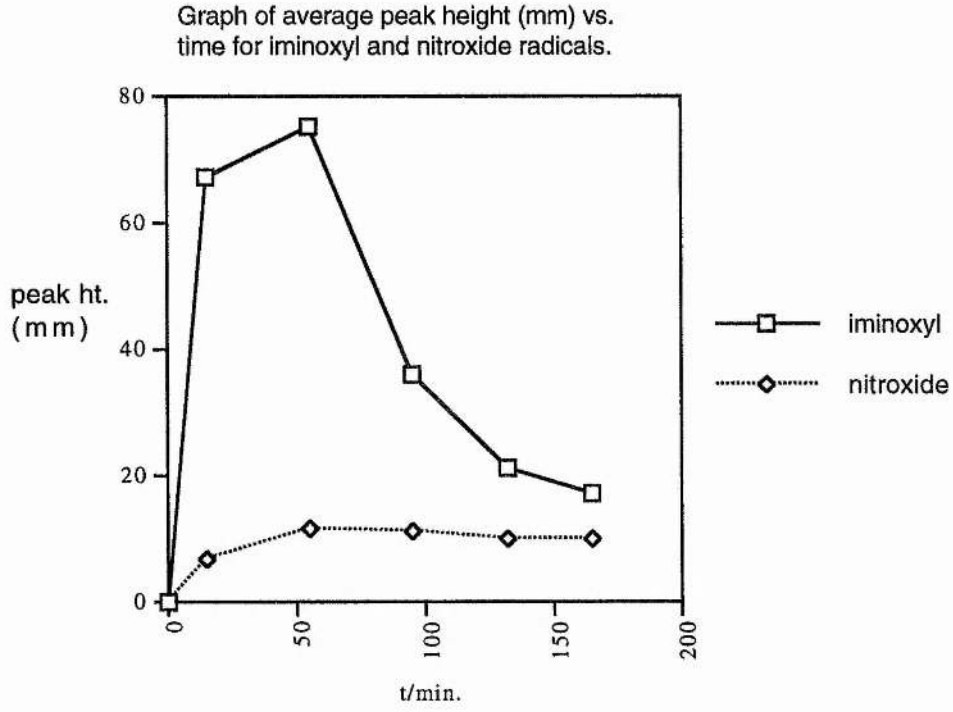


Fig. 2.4

The concentration of the NO/t-butylbenzene solution was not accurately known, but if a saturated solution is assumed, then it follows that with an increased amount of the solution, there will be an increased availability of NO.

The data shown in figure 2.5 represents the peak height of the iminoxyl and dialkyl nitroxides with time, but at varying nitric oxide availabilities.

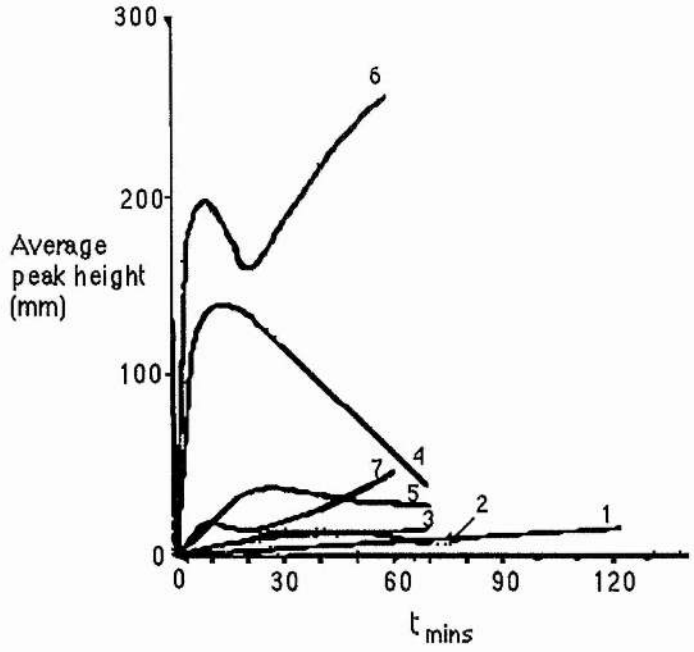


Fig.2.5

The numbers at the side of the graph correspond to the different quantities of NO/PhBu^t solution that were mixed with the standard solution of the diazo spin trap, and are summarised below:

1. Nitroxide: 25µl NO/PhBu^t
2. Iminoxyl: 75µl NO/PhBu^t
3. Nitroxide: 75µl NO/PhBu^t
4. Iminoxyl: 250µl NO/PhBu^t
5. Nitroxide: 250µl NO/PhBu^t
6. Iminoxyl: 450µl NO/PhBu^t
7. Nitroxide: 450µl NO/PhBu^t

Although these results were somewhat erratic and poorly reproducible, they show a rough trend toward more intense signals for both iminoxyl and nitroxide as the number of moles of NO increased.

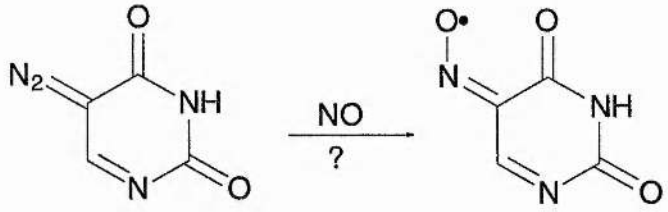
To test our spin trap under physiological conditions, 1.4×10^{-4}mol. of the spin trap was dissolved in t-butylbenzene. A saturated solution of NO in distilled water was made up after degassing with N_2, and an aliquot of this solution and the spin trap solution was extracted and mixed by vigorous shaking. This sample was then analysed by EPR spectroscopy. The first signals observed confirmed that the iminoxyl had been formed- a very strong 27G triplet was seen. However, when a control

sample was tested, i.e. without any NO, the triplet was observed here also. This meant that the diazo compound had been oxidised, probably by molecular O_2. Another batch was distilled, and a repeat experiment was carried out. This time, there was no signal from the control as expected, and there was also no evidence of radical production from the aqueous solution of NO. This was carried out twice to confirm that there had been no errors. The results stayed the same. It appeared that 2-diazocycloheptanone did not trap NO from water. A variation of this experiment involved dissolving the potential trap in distilled water, but it was found to be only sparingly soluble. After degassing with N_2, an aliquot was removed and combined with an aliquot of the NO/H_2O solution. When analysed by EPR spectroscopy, this gave no spectra.

Consequently, a different approach was tried. This involved the use of two known NO-releasing agents. The first of these, S-nitroso-N-acetyl penicillamine (SNAP), decays thermally and photochemically, and in the presence of Cu^{2+} to liberate NO[137]. A small quantity of SNAP (0.0042g, $2x10^{-5}$mol) was dissolved in $5cm^3$ t-butylbenzene and degassed. (It should be noted that SNAP is only sparingly soluble in this solvent.) Aliquots were then removed from this solution and that containing the diazo compound (0.003g, $2x10^{-5}$mol) in t-butylbenzene and mixed. They were then analysed by EPR spectroscopy. No EPR signal denoting a radical presence was obtained from these experiments. The next step was to encourage decomposition. On heating, still no EPR data was obtained, and after photolysis for 10 minutes no radical species were detected. Changing the solvent to MeCN had no discernible effect. The second NO donor investigated was isoamyl nitrite. The same series of tests was adopted; 0.035g ($3x10^{-4}$mol) of the nitrite was dissolved in $5cm^3$ t-butylbenzene and degassed. An aliquot of the diazo compound was combined with the nitrite solution. No signals were observed by EPR spectroscopy. Here too, encouragement to decompose was given by gentle heating and photolysis, but neither action produced a spin adduct detectable by EPR spectroscopy.

The results of the 2-diazocycloheptanone work thus appear to be inconclusive and somewhat contradictory. When a saturated solution of NO in t-butylbenzene or MeCN is mixed with the spin trap, there are radical species generated, but when known NO donors are used, there was no evidence of spin trapping activity.

2.2.4.3. *5-Diazouracil.*



5-Diazouracil was synthesised as described in the experimental section. In the primary experiment, 0.02g ($1.5x10^{-4}$mol) was dissolved in $5cm^3$ distilled water and degassed. As described in section 2.2.4.1., a degassed solution of NO/distilled water was made, and aliquots of each were extracted and combined. At room temperature, there was no reaction between NO and 5-diazouracil. The subsequent solutions were photolysed, but this also failed to generate an observable EPR signal. When the solvent was changed to MeCN, the temperature lowered to 225K, and the solution photolysed, an EPR spectrum comprising >11 lines was observed. This probably indicated that two or more radicals were formed. One had a triplet of a(N)= 14.8G with further splitting which could indicate that t-dialkyl nitroxide had been produced. A second radical had splitting of a(N)= 9.6G, but this was too high for a carbonyl nitroxide. It seems therefore that 5-diazouracil traps NO, but does not give the expected iminoxyl radical. These experiments were not taken further because of the weakness and complexity of the EPR signals.

2.3. Conclusions.

From the work on spin traps, it can be seen that NO is quite a difficult molecule to trap and therefore analyse. It seems that the best results arise from traps that are specifically designed, although there is potential for further development of traps analogous to those that we have used. Generally, only nitromethane was useful as a

spin trap, as nitroethane, despite forming an adduct, did not give the expected result. The high pH required may be countered by using buffered solutions, or utilising nitroalkanes with a less acidic β-hydrogen. The diazo compounds promise to be a good source of traps, as there is a strong and quite persistent EPR spectrum produced. This would be advantageous for the detection of small quantities of NO. A problem still to be overcome is the poor reproducibility of the results, which might be due to undiscovered impurities in the diazo compounds. The major drawback of the diazo compounds is their low water solubility. This could be overcome by the addition of functional groups which would promote their dissolution.

2.4. Experimental.

^{1}H and ^{13}C NMR spectra were obtained on Varian Gemini 200MHz, and Bruker 300MHz spectrometers. Samples were dissolved in $CDCl_3$; Me_4Si was used as the internal standard. GC-MS analyses were carried out with a Finnigan Incos 50 quadrupole mass spectrometer coupled to a Hewlett-Packard HP5890 capillary gas chromatograph fitted with a column coated with methylsilicone stationary phase. UV/vis spectra were obtained on a Philips PU8730 scanning spectrometer operating between 200 and 600nm, and a Shimadzu UV2101PC spectrophotometer operating at 390nm. A Perkin-Elmer 1710 FT-IR spectrometer was used for infra-red analysis. Elemental analysis was carried out by the University of St. Andrews microanalysis service.

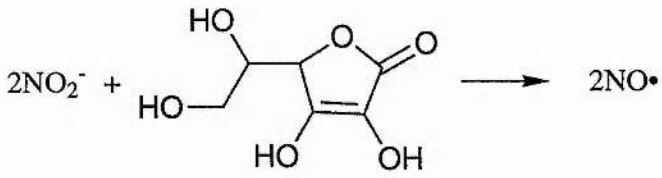
The main method of analysis for these compounds was by EPR spectroscopy. Spectra were recorded at either 9.15 GHz with a Bruker ER 200D instrument (analogue trace), or 9.45GHz with a Bruker ER200D controlled by a PC with appropriate (digital spectrum) software. Samples were prepared in quartz tubes with cyclohexane or *tert*-butylbenzene as solvent, and degassed by bubbling N_2 for 10 minutes. For more polar solvents, such as water or MeCN, capillary tubes (~ 3mm i.d.) were used. The EPR spectrometer was coupled to a Bruker temperature controller connected to a liquid nitrogen Dewar required for low temperature work.

Photolysis was carried out by a 500W super pressure Hg lamp focused into the cavity. Spectra were simulated using an Opus Technology PCV386 computer running the XESR program for exchange broadened EPR spectral simulation[138] on the Micro VAX-II SAVC system.

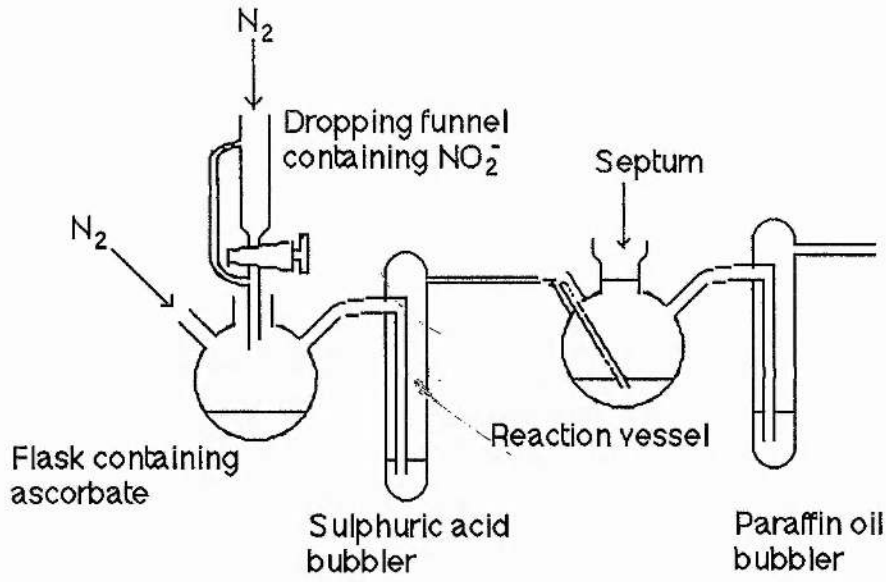
2.4.1. Preparations.

2.4.1.1. *Nitric oxide.*

This was produced by the action of sodium nitrite ($NaNO_2$) on ascorbic acid, according to the following equation, as described by Williams[139] and Nocek and Kurtz[140].



This is a quantitative reaction, therefore molar equivalent amounts were used, i.e. $NaNO_2$ (3.45g, 0.05 mol) in DIW ($25cm^3$) and L-ascorbic acid (3.52g, 0.02 mol) in DIW ($20cm^3$) produces two mol of NO. The ascorbic acid solution was placed in a three-necked flask and the nitrite solution was added from a dropping funnel. The flask was connected to a bubbler containing concentrated sulphuric acid to dry the evolved NO. This bubbler was connected to a second three-necked flask containing the solvent and prospective substrate. A second bubbler containing paraffin oil was connected to the flask to prevent oxygen intake and a rubber septum was attached to facilitate removal of the reaction mixture via a syringe (see diagram).



If the reaction required an increase in temperature, the second flask was placed in an oil bath on a magnetic stirrer/hot plate. The whole system including the nitrite in the dropping funnel was then degassed using N_2 for 15 minutes prior to starting each experiment.

2.4.1.2. o-*Quinone.*

o-Quinone was synthesised by the oxidation of catechol as described in Vogel[120], but different quantities of reagents were used. Celite was purified by successive washing with MeOH containing 10% concentrated hydrochloric acid and then distilled water until neutral. This was then dried at 120°C. Purified celite (15g) was added to a stirred solution of silver nitrate (17g, 0.1mol) in 100cm^3 distilled water. A solution of potassium hydrogen carbonate, (10.5g, 0.105 mol), in distilled water was then added slowly to the suspension. Stirring was continued for 10 minutes and a yellow/green precipitate was formed. This was filtered off and dried on a rotary evaporator over a period of several hours. Azeotropic distillation with toluene was used to free the product from residual water. Catechol, (1.0g, 9 x10^{-3}mol), was then refluxed in 200cm^3 toluene, and the reaction monitored by TLC. When the reaction was complete, the product was filtered off, and the residual solvent evaporated. Pure *o*-quinone was obtained.

2.4.1.3. *p-Toluenesulphonyl azide.*

This was prepared according to a literature method[141]. A solution of 71.5g, (1.1 mol) sodium azide in 200cm^3 distilled water was placed in a 2l Ehrlenmeyer flask and diluted with 400cm^3 90% aqueous EtOH. To this solution was added with stirring a warm (45°C) solution of 190.5g, (1.0 mol) *p*-toluenesulphonyl chloride in 1l 99% EtOH. The reaction was left stirring for 2.5 hours, after which time the solvent was removed on a rotary evaporator. The residue was mixed with 1.2l distilled water in a separatory funnel, and the oily layer was removed. The oil was washed with two 100cm^3 portions of distilled water, and dried over anhydrous sodium sulphate. Filtration yielded a colourless product which crystallised on standing. The yield was 76%. Analysis by ^{1}H NMR spectroscopy revealed aromatic peaks in the 7.4-8.2 ppm region, and IR analysis showed the azide functionality at 2250 cm^{-1}.

2.4.1.4. *2-Hydroxymethylenecycloheptanone*

2-Hydroxymethylenecycloheptanone was also prepared according to the literature[142] but initially with reduced quantities. This gave a product that was too impure to use, so the original amounts were used in a second preparation. A mixture of 23g (1mol) of sodium cut into 1cm cubes, 2l of dry ether, 112g, (1 mol) of cycloheptanone and 110g, (1.5 mol) of ethyl formate was placed in a 5l three-necked flask, equipped with overhead stirrer, stopper, and vent tube. The reaction was initiated by addition of 5cm^3 EtOH, and the flask was cooled in a water bath. After stirring for 6 hours, the mixture was left to stand overnight. After this, a further 25cm^3 EtOH was added, and the mixture stirred for a further 30 minutes. After addition of 200cm^3 distilled water, the solution was transferred to a 3l separating funnel and the ether layer washed with 50cm^3 water. The combined aqueous extracts were washed with 100cm^3 ether, and the aqueous layer acidified with 165cm^3 6 M hydrochloric acid. This was then extracted twice with 300cm^3 ether. The ethereal extract was then washed with 25cm^3 saturated sodium chloride solution and dried over anhydrous magnesium sulphate. The drying agent was removed by filtration and the ether removed on a rotary evaporator. The residue was distilled under reduced

pressure using a 6 inch Vigreux column. Analysis by 300MHz ^{1}H NMR spectroscopy gave δ_H 1.5-1.8 (6H, m, 3x CH_2), δ_H 2.4-2.6 (4H, m, 2x CH_2), δ_H 7.3 (1H, m, 1x -CHC=), and δ_H 14.7 (1H, d, 1x CHO), indicating the presence of the aldehyde tautomer. IR Spectroscopy confirmed the presence of the -OH (3500 cm^{-1}).

2.4.1.5. *2-Diazocycloheptanone*

The preparation was carried out by the method of Regitz *et al.*[143]. A mixture of 73g, (0.525 mol) 2-hydroxymethylcycloheptanone, 400cm^3 dichloromethane, and 106g, (1.05 mol) of triethylamine was placed in a 2l Ehrlenmeyer flask and cooled in an ice-salt bath to between -12 and -15°C. To this was added 98g, (0.5 mol) *p*-toluenesulphonyl azide, with vigorous stirring over a period of 1 hour. The azide was added at such a rate that the temperature did not rise above -5°C. Stirring was continued for a further 2 hours. A solution of 30.8g, (0.55 mol) of potassium hydroxide in 400cm^3 distilled water was added, and the mixture stirred at room temperature for 15 minutes. The resulting emulsion was then placed in a separating funnel and the dichloromethane layer removed. The aqueous alcoholic layer was washed with two 100cm^3 portions of dichloromethane. These extracts were combined and washed with a solution of 2.8g (0.05 mol) potassium hydroxide in 200cm^3 water, and then 200cm^3 water. The solution was then dried over anhydrous sodium sulphate. The solvent was removed on a rotary evaporator, and the crude orange oil was refrigerated until required. Distillation of the crude product was carried out under reduced pressure using a small air-condensed apparatus. This provided pure product which was analysed by NMR and IR spectroscopy. ^{1}H 300MHz NMR analysis revealed δ_H 2.4-2.6, (4H, m, 2x CH_2), and δ_H 1.6-1.8 (6H, m, 3x CH_2). The diazo and carbonyl functionalities were observed at ~2150cm^{-1} and 1650cm^{-1} respectively when examined by IR spectroscopy.

2.4.1.6. *5-Diazouracil*

5-Diazouracil was prepared by the method of Thurber and Townsend[144]. A solution of 2.0g, (1.6 x10^{-2} mol) of 5-aminouracil in 40cm^3 1M hydrochloric acid was cooled to 0°C in an ice-salt bath. To this was added dropwise 18cm^3 of a 6.9% sodium

nitrate solution over a period of 30 minutes. The temperature was kept to less than 3°C. The mixture was stirred for 5 minutes after the final addition of nitrate. The solid formed was collected by filtration and washed with ice water until a silver nitrate test for chloride ion proved negative. The remaining solid was added to boiling water and stirred at reflux temperature until solubilised. Activated charcoal (150mg) was added to this solution, and stirred. This was then removed by filtration, and the filtrate allowed to stand for 18 hours. After this time, yellow crystals were observed and collected by filtration. These were dried by azeotropic distillation with toluene at reflux temperature for 8 hours, m.p. 195°C (lit[144] 195-197°C). IR analysis showed the N=N stretch at 2150cm^{-1}. Analysis by 300MHz ^{1}H NMR in D_2O with dioxan as standard revealed peaks which coincided with those in the literature.

2.5. Details of reactions.

2.5.1. Reaction NO with NOCT-type compounds.

2.5.1.1. *Anthracene.*

The apparatus was set up as described above and anthracene (0.5g, 0.002mol) was dissolved in toluene (20 cm^3). The flask containing the anthracene and toluene was placed in an oil bath and heated to the desired temperature. The solution was then degassed by bubbling N_2 for 15 minutes. Other solvents used for this experiment were cyclohexane, 1,2-dichlorobenzene and *tert*-butylbenzene. Samples were taken after 1 hour and sent for GC-MS analysis. The chromatograms of the room temperature reactions showed only starting material. The reactions at 80°C showed anthracene, together with minor amounts of a single product with a MS similar to that of benzophenone.

2.5.1.2. *o-Quinone* .

Fresh o-quinone (0.5 g, 0.005 mol) was added to *tert*-butylbenzene (20 cm^3). The solution was degassed by bubbling N_2 through it, then NO was bubbled through. Samples were taken 1 hour after the initial addition of NO and then again after a further

1 hour. GC-MS analysis showed only starting material, but an EPR spectrum showed a weak three line signal characteristic of nitroxides.

2.5.1.3. *2-Acetylcyclohexanone.*

A solution of 0.5g (3.57 $x10^{-3}$ mol.) of 2-acetylcyclohexanone in 20 cm^3 cyclohexane was made up and NO was bubbled through. A sample was taken after 3.5 hours and submitted for EPR spectroscopy. No paramagnetic species were present in the solution, or in any subsequent samples taken at 5, 23 and 25 hours.

2.5.2. Reaction of NO with nitromethane ion-related compounds.

2.5.2.1. *Nitromethane.*

A 50mM solution of nitromethane in 0.5M NaOH was degassed by bubbling N_2 through the solution. Nitric oxide was then bubbled vigorously through this solution. No EPR signal was observed when a sample was taken after 1 hour. After 5 hours in contact with NO, a second sample was removed and analysed by EPR spectroscopy. A signal comprising 18 lines was observed.

2.5.2.2. *Nitroethane.*

Nitric oxide was bubbled through a 50mM solution of degassed nitroethane in 0.5M NaOH for a period of 24 hours. Samples were taken after 0.5, 3, and 20 hours. The only sample to show any sign of spin trap activity was that taken at 20 hours. A spectrum of greater than 10 lines was observed. On addition of 2M NaOH, the spectrum increased in intensity. Analysis by GC-MS showed that no identifiable products were present.

2.5.2.3. *Methyl nitroacetate.*

A solution of methyl nitroacetate was prepared in the same concentration as that used for nitromethane, i.e. 50mM. The solvent was 0.5M NaOH. The solution was degassed and NO was bubbled through for 5 minutes. No EPR signal was observed. The pH was raised by addition of $5cm^3$ 2M NaOH but the expected spectrum was absent. Analysis by GC-MS did not reveal any trace of the expected spin adduct **9** or its degradation products.

2.5.2.4. *Diethyl malonate.*

A 50mM solution (0.160g, $1x10^{-3}$ mol) of diethyl malonate [$C_2H_5O_2CCH_2CO_2C_2H_5$] in 0.5M NaOH was degassed for 1 hour. Nitric oxide was bubbled through for 1 hour. and a sample taken for EPR. No paramagnetic species were present. Analysis by GC-MS indicated that no reaction had taken place.

2.5.2.5. *Malononitrile.*

A solution of malononitrile (0.5g, $7.5 x10^{-3}$ mol.) in $20cm^3$ 0.5M NaOH was degassed for 1 hour. Nitric oxide was bubbled through for 2 minutes, and a sample was taken. No EPR signal was observed . The basicity was increased by addition of 2M NaOH ($5cm^3$) and NO bubbled for a further 2 minutes. There was still no signal observed. After 3 hours of gassing, no EPR spectrum was obtained.

2.5.2.6. *2-Nitropropane.*

A 50mM (0.089g, $1x10^{-3}$mol in $20cm^3$ 0.5M NaOH) solution of 2-nitropropane was degassed and then NO was bubbled through. Samples was taken after 21 and 22 hours but these showed no paramagnetic activity. Even after air was introduced there was no EPR signal.

2.5.2.7. *Ethyl nitroacetate.*

Nitric oxide was bubbled through a degassed 50mM solution of 0.133g ($1 x10^{-3}$mol) ethyl nitroacetate in $20cm^3$ 2M NaOH. No EPR signal was observed. The basicity was increased to 5M NaOH, and NO was again bubbled through a 50mM solution of ethyl nitroacetate. After 22 hours, there was no sign of a reaction. Analysis by EPR spectroscopy showed no adduct had been generated.

2.5.3. Reaction of NO with dienes.

2.5.3.1. *Trans,trans-1,4-diphenyl-1,3-butadiene.*

The substrate (0.5g, 0.002 mol) was dissolved in toluene (20 cm^3). The solution was degassed using N_2, then nitric oxide was bubbled through the solution for 1 hour at room temperature. Other solvents used were *tert*-butylbenzene and 1,2-dichlorobenzene, again at room temperature and cyclohexane, which was heated to 60°C to aid solvation. Yellow crystals separated from the solution on cooling. MS

analysis indicated that these were the starting material. EPR spectra of the solution and crystals showed no paramagnetic species had formed.

2.5.3.2. *2,5-Dimethyl-2,4-hexadiene.*

This was reported to have formed a spin trap by cyclisation[105] in anaerobic conditions. Further work on this area[106] disagreed with this, and so we decided to test both theories. Nitric oxide was bubbled through a degassed solution of 0.4g (3.6×10^{-3}mol) of the hexadiene in 20cm^3 *tert*-butylbenzene and a sample was taken for EPR. This gave rise to 3-line nitroxide signal, along with a second radical which gave a triplet of doublets.

2.5.4. Reaction of NO with diazo compounds.

2.5.4.1. *2-Diazocycloheptanone*

After the successful synthesis of the diazo compound, solutions comprising 0.227g (1.6×10^{-3}mol) of 2-diazocycloheptanone in 0.6cm^3 *tert*-butylbenzene was made. Each was placed in an NMR tube fitted with a septum and degassed with N_2. One was used as a control and was placed in the cavity of the EPR and showed no signal at room temperature, 325K, or 260K. Upon photolysis, a very weak 3-line spectrum appeared. Nitric oxide was then bubbled through the second tube for 5 minutes at 275K and this gave a weak signal. Further exposure to NO (15 minutes) at 260K led to a very strong signal. The major triplet (ca.30G) was split into a complex pattern. Second derivative studies resolved most of the lines and the analysis was confirmed by simulation. On raising the temperature to 330K, the signal became much weaker. After leaving the sample overnight, the signal had decayed almost to zero. Upon photolysis, the wide iminoxyl spectrum disappeared and was replaced by a 5-line spectrum, possibly of two radicals.

2.5.4.2. *5-Diazouracil.*

A solution of 0.02g (1.5×10^{-4} mol) of 5-diazouracil in 5cm^3 distilled water was degassed and an aliquot combined with a sample taken from a NO/distilled water solution. At room temperature there was no reaction, but on lowering the temperature

to 225K and photolysing the reaction mixture a radical was generated giving an EPR signal of >11 lines.

2.5.5. Reaction of 2-diazocycloheptanone with NO-releasing compounds.

2.5.5.1. *S-Nitroso-N-acetylpenicillamine.*

This molecule releases NO in the presence of Cu^{2+} in aqueous solution quite rapidly, and also when exposed to UV light. Two flasks were prepared, one containing a solution of 0.02g (1.45 $x10^{-4}$mol) 2-diazocycloheptanone in $3cm^3$ *tert*-butylbenzene, the other containing 4.2 $x10^{-3}$g (2.03 $x10^{-5}$mol) SNAP in *tert*-butylbenzene. The solutions were degassed with N_2 and aliquots of each were removed and mixed in a quartz EPR tube. This was then placed in the cavity of the EPR spectrometer and examined at room temperature. No signals were observed under these conditions. As SNAP was only sparingly soluble in *tert*-butylbenzene, the solvent was changed to acetonitrile. This had no effect on the results obtained. Further experiments were carried out under different conditions. These included heating the tubes and exposure to UV light for a period of 10 minutes.

2.5.5.2. *Isoamyl nitrite.*

A similar protocol to the above was employed, i.e. two flasks were prepared and degassed with N_2, one containing 0.0240g (1.74 $x10^{-4}$mol) of the diazo compound in $3cm^3$ *tert*-butylbenzene, the second with 0.0351g (3.00 $x10^{-3}$mol) of isoamyl nitrite in $3cm^3$ *tert*-butylbenzene. Again aliquots were removed and combined in quartz tubes. At room temperature, there was no signal from the reactants. Upon photolysis for 10 minutes, a weak signal comprising 5 lines was observed. This did not correspond to any of the expected radicals and must therefore have been a mixture. The only identifiable radical was the nitroxide (~14G splitting). The iminoxyl was absent.

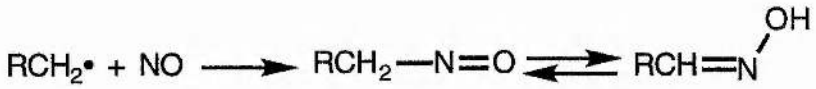
CHAPTER 3.

Nitric oxide and stable free radicals

3.1. The Reaction of NO with stable free radicals.

3.1.1. Stable free radicals.

The technique of spin trapping is obviously of fundamental importance when dealing with NO analysis. However, the new traps tried out in this study did not give a definite quantitative result for the amount of NO produced. Other methods of quantification were therefore sought. Although NO does not react with stable molecules, it has been known for some time[98,99,145] to react rapidly and efficiently with carbon-centred radicals in the gas phase to give nitroso compounds:



These can tautomerise to the oxime if there is an α-hydrogen present.

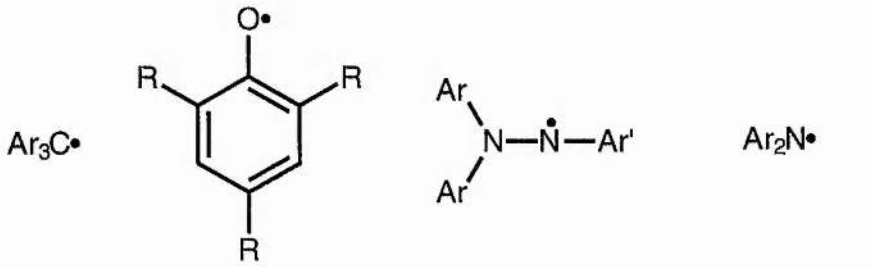
Nitric oxide has been used as a scavenger molecule for radicals in kinetic studies[146-149], and such precursors are well known in atmospheric chemistry[150,151]. We proposed to use the reaction of NO with a persistent free radical as a basis for a method of quantifying NO concentration. As the nitroso compounds formed from the radical precursors are coloured, it seemed possible that a long-lived free radical or free radical precursor would be suitable as an NO trap to provide a nitroso product which could be analysed by U.V. spectrophotometry. A series of investigations was begun into potential traps for the spectrophotometric determination and EPR analysis of the NO adducts. Two such materials were studied in depth.

A stable free radical can be synthesised via a conventional chemical pathway, whereas other more transient radicals are generated thermally, photolytically, mechanically, or through electron transfer reactions. These radicals are much more reactive unless they are trapped, (as explained in ch.2), or bound to a surface. Thus, a stable free radical can be defined as one which can be produced conventionally and be used in subsequent reactions or be studied statically by spectroscopic means. An inert medium is usually advantageous. However, the description 'stable' is now considered something of a misnomer, as thermodynamic stability is a quite different property than persistence. It is the bond dissociation energy of a molecule that measures the

thermodynamic stability of the radical, i.e. its ability to recombine, according to the equation:

$$R—R' \rightleftharpoons R\bullet + R'\bullet$$

Steric effects provided by bulky side groups such as phenyl help to shield the radical centre and thus affect the reactivity, and this is what promotes the persistence. Thus, there are two properties that affect the radical, thermodynamics (bond energies), and kinetics (reactivity). There are several types of long-lived radical which will be briefly mentioned here, including triarylmethyl, aroxyl, and hydrazyl. Nitroxide radicals have already been discussed, so the remainder can be described. A pattern for these radicals emerges when it is shown that they all possess bulky side chains, typically aryl (as shown below). Thus the increased steric hindrance enhances the persistence of radicals.

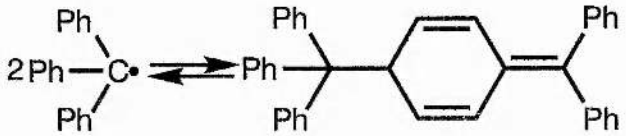


Triarylmethyl Aroxyl Hydrazyl Diarylaminyl

3.1.1.1. *Triarylmethyl radicals.*

Triarylmethyl radicals are known to exist in equilibrium with their dimers. At the turn of the century, Gomberg discovered the existence of a stable, persistent free radical, triphenylmethyl. It was thought that this radical was in equilibrium with its dimer, hexaphenylethane. Synthesis of other "hexa-arylethanes" was attempted, and this involved the reaction of a triarylmethyl chloride with a metal, usually silver, mercury, or zinc, at room temperature in a dry solvent. Benzene used to be the solvent of choice, but more commonly petrol, acetone or ether are employed. The reaction was carried out in the absence of both oxygen and preferably light, as the latter caused disproportionation. However, it was not until 1968 that it was realised that this assumption of the dimerisation was erroneous. Eventual investigation by Lankamp *et*

al.[152] using UV and NMR spectroscopy revealed errors in the influence of *para*-substituents on dissociation constants in analogous compounds, and this led to questions about the structure[153]. The structure of the dimer was eventually elucidated as 1-diphenylmethylene-4-triphenylmethyl-2,5-cyclohexadiene, **12**.



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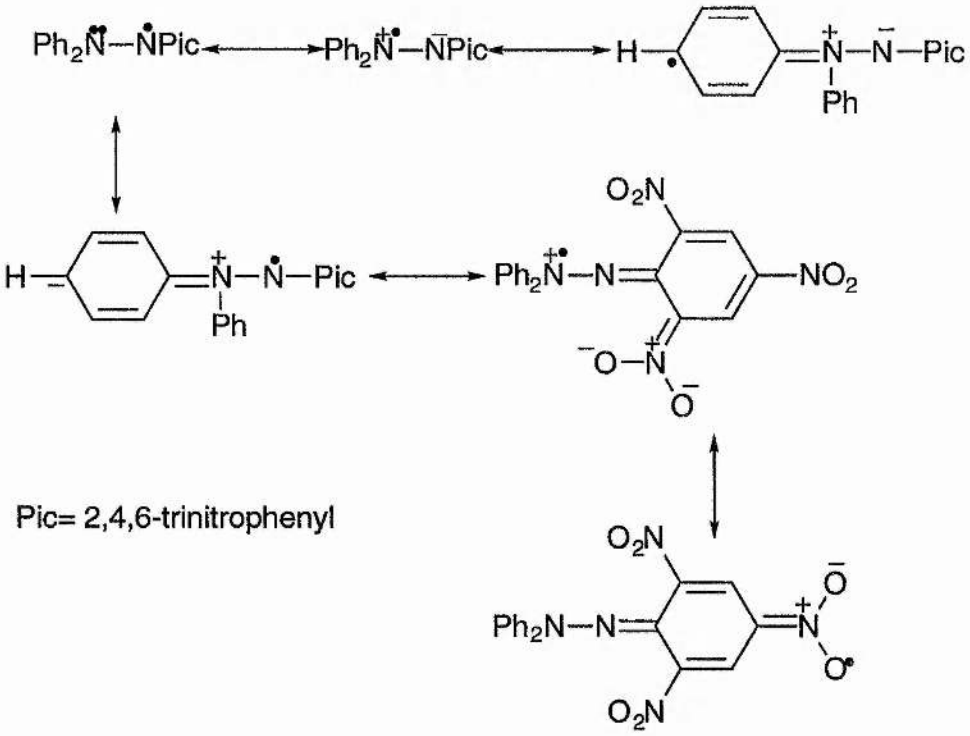
There are other methods of radical formation, for example, the reaction of triphenylacetyl chloride with silver gives the triphenylmethyl radical and carbon monoxide, and the oxidation of a triarylmethyl carbanion or the reduction of a triarylmethyl carbonium ion also leads to triarylmethyl radical formation. Dimerisation is the dominant reaction of $Ar_3C^{\bullet}$ radicals and leads to the formation of the head to tail dimer shown above. It is governed by steric strain in the dimer and mesomeric stabilisation of the radical. Both of these factors are themselves influenced by the substitution pattern of the dimer. Dissociation can be increased by *para*- or *ortho* -substitution of the aryl group. This leads to a non-planar conformation which will tend to stabilise the radical. On heating or exposure to light, triarylmethyl radicals tend to disproportionate, particularly if they contain primary or secondary alkyl groups. Hence, when using these radicals it is necessary to exclude light, reduce the temperature and work with freshly prepared solutions. Peroxides can be formed if triarylmethyl radicals are exposed to air. This provides a simple diagnostic test for the presence of triarylmethyl radicals, as the peroxide products are easily detectable. Triphenylmethyl combines with NO and NO_2[154], but no products have been isolated on addition of NO. Addition of NO_2 yielded triphenylmethyl nitrite and triphenylnitromethane. On heating, both reactions evolved oxides of nitrogen, implying that the reactions were reversible.

Triarylmethyl radicals appear to add conventionally to conjugated systems, e.g. the formation of 1,4-di-(triphenylmethyl) isoprene from isoprene via a 1,4-addition.

3.1.1.2. *Hydrazyl radicals.*

Hydrazyl radicals were discovered in the twenties by Goldschmidt and coworkers[155]. Possibly the most well known compound in this class is 2,2-diphenyl-1-picryl hydrazyl (DPPH). This is a remarkably stable radical, showing no tendency to dimerise in the solid state or in solution. It is used mainly as a scavenger in polymer chemistry, and as a standard for EPR spectroscopy. Hydrazyls are generally prepared by the oxidation of the corresponding trisubstituted hydrazine with PbO_2 or Ag_2O. DPPH has been investigated extensively, and it has been shown that it can exist in various forms due to the extent of delocalisation around the molecule. The unpaired electron can thus be distributed over a number of sites. This has led to the following canonical structures being proposed:



3.1.1.3. *Aroxyl radicals.*

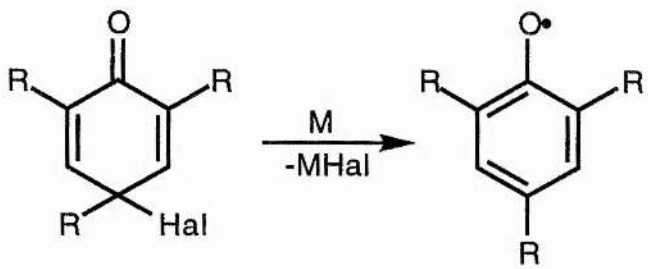
Stable aroxyl radicals were first recognised by Pummerer and coworkers in the early twentieth century[156] among the oxidation products of 2,2'-dihydroxy-1,1'-binaphthyl. Aroxyl radicals can be formed by irradiation or flash photolysis of a precursor, but only chemical procedures are useful for preparative purposes. Direct

methods can be used for synthesis, for example by electron removal from the corresponding phenoxide ion or hydrogen abstraction from the phenolic hydroxyl group, or indirectly by generation from related cyclohexadienones. Oxidising agents such as alkaline potassium ferricyanide, lead dioxide and silver oxide are frequently used. Phenols can also be oxidised with lead tetraacetate, benzoyl peroxide and ceric sulphate, but these are lesser used methods of production. Aroxyls can also be formed by dehydrogenation of phenols with aroxyls of higher redox potential. These form the symmetrical cyclohexadienone ether and regenerate the parent aroxyl. Similarly, if tri-*t* -butylphenol is shaken with an aroxyl of lower potential in the presence of manganese dioxide, then the cyclohexadienone ether will be generated. In this case, the parent phenol is regenerated and is immediately oxidised to the phenoxyl. Thus there is a high yield of product as a cascade of reactions is initiated.

An alternative method of preparation of aroxyls involves the oxidation of sodium phenoxide with halogens. Sodium salts can be formed from the reaction between triphenylmethylsodium and highly hindered phenols. Upon treatment with iodine in solution, aroxyl radicals are produced.

$$2ArO^-Na^+ + I_2 \longrightarrow 2ArO\bullet + 2Na^+I^-$$

The indirect method relies on the homolytic fission of related cyclohexadienones. Some aroxyl radicals exist as crystalline dimers and thus are in equilibrium in solution. Reaction of halocyclohexadienones (Cl or Br) with iodide ion or metals produces the aroxyl radical.



The most frequent type of reactions favoured by aroxyls are dimerisation and oxygen uptake. Mostly, stable aroxyls form peroxides in air, hence preparation is carried out under N_2. From experimental evidence, it would appear that the extent of

delocalisation and substitution affects the ability of the aroxyl radical to react with oxygen. Galvinoxyl[157], tetra-*t* -butylindophenoxyl[158], (a galvinoxyl analogue with a similar EPR spectrum), 2,4,6-triphenylphenoxyl[159], and polyhalophenoxyls[160,161], are all stable in air.

If NO_2 is bubbled through a solution of 2,4,6-tri-*t* -butylphenoxyl, the nitro adduct is rapidly formed. There are no reported reactions of aroxyls with NO, although pentachlorophenoxyl reputedly gave an isolable adduct with nitric oxide[162]. More recent work by Janzen *et al.*[162,163], has been carried out on the reaction of NO with sterically hindered phenols. The first to be investigated were 2,4,6-tri-*t* -butylphenol, 2,6-di-*t* -butyl-4-methylphenol, α-tocopherol (vitamin E), 4,4'-methylenebis(2,6-di-*t* -butylphenol), and phenyl-4,4-methinebis(2,6-di-*t* -butylphenol). It was reported that NO reacted with all of the above mentioned phenols to give the phenoxyl radical. This subsequently reacts with excess NO to generate, in the case of 2,4,6-tri-*t* -butylphenol, the nitrite and 2C-nitroso compounds (detected by TLC and ^1H NMR). When excess NO is removed (by bubbling N_2), the adduct dissociates back to the phenoxyl radical and NO. When analysed by EPR spectroscopy, all the compounds tested gave hyperfine splittings for the observed radicals which were comparable with those obtained from authenticated literature values, with the exception of 2,6-di-*t* -butyl-4-methylphenol. However, TLC and NMR studies showed that a new product had been formed. Thus, NO does appear to react with sterically hindered phenols to give phenoxyl radicals, and subsequently NO adducts. These adducts dissociate slowly in the absence of excess NO back to the phenoxyl radical.

The second investigation was very similar, the difference being that the reactions were carried out in sodium dodecyl sulphate (SDS) micelles to simulate a more biological environment. It was concluded that there are two possible mechanisms for phenoxyl radical production from the reaction of NO with phenols; hydrogen abstraction to form HNO, which in aqueous solution dimerises and is then reduced to give N_2O, or a one-electron reduction of NO leading to the nitronyl anion and the phenol radical cation. Phenoxyl would be formed by loss of a proton from the

phenol radical cation. This could be of biological significance as it has been shown that the nitronyl anion is also biologically active, reacting with protein sulphydryl groups[164] and with haem complexes. It may therefore be that phenols and other antioxidants attenuate the NO concentration *in vivo*. However, formation of HNO is thermodynamically unfavourable and it seems possible that the reactions are really initiated by hydrogen abstraction from the phenols by NO_2 (formed if traces of O_2 were present):

$$ArOH + NO_2 \longrightarrow ArO\bullet + HNO_2$$

The aroxyl radical then couples with the nitric oxide.

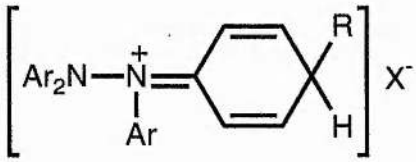
In summary, aroxyl radicals undergo many other reactions, including interaction with carbon-centred radicals, as used in antioxidant systems, dimerisation of the simpler aroxyls (due to steric effects and the extent of delocalisation), disproportionation of the bulkier aroxyls, reaction with other aroxyls to generate asymmetric cyclohexadienone ethers, addition to carbon-carbon double bonds, and their action as electron acceptors to form the aroxide ion. Aroxyls can also effectively remove hydrogen from a variety of functionalities, be they alkyl, amino, hydroxyl, or sulphydryl groups.

3.1.1.4. *Diarylamino radicals.*

In 1911, Wieland observed that a colourless solution of tetraphenylhydrazine turned green on heating and then faded on cooling[165]. Addition of $Ph_3C\bullet$ to the green solution gave the adduct $Ph_2N\text{-}CPh_3$, and the reaction with NO produced the nitrosamine Ph_2NNO. Thus, he concluded that the diphenylaminyl radical must be in equilibrium with its dimer.

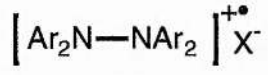
The dissociation of tetra-arylhydrazines depends upon the substituent groups present on the ring, particularly in the *para* -position, and on external factors such as light, temperature, concentration, and solvent. As in previous cases, the extent of delocalisation of the unpaired electron plays an important role in the stability of the radical. Electron donating groups such as OMe, $-NMe_2$, and Me are effective at increasing dissociation. Diphenylaminyl radicals purportedly disproportionate in hot

solution to yield diphenylamine and 9,10-diphenyldihydrophenazine, the latter via *ortho* -coupling. Dilution and the presence of light favours the reaction. Other work has shown that coupling can occur through the *para* - as well as *ortho*- positions. Diarylaminyl radicals do not react with oxygen, unlike triarylmethyls. They will react reversibly with NO and $Ph_3C^{\bullet}$ in solution to give diarylnitrosamines and triphenylmethyldiarylamines, respectively. Diarylnitrosamines are less stable, dissociating to NO and the disproportionation products of the radical. Diphenylaminyl radicals are also weak oxidising agents, and initiate the polymerisation of styrene and acrylonitrile[166], but inhibit the polymerisation of butyl acrylate[167]. A test for the presence of a tetra-arylhydrazine is to see if there is a colour change in the presence of an acid such as acetic , sulphuric, or hydrochloric. Wieland proposed that the quinonoid salt, **13,** had been formed:



13

It was proved that the N-N bond was intact, as the above salts could be converted back to the parent hydrazines by treatment with alkali. Evidence[168-170] showed that these salts are in fact hydrazinium radical ions, **14**:



14

Their structure was elucidated by preparation of tetrahydrazinium perchlorate and analysis of the structure and by its reduction to the hydrazine with mild reducing agents (KI, Fe^{2+} salts). EPR spectroscopy has now confirmed this structure. Hammond *et al.*[171] showed that Ph_2NNPh_2 reacts with strong acids to give Ph_2NH, N,N'-diphenylbenzidine, and a polymeric material derived from the latter by oxidation. The $Ph_2N^{+\bullet}H$ radical cation has been postulated as a transient intermediate in this reaction. Related to the diarylaminyl radicals are the phenazyls, phenothiazyls, pyrryls and

imidazyls. Phenazyl radicals are thought to be biologically important, as phenazines have been implicated in electron transfer reactions.

3.2. Stable free radicals and NO.

3.2.1. Synthesis of N-nitrosodiphenylamine.

The nitrosamine Ph_2NNO was synthesised as described in the experimental section. This was also analysed by UV because an authentic sample was needed for calibration purposes. The UV absorbance values were in the region 200-400nm which correlated well with those from the tetraphenylhydrazine. The results indicate that the diphenylaminyl radical is scavenging nitric oxide and thus forming the nitrosamine. The Beer-Lambert law was used to determine the molar extinction coefficient, ε. This was calculated as 6153.8 $M^{-1}cm^{-1}$ for a 3.25 $x10^{-3}$ M solution of Ph_2NNO in MeCN.

Infra-red spectroscopy was carried out on the nitrosamine synthesised from diphenylamine using KBr discs, and peaks were found at 1469, 1458, and 1440 cm^{-1}, indicating the presence of N=O. The spectra were compared with those obtained from the reaction between tetraphenylhydrazine and nitric oxide and a similar set of peaks were observed. ^1H NMR was carried out confirming the presence of aromatic rings. The lack of a signal at ~6ppm shows that there was no NH present so there was no diphenylamine in the sample. A solution of the nitrosamine was degassed using N_2 then heated to 130°C. No colour was observed in the flask or in the bubbler prior to or during heating, but when N_2 was used to flush the system, brown fumes (NO_2) were seen exiting from the second bubbler containing paraffin oil. It can therefore be concluded that nitric oxide was evolved from the nitrosamine on heating and reacted with atmospheric oxygen to form NO_2. This is consistent with findings in the literature[172].

3.2.2. Tetraphenylhydrazine.

Tetraphenylhydrazine is known to dissociate in solution to give an equilibrium mixture with the diphenylaminyl radical:

$$Ph_2NNPh_2 \rightleftharpoons 2Ph_2N\bullet$$

We envisaged, therefore, that the diphenylaminyl radical would trap NO to give N-nitrosodiphenylamine which is a known compound and which could be analysed spectrophotometrically. Indeed it is known that solutions of tetraphenylhydrazine absorb NO at 100°C to give quantitative yields of N-nitrosodiphenylamine[172].

Tetraphenylhydrazine was synthesised by the literature method[173], but difficulties were experienced in obtaining material of sufficient purity for analytical work. The techniques used for purification are described in the experimental section, along with the method of synthesis. A sample of the tetraphenylhydrazine synthesised first was dissolved in $20cm^3$ cyclohexane and degassed with N_2. Nitric oxide was then bubbled through the solution. Samples were withdrawn every 15 minutes, and monitored by UV analysis. No colour change was apparent. Analysis of the reaction mixture by GC-MS showed that no products were formed, but starting material and diphenylamine were identified. It was thought that the presence of diphenylamine may have inhibited the reaction. Consequently, further purification of the tetraphenylhydrazine was carried out by use of an adsorption column packed with alumina. After passing the tetraphenylhydrazine solution down the column, the solution was recovered and the tetraphenylhydrazine was used in the reaction with NO, again at room temperature and in cyclohexane. After leaving the reaction overnight, it was refrigerated and bright orange crystals were obtained. It was thought that these crystals were the nitrosamine. A sample of the crystalline material was sent for mass spectroscopic and ^1H NMR analysis. The latter revealed a series of small peaks in the 7.2-7.4 ppm. region, which were in a 4:1 ratio, consistent with the projected phenyl coupling. High resolution MS and GC-MS revealed no identifiable products, but again a small quantity of diphenylamine was detected. The ^{13}C NMR showed two main peaks, corresponding to the *meta* and *ortho* carbon atoms. These were too ill-resolved to see clearly, probably due the weakness of the sample. Pure tetraphenylhydrazine was obtained by recrystallisation; NMR and mass spectral analysis showed it to be free of diphenylamine. A sample of this pure

tetraphenylhydrazine was then used experimentally in the reaction with NO at room temperature in cyclohexane.

3.2.3. EPR analysis of tetraphenylhydrazine

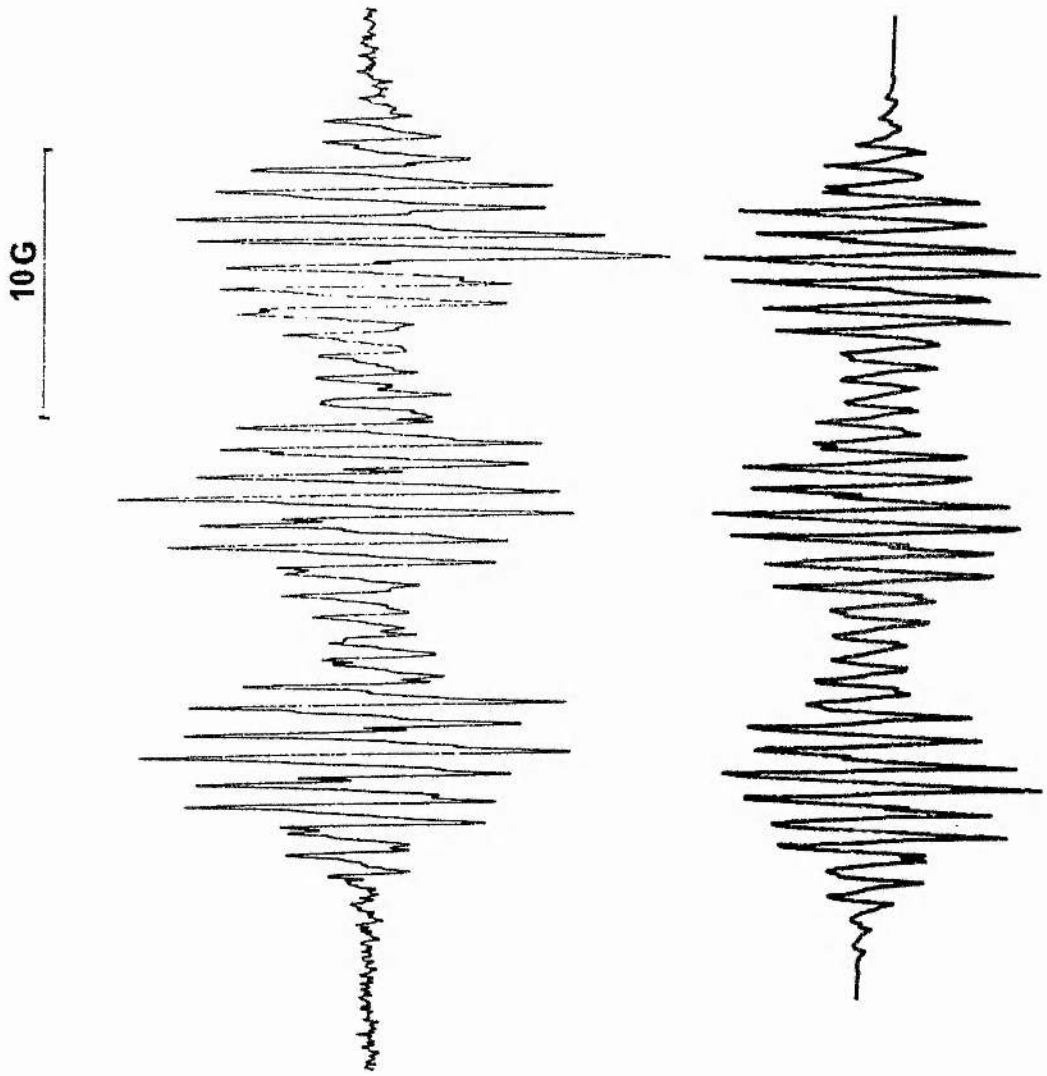
A degassed solution of tetraphenylhydrazine (10^{-2}M) in *tert*-butylbenzene was examined by EPR spectroscopy. The spectrum obtained is shown in figure 3.0. As stated earlier, tetraphenylhydrazine in solution is in equilibrium with the diphenylaminyl radical. The radical gives rise to a signal comprising three sets of multiplets. The conditions for this were: 1.25×10^5 gain; 0.16G modulation; 200s (sweep); 500ms scan time; 3335G; 100G sweep width; 9.13GHz; 16dB @ 330 K. It can be seen from the extreme right hand group of signals (see figure 3.0) that there is another signal underlying the diphenylaminyl radical. The lower portion of the figure represents a simulation of the spectrum observed using the values from Landolt-Bornstein obtained from authentic $Ph_2N^{\bullet}$ made by Marshal[174]. These were $\underline{a}$(N)= 9.68G, $\underline{a}$(6H)= 1.8G and $\underline{a}$(4H)= 0.8G. From the good correspondence between the experimental and simulated spectra, it can be concluded that the diphenylaminyl radical was obtained in high yield.

When NO was bubbled through the solution, no change was observed in the signal, indeed, the peaks appeared with greater resolution. This is unexpected as the NO should be scavenged by the diphenylaminyl radical, thus quenching the signal. A reason for this could be that NO was not bubbled through the solution for a sufficient length of time (~4mins.), although this should have reduced the signal to some extent.

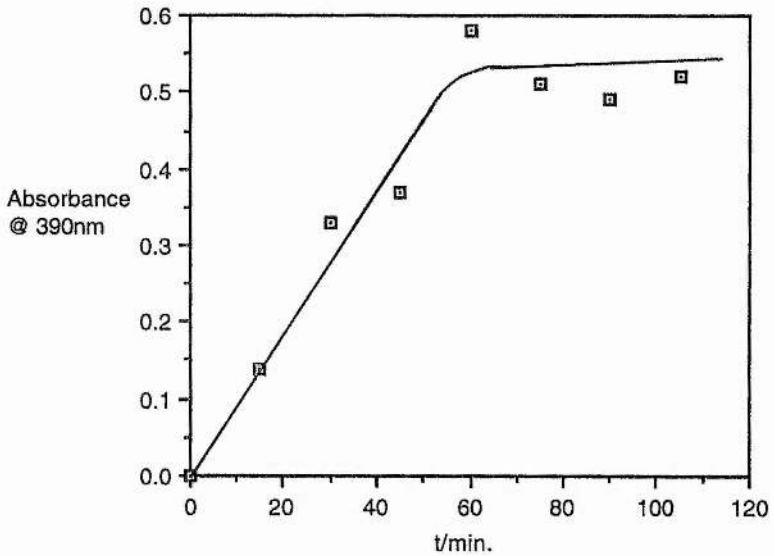
3.2.4. UV/vis analysis of tetraphenylhydrazine.

The reaction between tetraphenylhydrazine and nitric oxide was also monitored using UV/vis absorption spectroscopy. A standard solution of tetraphenylhydrazine was used as a blank against which all the other solutions were analysed. The samples taken from the reaction mixture all exhibited absorbance peaks between 350 and 400nm. The literature values[175] for the λ_{max} of the N=O functionality appear at 300 and 665nm for C-N=O, and at 218nm and 318-384nm for O-N=O. By comparison, the literature values for the absorbance of the nitroso group, N-N=O,[176,177] are λ_{max} at

Figure 3.0 Top; EPR spectrum of the diphenylaminyl radical obseved at 330K. Bottom; Computer simulation.



395nm and λ_{min} at 260nm. An amount of tetraphenylhydrazine was dissolved in cyclohexane to give a solution of known concentration. Nitric oxide was then bubbled through the solution and 0.5cm^3 samples were taken at timed intervals of fifteen minutes and diluted with cyclohexane to maintain the cuvette volume at 3cm^3. The absorbance was measured at 390nm and can be seen to be time-dependent, as the absorbance increased after 30 minutes, and then reached the λ_{max} after about one hour. This is illustrated in figure 3.1.



Graph of absorbance against time for the reaction of NO with tetraphenylhydrazine

Fig. 3.1

Another series of experiments was carried out using a continuous scan from 200 to 900 nm. Nitric oxide was bubbled through a degassed solution of 2 x10^{-5} mol Ph_2NNPh_2 in 20cm^3 cyclohexane. Samples (0.5cm^3) were taken every 15 minutes and made up to 3cm^3 with cyclohexane. A stock sample was used as a blank, and all subsequent samples referred to this. The results of this experiment are summarised in the figure 3.2.

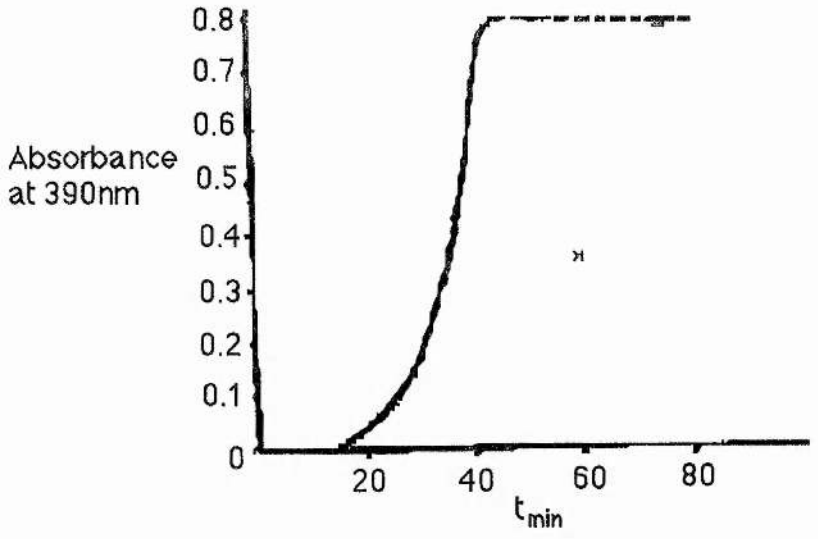


Fig. 3.2 Graph of absorbance against time at 390nm for the development of diphenylnitrosamine

It can be seen that apart from the erroneous point at 60 minutes, figure 3.2 shows an absorbance maximum at 390nm after approximately 40 minutes, after which it reaches a plateau. This is consistent with the above results.

For the next set of experiments, a known concentration of Ph_2NNPh_2 in *tert*-butylbenzene was used as standard. The amount of NO was then varied by means of a solution of NO in *tert* -butylbenzene. Aliquots of each were removed and mixed in a standard $3cm^3$ quartz cuvette. The preliminary experiments were set up and analysed at 415nm, and roughly done purely to determine the correct parameters. These experiments confirmed that there was a relationship between NO concentration and formation of Ph_2NNO, and that the formation of the product was very rapid, reaching a plateau in less than one minute, depending on the concentration. In comparison with the continuous scan experiments, it was decided that the absorbance at 390nm should be used. However, the major drawback with this set-up was keeping the Ph_2NNPh_2 concentration constant. As the volume of NO/$PhBu^t$ increased, so the Ph_2NNPh_2 concentration fell. To overcome this, an initial solution of NO/$PhBu^t$ was prepared, in conjunction with pure degassed $PhBu^t$. The NO/$PhBu^t$ solution was diluted by half each time; the same quantity of the Ph_2NNPh_2 solution was therefore used, and the

total volume in the cuvette remained constant. This gave improved results as shown below.

In the literature[178], it was reported that in a solvent such as toluene, the maximum NO concentration at 289K was 0.0123 mol dm^{-3}. In *m*-xylene, the NO concentration at 294K was 0.0111 mol dm^{-3}. Unfortunately, no data were given for PhBu^t. All the concentration values were similar for aromatic hydrocarbon solvents, so it could feasibly be assumed that the maximum NO concentration in PhBu^t would be comparable. The concentration of NO in MeCN was reported as 0.0152 mol dm^{-3} at 283K[178]. From this it was assumed that for our experiments, this was the starting concentration of NO in MeCN. The following graph (figure 3.3) displays the absorbance developing with time for the reaction of NO with tetraphenylhydrazine in PhBu^t, and the experimental parameters used.

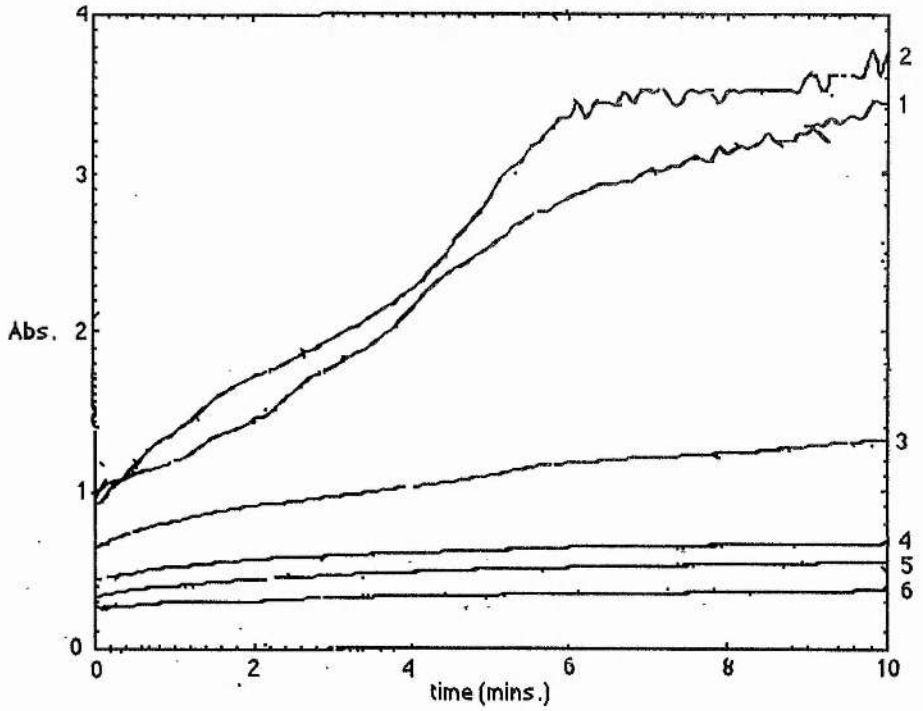


Fig. 3.3 Graph of absorbance against time for the development of diphenylnitrosamine in PhBu^t at 390nm.

The numbered lines on the right of the spectrum correspond to the tube numbers in the following table.

Tube	$PhBu^t/(Ph_2N)_2$* (cm^3)	$PhBu^t/NO$ (cm^3)	Dilution (cm^3)	Absorbance after 6mins
0	3.0	0.0	0.0	>4
1	1.5	1.5	0.0	2.8
2	1.5	1.5	3.0	3.3
3	1.5	1.5	6.0	1.2
4	1.5	1.5	12.0	0.6
5	1.5	1.5	24.0	0.5
6	1.5	1.5	48.0	0.4

* Aliquots of a 1.98×10^{-3}M solution

It can be seen that the reaction of NO with Ph_2NNPh_2 is dependant on the number of mols of available NO in the system, and the most concentrated solution of $NO/PhBu^t$ gave the highest absorbance, discounting the anomalous result of tube 2. By way of comparison, a different solvent, acetonitrile, was used in a parallel experiment using the same quantities and dilution factors. Figure 3.4 shows a graph of absorbance against time for the reaction of Ph_2NNPh_2 with NO in MeCN.

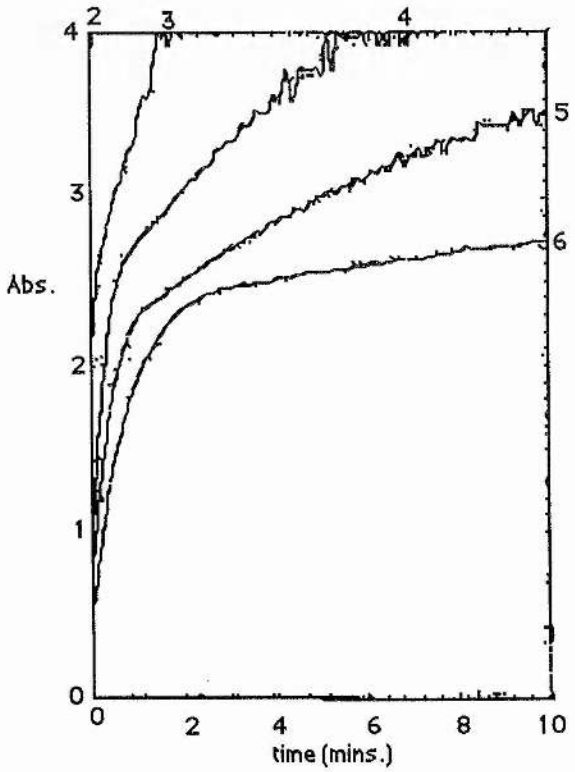


Fig. 3.4 Graph of absorbance against time for the development of diphenylnitrosamine in MeCN.

Tube	MeCN/$(Ph_2N)_2$* (cm^3)	MeCN/NO (cm^3)	Dilution (cm^3)	[NO](M)	Absorbance (1.5 min.)
0	3.0	0.0	0.0	-	-
1	1.5	1.5	0.0	$1.5x10^{-2}$	>4
2	1.5	1.5	3.0	$7.6x10^{-3}$	>4
3	1.5	1.5	6.0	$3.8x10^{-3}$	3.6
4	1.5	1.5	12.0	$1.9x10^{-3}$	2.8
5	1.5	1.5	24.0	$9.5x10^{-4}$	2.4
6	1.5	1.5	48.0	$4.8x10^{-4}$	2.1

* Aliquots of a $1.98x10^{-3}$M solution.

The graph shows that tube 1, which contained the most concentrated solution of NO, gave too high an absorbance to be recorded, and tube 2 was only just on the scale. The subsequent dilutions are all within the reaction time, and show a definite relation between NO and Ph_2NNPh_2. For the same concentration of Ph_2NNPh_2, the same dilutions, and assuming a saturated solution, it was found that the rate of reaction of NO and Ph_2NNPh_2 in MeCN was much faster than in $PhBu^t$. This could be due to the increased polarity of MeCN, which could exert a solvent effect on the dissociation of Ph_2NNPh_2 as indicated above. The concentration of Ph_2NNO produced in the reactions was calculated using the Beer-Lambert law, $A=\varepsilon cl$, where A= absorbance, (dimensionless), ε= molar extinction coefficient, (mol $dm^{-3}cm^{-1}$), c= concentration, (mol dm^{-3}), and l= path length (cm). The molar extinction coefficient, ε, was evaluated using synthetic nitrosamine (see above), and thus the concentration of Ph_2NNO could be determined. The following table displays the results.

Tube	$[Ph_2NNO]$/ (M) in $PhBu^t$	in MeCN	[NO]/* (M) in MeCN
1	$5.2x10^{-4}$	---	$1.52x10^{-2}$
2	$5.4x10^{-4}$	$>6.5x10^{-4}$	$7.6x10^{-3}$
3	$2.0x10^{-4}$	$6.5x10^{-4}$	$3.8x10^{-3}$
4	$9.7x10^{-5}$	$6.2x10^{-4}$	$1.9x10^{-3}$
5	$8.1x10^{-5}$	$5.5x10^{-4}$	$9.5x10^{-4}$
6	$6.5x10^{-5}$	$4.0x10^{-4}$	$4.75x10^{-4}$

* Assuming literature value for saturated solution.

The table represents the final concentrations of Ph_2NNO in the experiments with $PhBu^t$ and MeCN as solvents. The final absorbance readings were obtained from the above absorbance vs. time graphs at that point when each line began to reach a

plateau, i.e. there was no more formation of the nitrosamine. Tubes 1 and 2 were the exceptions, as tube 1 was off the scale, and tube 2 was only just on scale. Hence, the maximum absorbance value was used to calculate ε for the latter. The final column of NO concentrations was calculated from the solubility data, i.e.assuming that 1.52×10^{-2} mol dm^{-3} was the maximum concentration of NO in MeCN for tube 1. It was seen that the quantity of Ph_2NNO formed in the first two tubes was much lower initially than the concentrations or potential availability of NO. This was due to the actual number of moles of available NO being greater than the available diphenylaminyl. The results showed that for tubes 1-4 insufficient tetraphenylhydrazine was present to ensure complete scavenging of NO, but that at lower NO concentrations, (tube 6), good quantification was achieved.

$$Ph_2NNPh_2 \rightleftharpoons 2Ph_2N\bullet \xrightarrow{2NO} 2Ph_2NNO$$

From the above equation, it can be seen that 1mol. of tetraphenylhydrazine gives 2mol. of diphenylaminyl radical. The maximum available number of moles of diphenylaminyl in $PhBu^t$ and MeCN is 3.86×10^{-3}, so the quantity of NO available in tube 1 is an order of magnitude greater than the quantity of diphenylaminyl. It seemed that at low concentrations of NO, the concentration of diphenylnitrosamine was produced almost quantitatively. The low nitrosamine concentration in $PhBu^t$ could be due to the lowered solubility of NO in this solvent, or a slower dissociation of the hydrazine in this solvent. The general result is that there is definite generation of a coloured product, diphenylnitrosamine, when NO is bubbled through the system. The diphenylaminyl radical is an excellent scavenger of nitric oxide. Our results demonstrate that this approach could be used as a spectrophotometric method for NO quantification, especially at lower concentrations of NO. The sensitivity of the method means that [NO] in the range 10^{-4} - 10^{-7} could be detected.

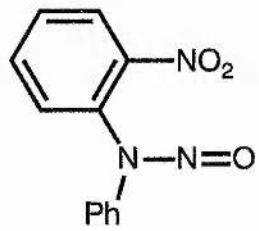
3.2.5. Reaction of NO and NO_2 with Diphenylamine.

An attempt was made to determine if NO reacted with the parent compound, diphenylamine. A solution of 3 $\times 10^{-3}$ mol Ph_2NH in $15cm^3$ cyclohexane was

degassed for 1 hour. After this time, NO was bubbled through the solution for 2 hours. A sample was taken for EPR analysis and showed no signals. A sample was also analysed by GC-MS. The chromatogram revealed only solvent and starting material. A further sample was taken and no spectra were observed by EPR analysis. When the solvent was decanted off, crystals remained in the reaction vessel. More crystals were obtained on evaporation of the solvent. Analysis of these crystals by ^1H NMR and GC-MS revealed them to be starting material. It can be said therefore that Ph_2NH does not react directly with NO to generate a nitroso compound.

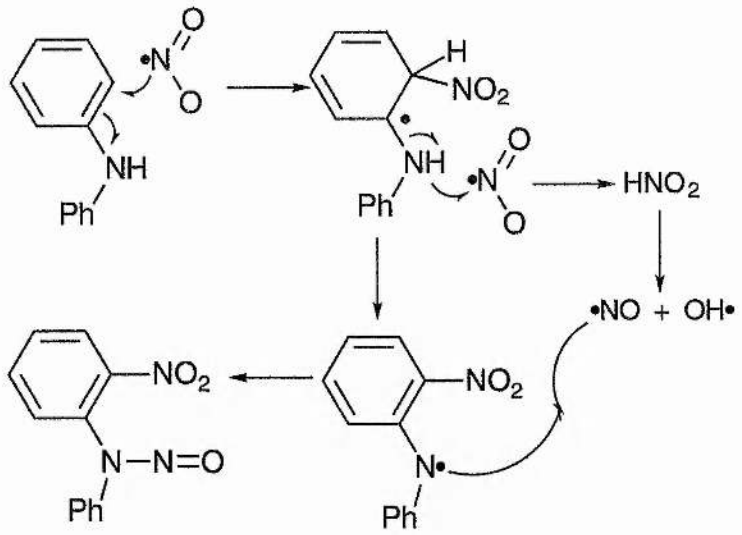
As an alternative, the reaction of NO_2 with diphenylamine was investigated. A solution of 3 $x10^{-3}$ mol Ph_2NH was prepared but not degassed. Concentrated HCl was dripped onto solid $NaNO_2$, liberating NO_2. This was bubbled directly through the Ph_2NH solution. After about 20 minutes, the solution turned from colourless to a greeny/yellow, an indication that a nitroso compound had formed. A sample was sent for EPR analysis, and this, as expected, showed no spectrum. Analysis of the solution by ^1H NMR showed only cyclohexane was present.

When the solution was decanted off, crystals were observed in the flask. These, along with the mother liquor, were analysed by GC-MS. The chromatogram of the solution showed little sign of starting material, and what appeared to be many minor products. The only major product was identified as 2-nitro-N-nitroso-N-phenylbenzenamine (**15**), with m/z 243.



15

Scheme 3.0 shows a possible mechanism for this reaction if nitration was followed by abstraction of a proton to generate the aminyl radical.

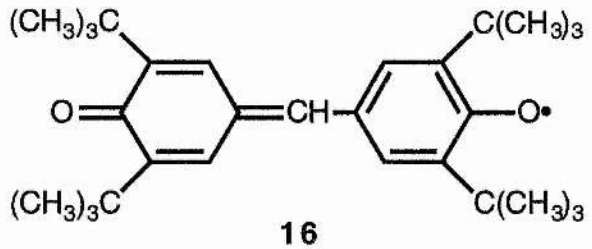


Scheme 3.0

The chromatogram of the flask crystals revealed some starting material, the above nitroso compound and other high molecular weight products, such as 4-nitro-N-phenylbenzenamine, 2-nitro-N-(2-nitrophenyl)-benzeneamine, and 2-nitro-N-(4-nitrophenyl)-benzenamine. The third analysis, the crystals from the evaporated solution, revealed a similar set of products. Thus it can be seen that NO_2 reacts with PH_2NH to give direct nitrosation, and even some nitration products. This supports the work of Alm[179] who found that NO was inert toward Ph_2NH and its derivatives, whereas NO_2 gave degradation products and in some cases, liberated NO_2.

3.2.6.Galvinoxyl.

A second radical source which was investigated was 2,6-di-*tert* -butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadiene-1-ylidene)-ρ-tolyloxy, hereafter referred to as "galvinoxyl", **16**. This is a stable, long-lived free radical which can act as a scavenger for other transient free radicals[180].



16

A solution of galvinoxyl in *tert* -butylbenzene was submitted for EPR spectroscopy. A signal was obtained which became less resolved as the temperature was raised. Nitric oxide was bubbled through the solution for ~5mins. No immediate change was observed in the EPR trace. This could mean that galvinoxyl does not rapidly scavenge NO, or that there was excess galvinoxyl in the solution. The EPR analysis was repeated after four days. The signal had decreased in intensity by a factor of ~50, possibly due to the gradual combination of NO with the galvinoxyl. It is not clear at which site nitric oxide attacks the galvinoxyl. It could be at the oxyl radical centre or at the =CH-, breaking the double bond, or at a double bond in the phenyl ring. Infra-red analysis was carried out on the reacted sample, giving a peak at 1675 cm^{-1}. If the nitric oxide was bound to the radical centre to give O-N=O, then this would give a peak between 1680 and 1650 cm^{-1}. However, the peak would be masked by the presence of the carbonyl group which also appears in this region.

3.2.7. Conclusions.

From the experimental data obtained, it was observed that the reaction between R•, (where R• = Ph_2N•), and NO was a very rapid process, being completed in some cases where the NO concentration was high, in less than 30 seconds. The EPR spectroscopy experiments revealed the presence of the diphenylaminyl radical and this was very persistent; it reacted with NO to form a coloured nitroso compound, N-nitrosodiphenylamine. This compound was easily detected by UV/vis spectroscopy. By comparison with authentic N-nitrosodiphenylamine, the molar extinction coefficient, ε, was evaluated and thus the concentration of product was calculated. This type of experiment could form the basis of an effective diagnostic test for the presence and concentration of NO.

An attempt was also made to generate a different type of stable radical from the bispentamethylcyclopentadiene dimer, but this was unsuccessful.

3.3. Experimental.

UV/vis spectra were obtained on a Philips PU8730 scanning spectrometer operating between 200 and 900nm, and a Shimadzu UV2101PC spectrophotometer operating at 415 and 390nm. Spectra were plotted directly from a HP440LC laser printer. Samples were analysed in $3cm^3$ quartz cuvettes. All solutions were degassed by bubbling N_2 through for at least 15 minutes.

3.3.1. Preparations.

3.3.1.1. *Tetraphenylhydrazine.*

Tetraphenylhydrazine was prepared according to the synthetic method of Gatterman[173]. The initial quantities were $^1/_5$ of those quoted, but this gave rise to an impure product. Consequently, the following preparation was used. Diphenylamine, (34g, 0.2 mol.), was dissolved in $200cm^3$ AnalaR acetone in a $500cm^3$ Buchner flask. The solution was kept cold in an ice-water bath while finely ground potassium permanganate was added, each portion added only after the colour of that preceding had disappeared. After each addition, the flask was shaken vigorously to mix the contents. After a time of 80 minutes, 16g of the permanganate had been added. The solution was then removed from the ice-water bath, and a further 10g of permanganate was introduced portionwise. There was no loss of colour after the last addition of oxidant. The solution was decolourised with $0.5cm^3$ EtOH and then filtered at the water pump. The manganese dioxide formed was washed twice with $20cm^3$ of warm acetone. The solvent was then removed on a rotary evaporator. To the crude product was added $30cm^3$ ether to dissolve any oily material. This solvent was then removed on a rotary evaporator. These conditions vastly improved the crude product yield, but the material was still too impure to use. Melting point tests were carried out on each sample but these were up to 25°C lower than that in the literature[172]. The stated method of recrystallisation involved benzene and ethanol, but this was not used because of the carcinogenic properties of the former.

First, attempts were made to recrystallise from dichloromethane and ethanol. These were unsuccessful as the product degraded when subjected to excess heat. No

crystals were obtained in usable quantity by this method. Petroleum ether was the next choice of solvent. This yielded better crystals, although still impure. A sample of this tetraphenylhydrazine, having a melting point of 134°C, was submitted for mass spectrometric analysis, resulting in m/z 336(11 m+), 169 (72), 168 (100), 167 (94), 166 (20), and 77 (32). Conditions were altered; the solvent was evaporated off under reduced pressure, (no heat), and in a foil-wrapped flask to prevent light entry. NMR spectra showed definite aromatic peaks without the amine peak. However, contamination was still a problem.

Simple recrystallisation appeared to be a dead end, so separation via a column was employed. Petroleum ether was the solvent and silica made up the adsorption surface. The column was foil-wrapped again to bar light. Increasing the solvent polarity from petroleum ether to ether to methanol had no effect on the purity of the product. Consequently, the silica was replaced by alumina, whilst again using petroleum ether as the first solvent. This gave fractions of a markedly increased purity. Characterisation of the products involved mass spectrometry, NMR and FT-IR analysis. The mass spectrum for this product was as follows: m/z 336 (18 m+), 169 (82), 168 (100), 167 (94), 166 (19), 77 (18). FT-IR analysis carried out on the sample revealed peaks at 801, 745, 690, and 668 cm^{-1}, indicating an aromatic presence. Material eluted from the column yielded a melting point of 136°C and mass spectrometry revealed a molecular ion of mass 336, probably tetraphenylhydrazine.

The tetraphenylhydrazine was still not pure so the literature method of recrystallisation using benzene and ethanol was eventually employed. This gave rise to a product of increased purity. Again this was characterised by FT-IR, NMR, and mass spectrometry, this latter spectrum giving m/z 336(52 m+), 169(100), 168(74), 167(58).

3.3.1.2. *N-Nitrosodiphenylamine·*

An alternative method of synthesis of the nitrosamine was attempted. This was carried out according to the method in Vogel[181], and involved the direct nitrosation of diphenylamine. Solutions of 17g (0.1 mol.) of diphenylamine in 140cm^3 of warm,

(30°C), EtOH and 8g (0.116 mol.) of sodium nitrite in $12cm^3$ distilled water were cooled in an ice bath to 5°C. To the ethanolic solution was added $12cm^3$ of concentrated hydrochloric acid, slowly and with stirring. The sodium nitrite solution was introduced immediately after this to prevent formation of diphenylamine hydrochloride. As the temperature rose, yellow nitrosodiphenylamine crystallised out. The mixture was cooled in an ice-water bath for 20 minutes and filtered at the water pump. The resulting crystals were washed with water and recrystallised from EtOH. These crystals had melting point 60°C (lit.[181] 68°C), and elemental analysis gave theor. C72.71; H5.09; N14.13: found, C73.58; H4.59; N14.68. Analysis by FT-IR, MS, and 200MHz ^{1}NMR were in agreement with the literature. The yield was 15.8g, 79%.

3.3.2. Reactions.

3.3.2.1. *Reaction of Tetraphenylhydrazine with NO*

Pure Ph_2NNPh_2, (0.1g, $2x10^{-4}$mol), was dissolved in cyclohexane ($20cm^3$). The solution was degassed using N_2 and NO was bubbled through for 5.5 hours. One sample was taken after this time. The experiment was repeated using Ph_2NNPh_2 (0.3g, $8x10^{-4}$mol) in cyclohexane ($20cm^3$). The flask was foil-wrapped to prevent light entry, and degassed for 15 minutes using N_2. Nitric oxide was then bubbled through for 6 hours. One sample was taken after this time and a second after 24 hours. A different solvent was used for the next experiments. A solution of Ph_2NNPh_2 (0.01g, $2.9x10^{-5}$mol) in 1,2-dichlorobenzene ($20cm^3$) was prepared. The solution was degassed for 15 minutes using N_2 and NO was bubbled through for 5 hours. One sample was taken after this time. For the UV/vis analyses, a range of Ph_2NNPh_2 solutions was made of varying concentrations. These were: 0.02g, $5.9x10^{-5}$ mol; 0.03g, $8.9x10^{-5}$ mol; 0.04g, $1.2x10^{-4}$ mol; 0.05g, $1.48x10^{-4}$ mol, all in cyclohexane ($20cm^3$). These reactions were degassed for 15 minutes, then NO was bubbled through for 2 hours. Yellow/orange crystals were obtained from this set of reactions and these were analysed by various methods. The ^{1}H NMR spectrum was very similar to that of authentic N-nitrosodiphenylamine. UV spectra also showed

peaks in the area expected for the nitrosamine. For the continuous scan work, a solution of 6.7 x10^{-3}g (2 x10^{-5} mol) Ph_2NNPh_2 in 20cm^3 cyclohexane was prepared, degassed for 15 minutes with N_2 and then NO was bubbled through the solution. A stock sample (2cm^3) was used as a blank, and 0.5cm^3 samples were withdrawn every 15 minutes and made up to 2cm^3 by addition of degassed cyclohexane. These were then analysed by UV/vis spectrometry. This revealed a maximum absorbance at 390nm, which did not appear to be time dependent. In other UV experiments, degassed solutions of 0.01g (2.97 x10^{-5}mol) Ph_2NNPh_2 in 15cm^3 *tert* -butylbenzene, and 0.01g Ph_2NNPh_2 in 15cm^3 acetonitrile were used. The concentration of NO was reduced by diluting a 3.0cm^3 solution of *tert* - butylbenzene (or MeCN) by half each time. Thus, if there were six samples taken then the total dilution factor would be $^1/_{32}$. The latter reactions proceeded much faster in acetonitrile than in *tert* -butylbenzene. All were analysed at 390nm.

3.3.2.2. *Reaction of Ph_2NH with NO.*

A sample of 0.5g (3 x10^{-3}mol) Ph_2NH was dissolved in 15cm^3 cyclohexane and degassed for 1 hour. Nitric oxide was the bubbled through the solution for 2 hours, and a sample was taken 30 minutes after final bubbling. No spectra indicating the formation of products were obtained by EPR, ^{1}H NMR, or GC-MS analysis.

3.3.2.3. *Reaction of Ph_2NH with NO_2.*

Concentrated HCl was added dropwise to 20g (0.29 mol) solid $NaNO_2$, producing NO_2. This was bubbled into a flask containing a non-degassed solution of 0.5g (3 x10^{-3} mol) Ph_2NH in 15 cm^3 cyclohexane. When the NO_2 had been bubbling for about 20 minutes, the solution turned from colourless to greeny/ yellow. No spectra were observed by EPR analysis, but samples submitted for GC-MS revealed major and minor products. The major product identified was 2-nitro-N-nitroso-N-phenylbenzenamine. Minor products revealed by GC-MS analysis included 4-nitro-N-phenylbenzenamine, 2-nitro-N-(2-nitrophenyl)-benzenamine, and 2-nitro-N-(4-nitrophenyl)benzenamine. These were direct nitrosation products from addition of NO_2.

3.3.2.4. *Reaction of Galvinoxyl with NO.*

The substrate (0.1g, 2.37×10^{-4}mol) was dissolved in *tert*-butylbenzene ($20cm^3$). The solution was degassed using N_2 and its EPR spectrum was observed. NO was then bubbled through the solution for ca. 5 minutes at room temperature. A sample was taken after this time and analysed by EPR spectroscopy. No change was observed initially in the EPR trace but after 4 days the spectral intensity had decreased by a factor of about 50.

CHAPTER 4.

Nitric oxide and cellular components

4.1. Reaction of NO with model cell components.

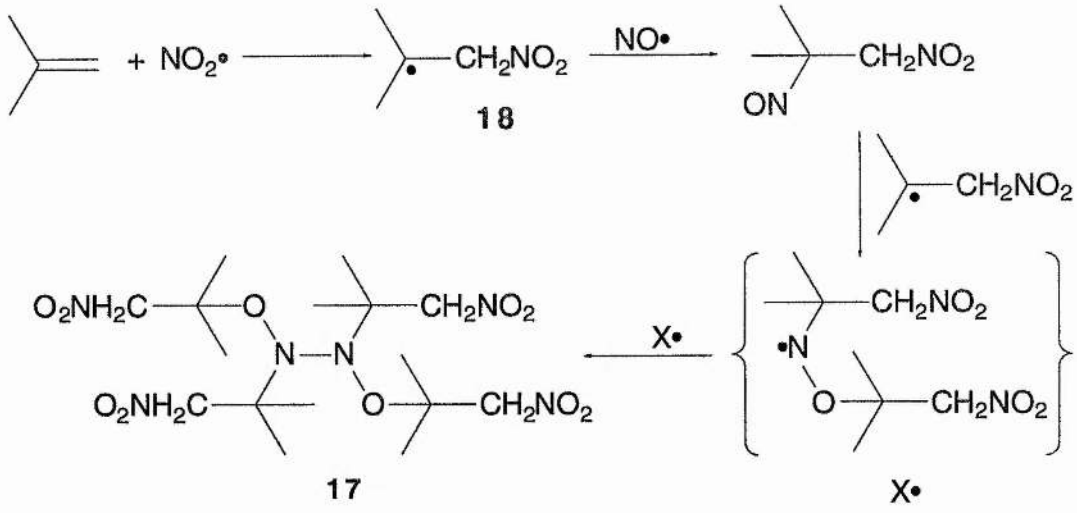
4.1.1. Nitric oxide and organic compounds.

There is little in the recent literature about the reaction of NO with organic compounds. The majority of experimental investigations have been concerned with the reaction kinetics of NO as a scavenger of other radicals generated by flash photolysis techniques[147,148,151], or its involvement in charge transfer reactions[182,183]. Yet more investigations have been carried out on its role as a pollutant.

The studies on NO as a reactant began in the latter half of the Nineteenth Century, and even up to the late 1930's it was thought that NO was inert toward many reaction systems, especially the ethylenic bond. It was not until a decade after this that NO was proved to react with alkenes.

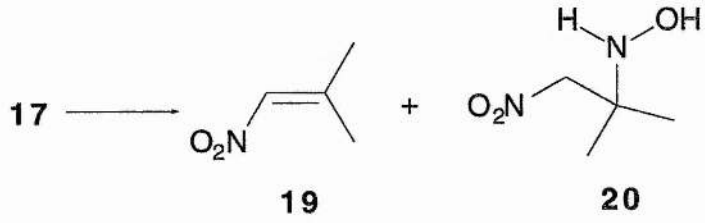
The work was carried out by Bloomfield and Jeffrey[184], who reacted NO with a variety of alkenes, including cyclohexene, 1-methylcyclohexene, 2,6-dimethyl-2,6-octadiene (dihydromyrcene), and rubber. It was found that all of the substrates tested yielded nitro compounds of various types. Cyclohexene gave nitrosocyclohexene, a mixture of isomeric nitrocycloalkenes, mainly the 1-nitrocyclohexene, and a major oily component. This latter contained a little impure nitrocyclohexene, but was mainly unidentified. There were no proposals for a mechanism at this stage.

Later work[185], concentrated exclusively on isobutene (2-methylpropene), and from this, three major characteristics of the reaction were established. First, NO_2 is required as an initiator of the reaction. Purified NO can be stored in contact with liquid alkenes without reacting. Second, the products of the reaction are a nitroso adduct and a liquid mixture, as found previously. Third, the distilled reaction products consist mainly of nitroalkenes. Linear alkenes gave only average yields of nitroalkenes and nitroso adducts, whereas branched types produced poor quantities of nitroso compounds, but good total yields of nitroalkenes. The structure shown at **17** was proposed for the primary product from the reaction between isobutene and NO. This was thought to be formed as shown in scheme 4.0.



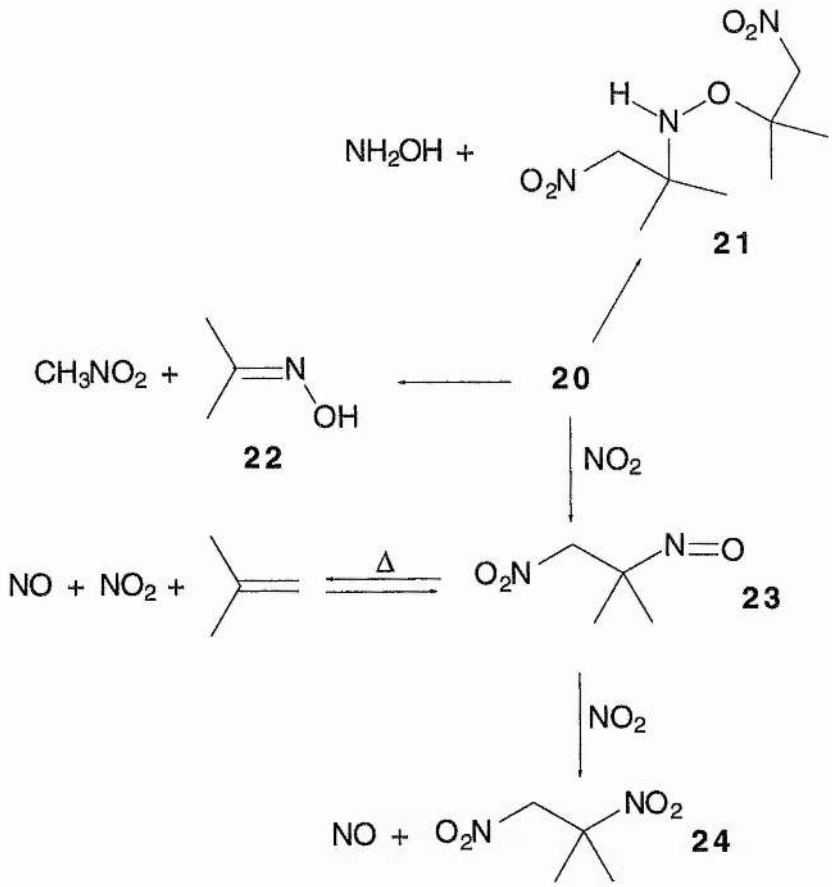
Scheme 4.0

Upon warming, **17** underwent elimination and decomposition to give 1-nitro-2-methyl-1-propene (**19**), and the nitro-hydroxylamine (**20**), (scheme 4.1).



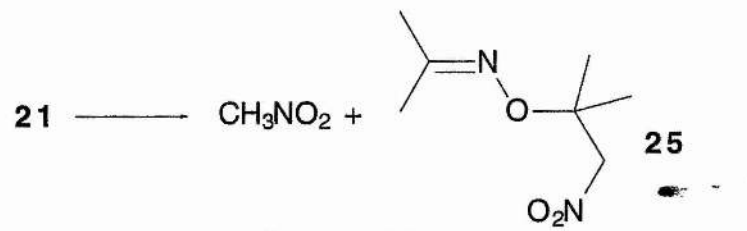
Scheme 4.1

The hydroxylamine **20** was also rather unstable and decomposed in three ways: disproportionation, dealdolisation, and reversion to the starting materials. Scheme 4.2 represents these pathways.



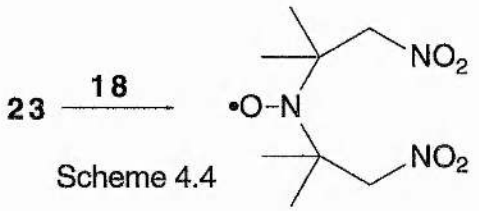
Scheme 4.2

Self-disproportionation gave O,N-bis-(1-nitro-2-methylpropane)-hydroxylamine (**21**) and hydroxylamine. Dealdolisation occurred on heating and resulted in the cleavage of **20** into nitromethane and acetoxime (**22**). The reversion to starting materials was an autocatalytic process induced by heating with nitrogen oxides, and resulted in the formation of 1-nitro-2-methyl-2-nitrosopropane (**23**), and dinitroisobutane (**24**), along with other gaseous products. Scheme 4.3 shows a similar reaction to the dealdolisation, whereby the dialkylhydroxylamine **21** gives O-(1-nitropropane)-acetoxime (**25**).



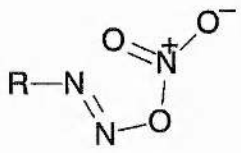
Scheme 4.3

It was noted that the reaction of isobutene with NO gave similar products to those obtained from more conventional methods of nitration, such as by treatment with N_2O_3, N_2O_4, or nitric acid. As an example, isobutene was treated with N_2O_4 in ether and the reaction produced 1,2-dinitroisobutane (**24**), 1-nitro-2-methylpropan-2-ol, 1-nitro-2-methylpropyl nitrate, and 1,3-dinitro-2-methylpropan-2-ol (**26**), all of which were generated by the reported action of NO on isobutene. Compound **26** was thought to have been produced by further oxidation of 1-nitro-2-methylprop-1-ene (**19**). The only product not identified from conventional nitration was the oil, **17**, but this may well have been due to a lack of analyses. The formation of this compound was thought to proceed via a radical pathway, as shown in scheme 4.0. This was a general pathway and was utilised in many similar reactions. We note here that a favourable alternative mode of reaction for the nitroisobutyl radical (**18**) would be formation of the ditertiary nitroxide as shown in scheme 4.4.

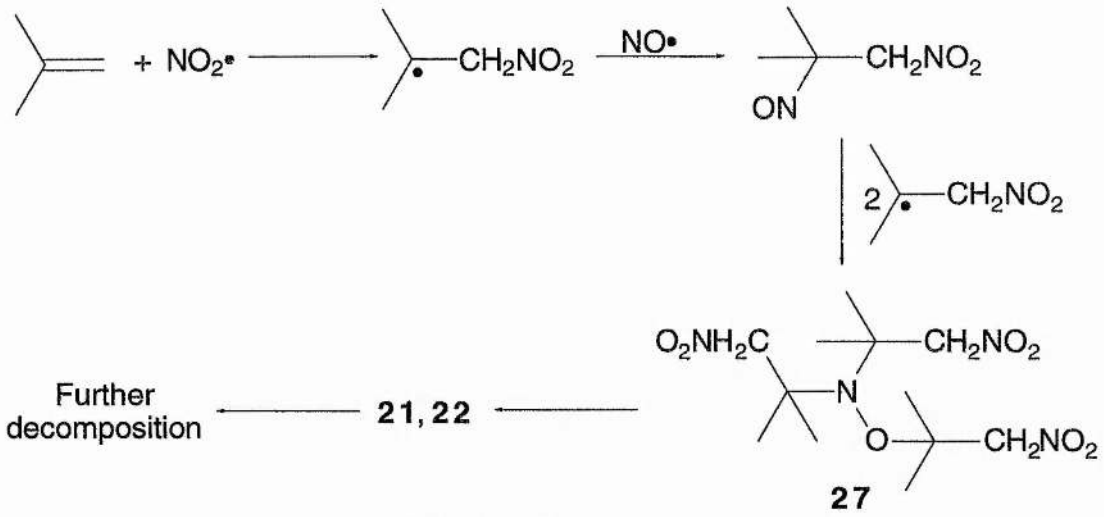


Scheme 4.4

The reaction between NO and isobutene relied on there being catalytic quantities of NO_2 present in the system. This was thought to be generated from the diazo nitrate, itself produced from the nitroso moiety.



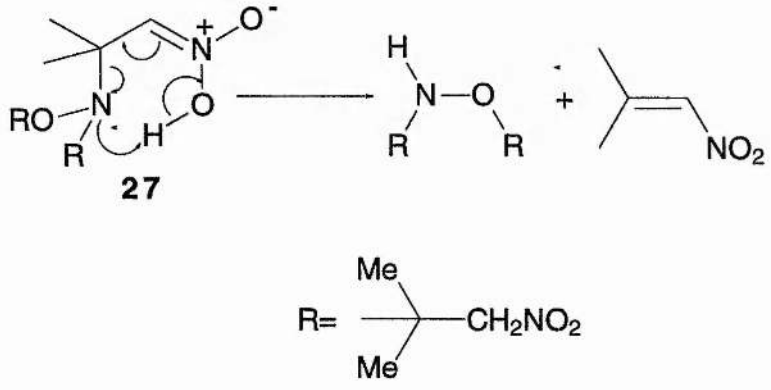
The resulting diazo nitrate then decomposed either homolytically or heterolytically, which effectively produced NO_2. A simpler explanation could be that oxygen had got into the system and combined with NO to generate NO_2. Further work on the reaction products of isobutene[186], revealed another reaction product, tris-(1-nitro-2-methylpropane)hydroxylamine (**27**). This was thought to be generated via formation of nitro-isobutyl radicals **18** and their subsequent reaction with NO (scheme 4.5):



Scheme 4.5

The hydroxylamine **27** decomposed rapidly, giving **21** and **22**, and a series of nitroisobutylenes plus traces of acetone and nitromethane. **21** also decomposed to nitromethane, nitroisobutene and **22**. These decomposition products suggested that **22** first decomposed to nitroisobutenes and **20**, which then decomposed to nitromethane and acetone.

It was proposed that thermal decomposition of the products was promoted by transition states involving a semi-six-membered ring intermediate. Here, the bis- and tris-compound nitro groups would be in *aci* form, whereas the mono-nitro group would be normal. The following structure represents the decomposition of the tris- form:

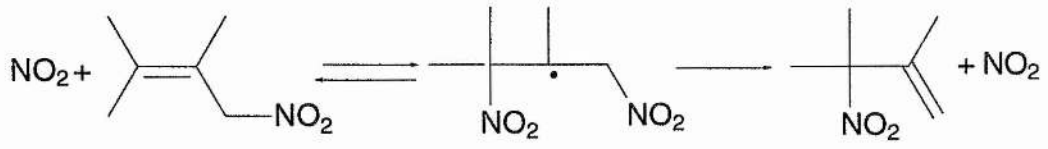


Scheme 4.6

An extensive study was carried out on the conversion of the nitro adducts and their decomposition products to precursors of methyl methacrylate. This is an

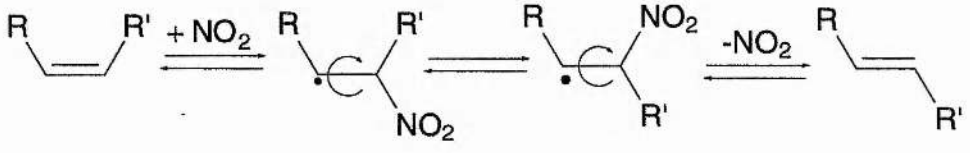
important polymer used in a wide variety of processes. The compounds, (adducts or decomposition products), were treated with HCl and MeOH to produce mixtures of methyl esters and hydroxylamine hydrochloride. The esters were mainly methyl-α-chloroisobutyrate and methyl-α-methoxyisobutyrate, but with traces of β-chloroisobutyrate, methyl-α-hydroxyisobutyrate, and methyl methacrylate. Methanolysis of the crude adducts gave a low yield of esters. If decomposition was carried out first, or if only α-nitroisobutylene was used as reagent, yields increased. In the presence of acids other than HCl, only low yields of esters and carboxylic acids were obtained. Further attention was given to generating methyl methacrylate precursors from nitroisobutylene. α-Nitroisobutylene was treated with anhydrous HCl to give α-chloroisobutyrylhydroxamyl chloride. Treatment of this with methanol in acid conditions yielded methyl-α-methoxyisobutyrate and methyl-α-chloroisobutyrate, plus hydroxylamine hydrochloride and chloromethane. If H_2O and HCl were used as reagents, α-chloroisobutyric acid, α-hydroxyisobutyric acid and hydroxylamine hydrochloride were formed. The esters and acids produced were both capable of acting as precursors for methyl methacrylate synthesis, all stemming from the reaction of NO with isobutene.

Once the work on isobutene had been fully elucidated, the way was paved for examination of other alkenes. Burkhard and Brown[187] studied the reaction of NO with tri- and tetra-methylethene. Their results indicated that the nitration of tri- and tetra-methylethene was very similar to that of isobutene, except that, in the case of tetra-methylethene, there was no formation of the α-nitroalkene. Also, the nitroso compounds generated appeared to be less reactive toward the alkyl radicals produced, and dimerisation occurred only slowly or not at all. Their results revealed that two isomeric β-nitroalkenes were obtained from tri- and tetra-methylethene, rather than just one. The following mechanism for this interconversion was proposed, as it was known that NO_2 was present.



Scheme 4.7

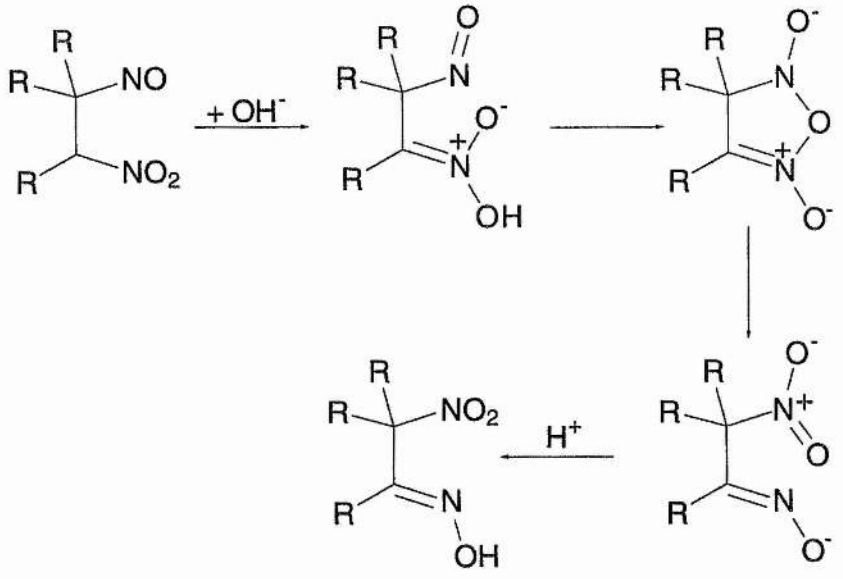
However, the 3-nitro substituted alkene would be less stable than the 1-nitro alkene, so the equilibrium would tend to lie on the left. These reactions were thought to be reversible for three reasons. First, nitrosonitroisobutene decomposed to its component parts on heating (see above). Next, it was observed that the photochemical chlorination of anhydrous nitro-paraffins[188] is very slow. If this reaction takes place via a free radical pathway, then that pathway must somehow be interrupted. This could occur through loss of NO_2 from β-nitroalkyl radicals. Third, the reversibility of the NO_2/alkene reaction provides a reasonable explanation as to why there is *cis-trans* isomerisation of alkenes. This is probably the oldest known isomerisation reaction[189], and was mainly used in the interconversion of unsaturated fatty acids. The reaction requires an NO_2-donating catalyst, such as HNO_3, NO_2, or NO, and is an equilibrium reaction. The usual nitroalkene adducts are always generated, and they are stable under the reaction conditions, only being converted back to alkenes on heating. These facts, coupled with the finding that the production of a β-nitroalkyl radical from addition of NO_2 to an alkene is reversible led to the proposal of the following mechanism:



Scheme 4.8

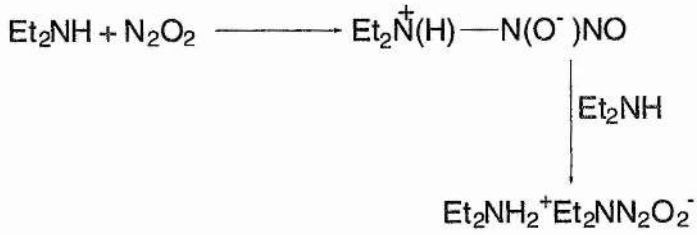
Previous work[190] had shown that the oxime was formed from 2-nitroso-2-methyl-3-nitrobutane, and reacted further in alkaline conditions to give substitution followed by loss of the nitro group. This all worked well until it was discovered that there were secondary nitro and tertiary nitroso functionalities present in the nitroso compound

which would not facilitate tautomerisation of the nitroso compound to the oxime. Thus, a nitrosite rearrangement was proposed which can be represented as in scheme 4.9:



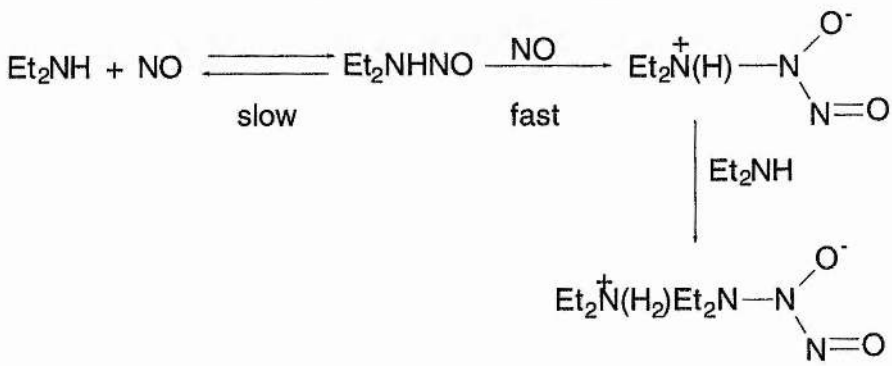
Scheme 4.9

In 1960, Drago and Paulik[75] reported that NO could act as an electron acceptor, and would react with a secondary amine, specifically diethylamine. A degassed solution of diethylamine in ether was cooled to -78°C and NO bubbled slowly through the solution. A precipitate was formed after ~15 hours. The lowering of the temperature facilitated dimerisation, a process in which NO was usually reluctant to participate. The product when isolated was assigned the empirical formula Et_2NHNO. The product was studied by EPR and IR spectroscopy, and this led to the proposal that the product was diamagnetic. Comparative reactions with hydrochloric acid showed the presence of the diethylammonium ion. The following scheme represented this formation:



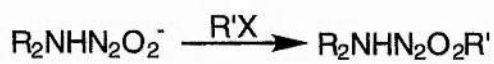
Scheme 4.10

The second molecule of amine removed the quaternary proton from the intermediate. Drago and coworkers[191] extended the secondary amine work to include the reaction of NO with a whole series of amines, both primary and secondary. They showed that there was a general reaction pathway whereby NO acted as a Lewis acid. They postulated that the products formed were of the general formulae $R_2NH_2^+R_2NNO_2^-$ and $RNH_3^+RNHN_2O_2^-$ for secondary and primary amines respectively. Further experimentation[192] clarified the mechanism, and was supported by kinetic data.



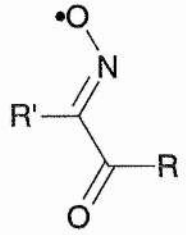
Scheme 4.11

The salts were able to release NO when the product was dissolved in water and acidified with HCl. Consequently, there has been much interest in these "Drago complexes" as pharmacological probes. However, as the decomposition to produce NO is spontaneous, this could be detrimental in a physiological environment, because tissues could become saturated causing unwanted injury. If the molecule were masked such that it only released NO when it was metabolised, this would be of major benefit. Longhi and Drago[193] showed that the ions could be alkylated

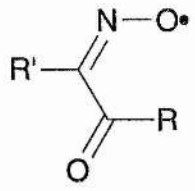


where R' was ethyl, n-butyl, or benzyl. A much later study[194], concluded that there was great potential for the preparation of precursors for NO-releasing agents from these compounds. One of the major revelations from their work involved the stereochemistry of the products. It was assumed that it was the interior oxygen which underwent alkylation, i.e. giving $R_2NN(OR')NO$. However, X-ray crystallography revealed that, at least for the larger product tested, it was the exterior oxygen which was alkylated, i.e. producing $R_2NN(O)=NOR'$. These compounds were found to be fairly resistant to hydrolysis, and thus cell types capable of accelerating the release of NO via enzyme action could benefit from these potential prodrugs.

Later work on alkenes was carried out by Fox McRae and Symons[195], who reported that compounds containing the methylene and carbonyl functionalities reacted with NO_2 at room temperature to give iminoxyl radicals. Other radicals were also formed, which were, according to their EPR spectra, nitroxides. Various alkenes were studied, and these all gave rise to an iminoxyl radical, the general structures of which are described in detail in Chapter 2. These radicals were all prepared by passing a mixture of dry NO_2 and O_2 directly into the substrate, or by distilling the condensed gas mixture onto the substrate surface. The iminoxyl was only generated when there was a methylene or vinyl group adjacent to the carbonyl. For saturated compounds, the reaction proceeded via attack on the enol, leading to a nitroso compound. This was then oxidised by NO_2 to give the iminoxyl. In the case of unsaturated molecules, it was thought that addition of the ubiquitous N_2O_3 across the double bond occurred, such that NO was adjacent to the carbonyl. All of the resulting radicals conformed to expected hyperfine splittings, i.e. the iminoxyls had nitrogen splittings of a(N)= 26-30G due to the two possible conformers, **28** and **29** and the nitroxides had a single nitrogen splitting of a(N)= 12-14G. In addition, a third type of nitroxide, given the general structure **30** was observed, having a(N) ~7G.



28



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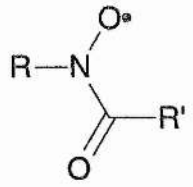
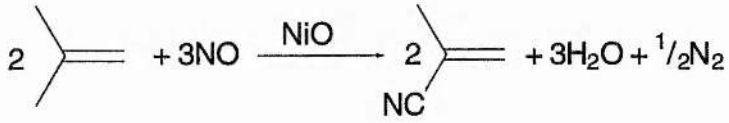


30

It was not known how this carbonyl nitroxide was generated.

Continuing investigations into the reaction of NO with alkenes, in particular isobutene, were carried out by Zidan and coworkers[196]. The reaction between NO and the alkene was carried out at 410°C and in the presence of a nickel oxide catalyst, supported by alumina or alumina-silica. The product of this was methacrylonitrile, in a highly selective reaction. A redox mechanism was proposed for this reaction. The overall equation can be summarised:



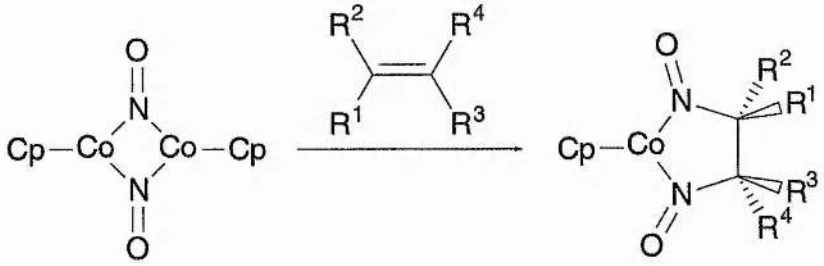
In 1985, X-ray crystallography[197] studies were carried out on the products of the reaction between NO and three alkenes. These were cyclohexene, styrene, and norbornylene. Pressures above atmospheric were required for a reaction to occur. In each case, the bis(1-nitroso-2-nitro)alkene was produced, for example, **31**, bis-(1-nitroso-2-nitro)cyclohexanone.

31

The structures were confirmed by crystallography. It was also discovered, (by mass spectrometry), that the only residual gas was N_2O; NO was completely absorbed. This indicated that there had been a disproportionation such that:

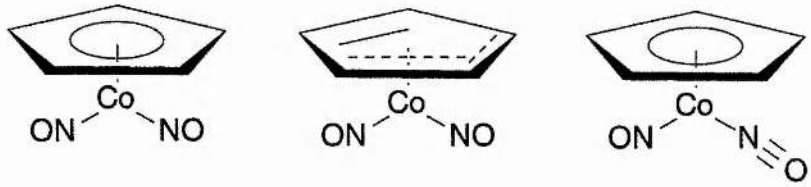
so once more NO_2 was required for the reaction.

The use of NO as a transition metal ligand has been known for many years. Much work was carried out by Russian chemists on the formation of nitrosyl complexes by reaction of dienes with NO in MeCN, water, or MeOH. The dienes were usually long-chain hydrocarbons with a halogen present in the complex. In 1980, a method of diaminating alkenes was established using a cobalt complex[198]. The complex, cyclopentadienylnitrosyl-cobalt dimer, $[(CpCoNO)_2]$, was reacted with a series of alkenes in the presence of excess NO. The $CpCo(NO)_2$ was generated by nitrosation of the $CpCo(CO)_2$, dimer, a similar process to that reported in chapter 1[84]. This gave rise, in fair yield, to the alkylnitroso complex (scheme 4.12), which was subsequently reduced by $LiAlH_4$ to the corresponding *vic*-diamine.



Scheme 4.12

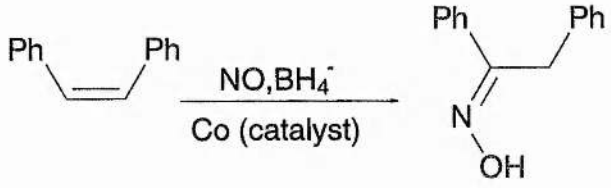
The mechanism of this reaction was at that stage unclear, but it was suggested that $CpCo(NO)_2$ was the reactive intermediate. Further experiments[199] to determine the kinetics of the reaction and subsequent analyses of the products by IR and ^1H NMR spectroscopy proved that this was indeed the correct intermediate. Only two bands were observed by IR spectroscopy, and the NMR spectrum revealed only one signal for the cyclopentadienyl group. However, these data alone could not conclusively prove the actual structure of the intermediate, so it was assigned 3 structures:



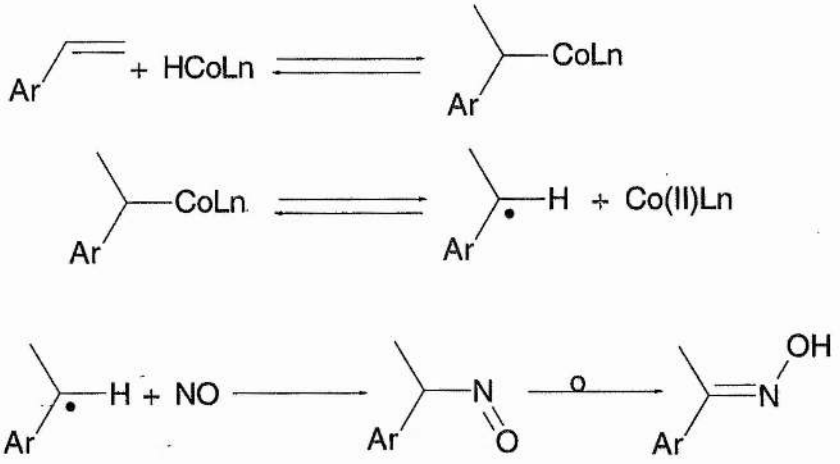
Analysis by X-ray crystallography would have identified the correct isomer but it was not isolable. It was, however, proved that NO reacted with a cyclopentadienylnitrosyl-

cobalt complex to generate a dinitroso compound capable of reacting further with alkenes to form cobalt dinitrosoalkanes. These were able to take part in alkene exchange reactions.

The interest in the chemistry of NO as a ligand was taken further by Okamato *et al.*[200] who proposed to react NO with aryl-substituted alkenes. This was attempted in the presence of tetrahydroborate anion, (BH_4^-), and was catalysed by a cobalt complex. The products isolated in each case were exclusively oximes.



The best catalyst used in conjunction with styrene and its substituted analogues was bis(dimethylglyoximato)-cobalt, (abbreviated as CoLn). Other catalysts were tried but these were less effective. By analogy with the oxygenation of alkenes using BH_4^- and a similar catalyst, the following mechanism (scheme 4.13) was proposed:

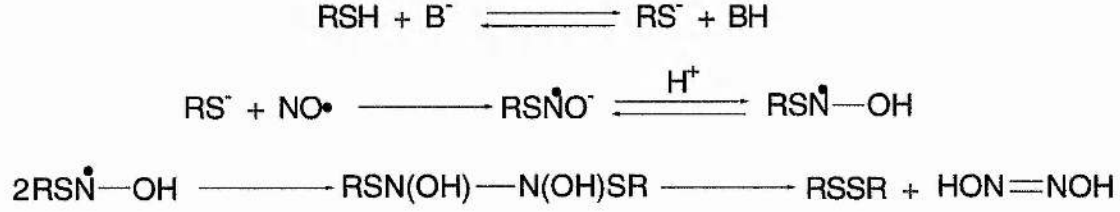


Scheme 4.13

The above proposal was based on the results obtained from the reaction between NO and styrene. The intermediate acetophenyl-cobalt complex [$CH_3CH(Ph)CoLn$] was synthesised and treated with NO in the presence of BH_4^-. In both cases, acetophenone oxime was isolated. It was postulated that because there was no vacant coordination

site available on the complex, the reaction had to proceed via a radical pathway, with NO combining with the phenylethyl radical generated in the reaction.

Nitric oxide does not only react across the ethylenic bond. As an extension to their work on secondary amines and NO, Longhi and coworkers[201] reacted NO with donor compounds such as aromatic and aliphatic thiols, triethylphosphite and triphenylphosphine. The thiol substrates were mixed with NaOMe prior to exposure to NO, whereas the phosphorus-containing compounds were dissolved in Et_2O and saturated with NO. From the reaction, the thiols produced the corresponding symmetrical disulphide in good yield; triethylphosphite produced triethylphosphate, and triphenylphosphine gave triphenylphosphine oxide, also in good yields. This appeared to be an excellent synthetic approach for the production of disulphides. In 1982, thiols were again under scrutiny, this time by Pryor *et al.*[80]. All the thiols tested gave rise to the corresponding symmetrical disulphide in quantitative yield. It was also observed that base catalysis promoted the reaction, and this ruled out the possibility of direct hydrogen abstraction from the substrate by NO. The eventual mechanism relied on the deprotonation of the thiol by base, and the formation of a radical anion by nucleophilic addition on NO. Radical coupling could then form the dihydroxyhydrazine, which would be unstable. Elimination of hyponitrous acid would then lead to the disulphide. The salts of hyponitrous acid decompose to N_2 and N_2O[80] which were the only gases detected by gas chromatography at the conclusion of the experiment.



Scheme 4.14

4.2. Results and discussion.

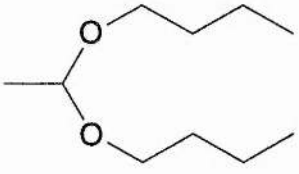
This aim of this part of the research was find out just how NO would react with selected organic molecules with varying functional groups, and some of more biological origin. If the products of the reaction could be isolated, it could give a target molecule for detection in a biological milieu, and a more detailed insight into the general reactivity of NO.

4.2.1. Substituted alkenes with electron donor groups.

The reaction of NO with several alkenes with electron-releasing functional groups were investigated by product analysis and EPR spectroscopy, in the presence and absence of oxygen.

4.2.1.1. n-*Butylvinyl ether.*

The reaction of n-butylvinyl ether was investigated solely at room temperature. A solution of the enol ether in t-butylbenzene ($PhBu^t$) was degassed using N_2, and NO was then bubbled through the system. The reaction vessel was left for a period of 16 hours, after which time the solvent was removed under reduced pressure. A yellow oil was obtained from the reaction. Samples of this oil were sent for analysis by ^1H NMR spectroscopy and GC-MS. The 200MHz ^1H NMR spectrum of the sample showed starting material, and that the extent of reaction was small. The GC-MS chromatogram revealed peaks for solvent, starting material, and a major product, having m/z 160 as its ion of highest mass. However, this was probably a fragment of a larger molecule, thought to be 1,1'-[ethylidenebis(oxy)]bisbutane (**32**):



32

A different major product was also observed that did have M^+ 160. This had a different mass spectrum than that reported above, but still possessed the n-butyl fragment. Another major component was identified as dioctyl phthalate. This material

is used as a plasticiser in tubing and was probably transported to the reaction vessel in the gas flow.

A second set of experiments was carried out using cyclohexane as solvent, as this was easier to remove from the reaction upon completion than PhBu^t. This solution was also degassed and NO bubbled through. After 2 hours, a sample was removed and analysed by GC-MS and ^{1}H NMR spectroscopy. After this time, it was noticed that there were brown fumes in the reaction vessel, indicating that there was NO_2 present in the system. The reaction was allowed to proceed and was terminated after a 72 hour contact period. A sample was then sent for GC-MS analysis. ^{1}H NMR of the previous sample, (taken after 2 hours), revealed a mixture of peaks, including those displaying aromatic coupling, and a number of side bands. From these it was only possible to tell that some reaction had occurred. GC-MS of the initial sample revealed starting material and solvent, but again, certain products were identifiable. One of these was the 1,1'-[ethylidenebis(oxy)]bisbutane, and the product with M^+ 160 was also present. Also observed was 1,1'-bicyclohexyl, presumably formed from abstraction of a hydrogen atom from the solvent and subsequent combination with a like radical. The plasticiser was also identified. The next sample also revealed the above products, which were significantly stronger on the chromatogram. Another major product shown was butan-1-ol. Minor products were observed and identified from their mass spectra as butyl acetate, butyl formate, 2-nitrobutane, a compound of possible empirical formula $C_9H_{18}O_2$, and two of $C_9H_{20}O_2$. The two potentially isomeric compounds came off the column at different times and both contained the n-butyl fragment. These three final compounds were not positively identified.

A third set of experiments was carried out in a similar manner. The solution was left to react with NO over a period of 48 hours. No NO_2 was observed in the system. The solvent, (cyclohexane), was removed under reduced pressure and the yellow residue analysed by GC-MS and ^{1}H NMR spectroscopy. The latter gave a spectrum similar to that obtained previously, i.e. very difficult to interpret, but this time

no aromatic peaks were observed. No structural information was gleaned from this data. GC-MS analysis again showed many products, all of which were named above.

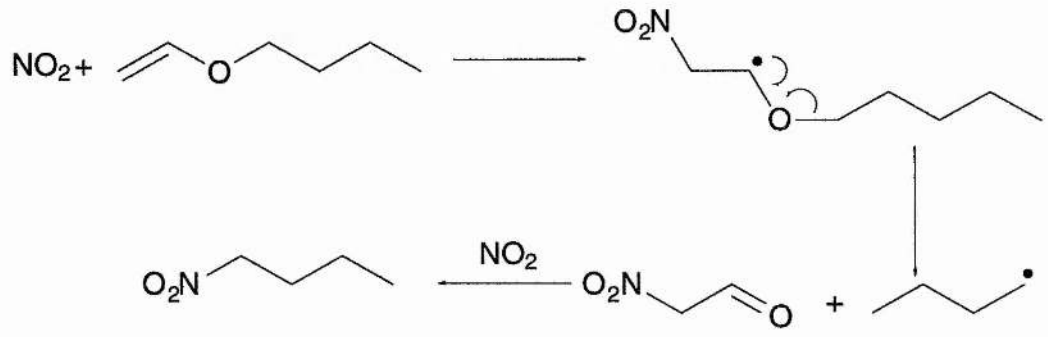
The fourth set of experiments using NO_2 was carried out by way of comparison. Nitrogen dioxide was bubbled through a non-degassed solution of the enol ether in cyclohexane. The solution was left in contact with the gas for 10 hours. Analysis of the samples removed was by GC-MS, ^1H and ^{13}C NMR spectroscopy. Preliminary 200MHz ^1H NMR spectra were similar to the aforementioned, also without aromatic signals. GC-MS showed fewer products, only 1,1'-[ethylidenebis(oxy)]bisbutane and the phthalate plasticiser. In an attempt to obtain a superior yield, this experiment was repeated. Analysis of the resulting residue was by GC-MS and 300MHz ^1H and ^{13}C NMR. spectroscopy. In this experiment there was suckback of the NO_2 produced which resulted in some of the reaction mixture entering the bubbler containing the drying agent, H_2SO_4. The cyclohexane layer that was formed was removed and the solvent removed. This was taken as the first sample. The second sample was taken from the solution remaining in the reaction vessel. Infra red analysis of the reaction mixtures revealed, for the sample retrieved from the acid bubbler, C-H (2900cm^{-1}), C=O (1650 cm^{-1}), and possibly C-O (1252cm^{-1}). For the sample in solution, IR analysis showed O-H (3400cm^{-1}), C-H (2900cm^{-1}). No C=O was observed. 300MHz ^1H NMR spectroscopy revealed that the phthalate plasticiser was in the cyclohexane (acidified) layer. The remaining solution gave peaks identified as CH_3CHO, remnants of the plasticiser, and 1,1'-[ethylidenebis(oxy)]bisbutane. There was also a possibility that butanol was present, which would account for the O-H stretch in the IR spectrum. A summary of the products identified by GC-MS analysis is shown:

Table 1: Summary of the products identified by GC-MS analysis.[a]

$t_{ret,min}$	Product	Fit	Type
1.3	Ethanal	978	major
2.0	Butan-1-ol	994	major
2.6	Butyl formate	983	intermediate
4.4	Butyl acetate	955	minor
7.4	2-Hexen-1-ol	956	minor
16.8	1,1'-[ethylidenebis(oxy)]bisbutane	866	major
19.2	1-Nitrobutane	908	intermediate
20.3	M^+ 160	858	minor
21.7	$C_9H_{20}O_2$	925	major
	Dioctyl phthalate		major

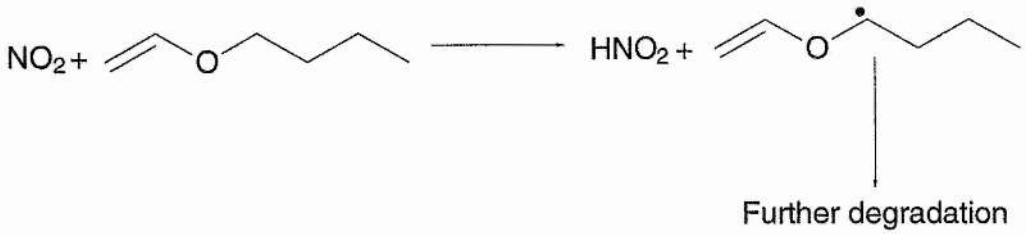
[a]Dioctyl phthalate was an impurity rather than a product in these experiments.

The presence of these products raises the question of how they were generated. A comparison with the spectrum of products in the presence and absence of oxygen with deliberately produced NO_2 indicates that reaction was probably initiated by NO_2. It is likely that this species both adds to the double bond and abstracts hydrogen from the n-butylvinyl ether. The adduct will probably undergo β-scission as shown to give nitroacetaldehyde together with an n-butyl radical which may combine with NO_2 to account for the 1-nitrobutane identified.



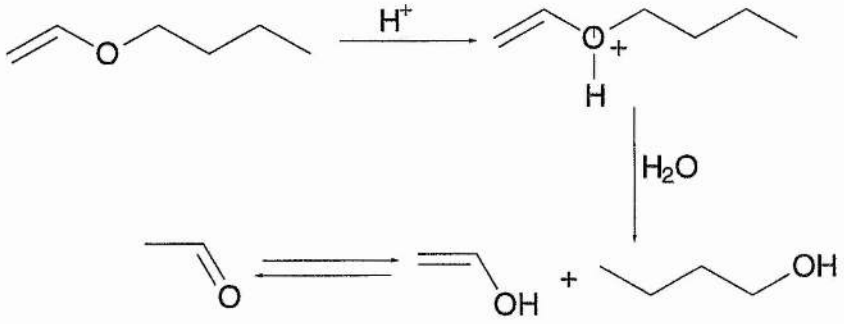
Scheme 4.15

Hydrogen abstraction will lead to the production of nitrous acid (scheme 4.16):



Scheme 4.16

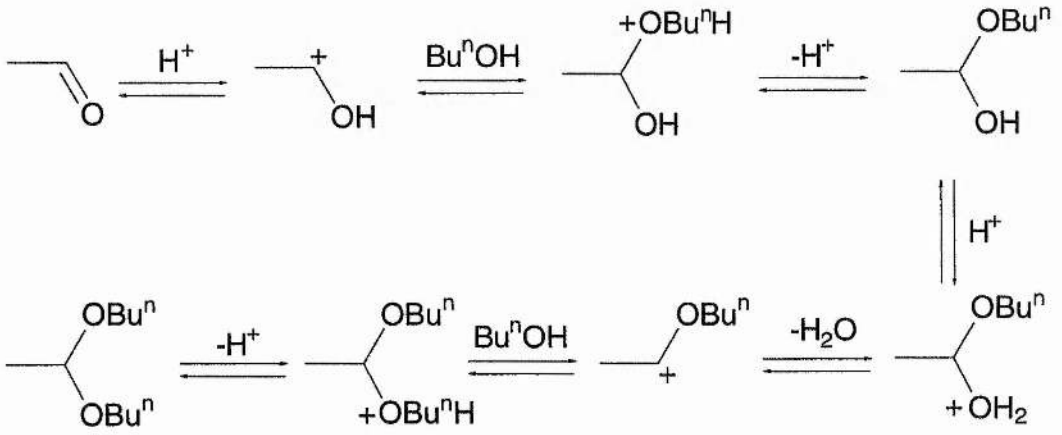
It may be this species which is responsible for the hydrolysis of the enol ether (scheme 4.17).



Scheme 4.17

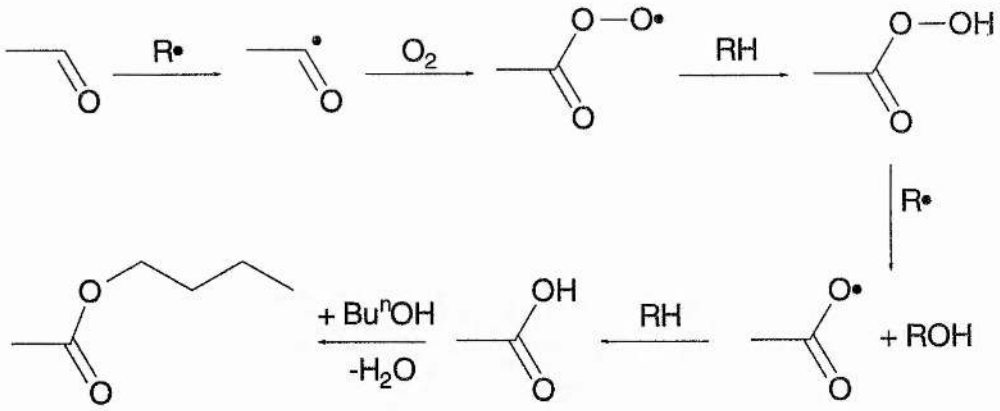
The source of the H_2O remains a mystery, because the gases and solvents were all dried prior to use. However, ethanal and butan-1-ol were both identified as major products.

Another major product, 1,1'-[ethylidenebis(oxy)]bisbutane was almost certainly formed by acid-catalysed ketalisation of the ethanal with butan-1-ol (scheme 4.18).



Scheme 4.18

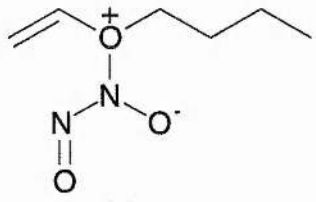
The ethanal probably undergoes autoxidation in the presence of oxygen to give ethanoic acid (scheme 4.19).



Scheme 4.19

The minor product, butyl acetate was plausibly formed as shown in scheme 4.19 by esterification of this acid with butan-1-ol. The presence of minor amounts of butyl formate suggests that further oxidative degradation of the ethanal or of the 2-nitroethanal produces formic acid, which is esterified in a similar way. The structure of the remaining major product, of t_{ret} 21.7 minutes, was not established, and its formation is unexplained.

As was reported above, NO reacts with Lewis bases to give charged adducts. If this were the case in this reaction, then it would be expected that a product of the following structure (**33**, RMM=160), would be observed.



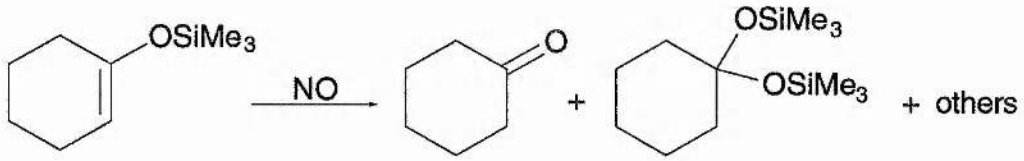
33

From the GC-MS analyses, i.e. t_{ret} 20.3 minutes in Table 1, there appeared a product from each reaction which had M^+ 160 and also contained the n-butyl fragment. This could possibly be the Lewis adduct. The mass spectrum also showed fragments of mass 30 and 60, which could indicate the loss of one or both NO groups.

4.2.1.2. *1-Cyclohexenyloxytrimethylsilane.*

Although the silyloxy group is not likely to occur in biological situations, 1-cyclohexenyloxytrimethylsilane containing this electron releasing group was next tested for comparison purposes.

A solution of 1-cyclohexenyloxytrimethylsilane in $20cm^3$ cyclohexane was degassed and NO bubbled through. Three separate sets of experiments were carried out, and samples were analysed by EPR, ^1H NMR spectroscopy and GC-MS. Samples from the first experiment taken after 20 hours showed an EPR signal of two overlapping triplets. Two nitrogen splittings of a(N)= 13.4G and a(N)= 7.8G were initially assigned. GC-MS analysis showed that in the early stages of the reaction cyclohexanone and cyclohexanebisoxytrimethylsilane were the main products, but that after 20 hours contact with NO a complex series of products developed.

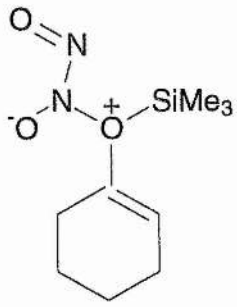


The ^1H NMR showed several distinct resonances for Me_3Si groups together with broad absorbances in the aliphatic region, which was consistent with the findings from the GC-MS analysis.

Table 2: Summary of the products identified from GC-MS of the long retention time mixture.

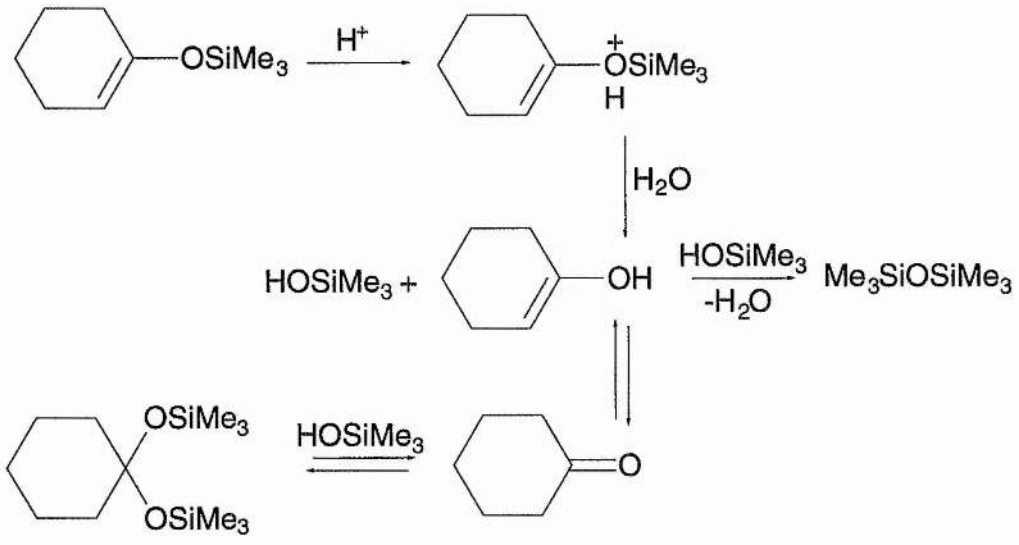
$t_{ret,min.}$	Product	Fit	Type
1.8	Cyclohexanone	945	Major
18.1	Cyclohexane(bis)oxytrimethylsilane		Major
1.6	Hexamethyldisiloxane	958	Major
16.6	Cyclohexaneoxytrimethylsilane	895	Major
18.3	1,1'-Bicyclohexyl	950	Minor

No evidence of the expected adduct **34** was obtained.



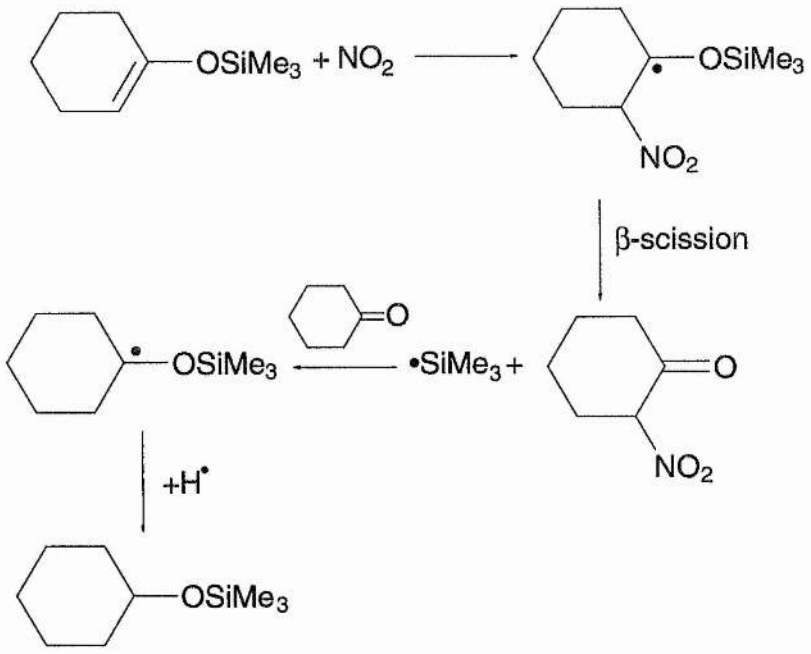
34

The main products in the early stages indicated a similar reaction to that of the n-butylvinyl ether, i.e., hydrolysis of the silyl enol ether:



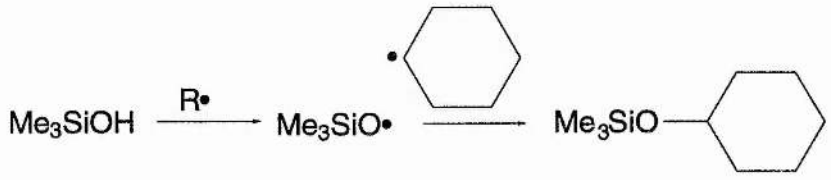
Scheme 4.20

Although trimethylsilanol itself was not detected, this is readily converted to hexamethyldisiloxane[202] which was a major product throughout. Similarly, condensation to form the ketal with cyclohexanone explains the presence of the t_{ret} 18.1 product. Several plausible reactions can be written for the formation of the cyclohexaneoxytrimethylsilane, e.g., by analogy with the n-butylvinyl ether reaction (scheme 4.21):



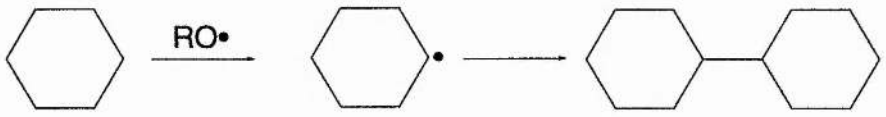
Scheme 4.21

However, no 2-nitrocyclohexane was detected, so an alternate must be considered. Hydrogen abstraction from trimethylsilanol would produce the trimethylsilyloxyl radical.



Scheme 4.22

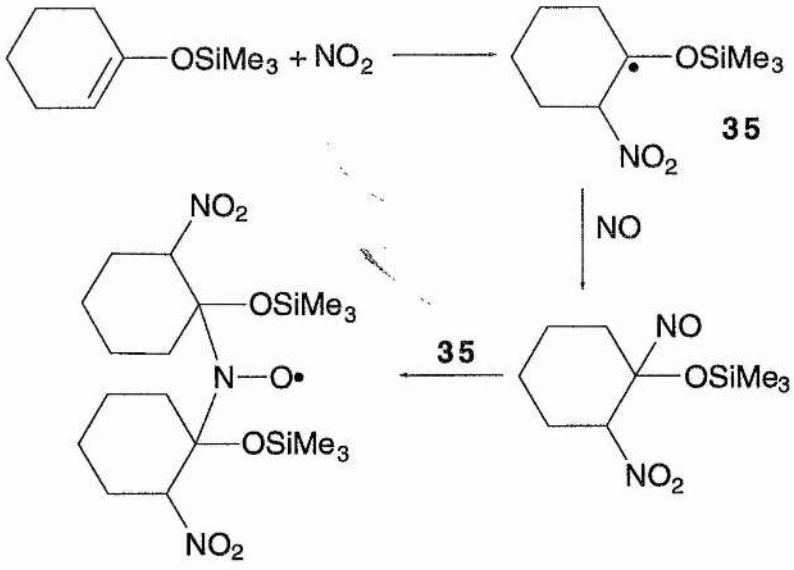
Combination of this with cyclohexyl radical derived from the solvent would also account for this product. The presence of cyclohexyl radicals was shown by the identification of bicyclohexyl.



Scheme 4.23

The EPR spectra showed the most promising results. As reported above, the nitrogen splittings obtained for the two radicals by comparison of experimental and

simulated spectra were a(N)= 13.6G and a(N)= 7.9G (figure 4.0). From previous results with hyperfine splittings, it appeared that a dialkyl nitroxide was formed



Scheme 4.24

having the larger splitting, and a carbonyl nitroxide was formed having the smaller splitting.

EPR analysis for the second set of experiments revealed a different spectrum. After 10 minutes exposure to NO, a wide triplet, a(N)= 29.5G was observed (figure 4.1). After 30 minutes exposure to NO, this radical was still seen, but a second radical had appeared, having a(N)= 13.6G, i.e., identical to the dialkyl nitroxide observed in the first sample. The wide ^{14}N splitting indicated that either an iminoxyl radical was formed, or an alkoxyl nitroxide, as the nitrogen hfs (29.5G) was right at the upper end of that expected for an alkoxyl nitroxide. This could be explained by the formation of the oxime, which underwent hydrogen abstraction by another radical, generating the iminoxyl radical. However, oxime formation is unlikely. It is more probable that the pathway shown in scheme 4.25 was the correct reaction. Thus, the 2-nitrocyclohexanone, formed by β-scission of radical **35**, undergoes addition at the nitro group by another radical, probably cyclohexyl or trimethylsilyl, to give an alkylnitroxide. This class of nitroxides also have a(N) values of ca. 30G.

Figure 4.0. EPR spectrum obtained from the reaction between 1-cyclohexenyloxytrimethylsilane and NO, with simulation (lower spectrum).

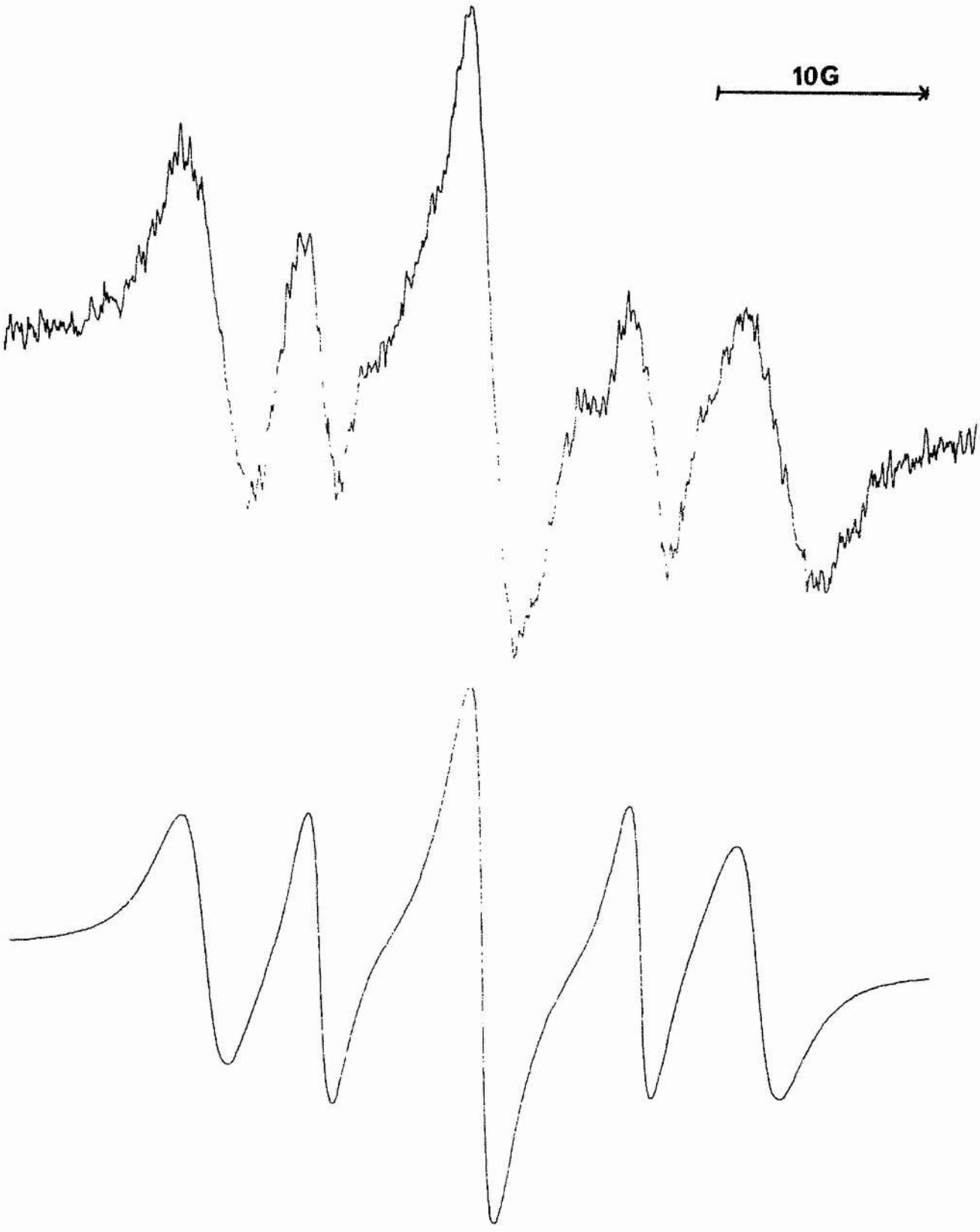
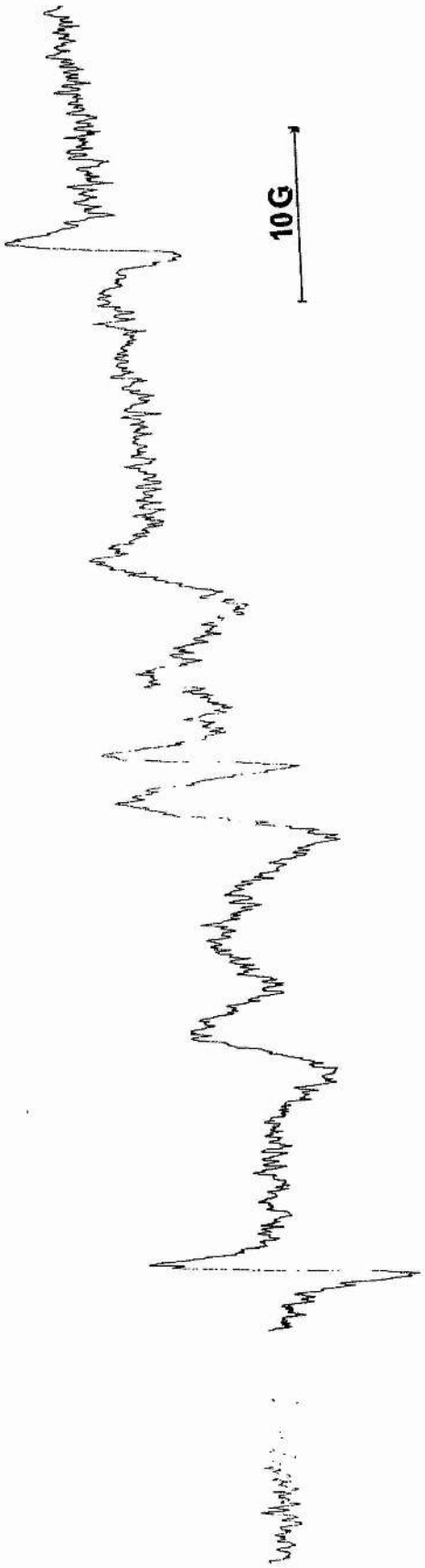
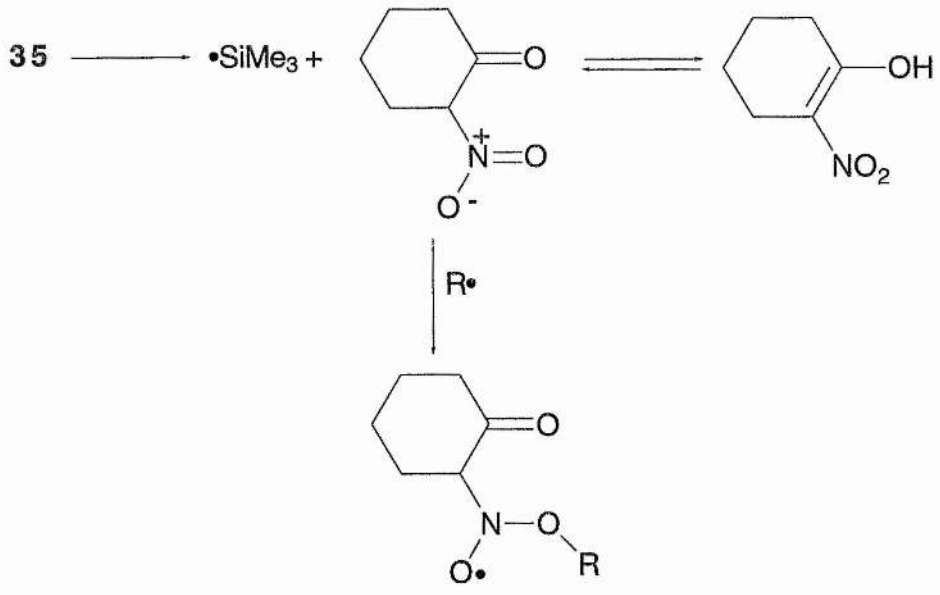


Figure 4.1. EPR spectrum at 210K showing wide additional signal.





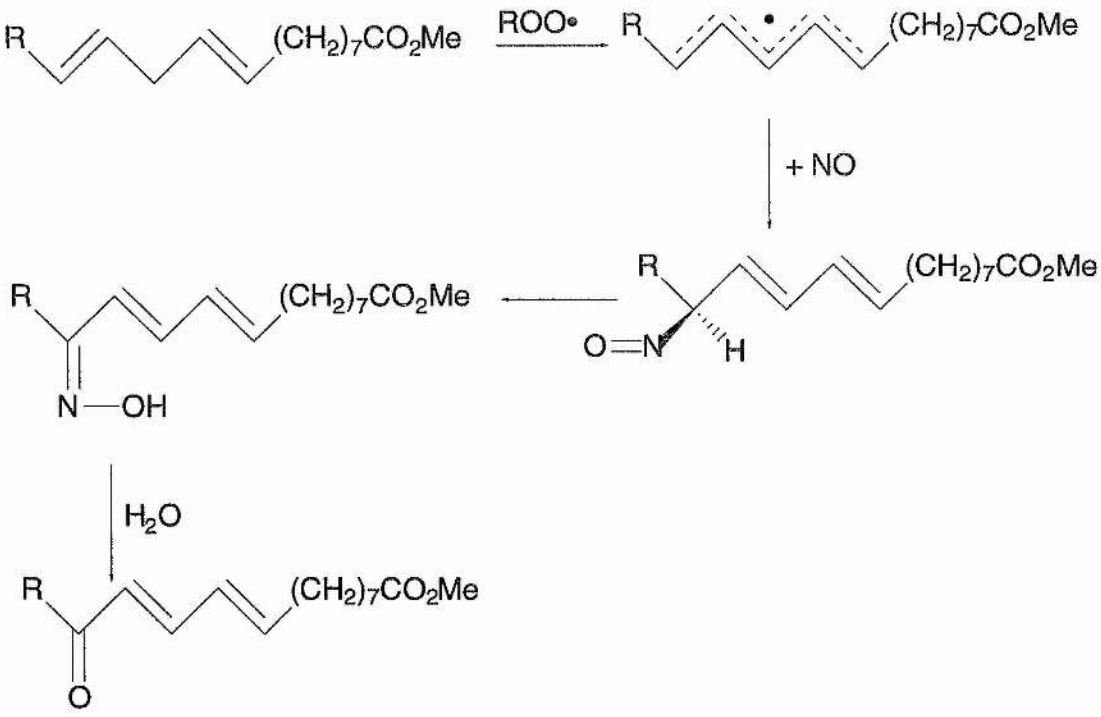
R•= cyclohexyl, $SiMe_3$

Scheme 4.25

The spectra were too broad for hydrogen hfs to be resolved.

4.2.1.3. *Methyl linoleate.*

Dienes were utilised because of their willingness to react with radical species. If a peroxide is added, there will be hydrogen abstraction at the bis-allylic site between the double bonds. If nitric oxide was present it was envisaged that the reaction shown below would occur, giving a nitroso compound. This would be reduced to the oxime, which would then hydrolyse to the ketone.



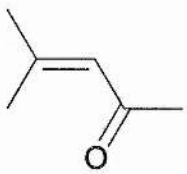
Scheme 4.26

The reaction of NO with methyl linoleate was carried out at room temperature in cyclohexane. Analysis by EPR and GC-MS showed no reaction. Addition of lauroyl peroxide as a radical initiator and a subsequent increase in temperature to 70°C had no detectable effect, i.e., the chromatogram showed only unchanged reactant. A stronger initiator, AIBN, was tried but this too had little effect. Only breakdown products of the initiators were found in the GC-MS analysis.

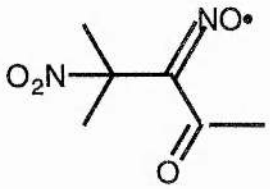
4.2.2. Substituted alkenes with electron acceptor groups.

By way of contrast to the above, it was decided to see if NO reacted with any of the molecules that possessed double bonds deficient in electrons. It has already been demonstrated that NO will form an adduct with the nitromethane anion, so there was a distinct possibility that a reaction would occur.

4.2.2.1. *Mesityl oxide.*



Mesityl oxide (4-methyl-3-penten-2-one) is an enone and was used in an attempt to trap NO and form a radical species. Symons *et al.*[195] reported EPR spectroscopic observations on this system in 1967. They identified iminoxyl and nitroxide radicals, and revealed that mesityl oxide formed a stable radical when reacted with nitric oxide in the presence of oxygen. The following structure was proposed for the stable intermediate:



Our work with mesityl oxide and NO was begun by using EPR spectroscopy. In each case, the reactions were carried out at room temperature, and the spectra were recorded at a frequency of 9.15GHz with 16dB power and 0.16G modulation. All the experimental work was carried out anaerobically by degassing the solutions with nitrogen for one hour prior to bubbling NO through the reaction mixture. In the complete absence of oxygen no spectra were obtained. However, after the introduction to the gas train of $2cm^2$ of air with a syringe, three radicals were observed developing with time. Two radicals appeared after 45 minutes, the stronger being a triplet of doublets (figure 4.2). This gave splittings of $\underline{a}(N) = 7.1G$, $\underline{a}(1H) = 1.6G$ (radical 1). The second radical observed was a triplet [$\underline{a}(N) = 30.0G$] of multiplets comprising a complex set of ~14 lines. The last to appear, after 60 minutes was also a triplet of multiplets, but made up of >8 lines. The triplet splitting for this radical was $\underline{a}(N) = 27.6G$, and the spacing of the multiplet was narrower than in the second radical, i.e. ca. 0.1 G.

The central multiplet of radical no. 2 was displayed under high resolution as a second derivative which showed a plane of symmetry about the central peak (figure 4.3). Splittings of $\underline{a}(3H)=0.49G$, $\underline{a}(3H)=1.22G$, and $a(3H)=1.75G$ were obtained by careful comparison of simulated and experimental spectra (figure 4.0). The formation of the three radicals was monitored as a function of contact time with NO by measuring the heights of their EPR signals and the data is given in the table below.

Figure 4.2. EPR spectrum showing the development of the three radicals derived from the reaction between mesityl oxide and NO with time.

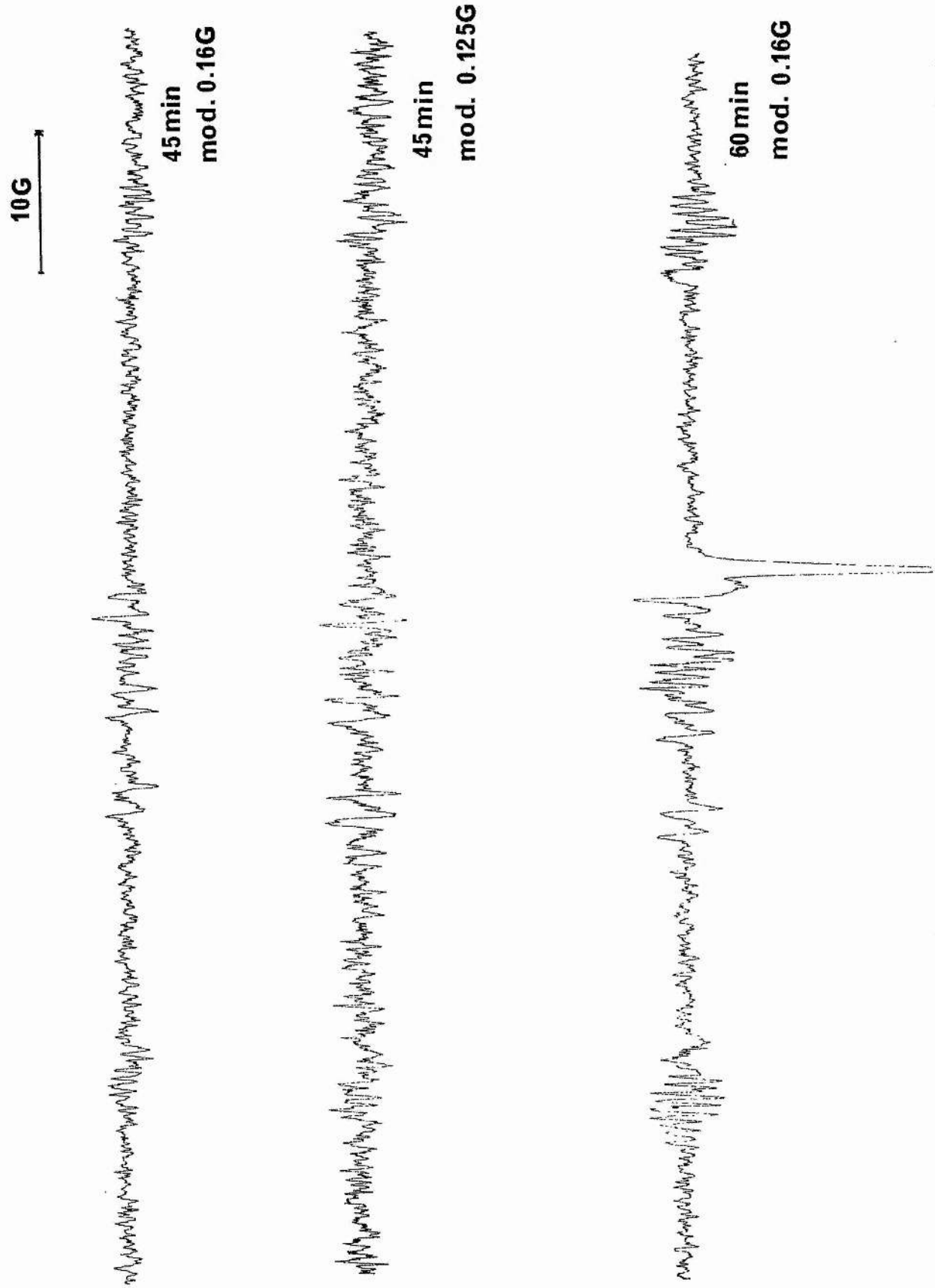


Figure 4.3. Top; EPR spectrum of central multiplet of radical no.2 from mesityl oxide with second derivative presentation. Bottom; computer simulation with hfs given in text.

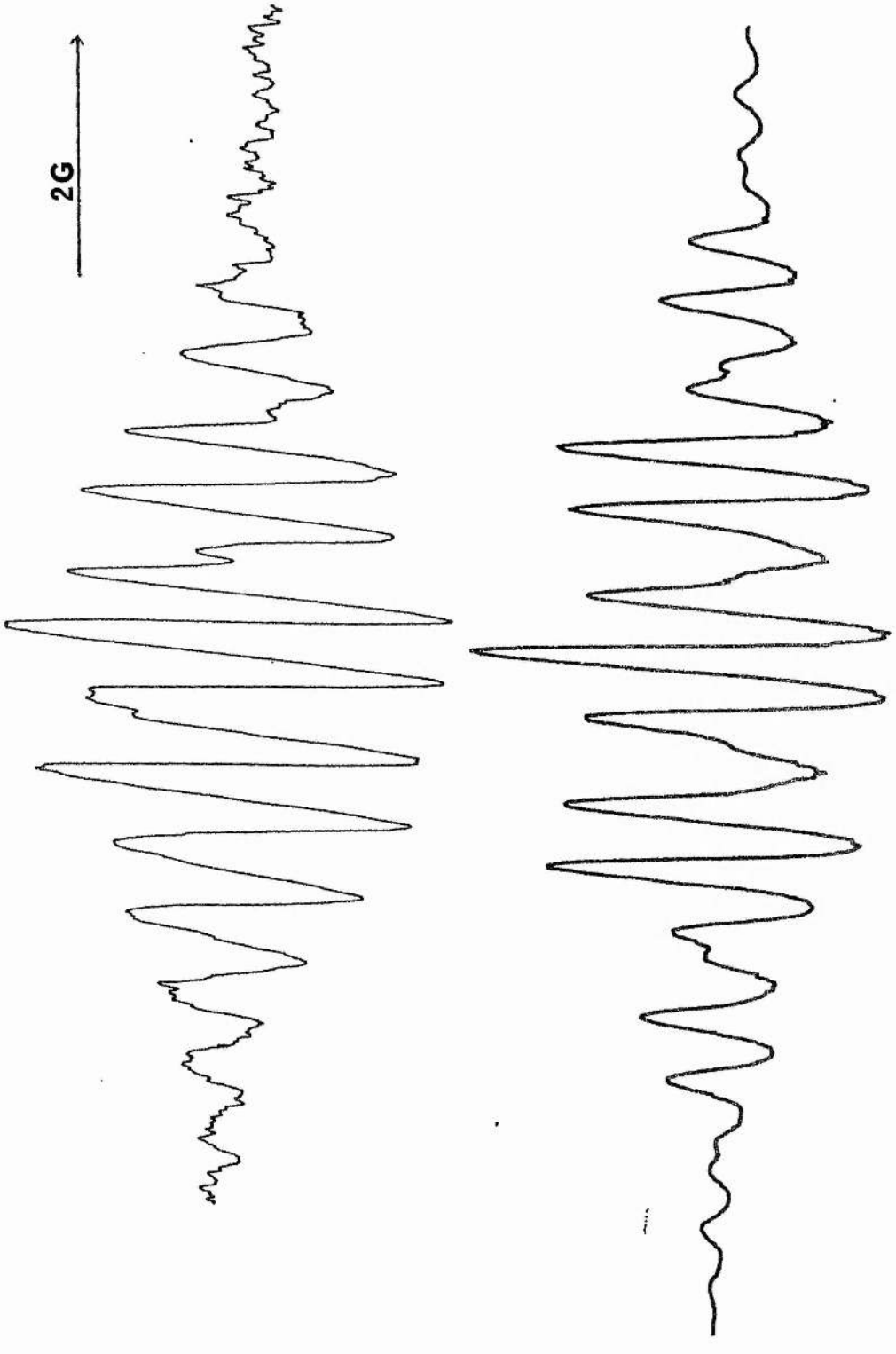
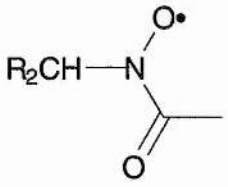


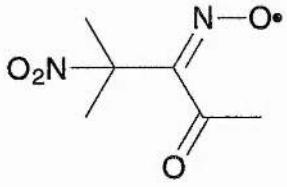
Table 3: Signal heights from reaction of NO with mesityl oxide as a function of time

Contact time/min.	Peak height (mm) Rad.1	Rad.2	Rad.3
15	0	0	0
30	0	0	0
45	11	8	0
60	15.5	20	11
90	25	8	0
105	9	0	0
120	9	0	0
135	11	6	7
150	24	5	9
180	12	8	7
240	7	6	9
300	27	13	25
360	16	0	30
1500	11	14	13

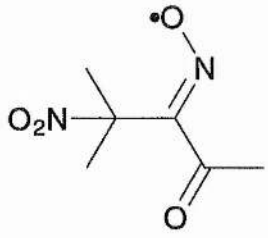
The heights of the signals of all three radicals go through a maximum after about one hour before decreasing and then increasing again. The first maximum was probably caused by admission of traces of oxygen which gave a burst of NO_2. Reaction of this with the mesityl oxide gave rise to the three radicals whose signals gradually decayed with time. Finally, after several hours, slight leakage of oxygen caused the gradual build-up of all three radicals.



Rad.1

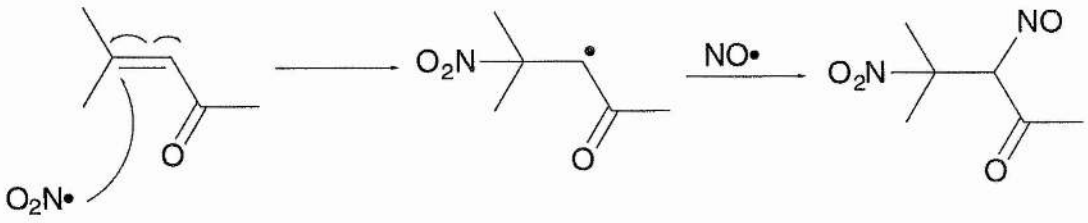


Rad.2



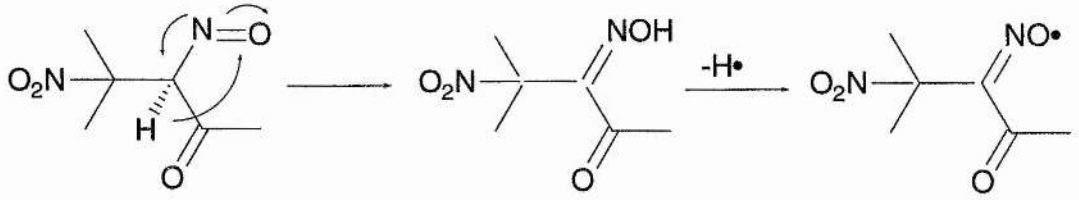
Rad.3

Radical 1 is a carbonyl nitroxide and radicals 2 and 3 are the **E** and **Z** conformers of the iminoxyl radicals, as shown above. The carbon-centred radical formed by addition of NO_2 would combine with nitric oxide to form the nitroso compound:



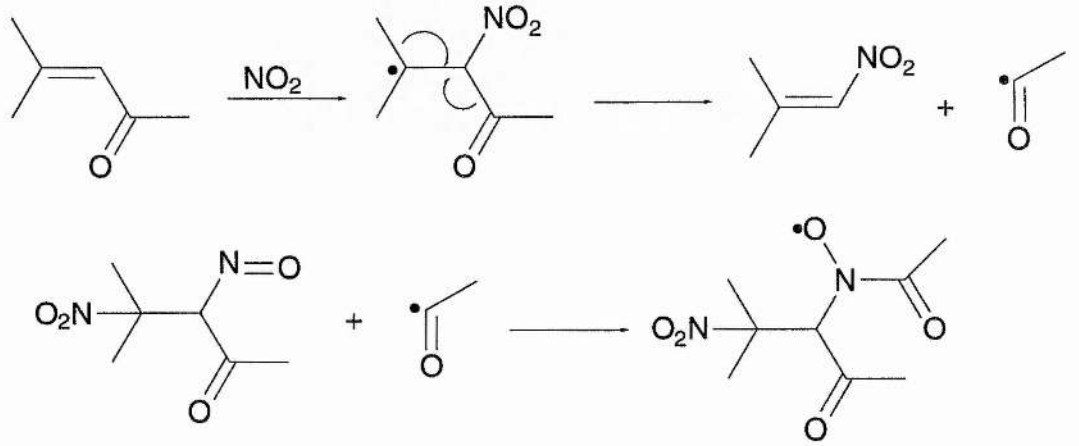
Scheme 4.27

The nitroso compound will equilibrate with its oxime tautomer and the latter will readily lose a hydrogen atom on attack by another radical, e.g. nitrogen dioxide, to give the observed iminoxyl radical.



Scheme 4.28

A possible explanation of the formation of radical 1, the carbonyl nitroxide, might involve formation of the acetyl radical. This could form by β-scission of the adduct from NO_2 and mesityl oxide at the less substituted end of the double bond. The nitroso compound formed as outlined above could then pick up the acetyl radical to give the carbonyl nitroxide (scheme 4.29):



Scheme 4.29

The small value of a(H) in radical 1, the carbonyl nitroxide, could be due to the H_β being almost in the nodal plane of the nitroxide π-orbitals containing the unpaired electron (figure 4.4).

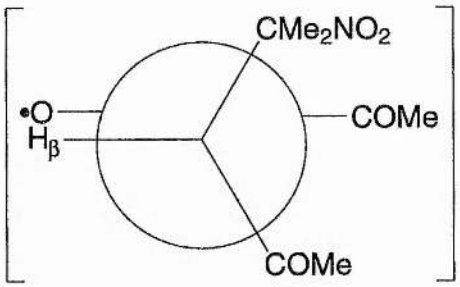
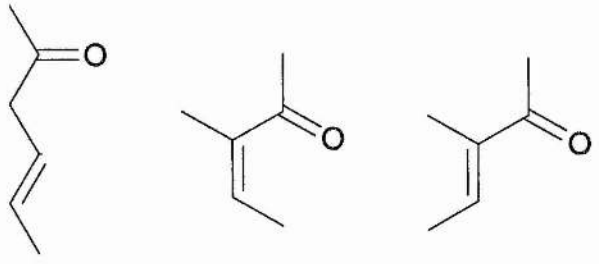


Figure 4.4

When mesityl oxide was reacted directly with NO_2, the same three radicals were detected after approximately 20 minutes. There was no induction period in the formation of the triplet of doublets (radical 1) as in the NO reaction. Radicals 2 and 3 were resolved into triplets of multiplets which had values of a(N) as above. The multiplets of radical 3 could not be resolved.

With the aim of understanding the final fate of nitric oxide the reaction mixtures were prepared on a larger scale and analysed by a variety of spectroscopic techniques. Samples of each experimental solution were analysed by GC-MS. The chromatogram showed unreacted mesityl oxide and solvent, together with several major and many minor products. Few, if any, of the individual components showed molecular ions in their mass spectra, which made identification difficult. Compounds with a molecular weight of 98, (as has mesityl oxide), were observed, and these were thought to be isomeric forms such as:



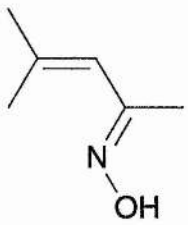
It was found that bicyclohexyl (f.w. 166), was present in some of the reactions, indicating that the solvent, cyclohexane, had undergone some reaction, probably

involving the abstraction of a hydrogen atom to form the cyclohexyl radical in a similar reaction to that shown in scheme 4.23.

The main fragment which appeared in many of the product spectra had m/z = 43, i. e. the CH_3CO^+ group. The chromatogram gave some indication that the major products were too volatile to be resolved from the solvent by GC, so an attempt was made to distil them from the solvent, but this was unsuccessful.

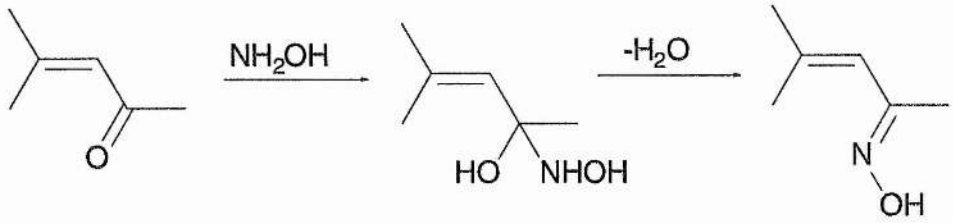
A silica gel column was used in an attempt to separate the less volatile products. Diethyl ether with increasing amounts of ethyl acetate was used to develop the column. Each fraction was examined by NMR spectroscopy but revealed no products. Finally, the column was stripped with methanol and this solvent was then evaporated off. Crystals were obtained and these were sent for analysis by mass spectrometry. The mass spectrum showed two major ions towards high molecular weight, the first with measured m/z = 139.07528 (calc. 139.0633). The fit of this ion for the empirical formula $C_7H_9NO_2$ was quite poor, however the other ion, at m/z = 181.98895 showed that of compounds containing C, H, N, and O, only formulas with very low hydrogen content fitted this mass, which suggested that the molecule contains a heteroatom, i.e. it is an impurity not derived from the reactants. Further columns were tried but these showed no products on analysis. Because of the low weights of all fractions from the columns it was concluded that the main reaction products were comparatively volatile materials from oxidative degradation of the mesityl oxide. The reaction was also carried out in t-butylbenzene solvent and acetonitrile, but in each case no significant product development was observed.

A possible intermediate in the mesityl oxide reaction appeared to be the oxime (**36**).



36

Authentic oxime was prepared, (scheme 4.30), and photolysed in the cavity of the EPR spectrometer in the presence of di-t-butyl peroxide (BOOB).

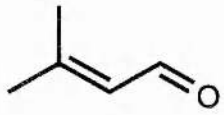


Scheme 4.30

The EPR spectrum obtained showed a wide triplet with a(N)= 33G, and two narrower triplets, one of a(N) ~15G, the other of a(N) ~7.5G. These results showed that photolysis appeared to generate the same types of radical as the reaction with NO/NO_2. This confirmed the identity of the iminoxyl radicals, and also the presence of the oxime intermediate.

4.2.2.2. *3-Methyl-2-butenal.*

We decided to examine more conjugated enones to establish if the presence of an extra electron acceptor group would make a difference to the reactivity of NO. The first of these compounds to be tested was 3-methyl-2-butenal.



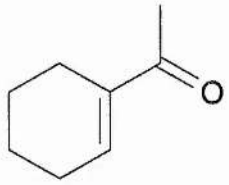
Nitric oxide was bubbled through a solution of 6.9 $x10^{-3}$ mol. 3-methyl-2-butenal in $20cm^3$ cyclohexane after being degassed for 1hour. No signal was observed when a sample was taken for EPR spectroscopy. When $2cm^3$ air was injected, there was still no EPR signal. Analysis by GC-MS showed only unreacted starting material.

4.2.2.3. *Cyclohexenone.*

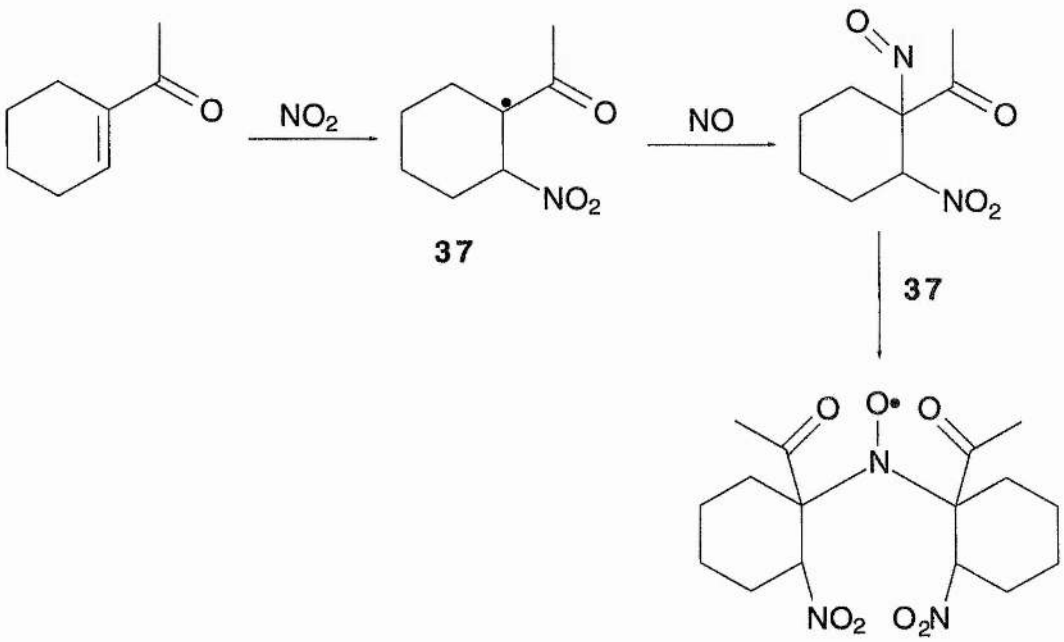
A solution of cyclohexenone in cyclohexane was degassed and NO bubbled through the solution for a period of 4 hours. A sample was then removed and analysed by EPR and ^1H NMR spectroscopy and GC-MS. No signal revealing that a reaction had occurred was recorded by EPR or ^1H NMR spectroscopy, and only

unreacted starting material showed on the GC-MS chromatogram and mass spectrum. The experiment was repeated twice, but the results were consistent.

4.2.2.4. *1-Acetylcyclohexene.*



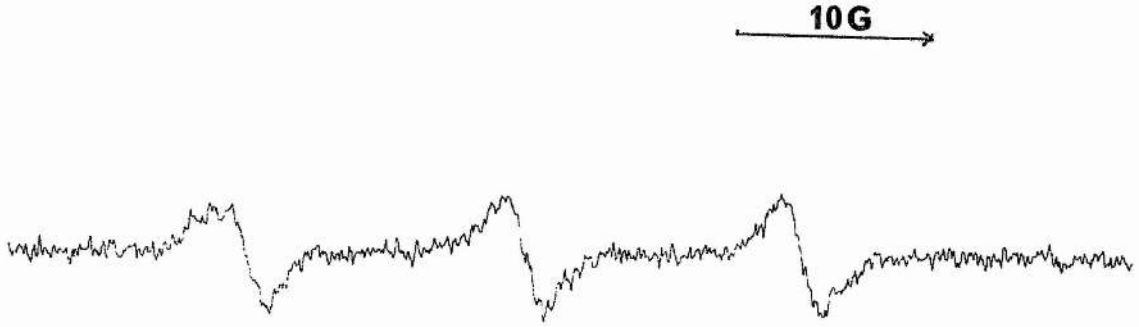
NO was bubbled through a solution of 4.03 $x10^{-3}$ mol. of 1-acetylcyclohexene in 20cm^3 cyclohexane. After bubbling, a colour change occurred (milky to pale yellow). No paramagnetic species were observed up up to 2 hours. When 2cm^3 of oxygen was introduced, a triplet was seen with a(N)= 14.5G (figure 4.5). The nitrogen hyperfine splitting suggested that this was due to a dialkyl nitroxide, produced as in scheme 4.31.

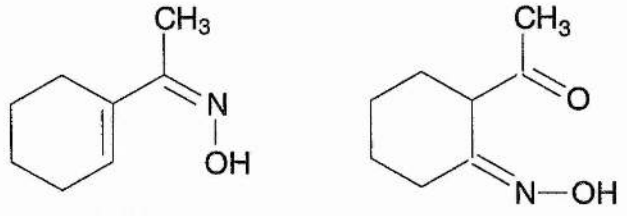


Scheme 4.31

All EPR spectra were recorded at room temperature. The chromatogram of the solution revealed a major product together with several other products having retention times greater than that of the starting material. The MS of the main product showed ions at m/z 138, 139 which suggested it may be the oxime derived from the reactant:

Figure 4.5. EPR spectrum of reaction between NO and 1-acetylcyclohexene after addition of air.





38

Another important product had m/z = 154 which is consistent with the oxime formed by attack at the double bond (see above). The ^{1}H NMR spectrum of the product mixture was consistent with this explanation.

By way of verification of the oxime formation, authentic 1-acetylcyclohexenyl oxime (**38**) was prepared, as described in the experimental section. A sample of this oxime was dissolved in 0.5cm^3 of PhBu^t and ~250μl of di-t-butyl peroxide was added. This was placed in the EPR spectrometer cavity and photolysed at room temperature for ~10 seconds. A signal comprising 3 lines was observed, and displayed on the oscilloscope screen. However, it was too transient to be recorded. Consequently, the temperature

was lowered to 210K and photolysed for ~1 second. This gave rise to a triplet of a(N)= 30.4G which also had a short lifetime, but the reduced temperature prolonged this considerably. The central peak of the of the observed triplet was examined with second derivative presentation, and this gave a complex splitting pattern of ~19 lines

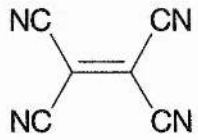
revealed that two radicals were present. The splitting pattern of this complex multiplet was simulated using the following parameters: a(3H) =1.4G, a(2H) =0.9G, a(1H) =2.3G (major multiplet); a(3H) =1.45G, a(2H) =0.8G, a(1H) =1.3G (minor multiplet), as shown in figure 4.6. From this it was deduced that the two conformers of the iminoxyl (E and Z) must be present, but the different ^{14}N hfs could not be distinguished.

Comparing this result with NO/1-acetylcyclohexane experiments indicates that any iminoxyl radical generated from the oxime would be too short lived at room temperature for EPR detection, and hence this explains why only the nitroxide was detected under these conditions. Furthermore, this result is entirely consistent with the intermediacy of the corresponding oxime. The nitroxide observed from the reaction must be entirely derived from the reaction between NO/NO_2 and the acetylcyclohexene, because it was not observed when the oxime was photolysed. Thus NO_2 was again required to start the reaction of NO.

The lack of hfs for the hydrogen meant that the structure of the nitroxide was not determined.

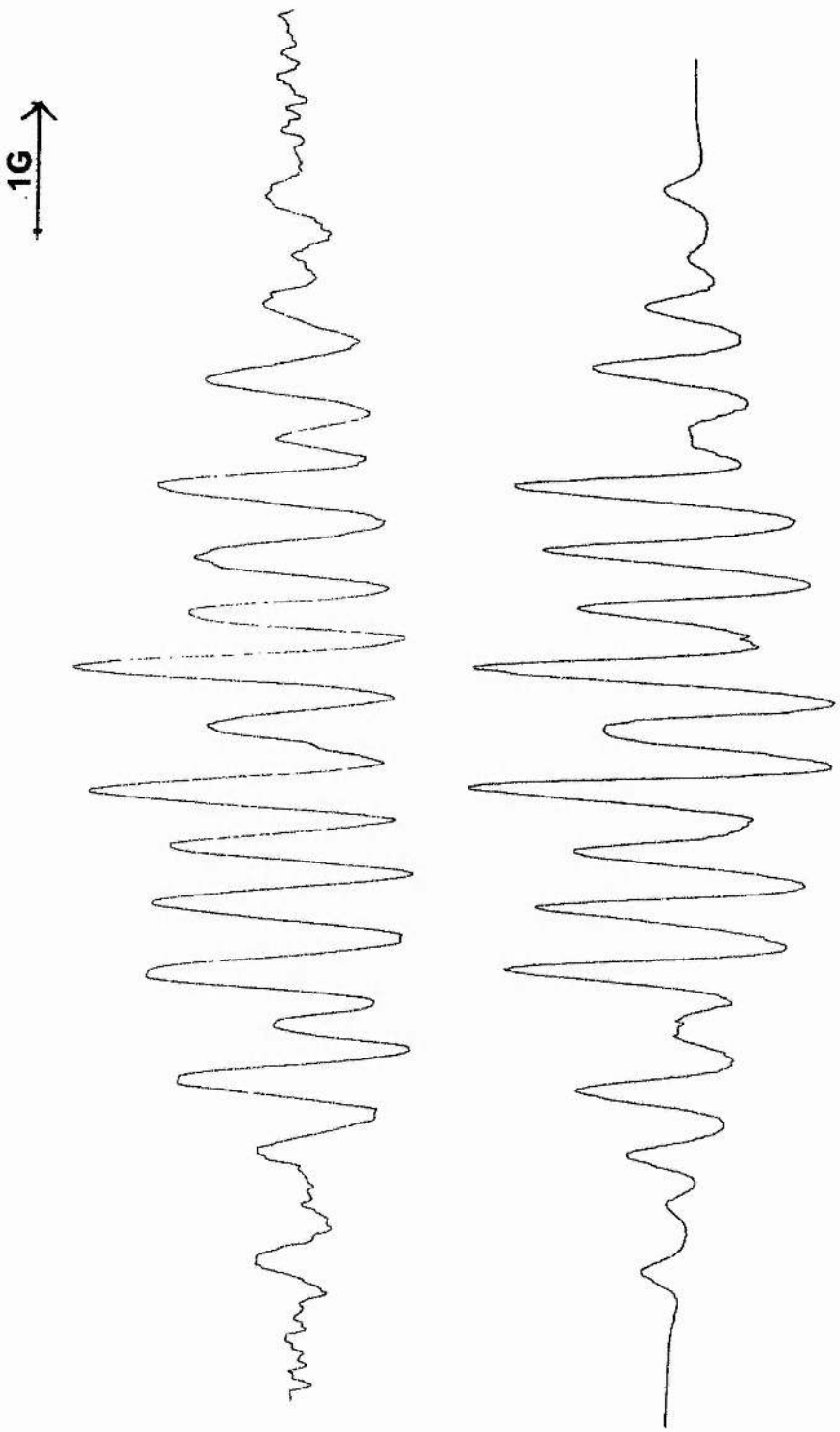
4.2.2.5. *Tetracyanoethene (TCNE).*

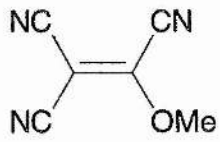
Direct reaction of NO with alkenes containing one electron-withdrawing substituent had not been observed and therefore it was decided to examine an alkene made much more electron deficient by the presence of four powerful electron-withdrawing groups.



A solution of 3.9 $x10^{-3}$ mol. of TCNE in $20cm^3$ MeOH was degassed for 1 hour. Nitric oxide was then bubbled though for 24 hours. A sample was taken and analysed by GC-MS. This indicated that the main product was the methyl ether (**39**).

Figure 4.6. Top; 2nd Derivative EPR spectrum showing the central peak of the wide iminoxyl signal obtained at 210K. Bottom; simulation of multiplet.





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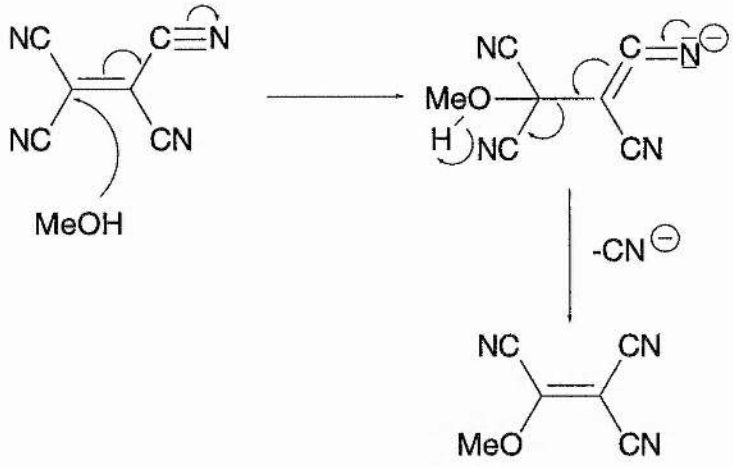
A possible mechanism for its formation is given in scheme 4.33. The cyano groups are so electron-withdrawing that they make the carbon δ^+ which makes it susceptible to attack from the methanol.



Scheme 4.33

There were no other identifiable products. Consequently, the solvent was changed to acetonitrile. Here, the solution (quantities as above) was degassed and reacted with NO as before. In this case, the reaction was left for 4 days. Samples were taken after 3 hours, 72 hours, and 96 hours. No products were identified by GC-MS except starting material and solvent.

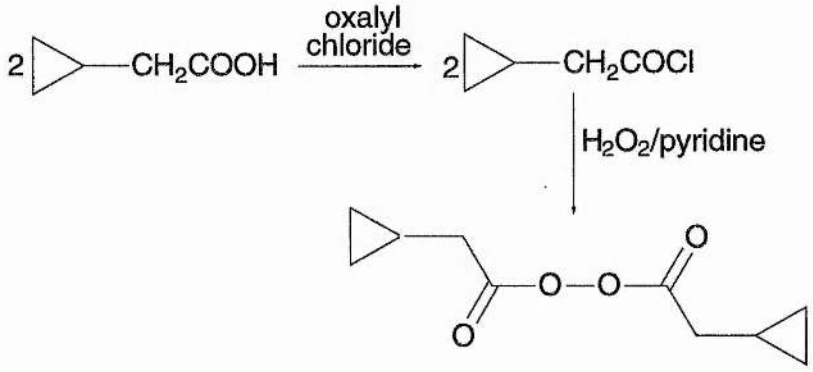
4.2.3. Reaction of NO with carbon-centred radicals.

From the results obtained in the first parts of this research it became apparent that nitric oxide was unreactive towards the majority of alkenes whether they contained donor or acceptor substituents. It was known from work in solution, and in atmospheric pollution studies, that NO reacted rapidly with carbon-centred free radicals. Carbon-centred free radicals are intermediates in several processes which occur *in vivo*, such as lipid peroxidation, so we next began a study of the behaviour of

nitric oxide with specifically generated free radicals. Diacyl peroxides are clean thermal and photochemical sources of carbon-centred radicals and therefore a diacyl peroxide was chosen as our initial radical precursor. The advantage of cyclopropyl acetyl peroxide was that thermal or photochemical decomposition gives initially the cyclopropylmethyl radical which rapidly rearranges to the butenyl radical and this can be utilised, under favourable circumstances, for timing individual steps[203]:

4.2.3.1.*Cyclopropylacetyl peroxide.*

The starting material used was cyclopropyl acetic acid which was first converted to the acid chloride according to scheme 4.34:



Scheme 4.34

Practical details are given in the experimental section. Because of its explosive nature, the peroxide was not isolated but was prepared and stored in ether. The ether was removed from the peroxide prior to use in the experiment. The peroxide (50 $x10^{-6}$ mol.) was dissolved in $10cm^3$ cyclohexane. The system was degassed for 1 hour, and then NO was bubbled through. The reaction mixture was heated to 60°C to cleave the peroxide. A sample was taken after 0.5 hours, and analysis by GC-MS suggested a very small extent of reaction. The experiment was restarted and another sample taken; the GC-MS indicated a product with principal ions in its mass spectrum at m/z 279, 280 and 391. This high molecular weight material was evidently not a primary product. In further experiments using cyclopropylacetyl peroxide, GC-MS analysis showed that

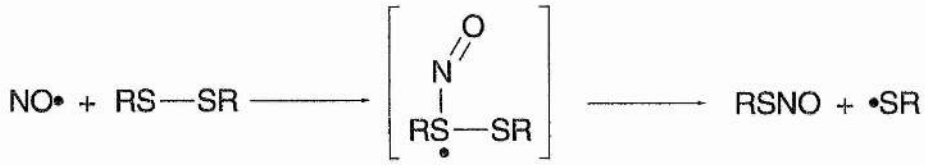
1,1-bicyclohexyl had been formed, presumably from radical attack on the solvent, and possibly also products from coupling of the cyclohexyl radicals with cyclopropylmethyl and/or butenyl radicals. No nitrogen containing products were detected, even in trace quantities.

An alternative method of radical generation was investigated. This involved dissolving the peroxide in 210cm^3 benzene. The solution was then degassed for 1 hour and exposed to UV light for 6 hours. Nitric oxide was bubbled through for the same length of time. The solution turned from colourless to yellow and samples were taken and concentrated for EPR spectroscopy. No signal was seen from the samples.

Preparative TLC (silanized silica plate) was used to separate the resulting reaction mixture. The mixture was dissolved in neat ethyl acetate and the plate was developed with a mixture of cyclohexane and ethyl acetate (60:40). Once the solvent had run its course, the plate was examined and three bands were observed, including the initial band. These were removed and dissolved in cC_6H_{12}/EtOAc solution, filtered through celite and analysed by ^{1}H NMR. This revealed that there had indeed been a reaction as the spectra were different to that of the starting material. Both product layers gave similar spectra which consisted of unresolved multiplets in the aliphatic region. This was consistent with the finding of the products of radical attack on the solvent, and with the cyclopropylmethyl and butenyl radicals coupling with cyclohexyl radicals. Due to an accident in the GC-MS laboratory, no GC-MS data was obtained for these samples.

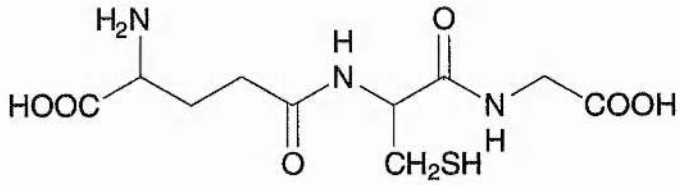
4.2.4. Reaction of NO with biological molecules.

Sulphur-containing compounds are important biological molecules, so it was decided to react NO directly with a thiol and with a disulphide to determine the extent of the reaction. It is known that thiols react with NO to give disulphides (see **4.1.**), but it is not known if NO acts as a direct nitrosating agent of thiols, or with disulphides. The reaction with a disulphide could involve a bimolecular homolytic substitution S_H2, which is known to occur readily with many radical types and disulphides (scheme 4.35).



Scheme 4.35

4.2.4.1. *Glutathione (GSH).*



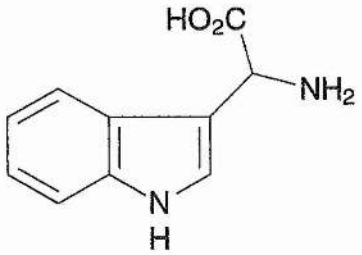
Nitric oxide was bubbled through an aqueous solution of GSH at room temperature. Samples taken were then submitted for UV/vis spectroscopy. This revealed peaks at 207.7 and 253.1nm, and was essentially the same as the reactant. Morris and Williams[204] found that S-nitrosothiols absorb at 330nm, but no band was observed in this region, and hence no reaction had taken place between nitric oxide and GSH. Nitric oxide did not act as a nitrosating agent.

4.2.4.2. *Dibenzyl disulphide.*

Nitric oxide was bubbled through a degassed solution of dibenzyl disulphide in cyclohexane at room temperature, and then at 70°C in cyclohexane. No products were observed in the room temperature reaction, but at 70°C two minor components were detected by GC-MS. These are currently unidentified. An alternative radical initiator, azoisobutyronitrile (AIBN) was used, the conditions being 80°C in toluene. Analysis showed one component that was likely to be the starting material $PhCH_2SSCH_2Ph$, and the breakdown products of AIBN.

Pure NO does not react directly with disulphides in aqueous solution at pH~7, or in hydrocarbon solutions.

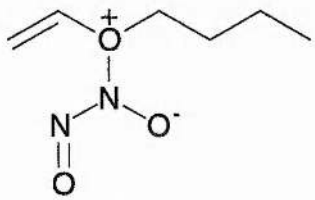
4.2.4.3. *L-Tryptophan.*



NO_2 was bubbled through a 50mM solution of L-tryptophan in water. A sample was taken after 25 minutes and analysed by EPR spectroscopy. A strong 3-line signal, with a(N)= 15.0G was observed, but these were not resolved any further. This radical adduct was not studied in depth. GC-MS analysis showed no identifiable products, only unreacted starting material.

4.3. Conclusions.

From the observed reaction between NO and model cell constituents, it was seen that in essentially every case, the presence of NO_2 as an initiator was required before NO would take part in any reaction. There was little evidence for the direct formation of any radical adduct from the compounds tested, but formation of an adduct did occur in several cases when traces of oxygen were introduced into the reaction system. It appeared to take part in extensive oxidative degradation of n-butylvinyl ether, and GC-MS analysis revealed the presence of the adduct **33**, (N-nitrosonitrite)-n-butylvinyl ether as expected.



33

Hydrolysis of enol ethers by the initial action of NO_2 to form HNO_2 gave a mixture of products, including the alcohol and the ketone, and ethers. Iminoxyl and carbonyl nitroxides were formed from enones in several cases, but the EPR spectra obtained were not well enough resolved to allow structure determination.

Carbon-centred radicals are usually highly reactive, and it was expected that the radical produced from the thermal or photolytic cleavage of cyclopropylacetyl peroxide would react with NO to form a radical adduct. However, under the conditions employed in this research the main process involved solvent derived species and no evidence of NO combination with carbon-centred radicals was obtained. Nitric oxide did not act as a direct nitrosating agent of thiols, and did not react with disulphides in aqueous solution or in organic solvent. Nitrogen dioxide did form an adduct with the amino acid L-tryptophan, and this was a reaction that could prove useful in the future.

4.4. Experimental.

All the compounds tested were used as purchased without further purification, except for mesityl oxime, 1-acetyl-1-cyclohexenyl oxime and cyclopropylacetyl peroxide, the syntheses of which are described below.

4.4.1. Preparations.

4.4.1.1. *Mesityl oxime.*[205]

Mesityl oxide (5g, 0.051 mol.) and hydroxylamine hydrochloride (5g, 0.07 mol.), EtOH ($5cm^3$) and pyridine ($5cm^3$) were refluxed on a water bath for 1 hour. The EtOH was removed on a rotary evaporator and $50cm^3$ of water was added to the cooled residue which was then cooled in an ice bath and stirred. A red oil was produced which did not crystallise. This was analysed by IR and ^1H NMR spectroscopy. υ/cm^{-1} 3250 (OH), 1680 (C=N); δ_H 5.6 (1H,d), 1.9 (6H,m), 1.7 (3H,m).

4.4.1.2. *1-Acetylcyclohexenyl oxime.*

A similar method to the above was used in this preparation[205]. A flask containing 0.5g (4×10^{-3} mol.) of 1-acetylcyclohexene, 0.5g (7×10^{-3} mol.) of hydroxylamine hydrochloride, $0.5cm^3$ EtOH and $0.5cm^3$ pyridine was set up on a water bath and the contents refluxed for 1 hour. After this time, the EtOH was removed on a rotary evaporator and $5cm^3$ water was added. This solution was then stirred in an ice bath for 15 minutes. Upon crystallisation from EtOH, the solid was

filtered off and washed with water. The product was then recrystallised from a 3:1 water:MeOH mixture. White crystals were obtained. υ/cm^{-1} 3250 (OH), 1670(C=N); δ_H 6.2 (1H,t), 2.1 (4H,m), 2.0 (3H,s), 1.8 (4H,m), (Calc. for $C_7H_{13}NO$: C, 69.030; H,9.413; N, 10.063; O, 11.494. Found: C, 69.10; H,9.75; N,10.18%).

4.4.1.3. *Cyclopropylacetyl peroxide.*

The synthetic method used[206] was carried out as follows; cyclopropyl acetic acid, (1g, 0.01 mol.) was dissolved in dry ether ($10cm^3$) in a three-necked flask. To this, oxalyl chloride (0.63g, 5 $x10^{-3}$ mol) was added dropwise, and the mixture was refluxed for 1 hour. The flask containing the solution was placed in an ice water bath and 27% H_2O_2 solution ($0.62cm^3$, 5 $x10^{-3}$mol.) was added, followed by pyridine (0.79g, 0.01 mol.). This was then left to stir for 2 hours. The solution was washed with dilute HCl, and then again with saturated $NaHCO_3$ solution. The ether layer was dried over $MgSO_4$ and filtered. The peroxide was left in the ether solution until ready for use. A sample was taken and the ether removed. The product was dissolved in $CDCl_3$, and was analysed by ^1H NMR, δ_H 2.2-2.4 (2H, d), 1.1 (1H, m), 0.6 (2H, t), 0.2 (2H, t).

4.4.2. Details of reactions.

4.4.2.1. n-*Butylvinyl ether.*

The experimental method involved bubbling NO through a degassed solution of n-butylvinyl ether (0.5g, 5 mmol) in cyclohexane. The reaction was left to proceed for 16 hours, then the solvent was removed and the resulting residue was analysed by GC-MS and ^1H NMR spectroscopy. The chromatogram showed a range of low and high molecular weight products. These included an adduct of the reaction with NO, the n-butylvinyl-(N-nitrosonitrite) ether, butan-1-ol, butyl formate, butyl acetate, 1,1'-[ethylidenebis(oxy)]bisbutane, 1-nitrobutane and an unidentified long retention time component of empirical formula $C_9H_{20}O_2$.

4.4.2.2. *1-Cyclohexenyloxytrimethylsilane.*

Nitric oxide was bubbled through a degassed solution of 0.5g (2.9 $x10^{-3}$mol) 1-cyclohexenyloxytrimethylsilane in 20cm^3 cyclohexane. A sample was taken after 20 hours and analysed by EPR spectroscopy. The spectra were obtained at a frequency of 9.05GHz with amplitude $5x10^5$ and 1.0G mod. The sweep time was 500 seconds, and the time constant was 500ms. This revealed a 3-line signal characteristic of a nitroxide. GC-MS analysis showed many products of oxidation (see text for details).

4.4.2.3. *Methyl linoleate.*

Methyl linoleate (0.5 cm^3, 1.69 mmol) was dissolved in *tert*-butylbenzene (20cm^3). The solution was degassed for 15 minutes using N_2, then NO was bubbled through at room temperature. Two samples were taken, 1 and 2 hours after initial addition of NO. These were exmined by EPR spectroscopy and GC-MS. No signal was obtained by EPR, and the GC-MS showed only unreacted starting material. In the next experiment, methyl linoleate (0.1cm^3 $3.39x10^{-4}$mol) was dissolved in cyclohexane (20 cm^3), with lauroyl peroxide (0.02g $0.5x10^{-3}$mol) as radical initiator. The solution was degassed for 15 minutes, and NO was bubbled through at 70°C. The first sample was taken after 4.5 hours, and the second sample 5.5 hours after initial NO addition. The experiment was repeated with the same conditions as the latter, but using AIBN (0.02g, $1.2x10^{-4}$mol) instead of lauroyl peroxide; samples taken after 6.25 and 7.25 hours were examined by GC-MS which showed unreacted starting material and breakdown products of the initiator.

4.4.2.4. *Mesityl oxide.*

Solutions of varying concentrations were used in this series of experiments. Initially, mesityl oxide, (0.5g, $5x10^{-3}$mol) was dissolved in cyclohexane (20cm^3). This was later increased to 2.0g (0.02mol) to provide a better yield of products for analysis. After NO was bubbled through the solution, samples were removed for analysis at timed intervals. Two complex multiplets and a triplet were observed using EPR spectroscopy. The hfs were noted, and the spectra were examined further under second derivative. Analysis of the reaction mixture by GC-MS showed several major

and minor products. Few components showed their molecular ions in their mass spectra, which made identification difficult. Compounds with a molecular weight identical to that of mesityl oxide were observed, and it was thought that these were isomeric forms. Bicyclohexyl was also identified in the chromatogram, but many of the products were too volatile to be resolved from the solvent by GC.

4.4.2.5. *3-Methyl-2-butenal.*

Nitric oxide was bubbled through a solution of 3-methyl-2-butenal (0.5g, 6.9 $x10^{-3}$mol) in cyclohexane ($20cm^3$) after being degassed for 1 hour. No signal was observed when a sample was taken for EPR spectroscopy. When $2cm^3$ air was injected there was still no signal. Analysis by GC-MS showed only unreacted starting material.

4.4.2.6. *Cyclohexenone.*

A solution of cyclohexenone (0.5g, 5.2 $x10^{-3}$mol) in cyclohexane ($20cm^3$) was degassed and NO was bubbled through. Samples were taken at intervals of 30 minutes and analysed by EPR spectroscopy. No signals were observed, even after air was introduced into the system.

4.4.2.7. *1-Acetylcyclohexene.*

Nitric oxide was bubbled through a solution of 0.5g (4.03 $x10^{-3}$mol) of 1-acetyl-1-cyclohexene in $20cm^3$ cyclohexane. After bubbling, a colour change occurred (milky to pale yellow). No paramagnetic species were observed up to 4.5 hours, but after this time a triplet was seen with $\underline{a}(N)$= 14.5G. The nitrogen hfs suggested that this was due to a dialkylnitroxide. The gas chromatogram of the solution revealed a major product together with several other products having retention times greater than that of the starting material. The mass spectrum of the main product showed ions at m/z (%) 139 (1), 138 (23), 110 (30), 95 (20), 67 (95), 43 (100), 41 (32), 39 (60) which suggested it may have been the oxime derived from the reactant.

4.4.2.8. *Tetracyanoethene.*

A solution of 0.5g (3.9 $x10^{-3}$mol.) of TCNE in $20cm^3$ MeOH was degassed for 1 hour. Nitric oxide was then bubbled though for 24 hours. A sample was taken and

analysed by GC-MS. This indicated that the main product was a methoxide, resulting from the reaction between MeOH and TCNE. Analysis by GC-MS gave a fit of m/z (%), 133 (19), 107 (48), 92 (11), 76 (15), 54 (23), 38 (23), 15 (100).

4.4.2.9. *Cyclopropylacetyl peroxide.*

The peroxide (10mg,50 x10^{-6}mol) was dissolved in 10cm^3 cyclohexane. The system was degassed for 1 hour and then NO was bubbled through.. The reaction mixture was heated to 60°C to cleave the peroxide. A sample was taken after 0.5 hours, and when analysed by GC-MS revealed a small extent of reaction. Another sample was taken after 3 hours and analysed by GC-MS, and this showed a single product with principal ions of m/z 279, 280 and 391 (m/z (%) 279 (16), 167 (38),149 (100), 113 (22), 83 (18), 71 (36), 57 (47), 43 (35)). This high molecular weight material was not a primary product. Further experiments revealed the formation of 1,1-bicyclohexyl, possibly from radical attack on the solvent, and also products from the coupling of the cyclohexyl radicals with cyclopropylmethyl and/or butenyl radicals. 1-(Cyclopropylmethyl)cyclohexane m/z % 100 (8), 83 (12), 55 (100), 39 (13), 29 (24).

An alternative method of radical generation was investigated. This involved dissolving the peroxide in 210cm^3 benzene. The solution was degassed for 1 hour and then irradiated with UV light for 6 hours. Nitric oxide was bubbled through for the same length of time. The solution turned from colourless to pale yellow, and samples were taken for EPR analysis. No signals were observed. Preparative TLC (silanized silica plate) was used to separate the reaction mixture. The mixture was dissolved in neat ethyl acetate and the plate was developed with a mixture of cyclohexane and ethyl acetate (60:40). On completion, three bands were observed, including the initial layer. These were removed and dissolved in cC_6H_{12}/EtOAc solution, filtered through celite and analysed by ^1H NMR. Both product layers gave similar spectra which consisted of unresolved multiplets in the aliphatic region, δ_H 0.95 (nH,m).

4.4.2.10. *Glutathione.*

This reaction was first attempted in acetonitrile, but GSH is insoluble in this. Thus an aqueous solution was used. GSH (0.2g $6.5x10^{-4}$mol) was dissolved in DIW ($20cm^3$). The solution was degassed using N_2 for 15 minutes, then NO was bubbled through for 8 hours. The reaction was carried out at room temperature and one sample was taken after 24 hours. UV/vis analysis showed no reaction had taken place.

4.4.2.11. *Dibenzyl disulphide.*

To *tert*-butylbenzene ($20cm^3$) was added dibenzyl disulphide (0.5g, 0.002 mol). The solution was degassed for 15 minutes using N_2, and NO was bubbled through for 4 hours. The first sample was taken after this time, and the second sample 1 hour later. The reaction was carried out at room temperature. A second reaction, using identical quantities of the disulphide and cyclohexane was carried out at 70°C. In addition, lauroyl peroxide (0.2g, $5x10^{-5}$mol) was used as radical initiator. Nitrogen was used to degas the system, and NO was bubbled for 1.5 hours. One sample was taken 1.5 hours after addition of NO. Thirdly, dibenzyl disulphide (0.5g, 0.002 mol) was dissolved in cyclohexane ($20cm^3$), plus azoisobutyronitrile (AIBN) (0.1g, $6.1x10^{-4}$mol), another radical initiator. The reaction was carried out at 80°C and NO was bubbled through for 8 hours. One sample was taken after 24 hours. GC-MS analysis showed no products in the room temperature reaction, but at 70°C two minor components were observed. When AIBN was used, GC-MS showed one component that was likely to be starting material, and breakdown products of the initiator.

4.4.2.12. *L-Tryptophan.*

A 50mM aqueous solution of L-tryptophan in water was prepared and NO_2 was bubbled through. A 3-line nitroxide signal was observed when a sample was taken for EPR.

CHAPTER 5

The abstraction of hydrogen from and rearrangement of silylamines.

5.1. An EPR study of the abstraction of hydrogen from silylamines.

5.1.1. Introduction.

Molecular rearrangements have been extensively studied over recent times, although investigations of those involving silyl group migration are comparatively sparse. This is of particular importance because silyl groups are increasingly being used as synthons and as protecting groups, for example TBDMS. Thus, it is important for chemists to be aware of potential structural reorganisation of these compounds and what rearrangements are possible. Rearrangements can be initiated in a variety of ways. The major categories of rearrangement are 1,2, 1,3, 1,4, and 1,n shifts, where $n \geq 5$, and these can be subdivided into thermal, photochemical, anionic and cationic rearrangements. The majority of these reactions can be thought of as intramolecular nucleophilic substitutions at silicon, in which the nucleophile is an adjacent electronegative ion or atom. In certain cases, such as 1,2 anionic and 1,2 and 1,3 thermal shifts, there is retention of configuration of a chiral migrating group. This stereochemistry can be explained by the involvement of a 5-co-ordinate intermediate transition state[207].

Another type of rearrangement that involves migration of the silyl group is a free radical process. This area of work has also been extensively reviewed[208]. Several types of free radical rearrangement can occur, and these can be divided into group transfers, ring closures, ring openings, and isomerisations. The latter covers (i) the rotation of functional groups within the molecule to form, for example, *cis* and *trans* isomers of allylic radicals, and (ii) inversions which involve a change in configuration of the group attached to the radical centre, without any bond breaking or making. This is most often observed for organosilyl, organogermyl and carbon-centred radicals that possess electron withdrawing groups such as fluorine or alkoxy on the radical centre.

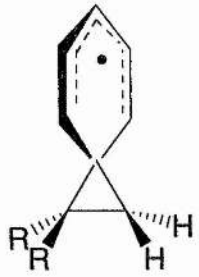
Early work on rearrangements was carried out by Urry and Kharasch, who discovered the neophyl rearrangement when studying the products of the cobalt chloride-catalysed reaction of neophyl chloride with phenylmagnesium bromide. The

neophyl radical (**40**) rearranges to the 2-benzylprop-2-yl radical (**41**) at higher temperatures. This has been observed by UV and EPR spectroscopy.

$$C_6H_5C(CH_3)_2\dot{C}H_2 \xrightarrow{O} C_6H_5CH_2\dot{C}(CH_3)_2$$

40 **41**

Experimental evidence has indicated that the rearrangement is intramolecular, so it was concluded that the reaction must proceed via an intermediate of structure (**42**), but no EPR evidence of this has been obtained.



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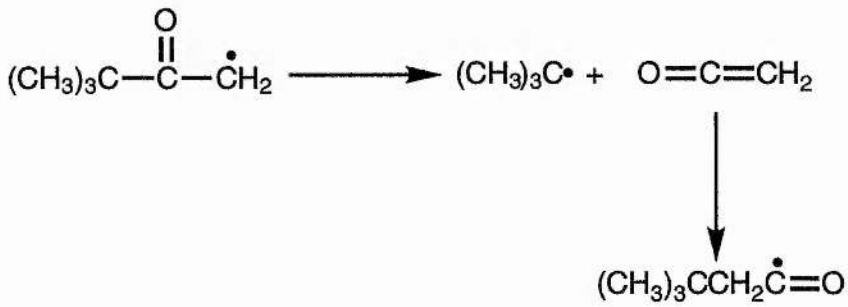
The rearrangement will be governed by the thermal stability of the unrearranged and rearranged species. Intramolecular group transfers are similar to bimolecular homolytic substitution reactions. This process occurs readily at carbon, provided that there is a low-lying unfilled orbital available to accept the unpaired electron. Consequently, aryl, vinyl, and other unsaturated groups migrate readily, but homolytic substitution at sp^3 hybridised orbitals is less likely. Thus, alkyl groups would tend not to migrate, but to undergo elimination and readdition, e.g.



To distinguish between these two types of process, the reaction must be carried out in the gas phase, or by means of trapping or crossover experiments. The notion that silicon can expand its valence shell from four to five may explain the fact that

several 1,2 migrations of the silyl group have been observed in the liquid phase. Much work has been carried out on the chemistry of group IVB radical reactions, and reviews have covered this work[209]. However, until recently, relatively few examples of rearrangements involving silicon and the other group IVB elements had been identified. It appeared that there were only selected groups that would migrate intramolecularly. In particular, the phenyl group did not seem to undergo a 1,2 shift either to or from silicon, but it was known to exhibit this behaviour with carbon radicals. It was found that no phenyltrimethylsilane was produced from benzyldimethylsilane, going through the intermediate benzyldimethylsilyl radical.

$$Ph_2CH_2Si(Me_2)H \longrightarrow Ph_2CH_2\dot{S}iMe_2 \not\longrightarrow \bullet CH_2Si(Me_2)Ph$$

In a similar study, the decarbonylation of triphenylsilylacetyl radical by methyl radical produced only triphenylsilylmethane, and none of the expected diphenylbenzylsilyl radical. When compared to the carbon analogue, phenyl migration here is the main route of rearrangement. This difference was thought to be due to a combination of factors, including lowered delocalisation in the phenylsilyl radicals; the Si-Ph bond being stronger than the Si-alkyl bond, or the lowered steric strain on the Si-Ph_3 system due to the large size of silicon.

Pyrolysis experiments by Sakurai *et al.*[210], on hexamethyldisilane revealed that there were two main products, trimethylsilane and trimethyl(dimethylsilylmethyl)silane. This showed that the trimethylsilyl group migrated from silicon to carbon. The following chain mechanism was proposed for the reaction:

$$Me_3SiSiMe_3 \longrightarrow 2Me_3Si\bullet$$

$$Me_3Si\bullet + Me_3SiSiMe_3 \longrightarrow Me_3SiSiMe_2CH_2\bullet + Me_3SiH$$

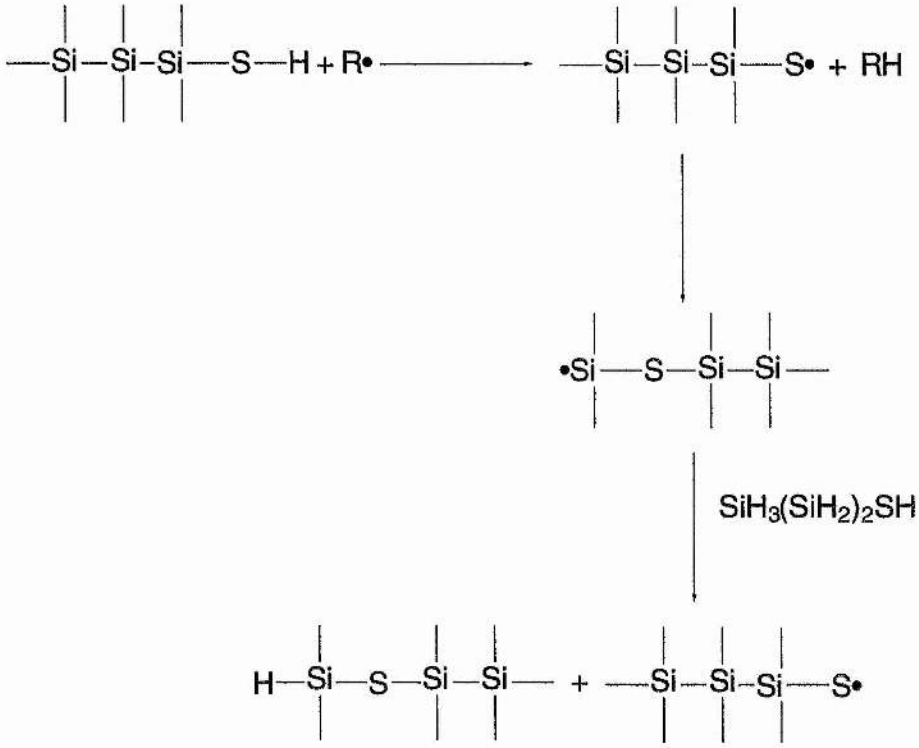
$$Me_3SiSiMe_2CH_2\bullet \longrightarrow \bullet SiMe_2CH_2SiMe_3$$

$$\bullet SiMe_3CH_2SiMe_3 + Me_3SiSiMe_3 \longrightarrow Me_3SiCH_2SiMe_2H + Me_3SiSiMe_2CH_2\bullet$$

The trimethylsilyl radical formed in this pyrolysis was also generated in the liquid phase by the decomposition of dibenzoyl peroxide in hexamethyldisilane. When the radical

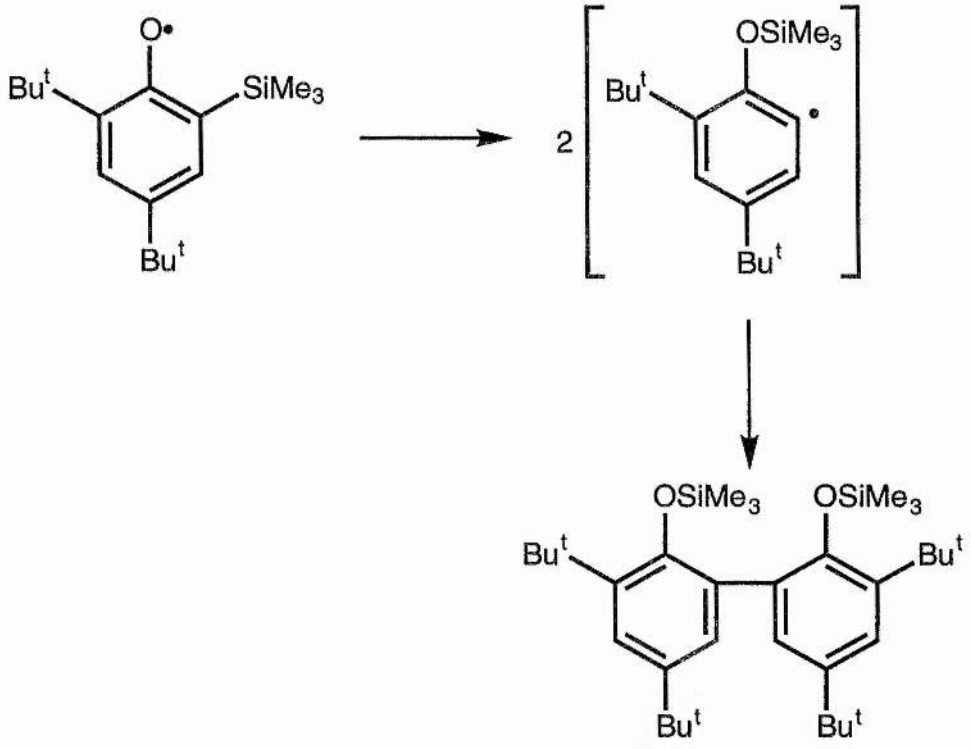
was generated in carbon tetrachloride, again in the presence of dibenzoyl peroxide, a chlorine atom was abstracted from the solvent, but there was no rearrangement of the resulting (pentamethyldisilanyl)methyl radical to the trimethyl[(dimethylsilyl)methyl]silane. This means that the rearrangement seen in the pyrolysis reaction must have a fairly high activation energy. It was also thought that the trimethylsilyl radical produced in the pyrolysis underwent dimerisation.

It was also reported[211] that certain trisilanethiols rearranged in the presence of AIBN. Heptamethyltrisilane-1-thiol underwent the following reaction on heating in cyclohexane:



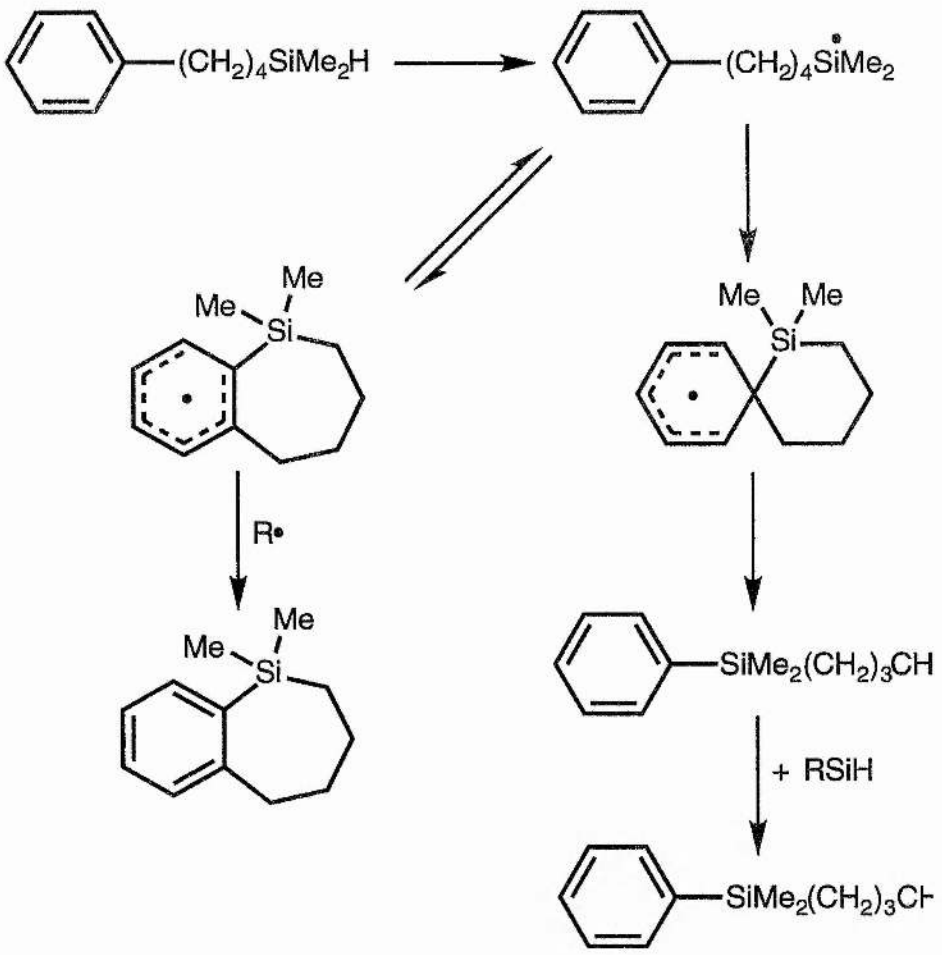
Trialkylsilyl group migrations from carbon to oxygen have been reported for the silylphenoxyl radical[212]. The oxidation of 2,4-di-t-butyl-6-trimethylsilylphenol in n-hexane or benzene gave the 2,4-di-t-butyl-6-trimethylsilylphenoxyl radical. This was analogous to the tri-t-butylphenoxyl radical (as described in ch.2). The silylphenoxyl radical was not isolated, but analysis of the conversion products was carried out, revealing the silyl migrated dimer, 2,2'-bis(trimethylsilyloxy)-3,5,3',5'-tetra-t-

butyldiphenyl. This compound was formed by migration of the trimethylsilyl group from the ring to aroxyl oxygen, followed by dimerisation:



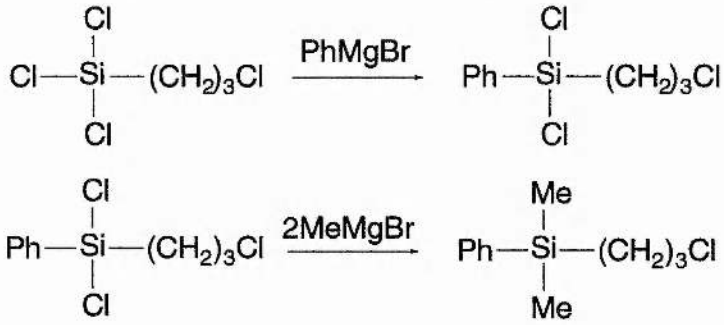
Two important group migrations were discovered almost simultaneously in the early 1970's. Sakurai and Hosomi[213] described the first 1,5 migration of phenyl from carbon to silicon, and 1,4 and 1,5 phenyl shifts from silicon to carbon were reported by Wilt and Dockus[214].

The migration from carbon to silicon entailed the thermal decomposition of (4-phenylbutyl)dimethylsilane in the presence of BOOB to give the rearranged product n-butyldimethylphenylsilane. Other compounds of the general formula $Ph(CH_2)_nSiMe_2H$ were also investigated, and it was found that only compounds where n=3 rearranged, and cyclisation only occurred for compounds of n=2 and n=3. This can be summarised:

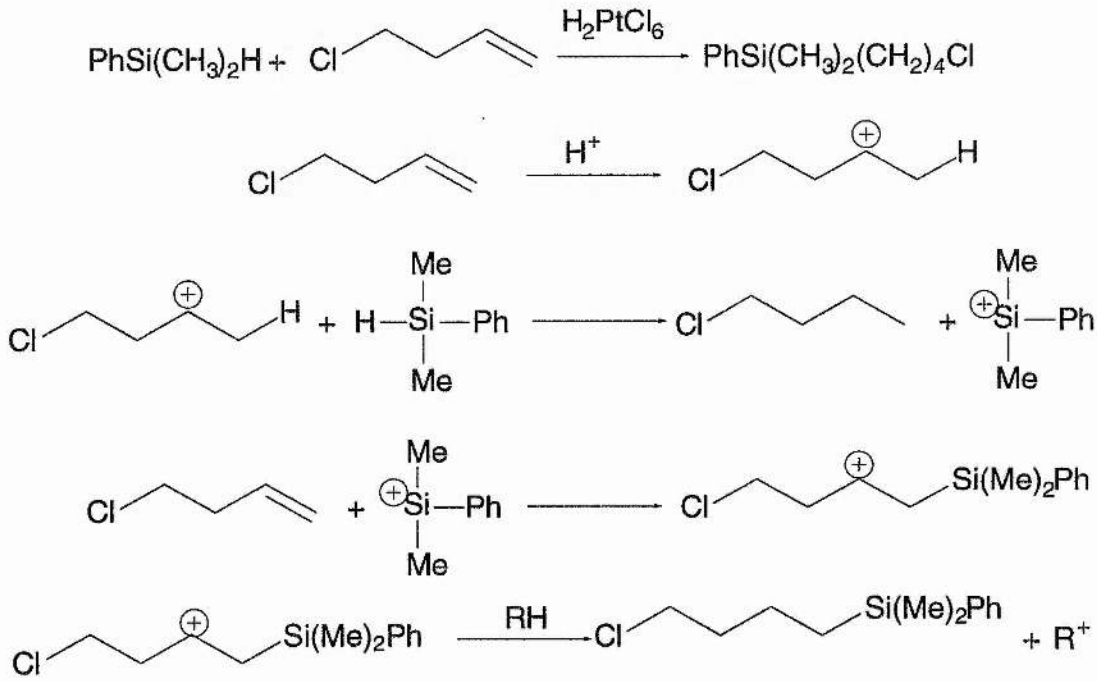


This scheme is acceptable, as n=0,1,2,4 and 5 would put too much steric strain on the ring system. For n=2, cyclisation may be preferred due to the collapse of the spiro radical to the cyclic intermediate, as the ring size favours the less strained five membered ring.

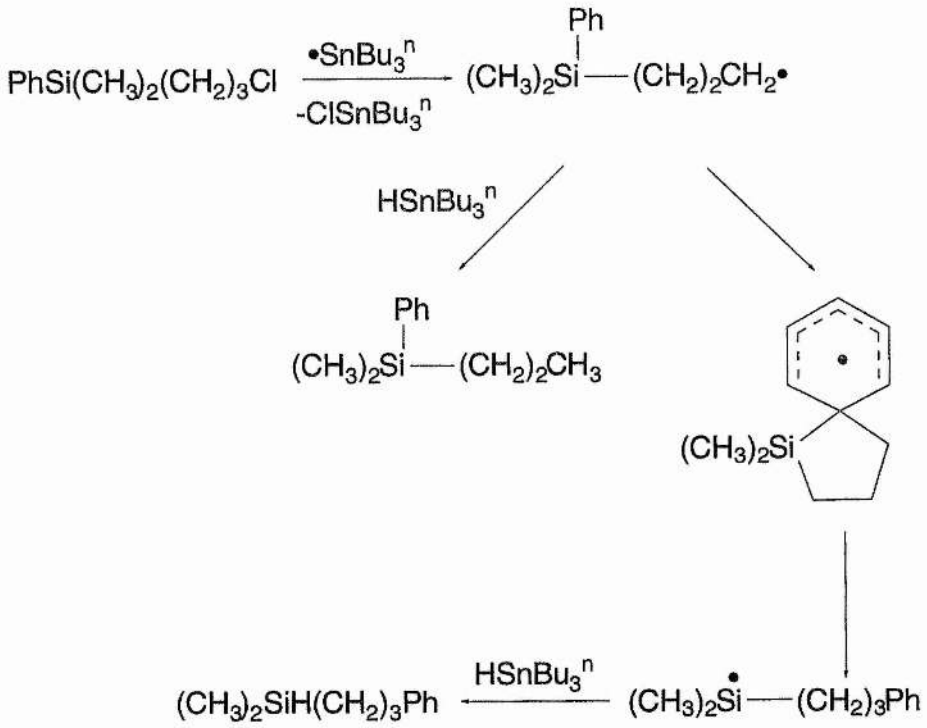
In the silicon to carbon migrations, 3-chloropropyltrichlorosilane was treated with one equivalent of a phenyl Grignard reagent, followed by two equivalents of a methyl Grignard reagent giving (phenyldimethylsilyl)propyl chloride:



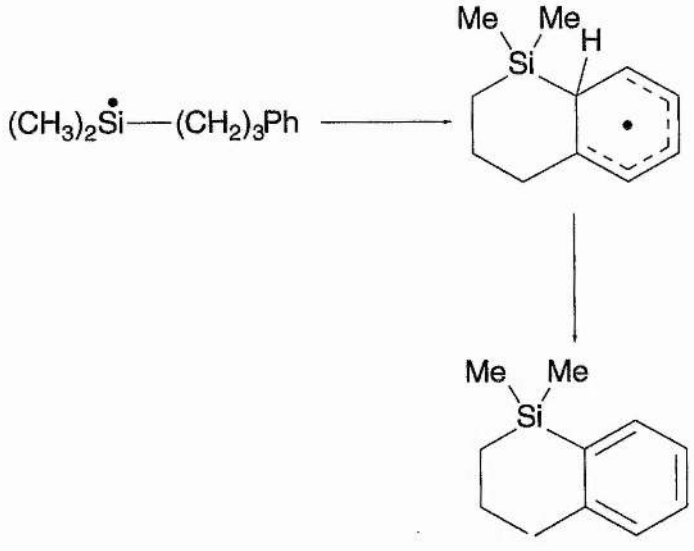
In a parallel reaction, addition of phenyldimethylsilane to 4-chloro-1-butene in the presence of chloroplatinic acid gave (phenyldimethylsilyl)butyl chloride:



The reduction of the chlorides with tri-n-butyltin hydride, (Bu^nSnH) produced the n-alkylsilanes, and also the rearranged silanes. From the n-propylchloride, phenyldimethyl-n-propylsilane and the rearranged phenylpropyldimethylsilane were isolated. No reaction occurred in the absence of Bu^nSnH. A general mechanism for this series of reactions is shown with (phenyldimethylsilyl)propyl chloride as an example:



An analogous mechanism applies to the butyl chloride. The reverse rearrangement of the radical did not occur, leading instead to the silatetralin when heated to 135°C in the presence of BOOB.

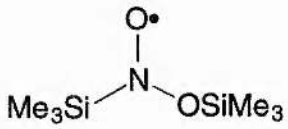


The ratio of the rearranged to cyclised products depended on the molar ratio of the starting material to initiator (BOOB). In both cases, an increase in [BOOB], i.e. a more dilute solution, increased the quantity of both types of product obtained. A general principle which applied to this system was that a good hydrogen donor, such as

a silane or Bu^nSnH, favoured the formation of the alkylsilane, whereas a hydrogen abstractor, e.g. t-BuO•, favoured the ring closure route.

Thus, it was shown that migration of phenyl from silicon to carbon and *vice versa* took place.

In 1971, West and Boudjouk[215] generated a novel class of radical, bis(organosilyl) nitroxides. These were of the general formula $(RMe_2Si)_2NO•$, where R= Me, Et, Ph, t-Bu, and were produced by electrolytic or oxygen oxidation of the bis(organosilyl)hydroxylamine anion or by hydrogen abstraction from the parent hydroxylamine. The nitroxides were analysed by EPR spectroscopy and had a(N) ~6.5G. Each was accompanied by a silyl splitting of a(Si) ~5.9G. When R=H, no Si-H coupling was observed (unlike dialkyl nitroxides). Addition of dry oxygen to a solution of $(Me_3Si)_2NO•$ caused disappearance of the narrow 6.5G nitrogen hfs to be replaced by a complex spectrum caused by the production of several radicals. The most stable of these replacement radicals had a triplet of equal intensity with a(N) ~15G. This was thought to be a result of insertion of oxygen into the side chain, which would cause an increase in the nitrogen hfs.



When solutions of the parent hydroxylamines $RMe_2SiNHOSiMe_2$ were photolysed in the presence of BOOB in the EPR cavity, the corresponding nitroxides, $(RMe_2Si)_2NO•$ were observed. The NH hydrogen was abstracted by t-BuO•, and the resulting hydroxylaminyl radical rearranged to the silyl nitroxide. The unrearranged nitroxide was not observed, even at low temperature.

Later work on the 1,2 migration of the silyl methyl group was carried out by Harris *et al.*[202]. As there were no other known 1,2 shifts of the silyl group apart from

those described above, (silicon to sulphur, oxygen to nitrogen), it was reasoned that as the Si-O and Si-N bond strengths were much greater than that of the Si-C bond, this would favour the migration of the trimethylsilyl group from carbon to nitrogen and from carbon to oxygen. Photochemically generated t-BuO• radicals were reacted with trimethylsilylmethylamine in the cavity of an EPR spectrometer. This gave rise to an aminoalkyl radical in the temperature range 150-230K.

$$Me_3SiCH_2NH_2 \xrightarrow{t\text{-}BuO\bullet} Me_3SiC\overset{\bullet}{H}NH_2$$

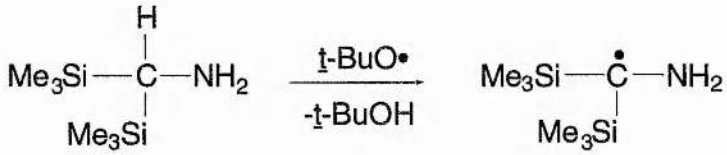
It was known that t-BuO• radicals abstract hydrogen from the nitrogen in addition to the carbon to give the aminyl radical, but this was not detected in solution:

$$Me_3SiCH_2NH_2 \xrightarrow{t\text{-}BuO\bullet} Me_3SiCH_2\overset{\bullet}{N}H$$

A further EPR spectrum was observed above 230K, and the EPR parameters indicated that it was the aminoalkyl radical generated from the 1,2 shift of the silyl group to give $\bullet CH_2NHSiMe_3$, the rearrangement product of the EPR silent $Me_3SiCH_2NH\bullet$. This was confirmed by isotope studies using deuterium. Product analysis was consistent with the initial formation of silylamines from the rearranged radicals.

When trimethylsilylmethanol was reacted with t-BuO• radical, the resulting EPR spectrum showed the presence of the $Me_3SiC^{\bullet}HOH$ radical, together with a second radical that had EPR parameters identical to that of the trimethylsilyloxymethyl radical, $\bullet CH_2OSiMe_3$. At first, it was thought that this was the radical $\bullet CH_2SiMe_3CH_2OH$ formed by abstraction of hydrogen from the trimethylsilyl group. Resolution of the γ hfs proved that it was not this radical. From the low temperature of the observations it was deduced that the trimethylsilyloxymethyl radical rearranges from the trimethylsilylalkyl radical very rapidly. Confirmation was obtained by generation of the trimethylsilylalkoxyl radical from the reaction of benzensulphenate with trimethyltin radicals. EPR spectroscopy showed the presence of the rearranged radical, and treatment of the product with Bu^nSnH gave the trimethylsilyl methyl ether, not trimethylsilylmethanol as would have occurred if rearrangement had not taken place.

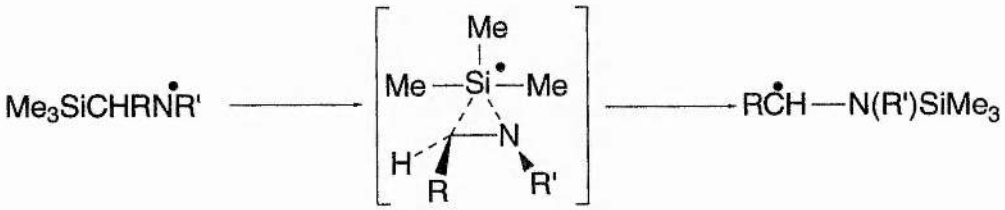
Following on from this work[216], a range of trimethylsilylamines was prepared and tested in addition to the original trimethylsilylmethylamine. These were N-methyltrimethylsilylamine, N-butyltrimethylsilylamine, bis(trimethylsilyl)methylamine, and deuterated versions of each. Photolysis in the EPR cavity in the presence of BOOB was carried out, and, as the temperature was raised, the spectra of the trimethylsilylaminyl radical became more complex. This was due to changes in the hydrogen hfs. Selective line broadening was also observed due to restricted rotation about the H_2N-C• bond. The spectrum of the N-methyl substituted amine was more complex and weaker than that of the original amine. The weakness and complexity precluded identification of the rearranged radical. The photolysis of the N-t-butyl substituted amine gave a well-resolved spectrum of a single radical up to ~300K. Above this, a second minor radical started to appear, corresponding to the rearranged radical $Me_3SiN(Bu^t)CH_2$•. It was considered that the bulky t-butyl group inhibited hydrogen abstraction from the amino group. The only detectable radical on hydrogen abstraction from the bistrimethylsilyl substituted amine was the carbon-centred species $(Me_3Si)_2C^{\bullet}NH_2$.



A minor radical was also observed which made spectral interpretation difficult. Analysis of the deuterated trimethylsilyl substituted species showed that hydrogen abstraction from the -CH group was much faster than from the amino group. For all the silylamines studied, the ratio of aminoalkyl radical:rearranged aminoalkyl radical was large. To account for this, hydrogen abstraction by t-BuO• from the CH group must have been faster than from the amino group, even when the amino hydrogen atoms were more sterically exposed. Several factors were thought to contribute to this. The t-BuO• radical is electronegative, and as the electronegativity difference between carbon and oxygen is greater than the difference between carbon and nitrogen, hydrogen abstraction from C-H would be enhanced by a polar effect. Also, the

trimethylsilyl group is known to be electron withdrawing in free radical reactions. Thus, the carbon-centred radical would contain both electron attracting and releasing groups, possibly aiding in stabilisation.

It was thought that the 1,2 silyl migration would occur via a transition state in which the silicon atom expands its valence shell to five.



Alternatively, it could take place by β-elimination of a trimethylsilyl radical and imine formation and readdition of the silyl group at the N-terminus:

$$Me_3SiCHR\dot{N}R' \longrightarrow Me_3Si\bullet + RCH{=}N(R') \longrightarrow \bullet CHRN(R')SiMe_3$$

If this was the case, then it could feasibly be expected that some $Me_3Si\bullet$ would leak from the solvent cage prior to readdition. In an attempt to confirm either mechanism, the affinity of $Me_3Si\bullet$ for halogens was exploited. The expected reaction,

$$Me_3Si\bullet + Me_3CCl \longrightarrow Me_3SiCl + Me_3C\bullet$$

did not occur. Also, no $Me_3Si\bullet$ spin adducts were detected by EPR spectroscopy when 1,3,5-trinitrobutane (TNB) was added. Thus, it appeared that the intramolecular route was most favourable. In an attempt to quantify the results, it was assumed that the temperature at which the rearranged radicals reached half their final concentration was at that temperature when their spectra became distinguishable. From this, and in conjunction with assumptions from previous experiments carried out by other workers, rate parameters that indicated silyl migration was sensitive to steric effects were calculated. It was found that the bistrimethylsilyl substituted rearranged radical had a rate constant of almost twice that of the monosubstituted silylamine. The rate constant for the N-t-butyl substituted amine was much smaller.

More recent work[217] was carried out in an attempt to confirm the above results and also to observe the unrearranged radicals by EPR spectroscopy. The trialkylsilylmethylaminyl radicals were generated by several methods. The first was by

trialkylsilyl radical addition to N-methylene-t-butylamine (MTBA). The trialkylsilyl radicals were obtained by hydrogen abstraction from the trialkylsilane using t-BuO• radicals. These gave radicals of the general structure $R_3SiCH_2N^{\bullet}Bu^t$, where R=Me, Et and i-Pr. When the radicals were examined by EPR spectroscopy, it was found that for a given temperature, a($2H_\beta$) increased in the order $Me_3Si<Et_3Si<$i-Pr. This was due to steric effects which increase the repulsion between the t-Bu group and the trialkylsilyl group.

The next method whereby trialkylsilylaminyl radicals were produced was by displacement of same from trivalent phosphorus. Alkoxyl radicals will displace dialkylaminyl radicals from dialklyaminophosphanes[218], according to the equation:

$$(RO)_2PNR'_2 \xrightarrow{R''O\bullet} R''O(RO)_2\overset{\bullet}{P}NR'_2 \longrightarrow R'_2N\bullet + (RO)_2POR''$$

This is the major pathway of production, but interference with EPR spectra can arise from generation of the R"• radical via β scission and readdition. Consequently, it was necessary to select a source of alkoxyl radicals which, if there were formation of the alkyl radical R"•, would give unobtrusive or at least identifiable spectra. Two aminophosphanes, N-t-butyl and N-Me were prepared and photolysed in the presence of a selection of peroxides, all of which afforded good EPR spectra of the desired trimethylsilylaminyl radicals. The spectra exhibited identical parameters to those obtained from the addition to MTBA.

To confirm the 1,2 silyl group migration from carbon to nitrogen, authentic $\bullet CH_2N(Bu^t)SiMe_3$ was prepared by hydrogen abstraction from the parent amine by t-BuO• radicals. The spectrum obtained from the photolysis of the aminylphosphane revealed the presence of both rearranged and unrearranged radicals at 350K. Similar observations were made for the N-Me substituted radical. When the N-Me aminophosphane was irradiated, the EPR spectrum recorded showed the presence of the aminyl radical and the carbon-centred radical, proving that the rearrangement did actually take place.

In agreement with Harris *et al.*, no $Me_3Si\bullet$ adducts were detected by EPR spectroscopy when the aminophosphanes were photolysed in the presence of dicumyl

peroxide and 1,1-di-t-butylethene, a known trimethylsilyl radical scavenger. Thus it was concluded that the 1,2 silyl group migration occurs via an intramolecular pathway, not through elimination and readdition.

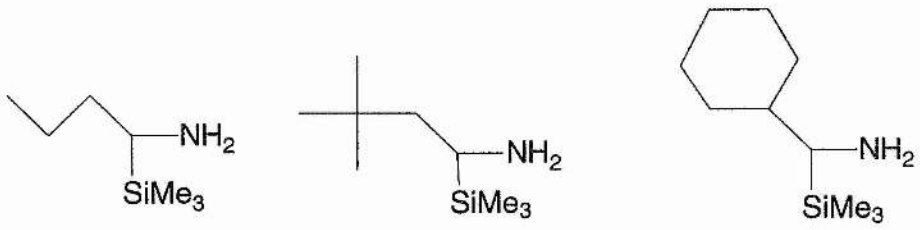
The final piece of experimental data came from hydrogen abstraction from the N-alkyltrimethylsilylmethyl amines (Me_3SiCH_2NHR). Photolysis of Me_3SiCH_2NHMe in cyclopropane in the presence of BOOB yielded a mixture of carbon- and nitrogen-centred radicals, indicating that hydrogen abstraction takes place at both sites. Similar results were obtained for the N-*t*-butyl substituted amine which gave a mixture of $Me_3SiC^{\bullet}HNHBu^t$ and $Me_3SiCH_2N^{\bullet}Bu^t$. One major difference between the work of Harris *et al.* and Roberts and Vasquez-Persaud, was that Roberts found that to satisfactorily simulate the spectrum of $Me_3SiC^{\bullet}HNHBu^t$, an additional splitting for 9H was required, indicating that both Me_3Si and the t-butyl groups can couple with the single electron.

In general, it can be seen that silyl group migration has been proved to occur through an intramolecular rearrangement. It can undergo shifts from carbon to nitrogen, carbon to oxygen, silicon to sulphur, oxygen to nitrogen, silicon to carbon, and carbon to silicon. It is an important and understudied process, and there is great potential for its application in the field of synthetic chemistry.

5.2. Results and discussion.

5.2.1. Hydrogen abstraction from trimethylsilylamines.

Previous work on hydrogen abstraction from, and silyl group migration in silylamines appeared to be influenced by steric effects. To investigate further, a hydrogen abstraction study was undertaken on three trimethylsilylamines with primary and secondary alkyl side chains. These were investigated by low temperature EPR spectroscopy and were photolysed in the presence of di-t-butyl peroxide (BOOB).



43 **44** **45**

Table 5.1 EPR parameters of radicals derived from silylamines.

Radical	*T*/K	hfs/G				Ref
$Me_3SiCH^{\bullet}NH_2$	220	16.0 ($1H_\alpha$)	6.30 (1N)	0.72 ($1NH_2$)	0.36 ($1NH_2$)	0.36 (9H)	217
$Me_3SiCH^{\bullet}ND_2$	260	15.7 ($1H_\alpha$)	6.20 (1N)			0.35 (9H)	"
$Me_3SiCH^{\bullet}NHMe$	240	16.0 ($1H_\alpha$)	6.30 (1N)			0.30 (9H)	"
$Me_3SiCH^{\bullet}NHBu^t$	225	15.0 ($1H_\alpha$)	7.20 (1N)	2.50 (1H)		0.34 (9H)	"
$(Me_3Si)_2C^{\bullet}NH_2$	220		6.30 (1N)	2.80 (1H)	0.28 ($1NH_2$)	0.30 (18H)	"
$(Me_3Si)_2C^{\bullet}ND_2$	280		6.40 (1N)	0.30 (1D)	0.30 (1D)	0.30 (18H)	"
$Me_3SiNHCH_2^{\bullet}$	260	16.3 (2H)	2.40 (1N)	2.40 (1H)			"
$Me_3SiNDCH_2^{\bullet}$	260	16.0 (2H)	2.40 (1N)	0.40 (1D)			"
$Me_3SiN(Bu^t)CH_2^{\bullet}$	300	14.8 (2H)	3.70 (1N)				"
$Me_3SiCH^{\bullet}NHSiMe_3$	280	14.4 (1H)	4.50 (1N)	1.80 (1H)	0.30 (9H)		"
$Me_3SiCH^{\bullet}NDSiMe_3$	280	14.4 (1H)	4.50 (1N)	0.30 (1D)	0.30 (9H)		"
$Pr^nC^{\bullet}(SiMe_3)NH_2$	240		9.20 (1N)	1.50 (1H)	3.20 (2H)	0.45 (9H)	a
$Bu^tCH_2C^{\bullet}(SiMe_3)NH_2$	160		8.70 (1N)	0.40 (mult)			a
$Bu^tCH_2CH^{\bullet}NH(SiMe_3)$	260	15.0 (2H)	1.70 (1N)	16.7 (1H)	0.88 (9H)		a
$Bu^tCH_2CH^{\bullet}NDSiMe_3$	260	15.1 (2H)	1.70 (1N)	16.7 (1H)			a

[a] this work

5.2.1.1. n-*Propylsilylamine.*

The EPR spectra recorded for **43** were very complex. A simulation of the entire spectrum using the values from table 5.1 is shown in figure 5.1. The complicated pattern arose from the rotation of the alkyl side chain which increases as the temperature is raised. The rearranged radical structure was not distinguished. The radical studied is shown below. The arrow indicates the rotation of the side chain.

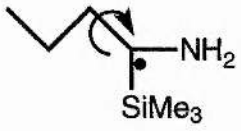
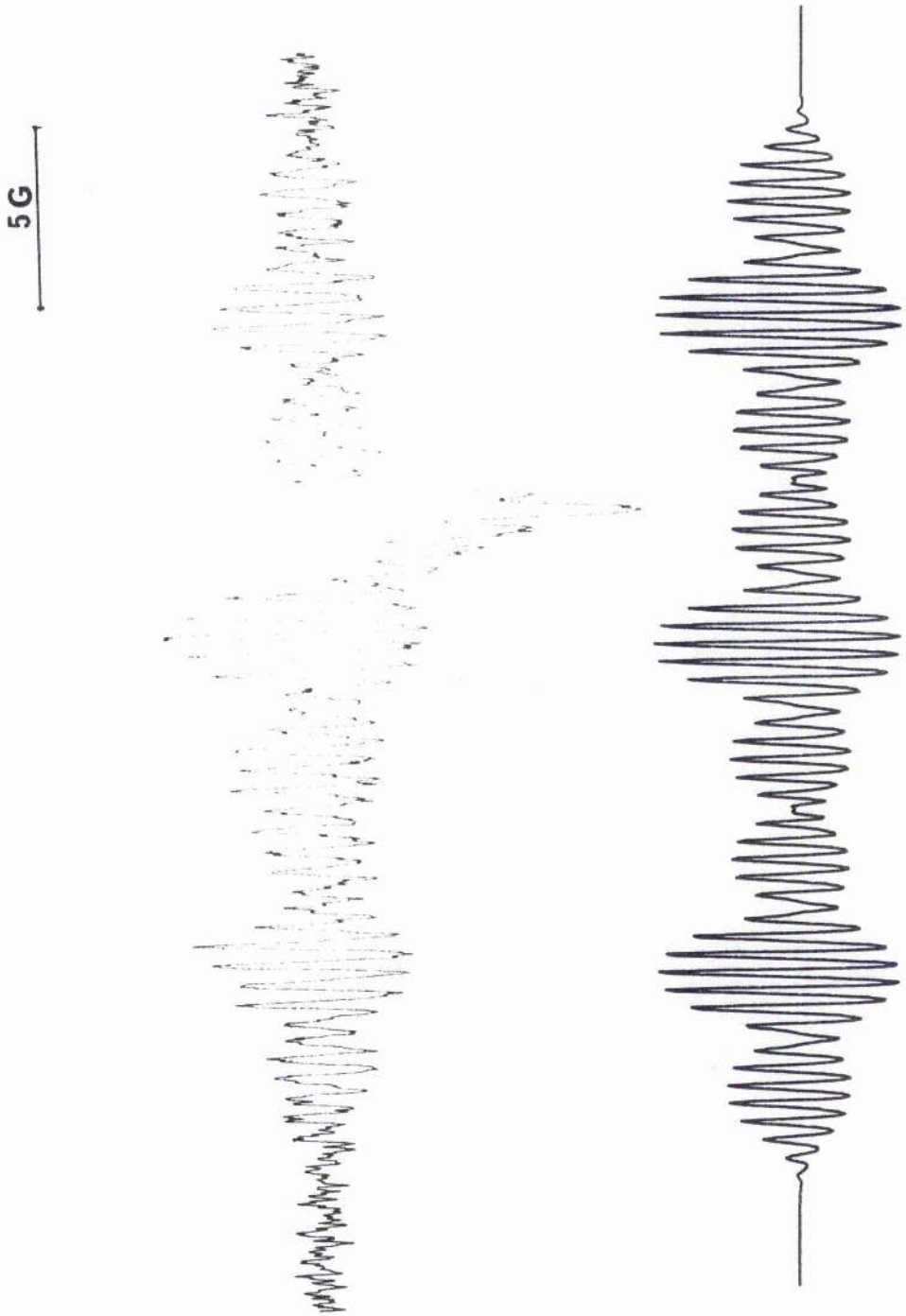


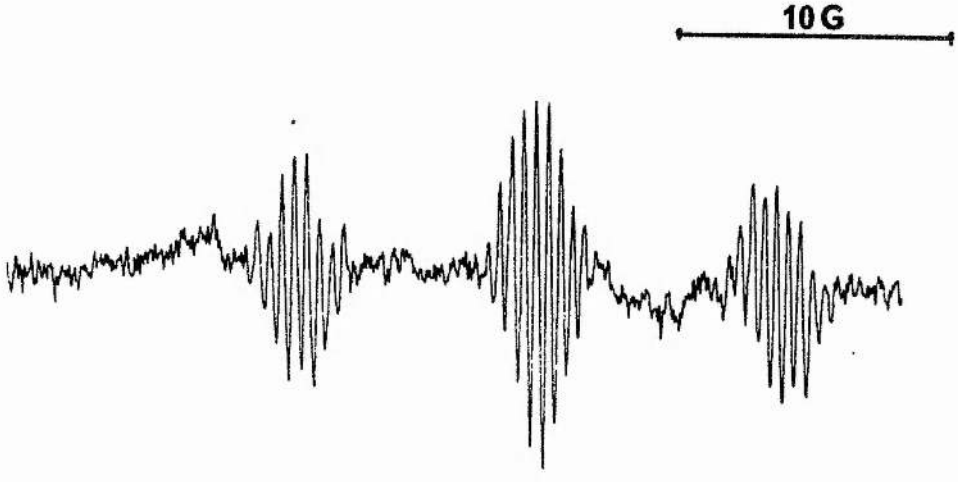
Figure 5.1. Top; 2nd derivative EPR spectrum obtained from the phoyloysis of **43** at 240K in cyclopropane. Bottom; computer simulation using parameters from text.



5.2.1.2. *Neopentylsilylamine.*

The neopentyl-substituted silylamine (**44**) was dissolved in 500µl of BOOB and photolysed at 240K and this gave a weak spectrum of a possible 1:3:3:1 quartet. Each component was split into a complex and asymmetrical multiplet. Alternatively, it could have been a double triplet with a(2H)=15.6G and a(1H)~18G; each component being a complex multiplet. In addition, there was a second spectrum with four broad lines with a possible spacing of 14.5G. This would indicate that there were two radicals formed, and an attempt was made to separate these species. To facilitate this, the experiment was carried out at a lower temperature. The amine was dissolved in cyclopropane and sealed under vacuum. The temperature was lowered to 160K and at this temperature, two radicals were observed, one with 4 multiplets. This could be a quartet of quartets with a(3H)=14.4G, a(3H)=1.6G. The second spectrum possessed 3 multiplets, but they were only partly resolved. The three multiplets were unequal in intensity at 160K, but as the temperature was raised they became more equal (figure 5.2).

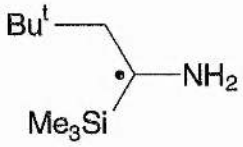
Figure 5.2 EPR spectrum of multiplets observed on photolysis of **44** at 160K.



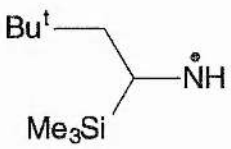
T/K	a(N)/G	a(mult)/G
160	8.7	~0.4
230	"	"

From this it can be deduced that this radical will be the carbon centred aminoalkyl radical **44a** rather than the nitrogen-centred alkylaminyl radical **44b**. When compared

to the data in the table, the nitrogen hfs are quite similar. The nitrogen-centred radical was not observed directly in this system, possibly because at this low temperature there was an insufficient concentration of **44b** to be detected.

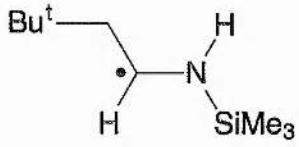


44a



44b

When the spectrum was recorded with a sweep width of 20G, the multiplet was seen to consist of greater than 13 lines of 0.48G spacing (figure 5.3). As the temperature was increased to 260K, a new spectrum appeared comprising 4 major multiplets. This was analysed as: a(2H)=15.0G, a(1H)=16.7G, a(N)=1.7G, a(1H)=0.8G (figure 5.4). These EPR parameters correspond to a new rearranged radical, (**44c**), as shown below, which is formed by 1,2-migration of the trimethylsilyl radical from carbon to nitrogen. For this to occur, there must be a certain amount of the nitrogen-centred radical **44b** present, but this was not observed by EPR spectroscopy. A possible explanation for this is that the actual rearrangement of **44b** to **44c** is very rapid.



44c

As a further confirmation of the rearrangement, the amine was mixed with D_2O to exchange the NH_2 hydrogen atoms.

At 165K, only the spectrum of three multiplets was observed [a(N)=8.8G, a(mult.)=0.4G], i.e. similar to the aminoalkyl radical observed with the protio-amine. This radical was still visible when the temperature was increased to 220K, but at 230K a rearranged radical began to appear. A scale expansion (s.w. = 20G) showed a 13-line multiplet of a(nH)=0.45G. This was very similar to the undeuterated sample. At

Figure 5.3 Top; EPR spectrum of central multiplet of fig.5.2 (**44a**) recorded at 160K. Bottom; computer simulation.

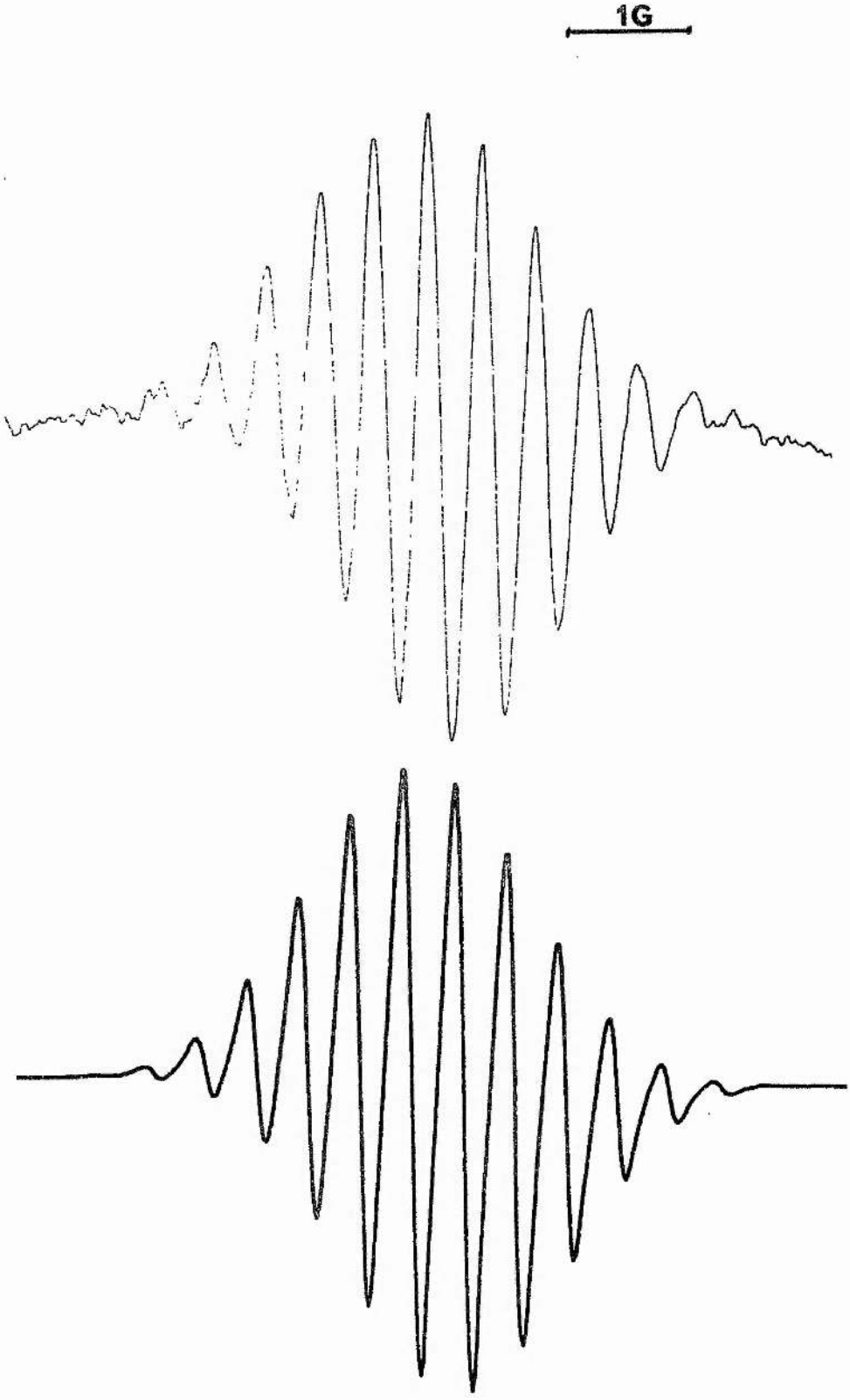
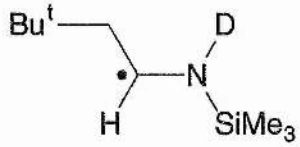


Figure 5.4 EPR spectrum of the rearranged radical **44c**, observed at 260K.

260K an EPR spectrum was observed with hfs of a(2H)=15.1G, a(N)=1.6G, and a(1H)=16.7G (figure 5.5). This radical was attributed the following structure (**44d**):



44d

The hyperfine splitting from the β-deuterium atom was too small to be resolved and this confirmed the nature of the rearrangement. On returning to 180K from 260K, this radical disappeared and the three line one reappeared.

In previous work[216], estimates of the rate of silyl migration were made. This method depended on the linear correlation between the midpoint of rearrangement, $T(=)$, (where [**44b**] = [**44c**]), and the Arrhenius activation energies[217]. The results are summarised in table 5.2. From a correlation of $T(=)$ vs. E_a for a range of rearrangements, activation energies may be deduced. In the case of **44b** to **44c** $T(=)$ was estimated to be ca. 240K from the EPR spectra, and hence E_a ~ 10.7 kcal mol^{-1}. Assuming log$k$ =11.5 -E / 2.3RT, then at 300K, log k =3.68, therefore k = 5x10^3 s^{-1}.

Table 5.2. Estimated kinetic parameters for 1,2 migration of the $Me_3Si^\bullet$ group from carbon to nitrogen in aminyl radicals.

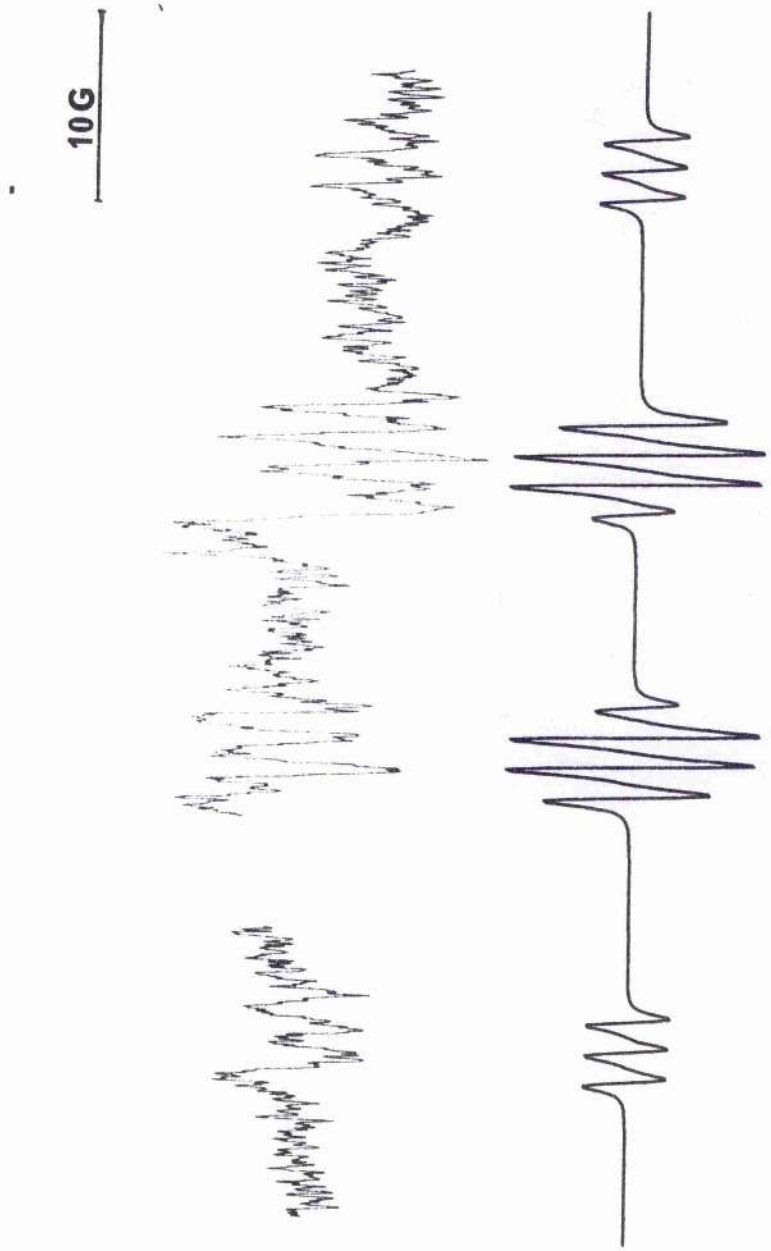
Migration in:	$T(=)$/K	E/kcal mol^{-1}	log[A/s^{-1}]	k/s^{-1} (300K)	Ref
$Me_3SiCH_2NH^\bullet$	245	11.0	11.5	3 x 10^3	217
$Me_3SiCH_2N^\bullet Bu^t$	310	13.8	11.5	3 x 10^1	"
$(Me_3Si)_2CHNH^\bullet$	235	10.5	11.5	3 x 10^3	"
$Bu^tCH_2CH(SiMe_3)NH^\bullet$	240	10.7	11.5	5 x 10^3	a

[a] This work.

5.2.1.3. *Cyclohexylsilylamine.*

This amine was investigated using similar conditions to the above, and spectra were observed confirming the formation of a radical. Upon photolysis of the amine at 160K, two radical spectra were observed, however the signals were very weak and this made accurate determination of the splittings difficult. One spectrum comprising three components of equal intensity was observed (figure 5.6), with a(N) =8.8G, and each

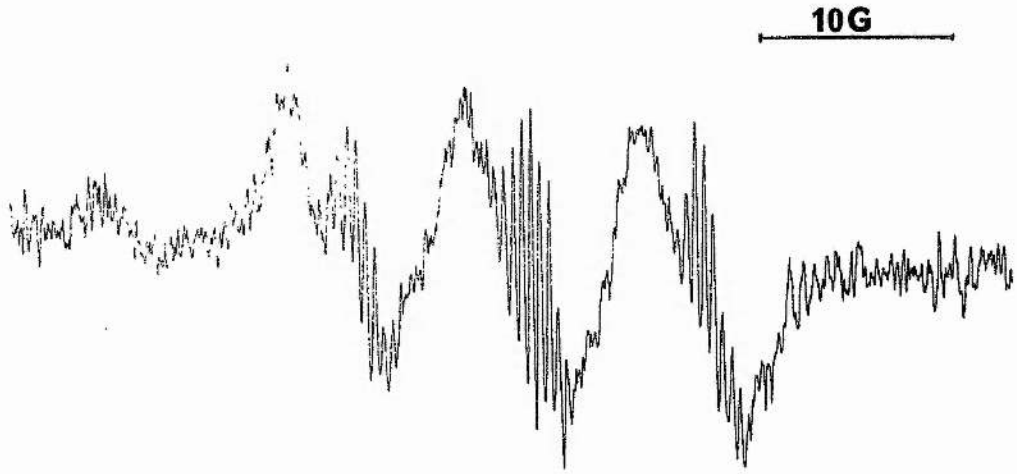
Figure 5.5 Top; EPR spectrum of radical **44d** observed at 260K after D_2O exchange. Bottom; computer simulation of same.



component was split into >11 lines, with $\underline{a}$ ≈0.4G. The radical observed was probably of the following structure:

The second spectrum had broad unresolved lines, with $\underline{a}$(N) =9.1G. Spectra were too weak and complex for the 1,2-silyl migration to be distinguished.

Figure 5.6 EPR spectrum of cyclohexylsilylamine after photolysis at 160K, showing broad- and narrow-lined spectra



5.3. Conclusions.

It can be seen from these experiments that hydrogen abstraction takes place from each of the silylamines. In the case of the neopentyl substituted amine, the EPR spectra showed the presence of two radicals, one given by direct hydrogen abstraction, and the other from the 1,2 shift of the trimethylsilyl group to the nitrogen. Photolysis of the cyclohexyl subsituted amine gave weak spectra that were poorly resolved. In addition, the rearranged radical was not observed, and this could be due to the bulk of

the side chain. Complex EPR spectra were also recorded for the n-propyl subsituted amine. As above, the rearranged radical structure was not distinguished.

5.4. Experimental.

All EPR spectra were recorded on a Bruker ER200D spectrometer with a Bruker B-ST100/700 temperature control unit. The samples of amine were synthesised by J-P. Picard and coworkers at the Laboratoire de Chimie Organique et Organometallique, Université Bordeaux. The samples were refrigerated until ready for use and the structures were checked by 200MHz ^{1}H NMR spectroscopy, and TLC was used to determine the purity. None of the amines were purified further. Samples were dissolved in cyclopropane in Suprasil quartz tubes, degassed on a high vacuum line using several freeze-pump-thaw cycles, and sealed under vacuum using an oxygen/natural gas torch.

5.4.1. Sample preparation.

5.4.1.1. *n-Propylsilylamine.*

A solution comprising 30µl of amine, 20µl of BOOB and 500µl of cyclopropane was prepared and photolysed at 160K. This gave rise to a strong but complex signal.

5.4.1.2. *Neopentylsilylamine.*

A solution of 30µl of t-butylsilylamine in 500µl BOOB was placed in a quartz EPR tube and degassed by bubbling N_2 through the solution for 10 minutes. The solution was then photolysed in the cavity of the EPR spectrometer. A weak spectrum was obtained showing the presence of two radicals. To separate the signals, photolysis was carried out at 160K, using 30µl of the amine, 20µl of BOOB and 500µl of cyclopropane as solvent. The tube was degassed as described above and sealed. Two radicals were detected at this temperature. On raising the temperature, a new spectrum appeared, corresponding to the rearranged radical. By way of confirmation of the structure of this radical, the amine was prepared as above, but with 30µl of D_2O

added to exchange with the amine hydrogens. On photolysis at 260K, the rearranged radical was observed.

5.4.1.3. *Cyclohexylsilylamine.*

Similar conditions to the above were used, i.e. 30µl of cyclohexylsilylamine, 20µl of BOOB and 500µl of cyclopropane. The solution was photolysed at 160K and a weak signal was observed.

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