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**Mechanistic Studies of Nitrosation Reactions of N-O- and
S-nitroso Compounds.**

by

Shirlene M. N. Y. F. Oh. B.Sc. (Dunelm)

St. John's College

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A Thesis submitted for the degree of Doctor of Philosophy

of the University of Durham

September 1990



To mum, dad
and
Sharon

"I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

L.T. More
(Sir Isaac Newton)
1934

M E M O R A N D U M

The work for this thesis has been carried out in the Department of Chemistry at the University of Durham between October 1987 and July 1990. It is the original work of the author unless otherwise stated. None of this work has been submitted for any other degree.

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Finally, I would like to thank Durham University for a Research Studentship, and the Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom for an Overseas Research Students Award, to fund this research.

ABSTRACT.

A kinetic study of the N-nitrosation of 2,3-diaminonaphthalene in acid solution at 25°C was undertaken. Reaction occurred via both the protonated and unprotonated forms of 2,3-diaminonaphthalene to yield 2,3-naphthotriazole. The reaction is acid-catalysed and is also catalysed by added nucleophiles. The mechanism is consistent with the rate-limiting formation of the diazonium ion (N-nitrosation followed by rapid proton transfer and loss of a water molecule), which undergoes a rapid cyclisation process, to yield the product.

Reactions of nitrosamines involving the N-N heterolytic cleavage with direct transfer of the nitroso group was also investigated. In particular, the denitrosation of N-nitrosoproline was studied. The reaction was carried out in the presence of an excess of nitrite trap to ensure irreversibility, in the presence of added nucleophiles, in high acidic conditions. All kinetic results are consistent with the mechanism involving a rapid reversible N-protonation followed by a rate-limiting attack by the nucleophile (added Br⁻, SCN⁻ or thiourea) giving the nitrosyl derivative, which is rapidly destroyed with the nitrite traps used in these experiments. However, at high nucleophile concentration and for reaction in ethanol, the reaction becomes independent of added nucleophile. This is because the rate-limiting step is now the proton transfer to the nitrosamine rather than the attack of the nucleophile. N-nitrosopyrrolidine, a heterocycle which does not contain a β-electron withdrawing group reacted less readily in the denitrosation reaction. N-nitrososarcosine, an acyclic compound containing a β-electron withdrawing group showed similar behaviour to NNP, suggesting that it is the β-electron withdrawing group, rather than the cyclic structure in NNP which is the dominant factor in the loss of the nitroso group.

The reaction of N-nitroso compounds containing an electron-withdrawing group was studied. The high reactivity of N-methyl-N-nitrosotoluene-p-sulphonamide (MNTS) with nucleophiles, with the loss of the nitroso group has been explained in terms of either a rapid bimolecular reaction between the nucleophile and the more susceptible nitrosamine, due to the β-electron withdrawing substituent, or the weakening of the N-N bond by -SO₂, resulting in extensive bond breaking. MNTS was reacted with powerful nucleophiles, L-cysteine, L-cysteine methyl and ethyl esters and N-acetyl-L-cysteine, in water at 25°C in the pH range 6-13. The pH dependence of the rate constant is consistent with a mechanism involving a direct nitrosation by MNTS with the thiolate anion of the thiol. A quantitative kinetic analysis yielded microscopic pK_a values for RSH ionisation in good agreement with literature.

Nitrosation by O- and S-nitroso compounds were also studied. The substrates used in this case include phenols, ascorbic acid and haems. Reaction of iso-amyl nitrite (IAN) (an alkyl nitrite) with phenols and ascorbic acid occurs via a rapid reversible acid-catalysed hydrolysis of iso-amyl nitrite giving nitrous acid, which then in its protonated form effects nitrosation. Reaction of S-nitroso-N-acetylpenicillamine (SNAP) with phenols and ascorbic acid, however, showed different characteristics: a possible radical mechanism is suggested on the basis of e.s.r. experiments. Preliminary investigations of the reactions of both IAN and SNAP with haems showed a complex reaction scheme: the reaction is not a simple single step reaction.

The properties of the N-, O- and S-nitroso compounds used in this study are also discussed.

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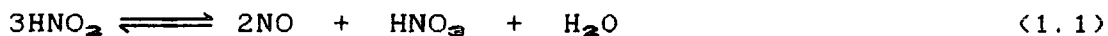
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CHAPTER 1
Introduction

1.1 Nitrosating agents

1.1.1 Acidic solutions of nitrous acid

The most common reagent used in nitrosation and diazotisation reactions is nitrous acid, a weak, unstable acid which is readily formed from nitrite salts and aqueous mineral acid. Decomposition occurs readily (equation (1.1)) in the presence of acid and must therefore be taken into account in quantitative work. Fresh solutions are always prepared and used immediately. Its pK_a has been determined by many workers to be ~ 3 , the most reliable being the values determined by Lumme and Tummavuori¹, who examined the variation with ionic strength, and quote a thermodynamic value of 3.148 at zero ionic strength at 25°C. Thus ionisation to the nitrite ion can be ignored for reactions carried out at $pH < \sim 2$.

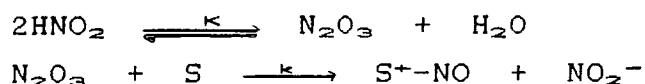


At reasonably high nitrous acid concentrations ($\sim 0.1\text{M}$) and at moderate acidities ($\sim 4\text{M}$), dinitrogen trioxide is formed in substantial quantities. The equilibrium in aqueous solution is shown in equation (1.2) and the equilibrium constant has been measured².



Dinitrogen trioxide is capable of effecting nitrosation in solution. The most studied reactions involving dinitrogen trioxide are the nitrosation, deamination and diazotisation³ of amines.

Early investigations gave apparently conflicting results. Hantzsch and Schumann⁴ studied the diazotisation of several amines in dilute acid using equal concentrations of nitrous acid and amine where a second-order reaction was observed. Taylor⁵ studied the nitrosation of a variety of primary aliphatic amines and dimethylamine and observed a third-order reaction. The mechanistic explanation for these results was given by Hammett⁶, and is shown in scheme (1.1).



Scheme (1.1)

S = Substrate

This mechanism has been confirmed by later work by Hughes, Ingold and Ridd⁷. The rate equation derived from the above mechanism is then given in equation (1.3) and predicts a second-order dependence on the nitrous acid concentration and a first-order dependence on substrate concentration.

$$\text{Rate} = k [\text{N}_2\text{O}_3] [\text{S}] = k K [\text{HNO}_2]^2 [\text{S}] \quad (1.3)$$

If the substrate is very reactive towards dinitrogen trioxide and the reaction occurs more rapidly than the hydrolysis of dinitrogen trioxide to nitrous acid, the rate-determining step would be the formation of dinitrogen trioxide (equation (1.4)).



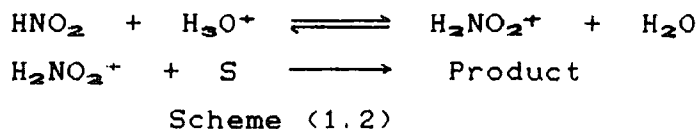
This limiting condition has been achieved with several substrates such as aniline⁷ and ascorbic acid⁸; the value of k' varies with the different conditions used but is approximately $9 \text{ l mol}^{-1} \text{ s}^{-1}$ at 25°C .

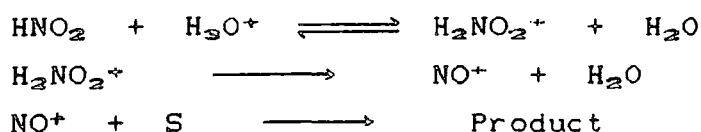
With less reactive substrates, the value of k can be determined if the equilibrium constant, K , is known (see equation (1.3)). K has recently been determined as $3.0 \times 10^{-3} \text{ l mol}^{-1}$ which is in agreement with the value calculated from thermodynamic cycles⁹. Using this value of K , k was determined for various amines³ to be $\sim 10^8 - 10^9 \text{ l mol}^{-1} \text{ s}^{-1}$ implying that reaction of amines with dinitrogen trioxide occurs at a rate close to the encounter controlled limit. The powerful nucleophiles, azide ion and 3-chlorophenylhydrazine as well as the reactive indole and indolizine derivatives also react upon encounter. N_2O_3 then is a more reactive species than previously thought¹⁰, because the equilibrium constant for its formation had been incorrectly determined as 0.2 l mol^{-1} as opposed to 0.003 l mol^{-1} .

At higher acidities and lower nitrous acid concentrations, dinitrogen trioxide is no longer the effective nitrosating agent. A different rate equation (see equation (1.5)) is observed, where the reaction is first-order with respect to nitrous acid, substrate and free hydrogen ion concentrations.

$$\text{Rate} = k [\text{HNO}_2] [\text{S}] [\text{H}_3\text{O}^+] \quad \text{S} = \text{substrate} \quad (1.5)$$

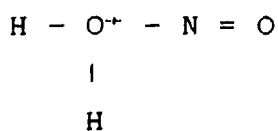
The mechanistic interpretation has been a subject of controversy as there are two possible mechanisms consistent with the rate equation (equation (1.5)). The first involves rate-limiting attack by the nitrous acidium ion shown in scheme (1.2), proposed by Hughes, Ingold and Ridd⁷, and the second involves the nitrosonium ion (see scheme (1.3)). They are kinetically indistinguishable.



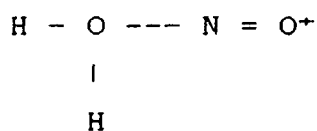


Scheme (1.3)

It has been shown¹² that the nitrosonium ion is the reactive species at very high acidities (~ 60% perchloric acid), the equilibrium constant for its formation has been variously determined¹¹ in the range 3.0×10^{-5} to 7.9×10^{-9} l mol⁻¹. There is, in contrast, no spectrophotometric or spectroscopic evidence for the presence of nitrous acidium ion, although it does nevertheless represent a reasonable possibility as the effective reagent in dilute aqueous acid solution. The other approach¹³ considers the two limiting forms (1.1) and (1.2) as the electrophile, the acidity of the medium determining the contribution of each form.



(1.1)



(1.2)

There is evidence for and against the proposed mechanisms above and this has been discussed in more detail¹⁴.

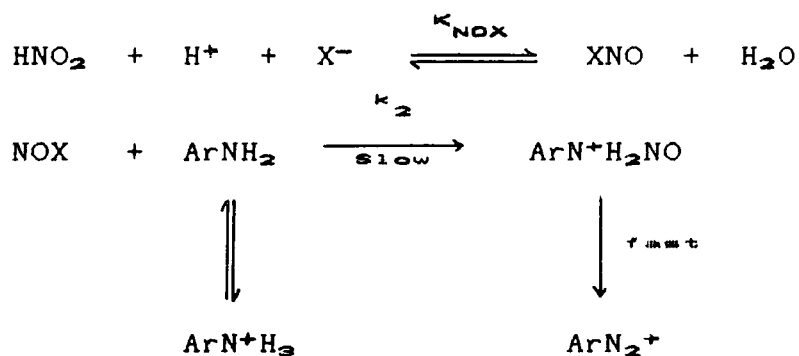
1.1.2 Nitrosyl halides

Nitrosyl halides have commonly been used as electrophilic nitrosating agents either in a range of organic solvents such as ether, acetic acid, chloroform, etc.¹⁵ where nitrous acid cannot be used, or in aqueous neutral and alkaline solutions¹⁶.

Nitrosyl chloride is the most commonly used nitrosating agent: the reaction with alkenes has been used synthetically particularly in terpene chemistry. Other substrates include carbonyl compounds, primary amines and alcohols. Schmid first observed the catalytic effects of halide ions in the diazotisation of amines by hydrochloric and hydrobromic acids^{17,18}, establishing the rate equation (1.6) which has also been established for other substrates¹⁹.

$$\text{Rate} = k [\text{ArNH}_2] [\text{HNO}_2] [\text{H}^+] [\text{X}^-] \quad (1.6)$$

This was mechanistically interpreted by Hammett⁶ in terms of rate-limiting attack on the unprotonated amine by the nitrosyl halide (see scheme (1.4)), formed in a pre-equilibrium step¹⁹⁻²⁶.



Scheme (1.4)

From scheme (1.4)

$$K_a = \frac{[\text{ArNH}_2] [\text{H}^+]}{[\text{ArN}^+\text{H}_3]} \quad (1.7)$$

$$K_{\text{XNO}} = \frac{[\text{XNO}]}{[\text{HNO}_2] [\text{H}^+] [\text{X}^-]} \quad (1.8)$$

Where K_a is the acid dissociation constant of ArN^+H_3 we can assume that $[\text{ArN}^+\text{H}_3] \sim$ total stoichiometric concentration of the amine and $[\text{XNO}] \ll$ total stoichiometric concentration of nitrous acid.

If less basic or non-basic reactants, such as alcohols or thiols, are used then appropriate modification is required. The derived rate equation (1.9) is shown below (c.f. the experimental equation (1.6)).

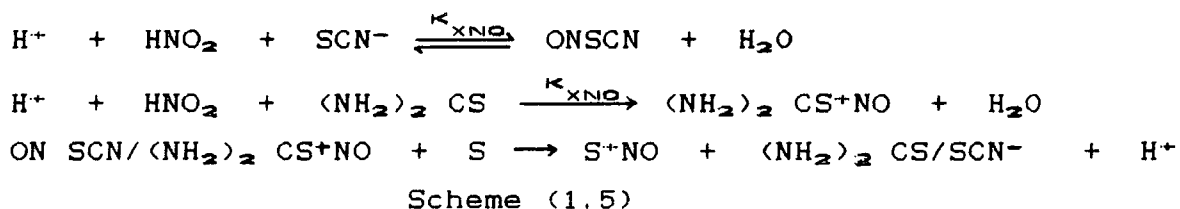
$$\begin{aligned} \text{Rate} &= k[\text{XNO}] [\text{ArNH}_2] \\ &= k_2 K_{\text{XNO}} K_a [\text{X}^-] [\text{HNO}_2] \tau [\text{ArNH}_2]_{\tau} \\ &\qquad\qquad\qquad \tau = \text{total (1.9)} \end{aligned}$$

If nitrosyl halide attack is rate-limiting, k_2 can be determined if K_{XNO} and K_a are known. K_{XNO} for nitrosyl chloride and nitrosyl bromide have been determined spectrophotometrically by Schmid and co-workers²⁷, with that for $\text{NOCl} < \text{NOBr}$, as expected from the nucleophilicity of the halogens. Nitrosyl chloride is more reactive than nitrosyl bromide as expected for an electrophilic nitrosation on the grounds of the electronegativity difference between chlorine and bromine. Bromide ion catalysis however, is always greater than chloride ion catalysis because the difference in the K_{XNO} value outweighs the difference in the k_2 values. For the more reactive species, k_2 approaches the encounter-controlled limit, and for the very reactive substrates under certain experimental conditions, the rate-limiting stage can be changed to that of the formation of the nitrosyl halide^{19, 28}. The rate equation for such reactions has been established (equation (1.10)) and the third-order rate constant can be readily obtained. The values determined for k are very similar and are close to those determined for a range of other anions²⁹, suggesting that the reactions occur at the encounter limit.

$$\text{Rate} = k [\text{H}^+] [\text{HNO}_2] [\text{X}^-] \qquad (1.10)$$

1.1.3 Nitrosyl thiocyanate and nitrosothiouronium ions

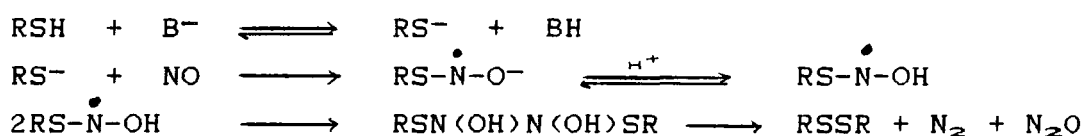
Nitrosyl thiocyanate has not been isolated, but exists as an unstable species in solution at low temperature. The S-nitroso species derived from nitrous acid and thiourea, the S-nitrosothiouronium ion, forms rapidly (equation (1.11)) with an equilibrium constant of $5000 \text{ l}^2 \text{ mol}^{-2}$ at 25°C ³⁰. Both nitrosyl thiocyanate^{19, 31-36} and S-nitrosothiouronium^{31, 34} have been identified kinetically as the effective nitrosating reagent generated *in situ* for reactions using nitrous acid and either the thiocyanate ion or thiourea. The mechanism can be compared to that for halides: the nitrosating agent is first formed, then rate-limiting attack of the substrate takes place (scheme (1.5)).



In general, catalysis by thiocyanate and thiourea is much more marked than catalysis by halide ions. The equilibrium constant for the formation of nitrosyl thiocyanate³⁸ and S-nitrosothiouronium ion³⁰ have been determined as $32 \text{ l}^2 \text{ mol}^{-2}$ at 20°C and $5000 \text{ l}^2 \text{ mol}^{-2}$ at 25°C respectively. Kinetic analysis reveals that the large catalytic effect of thiourea is due to its large equilibrium constant for its formation; the reactivity of S-nitrosothiouronium ion is actually lower than that of nitrosyl chloride, nitrosyl bromide and nitrosyl thiocyanate. Also, molecular orbital calculations based on the frontier orbital approach⁴⁰ predict that nitrosyl thiocyanate should be less reactive than nitrosyl chloride, which has been confirmed experimentally. Once again the powerful catalytic effect of thiocyanate ion is due to the large K_{ONSCN} value.

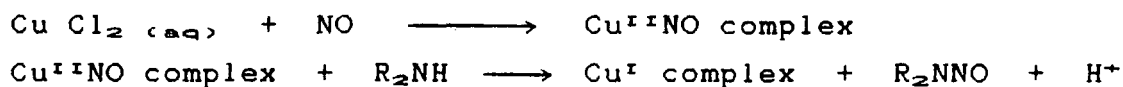
1.1.4 Nitric oxide

It is believed that the many literature reports of nitrosations brought about by nitric oxide in fact occur via dinitrogen trioxide or dinitrogen tetroxide, formed after aerial oxidation of nitric oxide. When oxygen is rigorously removed from the system, for example, in the nitrosation of amines in acetonitrile solvent, an extremely slow reaction takes place, probably due to the slow diffusion of air into the system^{41,42}. It has however been found that NO does react to give a nitroso product in its reaction with thiols in basic, oxygen-free solutions. The mechanism proposed involving free radicals⁴³ is shown in scheme (1.6).



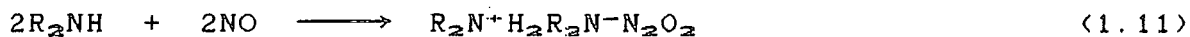
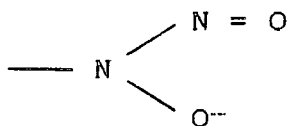
Scheme (1.6)

NO is an effective nitrosating agent in the presence of metals and other catalysts. Brackman and Smit⁴⁴ showed that the nitrosation of diethylamine by NO and copper (II) salts occurred via the intermediate copper-nitrosyl complex (see scheme (1.7)).

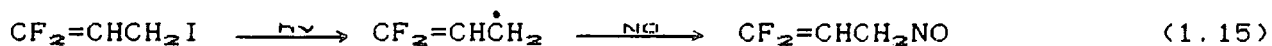
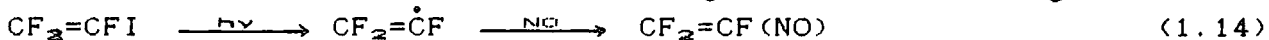
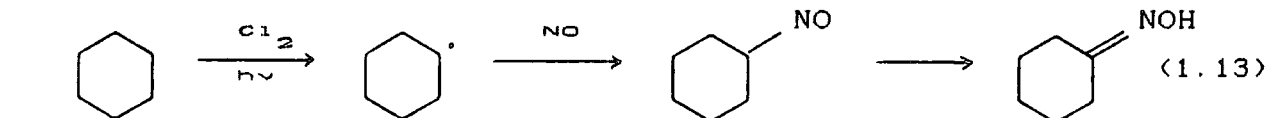
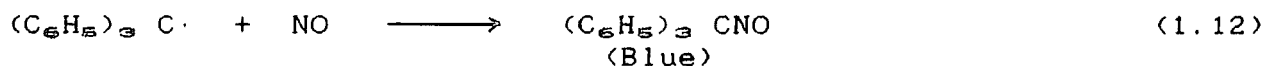


Scheme (1.7)

Nitric oxide forms stable complexes with amines⁴⁵, the 'Drago complexes' (see equation (1.11)) although no structure analysis is available. The anion is thought to contain the group:

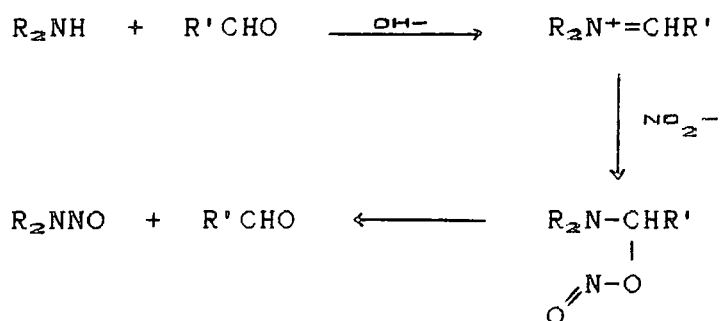


As nitric oxide is a stable free radical, nitroso compounds can also be formed by its reaction with other radicals. Examples include the triphenyl methyl radical⁴⁶ (see equation (1.12)), the cyclohexyl radical⁴⁷, a photochemical reaction with chlorine, (1.13), and reactions with alkenes (see equation (1.14) and (1.15)).



1.1.5 Nitrite Ion

The nitrite ion is not an effective nitrosating agent on its own. In the presence of a number of carbonyl compounds, however, nitrosation of secondary amines occurs, in basic and neutral solutions. Keefer and Roller⁴⁸ suggested a mechanism involving the formation of an iminium ion intermediate, which reacts with nitrite ion to form the dialkylamino nitrite ester which breaks down rapidly to give the dialkyl-nitrosamine (see scheme (1.8)).



Scheme (1.8)

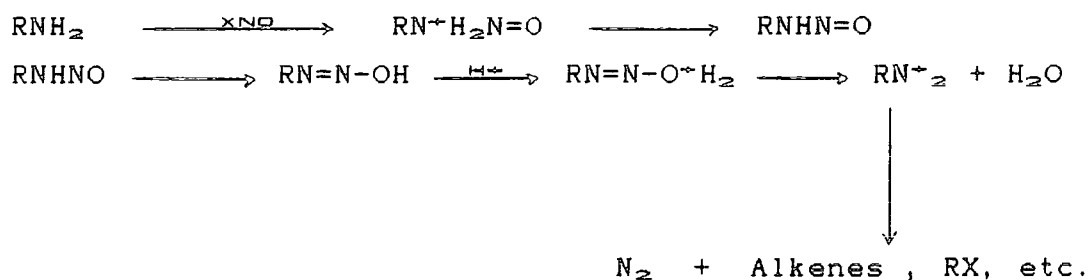
The same mechanism has been proposed for the nitrosation of amines by nitrite ion in halogenated solvents⁴⁹.

1.2 N-nitrosation

The nitrosation of amino compounds is by far the most widely studied nitrosation reaction. Since the discovery of deamination by Piria in 1846⁵⁰ (in the conversion of aspartic into malic acid), N-nitrosation reactions have attracted attention in connection with azo dyes, the generation of diazoalkanes and as a diagnostic test for amines. More recently, many N-nitroso compounds have been found to be carcinogenic, hence the growth in interest in their chemical and biological properties.

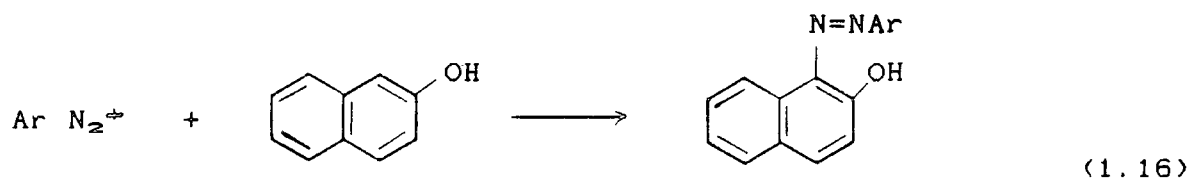
There has been much work done in the study of the mechanism of N-nitrosation and many reactive intermediates have been identified kinetically. The large range of mechanistic features which have been determined include acid catalysis, base catalysis, nucleophilic catalysis, reversibility, encounter-controlled reactions, intra-molecular rearrangements, C-C bond cleavage reactions and *ipso*-attack.

Nitrosation of aliphatic and aromatic primary amines using a range of nitrosating agents leads to deamination via an unstable diazonium ion intermediate, which reacts with nucleophiles to give substitution, elimination and rearrangement products such as alcohols, alkenes, alkyl and aryl halides and phenols (see scheme (1.9)).



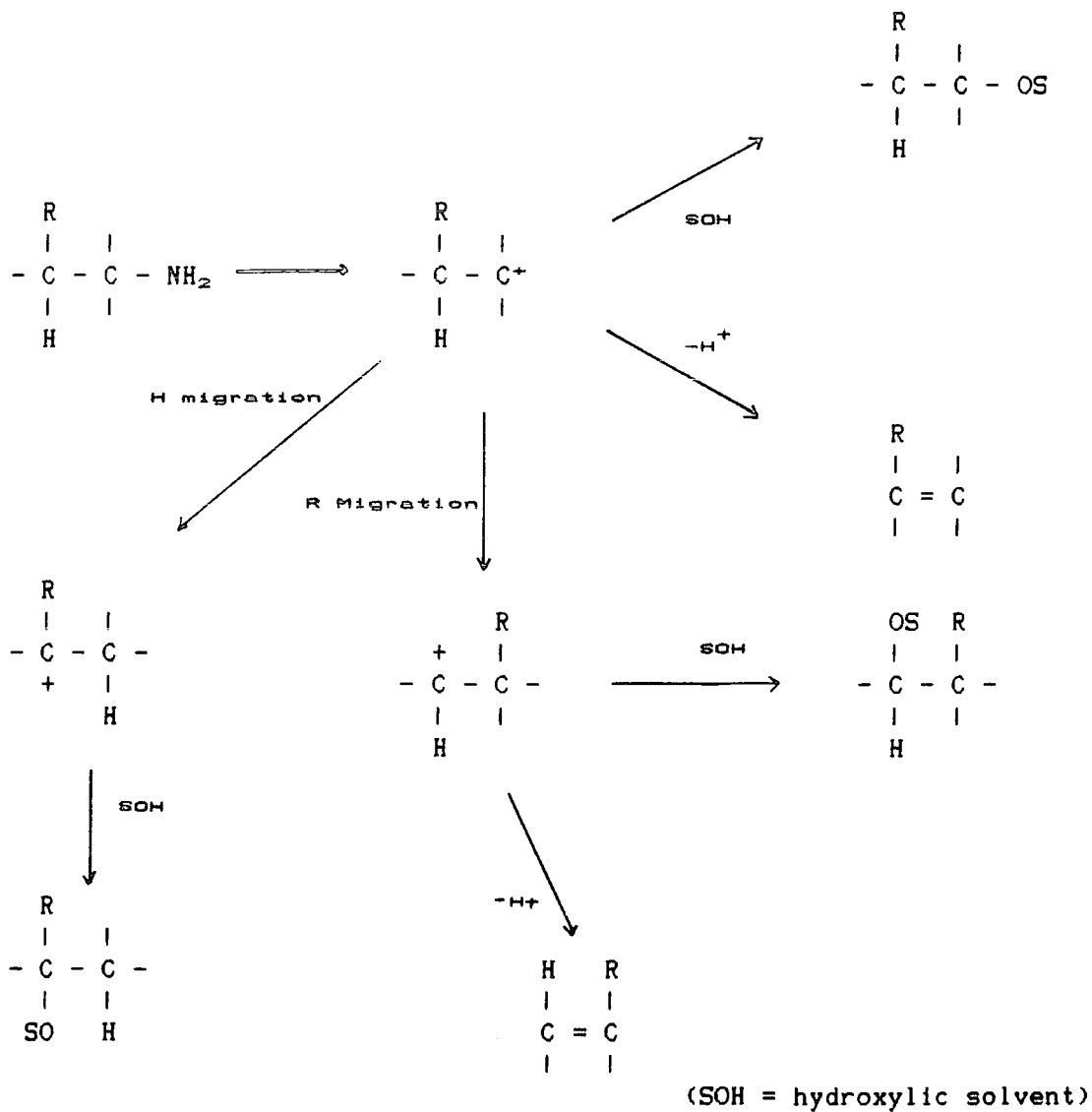
Scheme (1.9)

Aryl diazonium salts are much more stable than aliphatic ones as the positive charge formally on nitrogen can be delocalised into the aromatic systems. Thus aryl diazonium salts such as the tetrafluoroborate and hexafluorophosphate can be isolated. The electrophilic aromatic substitution by the diazonium ion (see equation (1.16)) is one of the important reactions in dye production on an industrial scale.



Aliphatic diazonium salts are very unstable towards loss of nitrogen, forming the carbocation. A range of products usually forms, in the nitrosation of primary aliphatic amines. These include alkenes, alcohols as well as those in which skeletal rearrangement has occurred (see scheme (1.10)). The use of aprotic solvents results in simpler product composition and is therefore more commonly used in synthesis.

Heterocyclic diazonium salts can also be stabilised by delocalisation of the positive charge into the heterocyclic system.

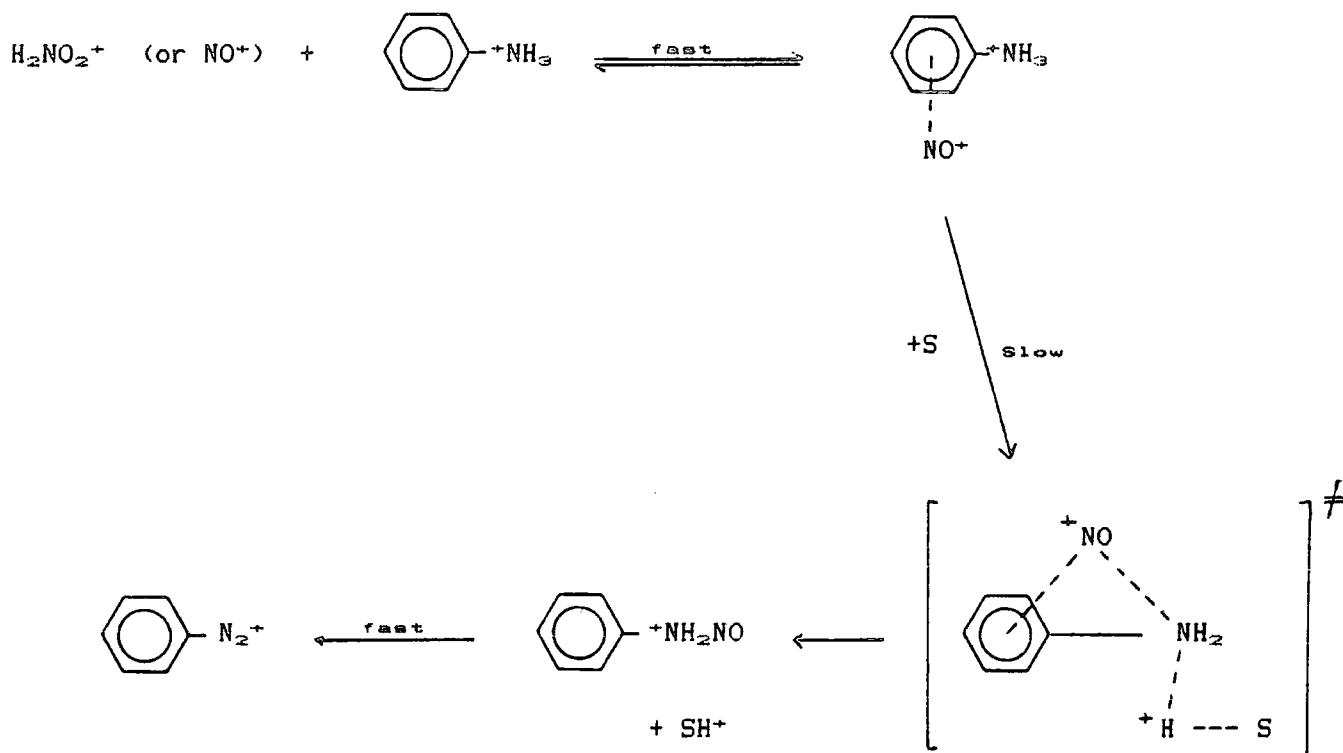


Scheme (1.10)

Kinetic studies of primary amine nitrosation have been useful in identifying specific nitrosating agents involved over the different experimental conditions used; as well as giving structure-reactivity correlations. A consistent mechanistic framework was established by Hughes, Ingold and Ridd in a series of papers⁵³ in 1958, and is summarised by Ridd in a review⁵⁴.

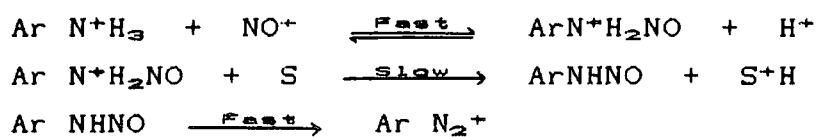
The reaction mechanism is dependent on the acidity of the medium. At low acid concentrations ($\leq 0.1M$) there are two possible pathways: one involves dinitrogen trioxide as the reactive species; and the other, the nitrous acidium ion (or the free nitrosonium ion which is kinetically indistinguishable, see Section (1.1.1)). At low acidity and high $[HNO_2]$ the pathway via dinitrogen trioxide is favoured. The bimolecular rate constant for the attack of dinitrogen trioxide can be determined as the equilibrium constant for the formation of dinitrogen trioxide is known², whereas that for the attack of nitrous acidium ion cannot be determined as the equilibrium constant for nitrous acidium ion has not been directly determined. For the more reactive amines at low acid concentrations and high amine concentrations, the rate of formation of dinitrogen trioxide becomes rate-limiting.

At higher acidities (0.1-6.5M perchloric acid) another mechanism occurs for aromatic, but not aliphatic amines. Acid catalysis occurs and the reactive species is now the protonated form of the amine and not the free base form. A suggested mechanism involves an initial rapid reversible formation of a complex (possibly a π complex), followed by a rate-limiting rearrangement of the nitroso group to the amino nitrogen atom, which occurs simultaneously with proton transfer to the solvent S (see scheme 1.11)).



Scheme (1.11)

At very high acidities (> 60% perchloric acid and 60% sulphuric acid), nitrous acid is virtually quantitatively converted to the NO⁺ ion. The mechanism involves the rapid and reversible formation of the protonated primary nitrosamine which undergoes a rate-limiting proton transfer (with a primary kinetic isotope effect) to the solvent (S) which then rapidly forms the diazonium ion (see scheme (1.12)).



Scheme (1.12)

Catalysis occurs in the presence of nucleophiles with catalytic efficiency: chloride ion < bromide ion ~ dimethyl sulphide < thiosulphate ion < thiocyanate ion < thiourea < iodide ion.

In all cases catalysis occurs due to the formation of a nitrosyl species XNO, which acts as the nitrosating agent usually in the rate-limiting process outlined in scheme (1.4). For very reactive aromatic amines at low acidity, the rate-limiting step then changes to the formation of XNO (see section 1.1.2 for further discussion).

The nitrosation of aliphatic and aromatic secondary amines is essentially the first reaction step in the nitrosation of primary amines. As the proton transfer cannot now occur, the secondary nitrosamines cannot be transformed into diazonium salts, and are therefore fairly stable.

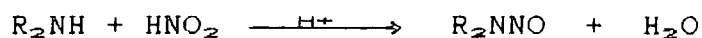
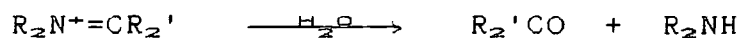
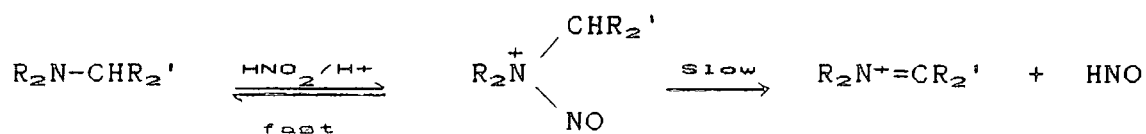
Nitrosation of secondary amines is important as it occurs in food, especially after fermentation or cooking⁵⁵. Many secondary amines are used as drugs and pesticides; nitrosamines can therefore be readily formed *in vivo* from these secondary amines and sources of nitrous acid, in particular, nitrite ion used in meat preservation and nitrate ion in water supplies. The nitrosation of secondary amines then has been much studied since the discovery in 1956 that nitrosamines and nitrosamides are powerful carcinogens in all animal species that have been tested.

The kinetic patterns are much the same as those established for primary amines since the rate-limiting step is usually the same in both cases. The more basic aliphatic secondary amines react with dinitrogen trioxide in nitrous acid solutions.

The effect of changing the acidity is the same as for primary amines. Aromatic secondary amines (e.g. N-methylaniline) react with dinitrogen trioxide at low acidities but with the nitrous acidium ion (or nitrosonium ion) at high acidities. Catalysis by nucleophiles such as chloride, bromide, thiocyanate and thiourea is also observed. In neutral or basic solution, nitrosation can be achieved with nitrite ion, in the presence of carbonyl compounds such as formaldehyde. Long chain secondary amines exhibit micellar catalysis⁵⁶, a function of the chain length which contributes to the electrostatic interactions on the micelle surface. Secondary amino acids readily give the corresponding nitrosamines with a range of nitrosating species.

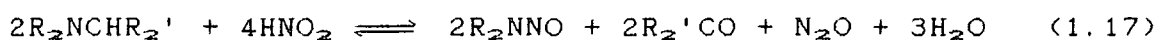
Tertiary amines were originally believed to be unreactive towards nitrous acid and as such their nitrosation reactions have not been studied as extensively as those of primary and secondary amines. However, the reaction of triethylamine with nitrous acid, to give diethylnitrosamine was reported as early as 1864⁵⁷. Other early work on the reaction of tertiary amines with nitrous acid has been reviewed by Hein⁵⁸ and Smith and Loepky⁵⁹. There has been more interest in these reactions recently because of a number of tertiary amine structures found in drugs and because of possible N-nitrosamine formation from tertiary amines in general⁶⁰.

The mechanism proposed by Smith and Loepky involves a rapid N-nitrosation, followed by a rate-limiting elimination of nitroxyl radical to give the iminium ion, which on hydrolysis gives the carbonyl compound and the secondary amine. The secondary amine then reacts with more nitrous acid to yield the nitrosamine. Later studies have shown that there is no nucleophile catalysis, which is consistent with the mechanism proposed (see scheme (1.13)).

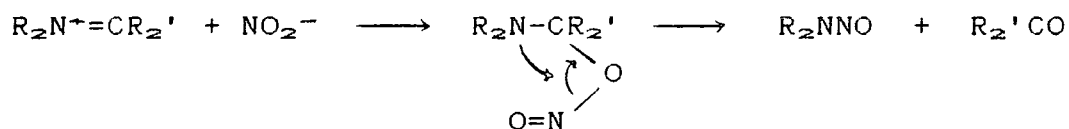


Scheme (1.13)

The stoichiometry of the reaction is given in equation (1.17)



Another suggestion has been made⁶¹ with regard to the fate of the iminium ion: it possibly reacts with nitrite ion, instead of being hydrolysed, to give an unstable alkyl nitrite derivative which rearranges to give a nitrosamine and elimination of a carbonyl compound (see scheme (1.14)).

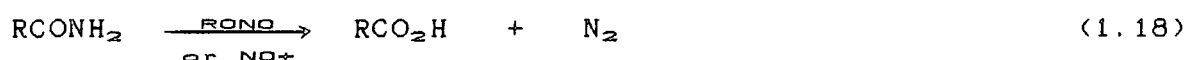


Scheme (1.14)

An alternative pathway involving electron transfer to form the iminium salt, rather than N-nitrosation, has been proposed by Michejda *et al* and this could be favoured for aromatic amines⁶².

Usually, stringent conditions (50-100°C) are required for the nitrosation of tertiary alkylamines, and it has been estimated⁶³ that these compounds are ~ 10 000 times less reactive than the secondary counterparts. Kinetic studies under these conditions are unreliable however, as nitrous acid decomposes.

Nitrosation of amides is generally much slower than that of the corresponding amines because of strong electron-withdrawing carbonyl group, discouraging electrophilic attack. Primary amides yield deamination products (see equation (1.18)) while secondary amides form nitrosamides in a reversible process (see equation (1.19)) upon nitrosation.



There is much interest in the nitrosation of ureas, guanidines and carbamates as a number are found in nature, where the ureas probably arise by bacteria-catalysed deimidation of guanidines or by carbamylation of amines⁶⁴. Many ureas and carbamates are also used as drugs and as insecticides. There is therefore, the possibility of the formation of carcinogenic nitrosamides within the human body.

Effective nitrosating agents for nitrosation of amides on the synthetic scale include alkyl nitrites in organic solvents⁶⁵, nitrosonium salts in acetonitrile⁶⁶, nitrosyl chloride⁶⁷ and dinitrogen tetroxide in carbon tetrachloride or acetic acid (containing sodium acetate). The mechanism of nitrosation of amides involves a rate-limiting proton transfer from the initially rapidly (and reversibly) formed N-nitroso species^{68 69}, rather than the rate-limiting attack by the nitrosating species as is the case with amines (see scheme (1.15)). For amides with powerful electron-withdrawing carbonyls, it is easier to achieve the limiting case of $k_{-1} [\text{X}^-] \gg k_2 [\text{B}]$.

Up to the early 1980's it was thought that bacterial reduction of nitrate to nitrite was the major contribution bacteria made to nitrosation. More recently, however, it has been shown that bacteria can participate directly in the nitrosation of amines^{76-78,82}. The bacterial genera capable of catalysing nitrosation include *Neisseria*, *Pseudomonas*, *Escherichia*, *Klebsiella*, *Proteus*, *Alcaligenes* and *Bacillus*^{76-79,81-83}. To facilitate the study of a number of bacterial strains under a wide range of reaction conditions, Ralt⁸⁴ et al have developed an assay based on the formation of a fluorescent product, 2,3-naphthotriazole, upon nitrosation of 2,3-diaminonaphthalene⁸⁵ as an alternative to the gas chromatographic-thermal energy analysis method⁸⁶ which is expensive and specific.

Wiersma⁸⁶ first described the use of 2,3-diaminonaphthalene as a spectrophotometric and fluorimetric reagent for the determination of nitrite ion. The fluorescent property of 2,3-naphthotriazole has been known and previously⁸⁷ used as a reagent for silver. Many methods are available for the determination of nitrite in environmental samples including spectrophotometric methods⁸⁸⁻⁹⁶ which may be subject to interferences by other ions, oxidants, coloured matter and turbidity, especially in biological samples; electrometric methods^{97,98}; HPLC of the free ion, ⁹⁹⁻¹⁰³ and GLC or HPLC of its organic derivatives¹⁰⁴⁻¹¹³. Some of these methods require close control of conditions, some have unsatisfactory detection limits and some, which are too complex and time-consuming, are unsuitable for routine application.

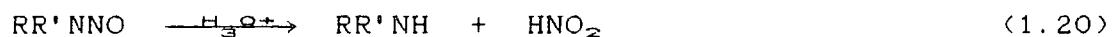
Fluorometric methods have been commonly used^{84,114-8} to determine trace levels of nitrite. The methods using 2,3-diaminonaphthalene^{114,115,117} are based on Wiersma's findings.

The mechanism for the reaction however, has never been elucidated. Also, much is known mechanistically about the diazotisation of aniline^{23,54}, and of naphthylamine^{119,120} but the corresponding reactions of diamines have not much been studied. The mechanism of the nitrosation of 2,3-diaminonaphthalene with acidified nitrite from 1M perchloric acid to pH~7 was studied.

1.2.2 Nitrosamines as Nitrosating Agents

The N-N bond of nitrosamines can cleave heterolytically or homolytically to yield a fragment capable of nitrosation, or a direct transfer of the NO group, without the intermediate formation of a free nitrosating agent can occur. We are concerned with the heterolytic cleavage of the N-N bond with direct transfer of the nitroso group.

The earliest recognised reaction of this type is the acid-catalysed denitrosation of a nitrosamine to give the secondary amine and nitrous acid (see equation (1.20)), the reverse reaction of the nitrosation of amines.



Denitrosation reactions are important industrially, in the disposal of waste chemicals which can be toxic or carcinogenic, as well as medically, as discussed later. The reaction has also been much used in the quantitative analysis of nitrosamines using a Thermal Energy Analyser: nitrosyl bromide is produced on treating a nitrosamine with hydrogen bromide¹²¹, which is then converted to nitric oxide, which is analysed quantitatively using a chemiluminescence detector following reaction with ozone when nitrogen dioxide is formed in an excited state.

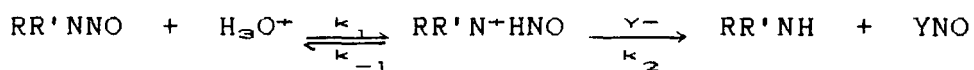
The kinetics of denitrosation has been studied for a number of nitrosamines, with a range of nucleophiles (Y^-) and solvents. To ensure the irreversibility of the process, nitrite traps are used^{1,2,2}. The traps used include hydrazine, hydrazoic acid, sulphamic acid, hydroxylamine, ascorbic acid, aniline and urea. The rate equation in water has been established (see equation (1.21)) assuming that the concentration of the nitrite trap exceeds a minimum threshold value so that the reaction is truly zero order in the nitrite trap, and thus effectively irreversible.

$$\text{Rate} = k h_o [\text{Nitrosamine}] [Y^-] \quad (1.21)$$

Because the reaction is acid catalysed the protonated form of the nitrosamine must be the reactive species. There are, of course two protonation sites: at the amino nitrogen atom and at the nitroso oxygen atom. N.m.r. studies^{5,1} and theoretical calculations^{5,2} favour the O-protonated form, although the principle of microscopic reversibility would require N-protonation. The mechanism for denitrosation is set out in scheme (3.3) involving a rapid reversible N-protonation followed by a rate-limiting attack by Y^- giving the nitrosyl derivative which is destroyed by the nitrite trap. The mechanism is also supported by kinetic isotope effects $k_{H_2O}:k_{D_2O}$ of ~ 0.3 . The reactivity of the nucleophile parallels that found for other nucleophilic reactions e.g. S_N2 substitutions. In the absence of nucleophiles the solvent acts as the nucleophile thus effecting the hydrolysis of the nitrosamine to give the secondary amine and free nitrous acid. This reaction is slower than the nucleophile-catalysed reactions. When nucleophiles are used, only the more powerful and non-basic ones are effective because of the fairly high acid concentrations required.

Denitrosation of some aliphatic heterocyclic nitrosamines has also been achieved and a number of such reactions have been studied kinetically, in the presence of added nitrite trap. 4-Methyl-1-nitrosopiperazine, mononitrosopiperazine and dinitrosopiperazine all undergo denitrosation in 3M hydrochloric acid at 50°C¹²³. There is no report establishing a direct reaction between a nitrosamine and an alcohol although ethyl nitrite is formed when denitrosation is carried out in ethanol solution¹²⁴.

Although the denitrosation reactions have been represented by scheme (3.3) and rate equation (1.21), a different rate law is observed when different conditions are used, where the nucleophile dependence disappears (see equation (1.22)). This usually occurs at high nucleophile concentration, for the more powerful nucleophiles, for nitrosamide substrates and those containing powerful electron-withdrawing groups, and for reactions in non-aqueous solvents, typically ethanol. An intermediate situation arises¹²⁵ where at low nucleophile concentration equation (1.21) is followed, and at high concentration, there is a zero-order nucleophile dependence. This has been observed for the denitrosation of nitrosamides^{70,71}, a nitrososulphonamide¹²⁶ and nitrosoureas^{68,69}, and for the denitrosation of N-methyl-N-nitrosoaniline in ethanol solvent. This is explained in terms of the rate-limiting proton transfer to the nitrosamine or nitrosamide rather than the attack of the nucleophile, Y⁻. This is achieved when $k_2 [Y^-] \gg k_{-1}$ (see scheme (1.16)).



Scheme (1.16)

Evidence in support of this comes from the observation of solvent primary kinetic isotope effects of 1.3 - 3.8.

Proline, an amino acid, is now used in the evaluation of human exposure to endogenously formed N-nitroso compounds. Ohshima¹²⁷ *et al* developed a sensitive procedure for the quantitative estimation of N-nitrosation in vivo in rats and in humans based on the findings that N-nitrosamino acids; nitrosoproline, nitrosohydroxyproline and nitrososarcosine, when administered orally, are excreted unchanged almost quantitatively (88 to 96% of the dose) in the urine and faeces. Because nitrosoproline has been reported to be noncarcinogenic and nonmutagenic^{129,130}, this method can be used to estimate the extent of endogenous N-nitrosation in high risk population, for example the high incidence of oesophageal cancer in some provinces in Northern China, thought to arise from the high levels of nitrate/nitrite in the drinking water, or from the intake of pickled vegetables and mouldy foods^{131,132}, or in high risk individuals, in which endogenous N-nitroso compounds have been associated with increased risk of cancers. That nitrosoproline is noncarcinogenic and nonmutagenic may be related to the fact that it appears to be neither absorbed nor metabolised in vivo, unlike N-nitrosodimethylamine, N-nitrosopyrrolidine and N-nitrosoureas. It was thought appropriate to investigate the denitrosation of nitrosoproline, an amino acid with a β -electron-withdrawing carbonyl function. The denitrosation of N-nitrosodimethylamine, a simple aliphatic amine, N-nitrosopyrrolidine, a heterocyclic amine without a β -electron withdrawing carbonyl group and N-nitrososarcosine, an acyclic amine containing a β -electron withdrawing group were also studied, and compared to the denitrosation reaction of NNP.

1.3 S-Nitrosation

S-nitrosation reactions are less common than N-nitrosation or C-nitrosation reactions due partly to the generally unstable S-nitroso products formed. As in alkylation and halogenation reactions, the sulphur atom in sulphur containing compounds is subject to electrophilic attack of nitrosating agents, such as nitrosyl chloride, nitrous acid, dinitrogen trioxide, dinitrogen tetroxide and alkyl nitrites.

The synthesis of S-nitrosothiols (equation (1.22)) has been known for some time; the products first isolated by Tasker and Jones¹³³ include phenyl thionitrite and ethyl thionitrite. The more stable thionitrites are those containing bulky groups attached to the carbon atom bonded to the sulphur atom: t-butylthionitrite¹³⁴, triphenylmethyl thionitrite¹³⁵ and S-nitroso-N-acetyl penicillamine¹³⁴. They are generally yellow, red or green and have a broad absorption band in the u.v.-visible region with a maximum at 330nm (extinction coefficient $\sim 10^3$).

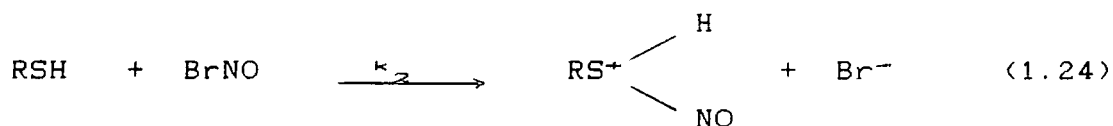


The kinetics of S-nitrosation has been established and the pattern is analogous to a range of other substrates. The rate-limiting step involves the attack by the nitrosonium ion or the nitrous acidium ion. The third-order rate constant, k (see equation (1.23)), for the more reactive thiols tends towards $7000 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$, taken to be the encounter-controlled limit. The reactions are generally fast, indicating that the overall reactivity of thiols is greater than that of nucleophilic but basic amines where protonation reduces greatly the concentration of the reactive free amine form. Thiols are not significantly protonated at moderate acidities.

$$\text{Rate} = k [\text{HNO}_2] [\text{RSH}] [\text{H}^+] \quad (1.23)$$

Thiols can therefore compete effectively with amines in nitrosation reactions; it is possible to suppress nitrosamine formation completely from a secondary amine in the presence of a sufficient excess of either cysteine or N-acetylpenicillamine¹³⁷.

Thiol nitrosation is as expected catalysed by added nucleophiles. The second-order rate constant k_2 (see equation (1.24)) for attack by nitrosyl chloride, nitrosyl bromide and nitrosyl thiocyanate have been obtained^{31,33} and the familiar reactivity sequence nitrosyl chloride > nitrosyl bromide > nitrosyl thiocyanate is demonstrated.

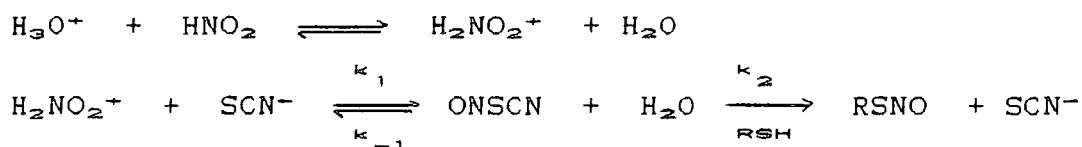


$$\text{Rate} = k_2 [\text{RSH}] [\text{BrNO}]$$

It is interesting that the k_2 values for the most reactive thiols and the most reactive reagent (nitrosyl chloride) do not approach the calculated encounter limit of $\sim 7 \times 10^9 \text{ l mol}^{-1} \text{ s}^{-1}$ but tend to a limit $\sim 2 \times 10^7 \text{ l mol}^{-1} \text{ s}^{-1}$. This is also true for aliphatic amines whose limit for k_2 approaches the value of $1 \times 10^7 \text{ l mol}^{-1} \text{ s}^{-1}$ over a wide range of pKa values. The reason why k_2 for nitrosation of aromatic amines tend to $7 \times 10^9 \text{ l mol}^{-1} \text{ s}^{-1}$ may have something to do with the aromatic π -electron system.

For most thiols the reaction is first-order in [RSH] over the ranges studied, with the exception of thioglycolic acid in the presence of bromide ion or thiocyanate ion where plots of the observed first-order rate constants against [RSH] are initially linear, but curve downwards and tend to level off at higher [RSH] so that the reaction is now zero order with respect to [RSH].

In this case, the rate of formation of nitrosyl bromide or nitrosyl thiocyanate is rate-limiting (see scheme (1.17)) i.e. $k_2[\text{RSH}] \gg k_{-1}[\text{H}_2\text{O}]$.



Scheme (1.17)

There are reports¹³⁸ of formation of S-nitrosothiols from thiols and nitric oxide, which is not in general an active nitrosating agent. In basic solution with oxygen being rigorously excluded, nitric oxide reacts⁴³ with the thiolate anion to yield the disulphide as outlined in scheme (1.6). This mechanism is analogous to that proposed¹³⁹ to account for disulphide formation from a nitrosamine and a thiolate anion.

Nitrososulphonamides also react with thiols in reactions which involve S-nitrosation¹⁴⁰. This is discussed later (Section 1.3.1) The denitrosation of nitrosamines is catalysed by both cysteine and glutathione, when the reaction takes place in the presence of an excess of a nitrous acid trap¹⁴¹, so that a direct S-nitrosation reaction occurs here. Cysteine and glutathione have similar reactivities to chloride ion. Similar reactions occur at sulphur sites in thioureas and sulphides. Both thio and dithiocarboxylic acids also react with nitrous acid to form S-nitrosothiols¹⁴² by reaction with the thiol group.

S-nitrosation is thought to play an important part in the mechanism of action of a number of vasodilatory drugs, which are used in the treatment of angina, heart failure and high blood pressure.

These drugs include alkyl nitrates, in particular, glyceryl trinitrate, alkyl nitrites and pentacyanonitrosyl-ferrate anion. These are capable of effecting *in vivo* S-nitrosation of tissue-bound thiol groups. There is some controversy as to whether the S-nitrosothiol or nitric oxide resulting from its decomposition activates the enzyme guanylate cyclase, which brings about smooth muscle relaxation, thus reducing the arterial blood pressure¹⁴³ (see section 1.5.3 for further discussion).

1.3.1 S-Nitrosation of cysteine and its derivatives

using N-Methyl-N-nitrosotoluene-p-sulphonamide.

N-methyl-N-nitrosotoluene-p-sulphonamide (MNTS) has been found to methylate DNA *in vitro*¹⁴⁴. Addition of cysteine, however, was found to decrease methylation by MNTS considerably, thereby acting as a protective agent against certain types of alkylating agents¹⁴⁵. MNTS is well-known as a source of diazomethane¹⁴⁶ but its reactions with nucleophilic reagents other than oxygen nucleophiles has yielded several products; N-nitrosopiperidine and diphenylnitrosamine both formed from the reaction of MNTS with piperidine¹⁴⁶ and diphenylamine¹⁴⁷ respectively. Schultz and McCalla first investigated the reaction between cysteine and MNTS¹⁴⁰.

Cysteine, a naturally occurring amino acid exists as different ionic species depending on the pH (see chapter 4 for further discussion). Its nitrosation using acidified nitrous acid in the absence¹⁴⁸ and presence^{31,33} of added nucleophiles has been studied. Nitrosation by alkyl nitrites in acidic conditions has also been studied¹⁴⁹ and the mechanism proposed involves the rapid reversible acid-catalysed hydrolysis of alkyl nitrites to give nitrous acid which then effects nitrosation of the thiols.

1.4 O-nitrosation

O-nitrosation is comparable to S-nitrosation with the O-containing compounds expected to be less reactive than their sulphur analogues as the oxygen atom is less nucleophilic. These reactions have been more extensively studied, the best known by far, is the formation of an alkyl nitrite from an alcohol and nitrous acid (see equation (1.26)).

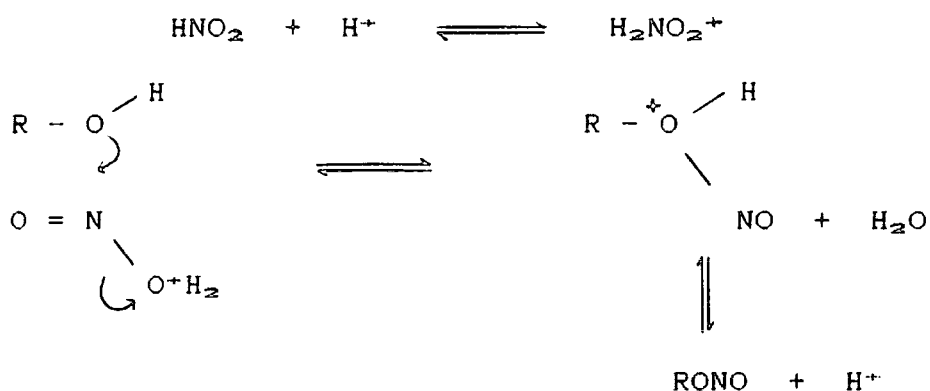


Allen¹⁵¹ established that optically active alcohols yield the corresponding alkyl nitrite without racemisation, indicating O-nitrosation rather than nitrite ion attack. Further, hydrolysis of alkyl nitrites in ¹⁸O-enriched water fails to produce ¹⁸O in the alcohol produced.

The reaction is general for any alkyl group R, but not for phenyl where aromatic NO substitution occurs; the reaction is reversible and the position of equilibrium depends on the electronic and steric properties of the alkyl group. Equilibrium constants for alkyl nitrite formation have been measured by direct spectrophotometric measurement¹⁵², by the indirect kinetic method of determining the rate constants for the nitrosation of morpholine in the presence of varying amounts of alcohol¹⁵² and also by a direct kinetic method using stopped-flow spectrophotometry³⁵. All three methods show that K (see equation (1.27)) decreases along the series R = CH₃ > C₂H₅ > i-C₃H₇ > t-C₄H₉ implying that steric effects are more important than electronic effects in the O-nitrosation of alcohols.

$$K = \frac{[\text{RONO}]}{[\text{ROH}] [\text{HNO}_2]} \quad (1.27)$$

The reaction mechanism is set out in scheme (1.19) and the reactivity of the alcohols is defined by the third-order rate constant (see equation (1.28)) as is the case with thiols.



Scheme (1.19)

$$\text{Rate} = k [\text{ROH}] [\text{HNO}_2] [\text{H}^+] \quad (1.28)$$

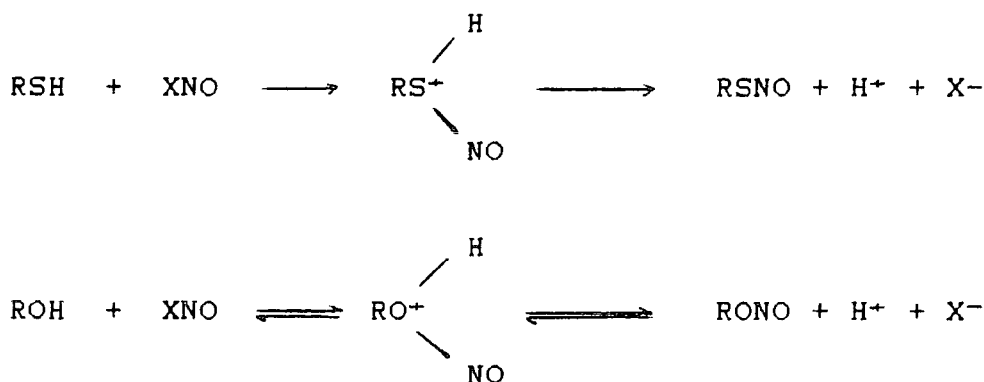
Because the pKa of the nitrous acidium ion is not known, the bimolecular rate constant for attack by nitrous acidium ion cannot be obtained. For very reactive species however, the values of k show a limiting value, taken to be the encounter rate between the two species¹⁵³. In general, alcohol nitrosation is a rapid process; in fact the reactivity of alcohols and amines are comparable even with allowances made for the protonation of the amines. Amines are several orders of magnitude more basic than alcohols; their comparable reactivity can be explained in terms of the HSAB principle¹⁵⁴, which designates both alcohols and aliphatic amines as hard bases, with similar reactivity towards a common electrophile. The reaction is catalysed by nucleophiles³⁵, as expected, although catalysis is less extensive than in the case with aromatic amines, such as aniline²³. This is explained once again in terms of the HSAB principle where the 'softer' nitrosyl halide binds less favourably with the 'hard' alcohol substrates compared to the more 'borderline' aromatic amines in particular.

Reaction with nitrosyl chloride, in non-aqueous solvents have also been investigated for example in acetic acid¹⁵⁵ and in carbon tetrachloride-acetic acid mixtures.¹⁵⁶ These require rapid measuring techniques including stopped-flow spectrophotometry and solvent-jump relaxation.

Other O-nitrosation reactions include those of ascorbic acid (see section 1.5.2 for further discussion), carboxylic acids^{157, 158}, and hydrogen peroxide¹⁵⁹⁻¹⁶².

The indications are that O-nitrosation is slower than S-nitrosation although no exact comparison has been made. N-acetyl-penicillamine, a relatively good model for t-butyl thiol is several orders of magnitude more reactive than t-butanol. O-nitrosation is significantly reversible whilst S-nitrosation is effectively quantitative. This is because oxygen is more basic than sulphur, in organic molecules ($\Delta pK_a \sim 5$) so that the rate of the reverse reaction is greater for oxygen than for sulphur.

Thus the forward reaction is governed by the nucleophilicity (S- > O-), whilst the reverse reaction is governed by basicity (O- > S-) (see scheme (1.20)).



Scheme (1.20)

1.5 Nitrosation by alkyl nitrites and thionitrites

Both alkyl nitrites and thionitrites can be used as electrophilic nitrosating agents quite generally, reacting with a range of substrates including amines, alcohol, thiols and a range of C-nitrosation reactions.

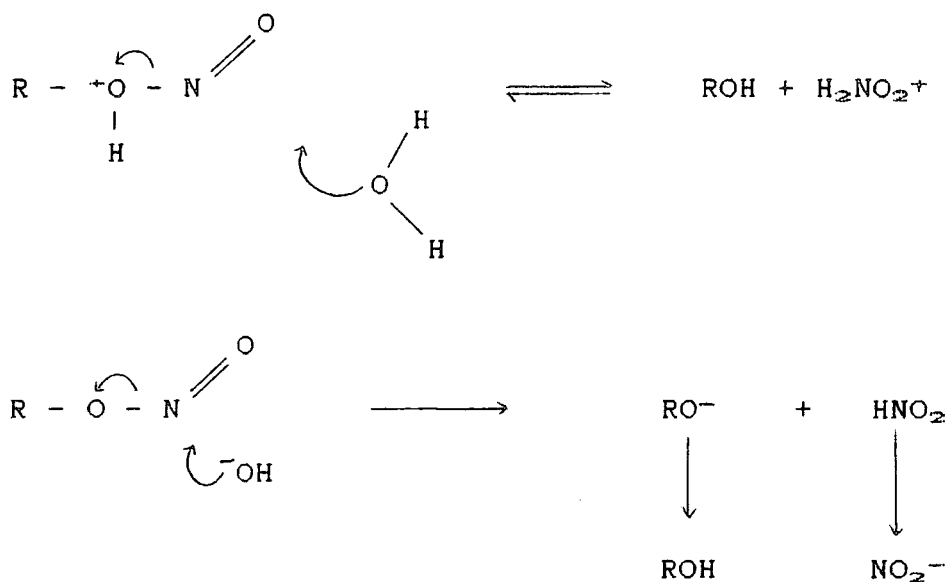
Alkyl nitrites undergo both acid-catalysed and base-catalysed hydrolyses. Acid-catalysed hydrolysis is rapid and the reactions of the protonated form of the alkyl nitrite and a water molecule occur at or close to the encounter-limit. The rate equation has been established by Allen¹⁵¹ (equation (1.29)).

$$\text{Rate} = k [\text{H}^+] [\text{RONO}] \quad (1.29)$$

The reactions are reversible and equilibrium constants have been determined. For tertiary nitrites such as t-butyl nitrite, hydrolysis proceeds virtually to completion due to steric hindrance for the reverse reaction³⁵.

Thus reaction of alkyl nitrites in water is accompanied by rapid hydrolysis and nitrosation by the intermediate nitrous acid formed may have to be taken into account, instead of a simple direct reaction involving the alkyl nitrite molecule.

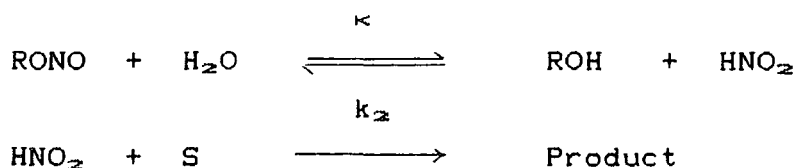
Base-catalysed hydrolysis of alkyl nitrites has been more extensively studied and has been found to be considerably slower than the corresponding hydrolysis of carboxylic acid ester, unlike the acid-catalysed hydrolysis. The reaction is irreversible as the nitrous acid produced is present as the inactive nitrite ion. O-N bond fission occurs^{151, 163}, as for the acid-catalysed reactions where protonation first occurs at the oxygen atom bound to the alkyl group (see scheme (1.21)).



Scheme (1.21)

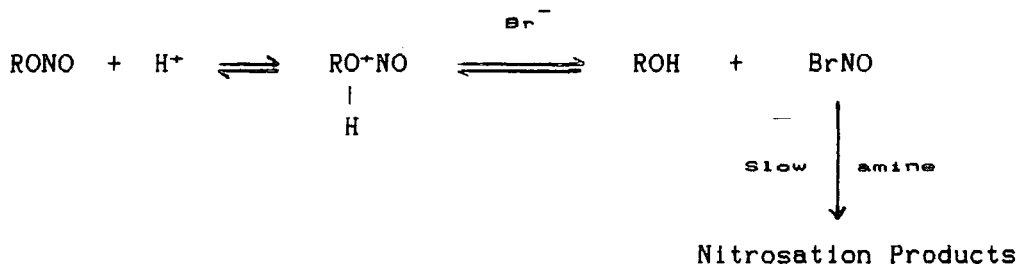
Alkyl nitrites are particularly useful in the nitrosation of substrates with low solubility in water, as alkyl nitrites can be used in a range of solvents, for example, the formation of diazonium ions in alcohols, acetic acid, dioxan, tetrahydrofuran, etc.¹⁶⁴. The chemistry of diazonium ions has been studied extensively¹⁶⁵.

In aqueous or mixed aqueous organic solvents, reactions of alkyl nitrites particularly in acid solutions are complicated by rapid hydrolysis. The reaction of 2-propyl nitrite with hydrazoic acid, sulphamic acid, thioglycolic acid and N-methylaniline involves a reversible hydrolysis of the alkyl nitrite, giving free nitrous acid, which effects nitrosation in the rate-limiting step¹⁶⁶ (see scheme (1.22)).



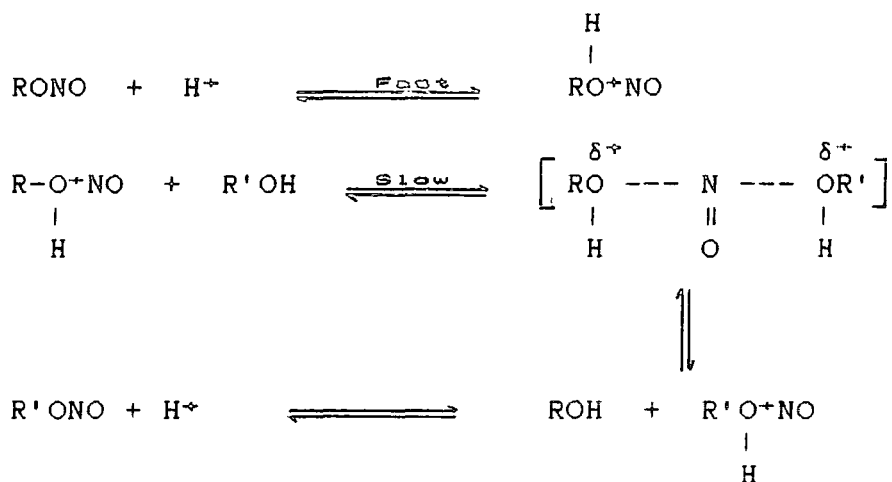
Scheme (1.22)

The reduction of the observed rate constant for nitrosation, with the addition of aliphatic alcohol, enables values of the equilibrium constant for alkyl nitrite hydrolysis, K , and k_2 , the rate constant for nitrosation with nitrous acidium ion/nitrosonium ion, to be determined. Nucleophile catalysis¹⁶⁷ is also observed, as expected for nitrosation by acidified nitrous acid. The reaction is first-order in [nucleophile] at low concentration and the mechanism involves the denitrosation of the alkyl nitrite by nucleophilic attack of the bromide or chloride ion at the nitrogen atom in the protonated alkyl nitrite. The nitrosyl compound then reacts with amine in the rate-limiting step (see scheme (1.23)). For less basic amines, acid catalysis is found (e.g. 4-nitroaniline) as expected, but for aniline and N-methylaniline, protonation of the alkyl nitrite is counter-balanced by the amine protonation.



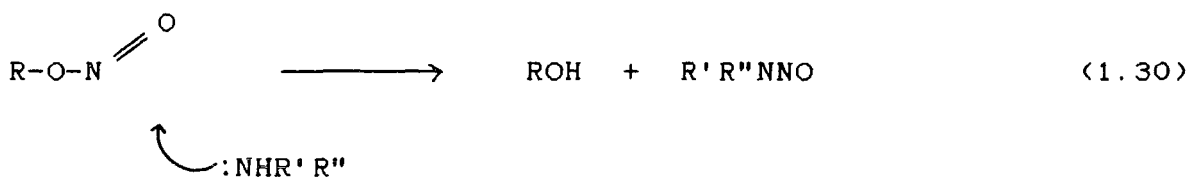
Scheme (1.23)

O-nitrosation, which involves the exchange of a nitroso group between alkyl nitrites and alcohols has been found to be reversible¹⁶⁸, and first-order in [RONO] and [H⁺]. There is no evidence for or against the involvement of a discrete intermediate, although chloride ion catalysis occurs, pointing to an additional nitrosyl chloride pathway in the scheme proposed, which only takes into account the protonated alkyl nitrite as a direct reagent (see scheme (1.24)).

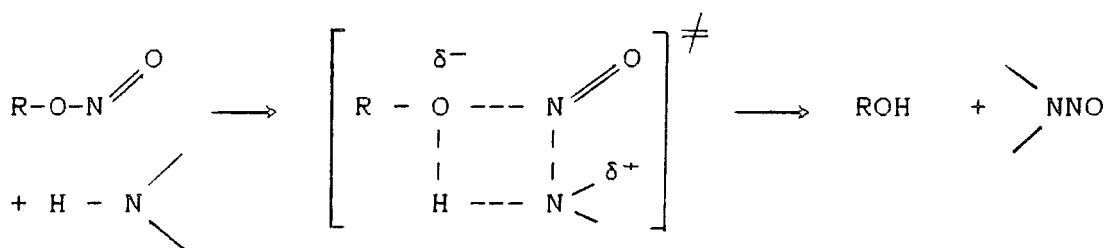


Scheme (1.24)

Nitrosation by alkyl nitrites under alkaline conditions has been achieved for amines, alcohols, ketones, nitro compounds, some hydrocarbons and thiols, to yield thionitrites and dialkyl sulphides. The most widely studied involves the reactions of amines. Direct nucleophilic attack by the amine occurs at the nitrogen atom of the alkyl nitrite (see equation (1.30)) to form the nitrosamine which is stable for secondary amines but which decomposes to give hydrocarbon products if the amine is primary¹⁶⁹.

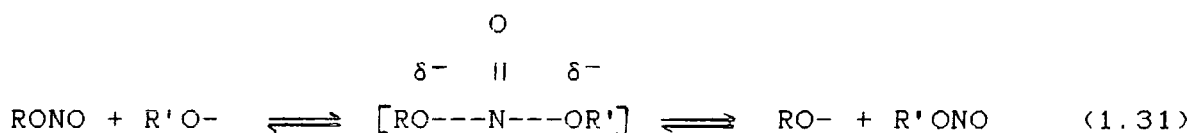


The reaction is unusually rapid and has been rationalised in terms of a concerted reaction, supported by ionic strength effects and the value of the entropy of activation. Reactivities of the amines correlate with their vertical ionisation potentials, rather than their basicities, indicating orbital-controlled reaction. The solvent isotope effect is $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} \sim 2$ implying proton transfer in the rate-determining step so that the proposed transition state is a cyclic structure (see scheme (1.25))



Scheme (1.25)

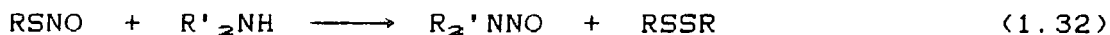
Nitrosation at oxygen in alkaline conditions involves nucleophilic attack by the alkoxide ion (see equation (1.31)) and there is no evidence for the existence of an intermediate species.



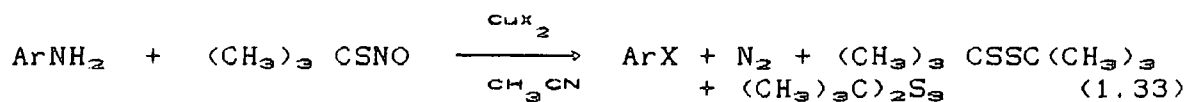
Nitrosation in non-hydroxylic solvents such as acetonitrile or chloroform appears to be rapid and quantitative and provides an excellent synthetic pathway for N-, O- and S-nitrosation generally¹⁷⁰.

Thionitrites are generally fairly unstable and have therefore not been as extensively used as alkyl nitrites as nitrosating agents. Thionitrites decompose thermally and photochemically to give the disulphide¹⁷¹. Reaction with another thiol yields the unsymmetrical disulphide; reduction yields the thiol; oxidation the corresponding thionitrate.

Nitrosation of secondary amines yield nitrosamines¹⁷² (see equation (1.32)) and aniline¹³⁶ derivatives to yield azo dyes after coupling with β -naphthol.



Thionitrites can also deaminate arylamines in acetonitrile in the presence of anhydrous copper (II) halide¹⁷¹ (see equation (1.33)).



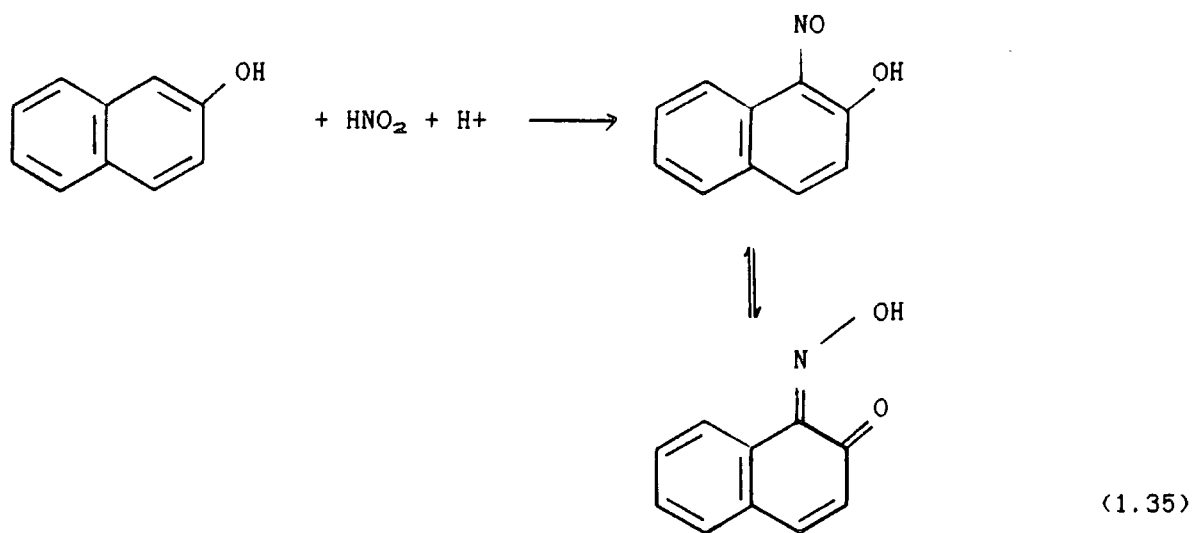
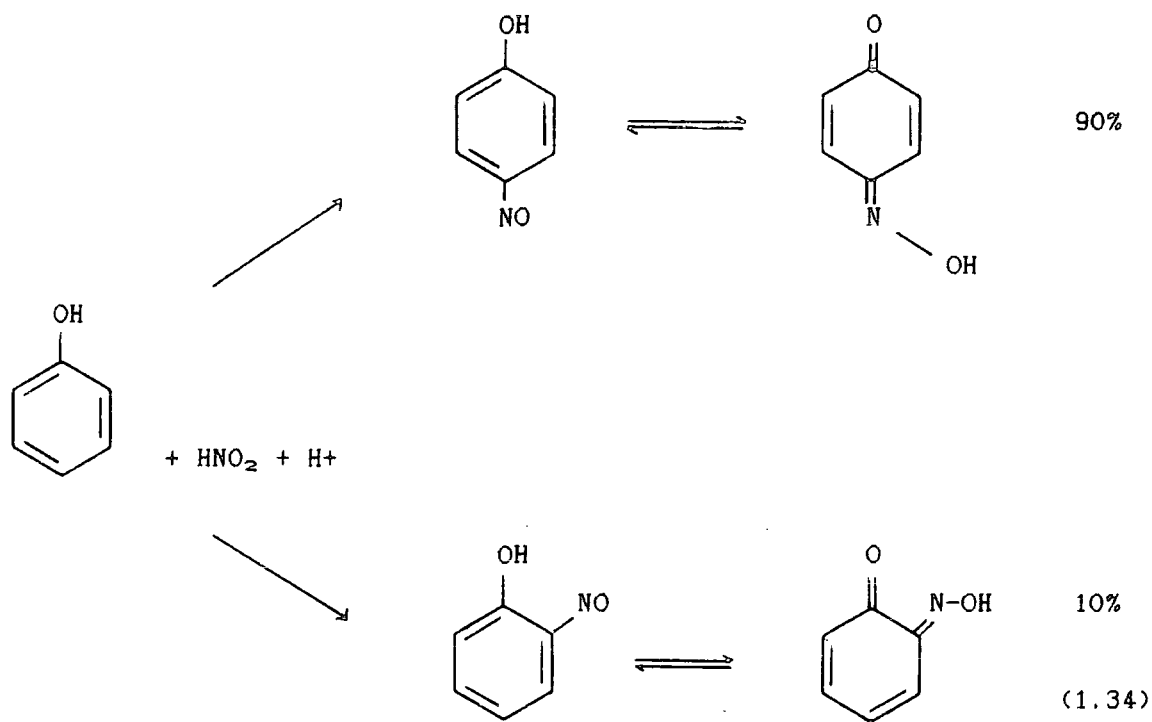
Thionitrites can also nitrosate alcohols forming alkyl nitrites although the yields are quite low¹⁷³.

The mechanism of all the above reactions is as yet unknown, although they have been much used synthetically. Nitrosation may occur directly or may involve prior reaction of the thionitrite to give a free nitrosating species.

It is known that thionitrites can transfer the nitroso group directly to nucleophiles such as water, halide ion, thiocyanate ion and thioureas, in catalysed reactions³⁹. Reaction can only occur at high acid concentration and only if the nitrosyl species is removed rapidly. Hydrolysis of thionitrites is much slower than that of alkyl nitrites probably because of the greater basicity of the oxygen atom.

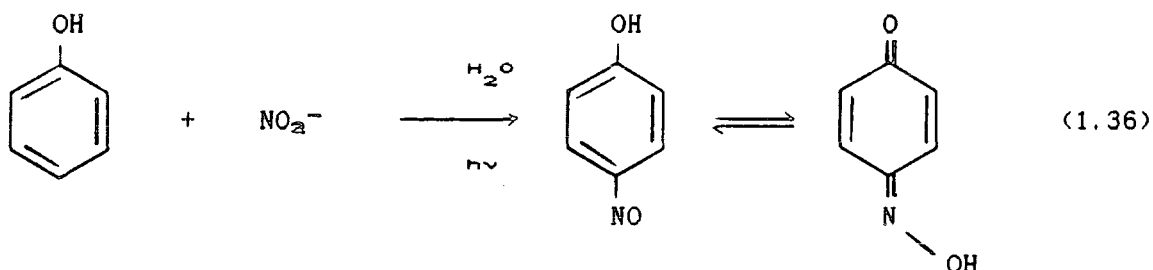
1.5.1 Aromatic C-nitrosation using O-nitroso and S-nitroso compounds

Aromatic C-nitrosation is not as widely studied as aromatic nitration, which is well-known synthetically and mechanistically¹⁷⁴. The most common nitrosation reactions are those of phenols and naphthols with nitrous acid in dilute acid solutions. The C-nitroso compounds which form exists primarily as the quinone monooximes. Phenol yields p-nitrosophenol (90%) and 2-nitrosophenol (10%)¹⁷⁵ and 2-naphthol yields 1-nitroso-2-naphthol¹⁷⁶ (see equations (1.34) and (1.35)).



Phenyl ethers also yield C-nitroso products, often accompanied by dealkylation so that for anisole and diphenyl ether¹⁷⁷, the main isolated product of nitrosation is p-nitrosophenol.

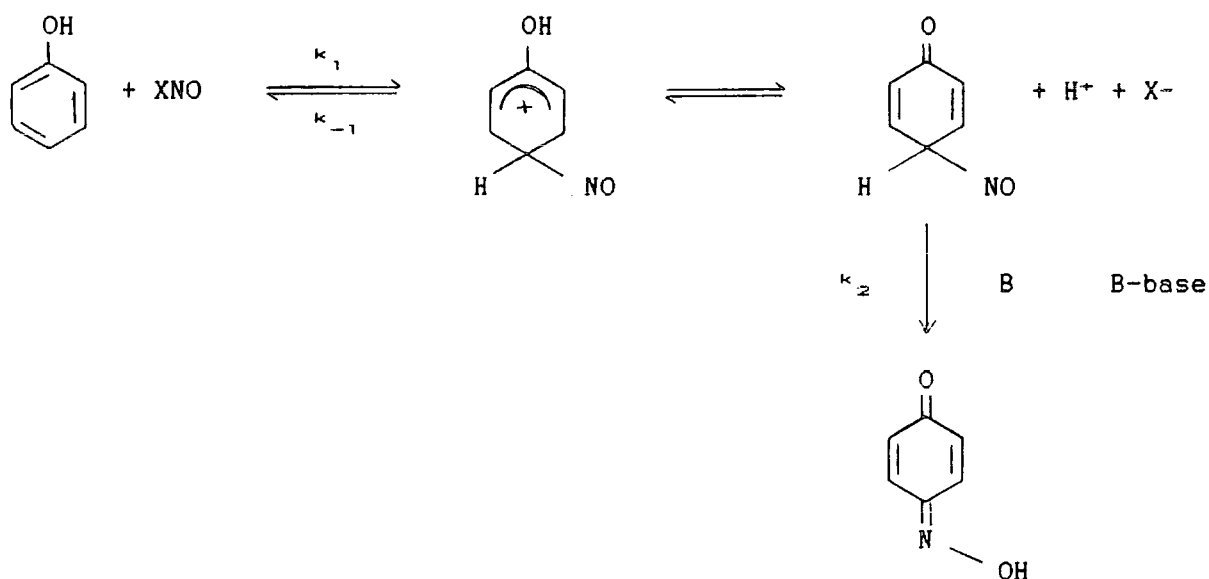
Phenols can also be nitrosated photochemically with aqueous nitrite ion in the presence of u.v. light. Phenol yields 4-nitrosophenol¹⁷⁸ probably via the reaction of nitroso hydroxy radicals generated photochemically (see equation (1.36)). Naphthols and anthrols also undergo photochemical nitrosation¹⁷⁹, using nitrosamines as a source of nitroso radicals.



Tertiary aromatic amines also yield the 4-nitroso product but secondary amines are initially nitrosated at the nitrogen atom, with subsequent rearrangement (Fischer-Hepp rearrangement) to form the 4-nitroso derivative.

Benzenes and alkylbenzenes react slowly¹⁷⁷ with nitrous acid to form diazonium salts¹⁸⁰ with the probable formation of nitrosoarenes initially. Nitrosophenols are also formed in the reaction of benzene derivatives with hydroxylamine, hydrogen peroxide in the presence of copper (II) salts or sodium pentacyanoammineferroate, involving free radical intermediates (Baudisch reaction¹⁸¹). Aromatic nitroso compounds can also be prepared by *ipso* substitution, replacing groups other than the hydrogen atom, for example¹⁸²: $-\text{CH}(\text{OH})\text{Ph}$, $-\text{CH}_2\text{C}_6\text{H}_4\text{NMe}_2$, $-\text{N}_2\text{Ph}$, $-\text{COMe}$ and $-\text{CHO}$ can be displaced from the 4-position in the reactions of 4-substituted N,N-dimethylanilines¹⁸².

The reaction mechanism of aromatic C-nitrosation has been studied for phenols, naphthols and aryl ethers. Challis and co workers proposed the well-known A-S_E2 mechanism for aromatic electrophilic substitution, involving the reversible formation of an intermediate followed by a proton transfer to the medium (see scheme (1.26)).



The expected rate equation is given in equation (1.37); the ionisation of nitrous acid to nitrite ion has not been included, for simplicity, although this has to be accounted for in pH>2 solutions.

$$\text{Rate} = \frac{k_1 k_2 [\text{PhOH}] [\text{H}^+] [\text{HNO}_2] [\text{B}] [\text{X}^-]}{k_{-1} [\text{H}^+] [\text{X}^-] + k_2 [\text{B}]} \quad (1.37)$$

In the limiting cases, in the absence of added X⁻, when $k_{-1} [\text{H}^+] \gg k_2 [\text{B}]$, a primary kinetic isotope effect and general base catalysis is predicted, together with the absence of acid catalysis.

This has been found in the pH range 1.0 - 4.5^{183,184} and there is a pronounced kinetic isotope effect, $k_H/k_D \sim 3.5^{183,184}$ for the reaction of phenol substituted with deuterium in the 4-position. When $k_{-1}[H^+] \ll k_2[B]$, the reaction should be acid catalysed with no kinetic isotope effect nor base catalysis. Further confirmation of this mechanism arises from the effect of added X^- such as chloride ion, bromide ion and thiocyanate ion when $k_{-1}[H^+][X^-] \gg k_2[B]$ there is no nucleophile catalysis as the final proton-transfer will be rate-limiting. At low acidity and low $[X^-]$ however, there is nucleophilic catalysis¹⁸⁵, in the expected order: chloride ion < bromide ion < thiocyanate ion.

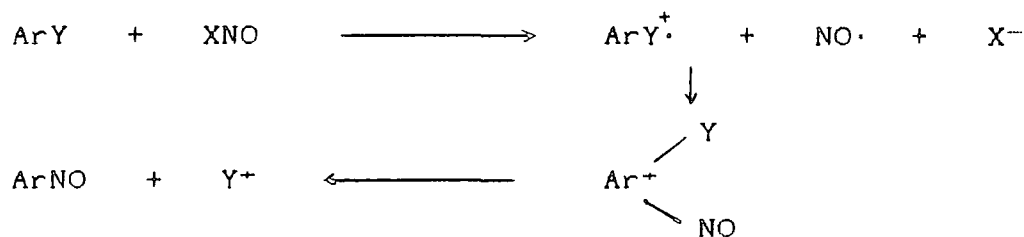
In the absence of added catalysts reaction at low acid concentrations involves the nitrous acidium ion (or nitrosonium ion), there being no evidence of reaction via dinitrogen trioxide. At acidities greater than $\sim 0.5M$ acid catalysis becomes a general feature of phenol nitrosation, believed to arise by the onset of another pathway for the decomposition of the dienone intermediate which is acid-catalysed. The dienone intermediate has not been detected spectroscopically, but may be too reactive to build up in a significant concentration. The rate-acid concentration profile has a maximum at $\sim 6-7 M$ perchloric acid because of the decreasing water concentration which acts as a base for the final proton removal.

Phenyl ethers react similarly to phenols with the exception of a lack of an acid-independent pathway at moderately low acidities. Kinetic studies for anisole^{177,183,186} and for diphenyl ether¹⁷⁷ fit the expected A-S_E2 mechanism, with an intermediate σ -complex as the dienone intermediate is not now possible.

C-nitrosation has also been studied mechanistically in the heteroaromatic indole systems¹⁸⁷ and is believed to be the A-S_E2 mechanism proposed for phenols and phenyl ethers. However, the rate-limiting step now depends on the reactivity of the indole: for the more basic compounds (with pK_a > - 3.5) formation of the intermediate is rate-limiting.

The nitrosation of benzene has been studied from a theoretical point of view¹⁸⁸. It is believed that electron transfer takes place from a benzene frontier orbital to a π^* orbital in a nitrosonium ion to give two interconverting π complexes of different symmetry. These complexes have been detected in reaction in solutions and in the gas phase.

The A-S_E2 mechanism is also likely to apply in those cases where a group other than a hydrogen atom is displaced. A different explanation has been suggested, nevertheless to account for the *ipso* nitrosation in 3-substituted indoles, N,N-dimethylanilines and 1-, and 3- substituted indolizines¹⁸², involving a one-electron transfer from XNO to give a radical cation and nitric oxide which react further by a free-radical reaction at an aromatic carbon atom to give the Wheland intermediate. It is quite possible that the first stage of any aromatic nitrosation, where significant nitrosonium ion concentrations exist, is the formation of the radical cation which then collapses to the Wheland intermediate (see scheme (1.27)). This could apply to *ipso* substitutions as well as to reactions where the proton is eliminated (Y = H).

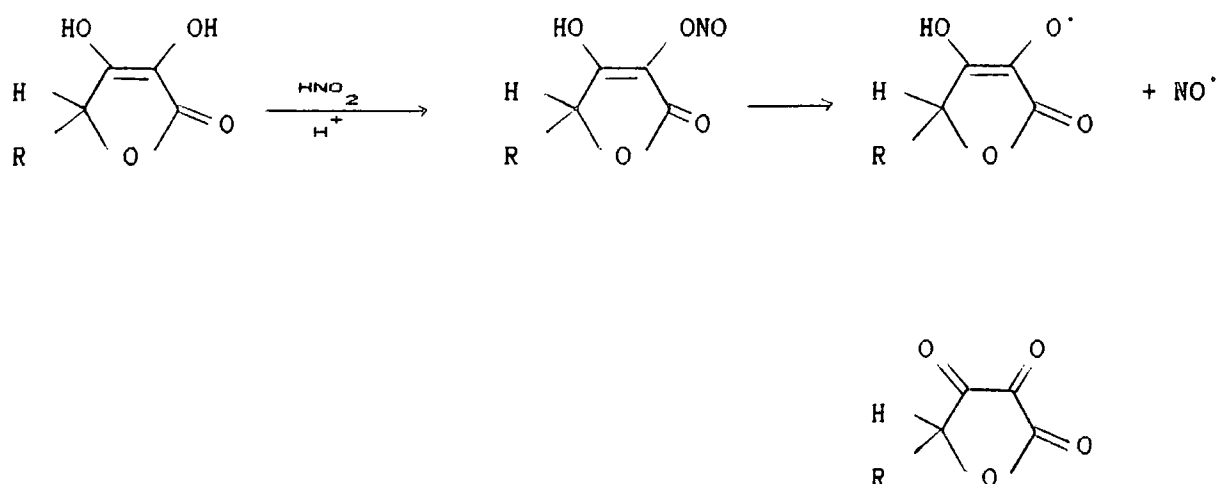


Scheme (1.27)

The nitrosation of phenols by alkyl nitrites and thionitrites have not previously been studied. A mechanistic study of these reactions was undertaken, including an attempt to compare the reactivities of alkyl nitrites and thionitrites.

1.5.2 Ascorbic acid nitrosation using O- and S-nitroso compounds

Ascorbic acid (vitamin C) is known to react readily with nitrous acid in acid solutions. The stoichiometry of the reaction has been established: two moles of nitrous acid reacts with one mole of ascorbic acid to give dehydroascorbic acid and nitric oxide. The mechanism that has been proposed involves O-nitrosation to give an mononitrite ester, which by homolytic fission gives nitric oxide and a semiquinone radical, which is then rapidly oxidised by another molecule of nitrous acid (see scheme (1.28)).



At pH < 1 the nitrosating agent is the nitrous acidium ion (or nitrosonium ion) and at higher pH values the reactive species is dinitrogen trioxide¹⁸⁹. The pK_a of ascorbic acid is ~ 4 and at pH 3-5, the reactive monoanion will exist in significant concentrations. Chloride ion, bromide ion and thiocyanate ion catalysis is observed, as for alcohols in general. Rate measurements on the acid-catalysed reaction show that the reaction of the ascorbate anion is likely to be at the diffusion-controlled limit, comparable to reactions of azide, acetate and nitrite¹⁵³. Molecular ascorbic acid is less reactive with rate constants comparable to that of hydrazoic acid and some substituted aniline derivatives¹⁵³.

Because ascorbic acid is non-toxic and with its high reactivity, it has been used to inhibit other nitrosation reactions for example, of amines, in a direct competition. It is more effective than alcohols and carbohydrates generally where the O-nitrosation is a reversible process. Reaction with ascorbic acid is irreversible due to the loss of nitric oxide. It has been suggested⁶⁴ that ascorbic acid be incorporated into drugs containing nitrosable secondary amine functions to reduce the extent of in vivo nitrosation in the stomach.

Ascorbic acid has also been used in the nitrite curing process of meats. Nitrite reacts with myoglobin, inhibiting the growth of the bacteria *Clostridium botulinum*¹⁹⁰ as well as giving it the characteristic pink cured-meat colour and flavour. The use of nitrite has recently come under considerable pressure due to its reaction with amines and amino acids found in meats to produce nitrosamines¹⁹¹. Ascorbic acid has been used successfully, both to enhance the formation of nitrosyl haemoprotein, the product formed on treatment of haemoproteins with nitrite or with nitric oxide¹⁹², as well as to prevent the formation of carcinogenic nitrosamines.

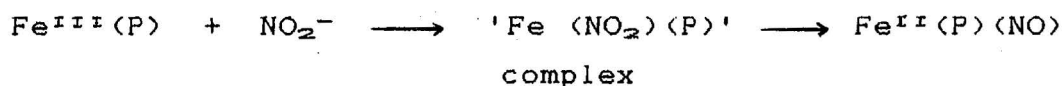
Nitrosyl haemoproteins have also been implicated as possible intermediates in the control of blood pressure. The activation of the enzyme guanylate cyclase causes relaxation of vascular smooth muscle. Activation of guanylate cyclase is believed to be the result of the binding of NO to Fe in the haem prosthetic group which is bound to the enzyme. Because alkyl nitrites such as iso-amyl nitrite and thionitrites are known to be vasodilators, their interaction with the haem group has been investigated (see section 1.5.3 for further discussion). As ascorbic acid is present *in vivo*, the prior interaction of alkyl nitrites and thionitrites with ascorbic acid, before their interaction with haemoproteins must also be investigated.

1.5.3 Nitrosation of haems using alkyl nitrites and thionitrites

Haemoproteins, such as myoglobin and haemoglobin basically consists of the protein or globin, and the porphyrin ligand, which surrounds the metal atom at the centre. The nitrosation reactions of haemoproteins are widespread: assimilatory nitrite reductases catalyse the six electron reduction of nitrite ion to ammonia¹⁹³, while dissimilatory nitrite reductases reduce nitrite ion to nitrous oxide, nitric oxide and dinitrogen¹⁹⁴.

Both types of enzymes possess a haem prosthetic group, which reacts with nitrite to form a nitrosyl complex of the haem¹⁹⁵. Nitrite interactions with haemoproteins also form important complexes in meat-curing processes, as described above.

Reactions of porphinato-iron (III) complexes with nitrite ion have been investigated^{196,197}; the stable product of the reaction is not the co-ordinated nitrite species but rather the iron-nitrosyl species, which probably forms¹⁹⁷ by single oxygen atom transfer from a co-ordinated nitrite ion to an unco-ordinated nitrite ion (see scheme (1.29)).



Scheme (1.29)

Furthermore, the stoichiometric formation of the nitrosyl using nitrites and Fe^{II} porphyrins has been found to be more efficient in the presence of a reducing agent¹⁹⁸ such as ascorbic acid, although the iron (II) porphyrin may fulfil this role in the absence of other reductants, with the consequent formation of the nitrosyl complex and the iron (III) complex in a 1:1 ratio^{198,199}. These investigations point to Fe^{II} porphyrins, rather than Fe^{III} porphyrins being the active oxidation state for the Fe atom in the nitrosylation/nitrosation process.

The reactions of haemoglobin with alkyl nitrites has also been studied²⁰⁰. The rate constant for the reaction of ethyl nitrite with haemoglobin from which the fourth oxygen has dissociated was found to be 45 times greater than the corresponding rate constant for oxidation of deoxyhaemoglobin, proposed to be a reflection of the oxidative susceptibility of the R and T conformational states of haemoglobin. To avoid this complication, the reaction of the metalloporphyrins without the protein/globin part of the molecule was undertaken. Its reactions with alkyl nitrites as well as thionitrites were investigated, in view of the vasodilatory properties of these compounds as well as the implication of nitrosylhaems in the control of blood pressure.

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CHAPTER 2

Nitrosation of 2,3-diaminonaphthalene

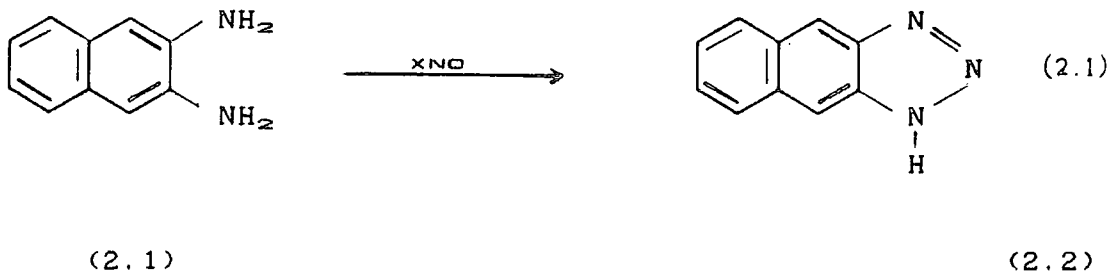
2.1 Introduction

2,3-Diaminonaphthalene (DAN) has mainly been used in analytical procedures, for following the nitrosation of secondary amines by bacteria¹. The assay is based on the formation of the fluorescent product, 2,3-naphthotriazole², and has been used to study the nitrosation rate of bacteria such as *Escherichia coli*³ and *Neisseria subflava*⁴. This is because bacteria have recently been found to be capable of participating directly in the nitrosation of amines⁵. For the study of bacterial production of N-nitroso compounds, the above technique is preferable to the otherwise specific and expensive requirement for a gas chromatograph linked to a Thermal Energy Analyser.

Although mechanistic details of the nitrosation of amines, both aliphatic and aromatic, have been well established, little is known about the reaction of diamines. It was therefore thought appropriate, given its usefulness in analysis, to study the nitrosation of DAN using nitrous acid, in a range of acid conditions.

2.2 Nitrosation of DAN

The diazotisation of aniline^{6,7} and of 1-naphthylamine^{8,9} has been studied mechanistically while the corresponding reactions of the diamines have not, although it is well known that aromatic 1,2-diamines do react with nitrous acid to give ring closed triazole products e.g. o-phenylenediamine reacts with nitrous acid. The nitrosation of DAN (2.1) results in the formation of 2,3-naphthotriazole (2.2) (equation (2.1)).



This reaction has now been examined kinetically in aqueous acid solutions (containing 5% methanol to dissolve DAN) at 25°C.

The reactions were rapid and measurements had to be carried out using a stopped-flow spectrophotometer, following the increasing absorbance at 355nm due to the product, triazole. Pseudo first-order conditions were used, with concentrations of DAN in large excess over the concentration of HNO_2 (typically $2 \times 10^{-3} \text{ mol dm}^{-3}$ and $1 \times 10^{-4} \text{ mol dm}^{-3}$ respectively).

Good first-order behaviour was found throughout and the first-order rate coefficients, k_{obs} , were reproducible to within $\pm 6\%$. The reactions were also first order with respect to [DAN]. Plotting k_{obs} vs $[\text{HClO}_4]$ gives a straight line, with a significant positive intercept at zero $[\text{HClO}_4]$. The experimental results are shown in table (2.1).

Table 2.1

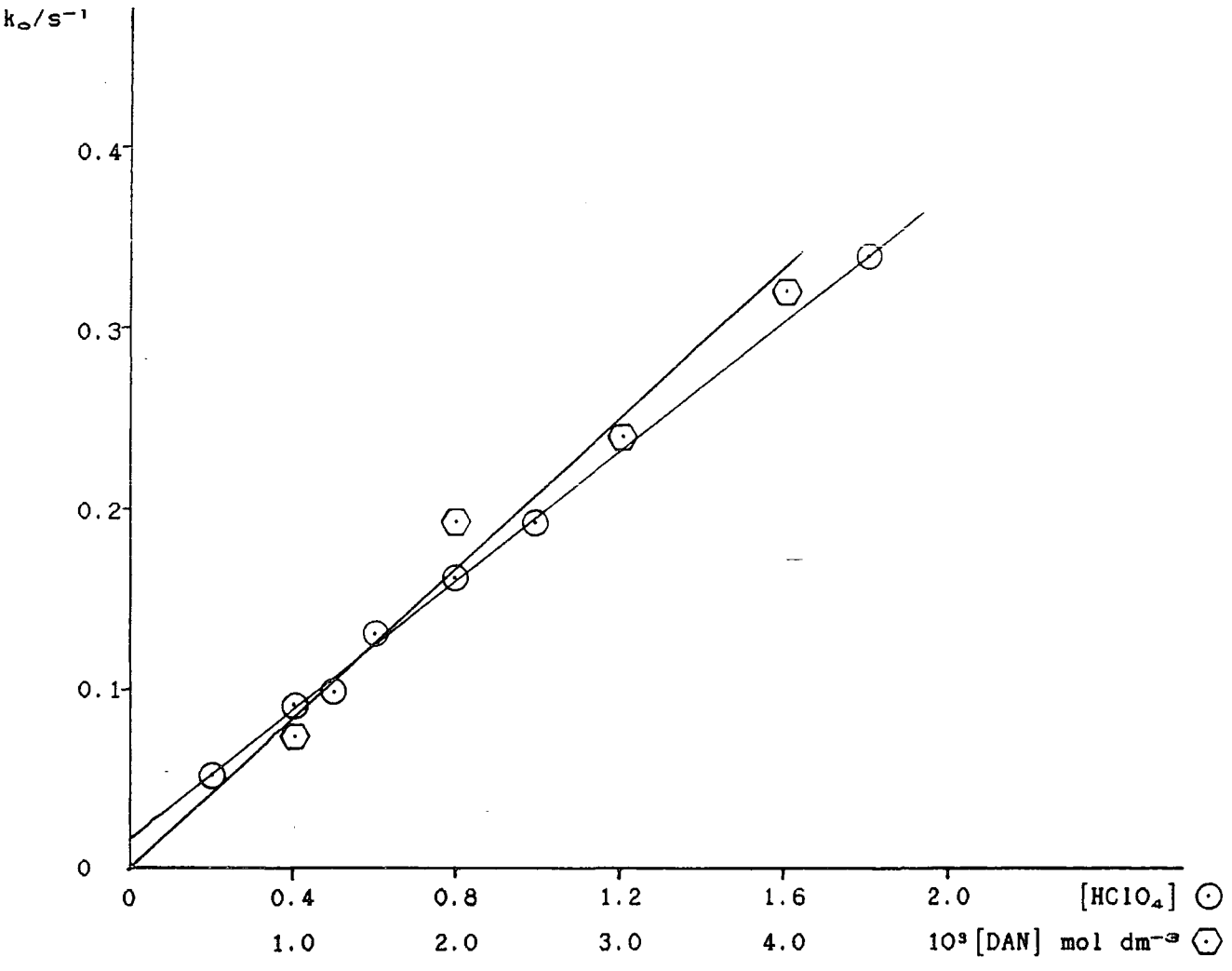
First-order rate constants (k_o) for the diazotisation of DAN with nitrous acid in aqueous perchloric acid.

$$[\text{HNO}_2] = 1.0 \times 10^{-4} \text{ mol dm}^{-3}$$

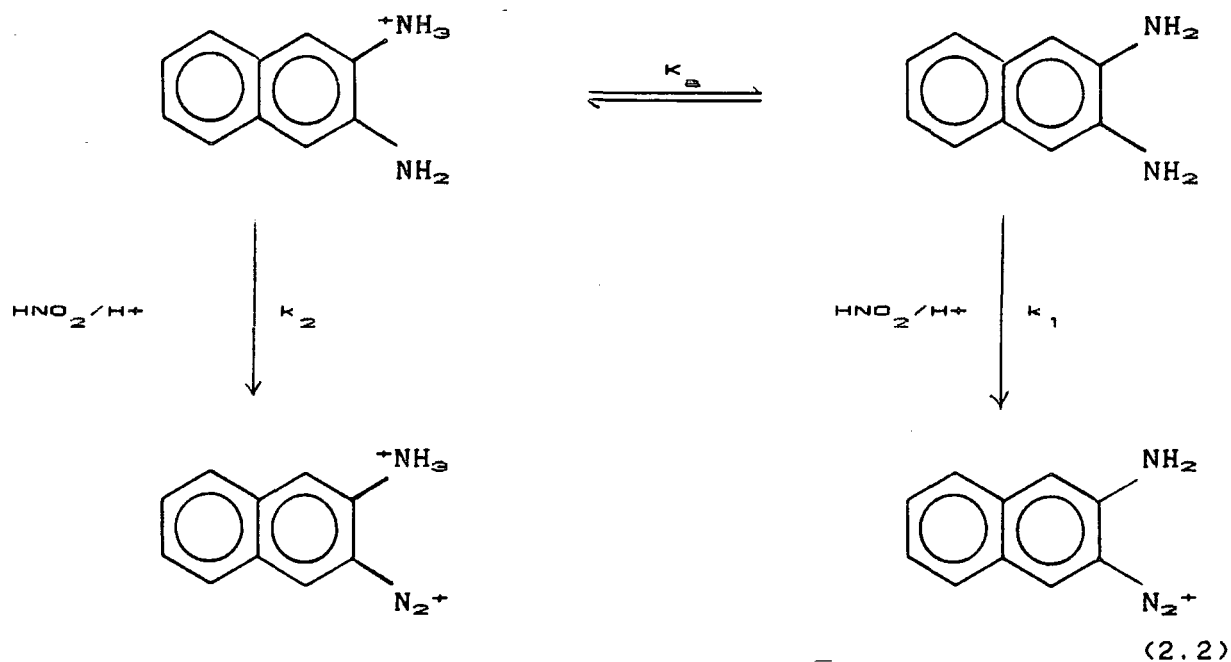
$10^3 [\text{DAN}]/\text{mol dm}^{-3}$	$[\text{HClO}_4]/\text{mol dm}^{-3}$	k_o/s^{-1}
2.00	0.200	0.055
2.00	0.400	0.089
2.00	0.500	0.098
2.00	0.600	0.129
2.00	0.800	0.161
2.00	1.00	0.192
2.00	1.80	0.340
1.00	1.00	0.066
3.00	1.00	0.240
4.00	1.00	0.323

Graph 2.1

Dependence of k_o on $[HClO_4]$ and on $[DAN]$



The dependence on acidity is characteristic of a reaction occurring simultaneously via the protonated and unprotonated forms. The scheme for such a reaction is shown in equation (2.2).



The expression for k_o from such a scheme is given in equation (2.3) where k_1 and k_2 are third-order rate coefficients, defined as $\text{rate} = k_1 [HNO_2] [H^+] [\text{unprotonated form}]$ and $\text{rate} = k_2 [HNO_2] [H^+] [\text{protonated form}]$. $[S]_T$ is the total stoichiometric concentration of DAN and K_a is the dissociation constant of the protonated form. The pK_a used in this analysis was taken as 3.90^{10} , although other literature values of 2.1^{11} and 3.99^{12} have been reported.

$$k_o = k_1 K_a [S]_T + k_2 [H^+] [S]_T \quad (2.3)$$

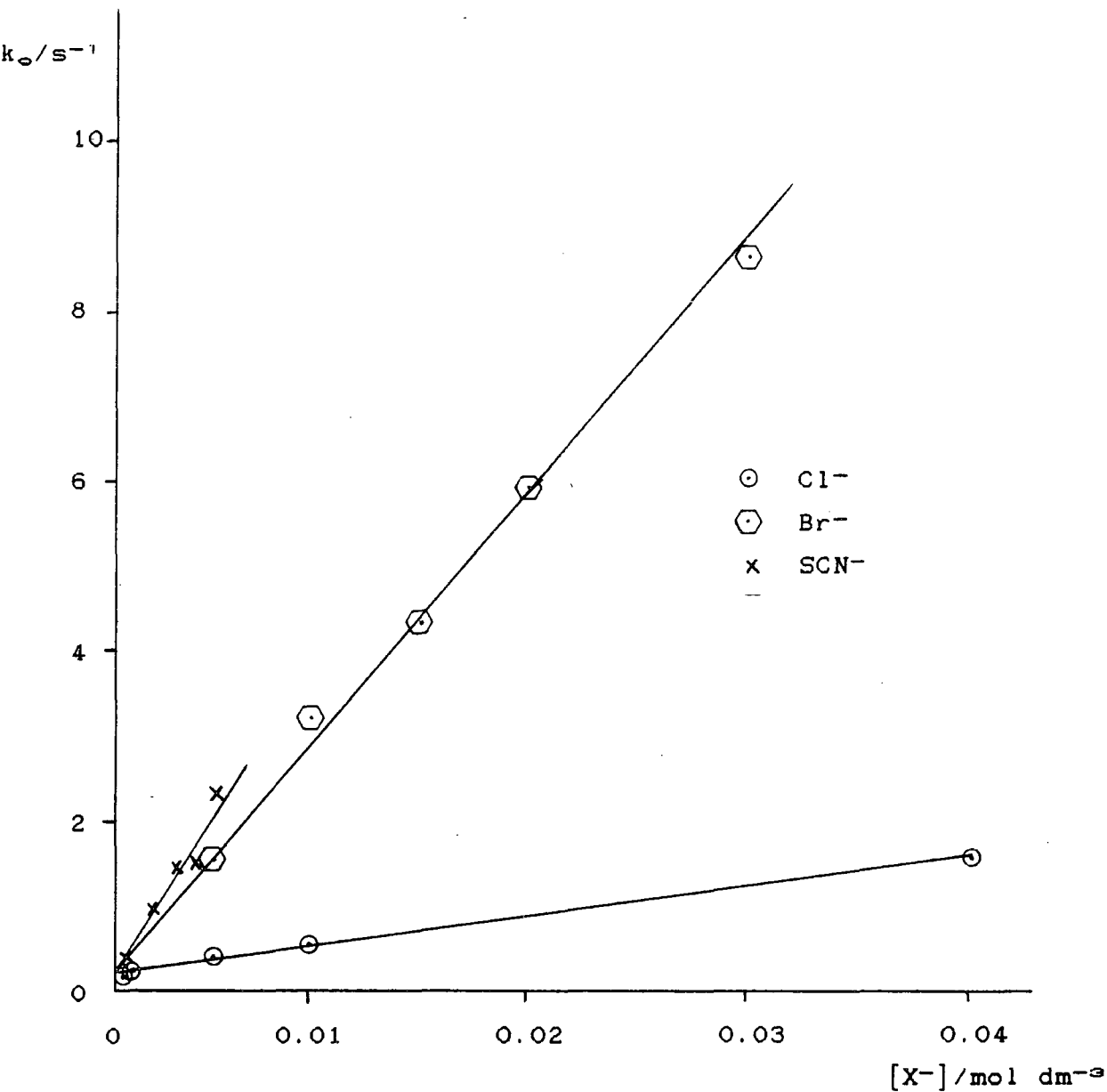
The values of k_1 and k_2 obtained were 7.5×10^4 and $86 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$ respectively. Thus, as expected the unprotonated species is the more reactive form. The value of k_1 is ~ 10 times greater than the generally assumed¹³ limit for a diffusion-controlled reaction.

It has been observed that the values of rate constants for reactive species tend to a limit, taken to be the encounter-controlled limit. For neutral substrates, it is $\sim 7000 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$ at 25°C ; for anionic substrates, it is $2000 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$ at 0°C ($11\ 700 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$ at 25°C for comparison) due to additional electrostatic factors; for a doubly charged anion, the limit is $\sim 18\ 000 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$.

A number of reactions e.g. nitrosation of certain substrates¹⁴⁻¹⁹, nitration and halogenation under certain conditions, involve small concentrations of very reactive intermediates, whose reactions may be subject to diffusion control, even though the overall reaction is relatively slow. In solution, two solute molecules coming together are effectively held within a cage of solvent molecules and would make a number of collisions with each other in this cage. This set of repeated collisions has been termed encounter¹⁴. Because the solvent molecules are themselves subject to diffusion, the life-time of each encounter is very short: $10^{-10} - 10^{-9} \text{ s}$ ¹⁵. If the reaction has a low activation energy, the probability of reaction at each collision approaches unity. In the limit then, the rate of encounter rather than the calculated rate of collision would be the limiting rate, since only the first collision of every encounter contributes to the reaction rate.

Graph 2.2

Dependence of k_o on $[X^-]$ in the diazotisation of DAN.



The value of k_1 above is crucially dependent on the determined K_a value and on the measurement of a relatively small intercept of course. Nevertheless, the indications are that the unprotonated form of DAN reacts with NO^+ or H_2NO_2^+ on encounter. The electron withdrawing 2- N^+H_3 substituent reduces the intrinsic reactivity by a factor of about 800.

The catalytic effect of added nucleophiles ($\text{X}^- = \text{Cl}^-$, Br^- and SCN^-) on the nitrosation of DAN has also been investigated. The results are given in table (2.2).

Table 2.2

Catalysis by Cl^- , Br^- and SCN^- in the diazotisation of DAN with nitrous acid in aqueous perchloric acid.

$$[\text{DAN}] = 2.0 \times 10^{-3} \text{ mol dm}^{-3}$$

$$[\text{HNO}_2] = 1.0 \times 10^{-4} \text{ mol dm}^{-3}$$

$$[\text{HClO}_4] = 1.0 \text{ mol dm}^{-3}$$

$[\text{Cl}^-]/\text{mol dm}^{-3}$	k_o/s^{-1}	$[\text{Br}^-]/\text{mol dm}^{-3}$	k_o/s^{-1}	$[\text{SCN}^-]/\text{mol dm}^{-3}$	k_o/s^{-1}
0.00050	0.215	0.0050	1.53	0.00050	0.364
0.0010	0.244	0.010	3.19	0.0020	0.982
0.0050	0.374	0.015	4.38	0.0030	1.46
0.010	0.527	0.020	5.95	0.0040	1.51
0.040	1.65	0.030	8.68	0.0050	2.28

The effect of added nucleophiles was found to be similar to that encountered for N-nitrosation and diazotisation: the reaction was catalysed with the expected efficiency of $\text{SCN}^- > \text{Br}^- > \text{Cl}^-$. In each case the plot of k_o vs $[\text{X}^-]$ is linear, with a common intercept (within the experimental error), which represents the uncatalysed reaction.

The full expression for k_o is now given by equation (2.4), where K_{XNO} is the equilibrium constant for XNO formation, and k_3 and k_4 are the second-order rate coefficients of XNO reaction with the unprotonated and protonated forms of DAN respectively, and k_1 and k_2 are as previously defined for the uncatalysed reaction.

$$k_o = k_1 K_{\text{D}} [\text{S}]_{\text{T}} + k_2 [\text{H}^+] [\text{S}]_{\text{T}} + k_3 K_{\text{D}} K_{\text{XNO}} [\text{X}^-] [\text{S}]_{\text{T}} + k_4 K_{\text{XNO}} [\text{X}^-] [\text{H}^+] [\text{S}]_{\text{T}} \quad (2.4)$$

The results of the acidity dependence for the X^- catalysed diazotisation of DAN for all three nucleophiles are given in table (2.3).

Table 2.3

Acidity dependence for the X^- catalysed diazotisation of DAN with nitrous acid.

$$[\text{DAN}] = 2.0 \times 10^{-3} \text{ mol dm}^{-3}$$

$$[\text{HNO}_2] = 1.0 \times 10^{-4} \text{ mol dm}^{-3}$$

$[\text{Cl}^-] = 0.04 \text{ mol dm}^{-3}$		$[\text{Br}^-] = 0.03 \text{ mol dm}^{-3}$		$[\text{SCN}^-] = 0.005 \text{ mol dm}^{-3}$	
$[\text{H}^+]/\text{mol dm}^{-3}$	k_o/s^{-1}	$[\text{H}^+]/\text{mol dm}^{-3}$	k_o/s^{-1}	$[\text{H}^+]/\text{mol dm}^{-3}$	k_o/s^{-1}
0.100	0.538	0.200	3.30	0.100	0.608
0.300	0.739	0.300	3.87	0.300	1.07
0.500	1.06	0.500	5.22	0.500	1.49
0.700	1.25	0.700	6.61	0.700	1.98
1.00	1.65	1.00	8.68	1.00	2.28

Again it is clear that reactions via XNO also occur simultaneously via the protonated and unprotonated forms of DAN.

Having obtained the value of k_1 and k_2 , the values of k_3 and k_4 can be calculated from the values of the slopes and intercepts of plots of k_o vs $[\text{H}^+]$ in the presence of a constant $[\text{X}^-]$. The deduced values of k_3 and k_4 for all three XNO species are given in table (2.4) (again assuming $pka = 3.90$).

Graph 2.3

Dependence of k_0 on $[H^+]$ for the X^- catalysed diazotisation of DAN.

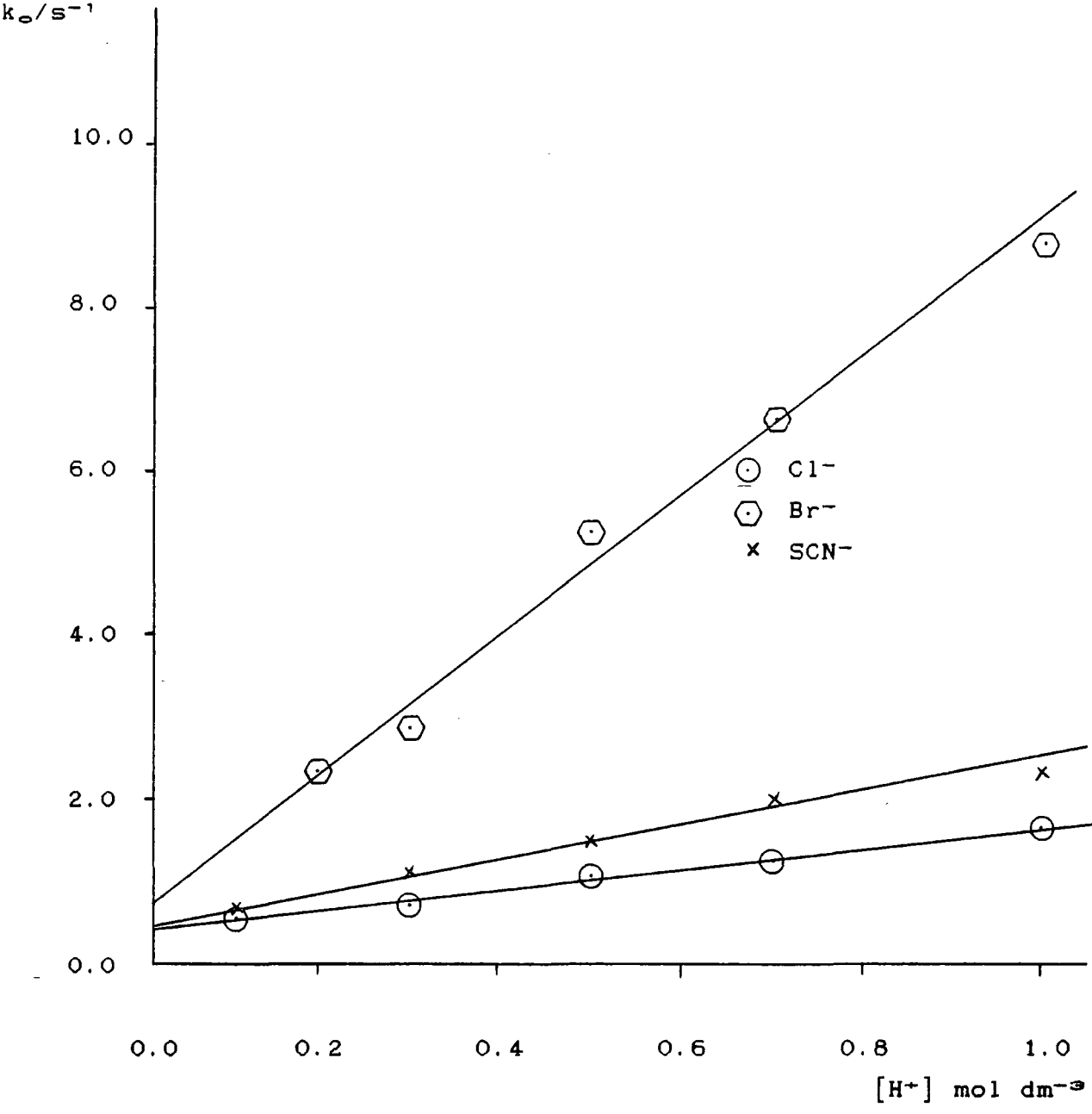


Table 2.4

Values of the derived second-order rate coefficients k_3 and k_4 for the reaction of XNO with the unprotonated and protonated forms of DAN.

<u>XNO</u>	<u>$k_3/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>	<u>$k_4/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>
ClNO	3.4×10^{10}	1.2×10^7
BrNO	4.8×10^9	2.2×10^6
ONSCN	2.2×10^6	6.5×10^3

The unprotonated form of DAN is clearly significantly more reactive towards all XNO species as expected; the reaction of ClNO probably occurs at the encounter. The well established²⁰ reactivity trend ClNO > BrNO > ONSCN is evident for both forms of DAN.

As an internal consistency check, the slopes and intercepts of the k_5 vs $[X^-]$ plots can be calculated from the values of k_1 , k_2 , k_3 and k_4 obtained. The results, shown in table (2.5) confirm the agreement within the experimental error between the calculated and observed values.

Table 2.5

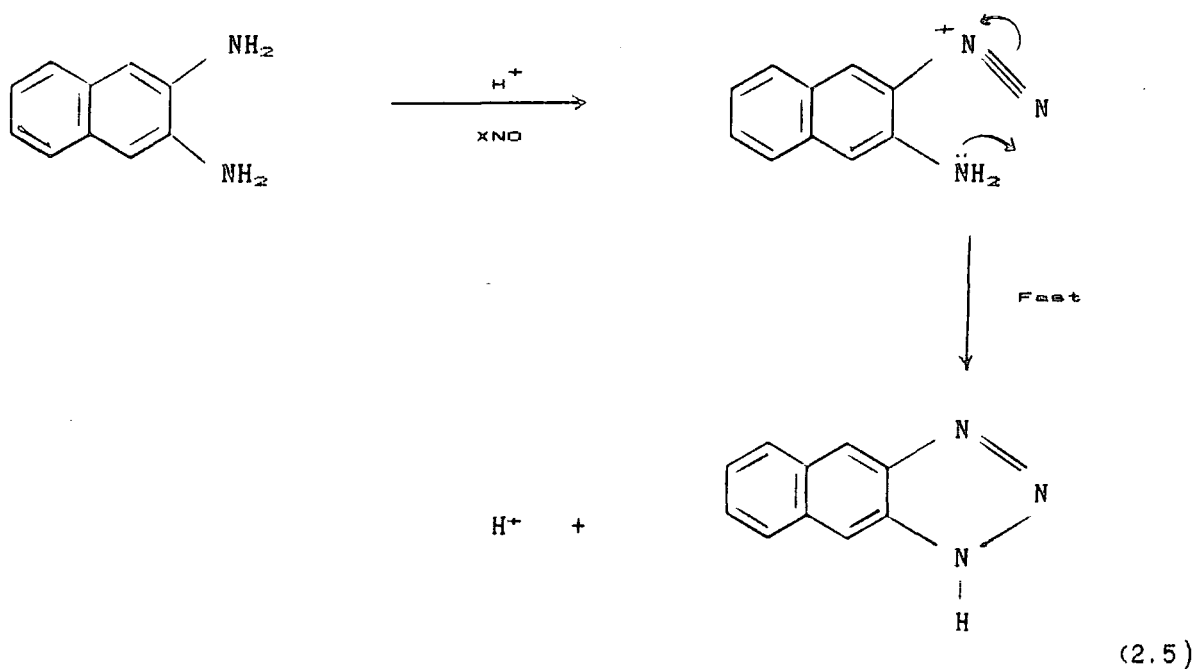
Calculated and observed slopes and intercepts of plots of k_{D} vs $[X^-]$ for the catalysed diazotisation of DAN.

X^-	Slope/dm ³ mol ⁻¹ s ⁻¹		Intercepts/s ⁻¹	
	Calc ^d	Obs ^d	Calc ^d	Obs ^d
Cl ⁻	36	36	0.19	0.19
Br ⁻	290	280	0.19	0.21
SCN ⁻	490	430	0.19	0.15

2.3 Discussion

The rate coefficients for diazotisation of aniline^{6,7} and 1-naphthylamine^{8,9} are, as expected, quite similar for a range of nitrosating agents, with the values for ClNO and BrNO approaching the diffusion limit. The present results for DAN show that the rate coefficients for the unprotonated form show only small changes (compared with aniline) arising from the effects of the ortho-NH₂ group, whereas the reactivity of the protonated form is much reduced and is consistent with reaction at the free NH₂ group, modified significantly by the presence of the powerfully electron-withdrawing ortho-NH₃⁺ group.

All of the results presented are consistent with the rate-limiting formation of the diazonium ion (N-nitrosation followed by rapid proton transfer and loss of a water molecule), which undergoes a rapid cyclisation process as outlined in equation (2.5) c.f. o-phenylenediamine. A similar scheme can be written for reaction of the protonated form of DAN.



The fraction of reaction via the protonated and unprotonated forms at different acidities are shown in table (2.6).

Table 2.6

Concentrations of protonated and unprotonated form of DAN at different acidities.

<u>[HClO₄]/M</u>	<u>[Unprotonated DAN]/M</u>	<u>[Protonated DAN]/M</u>
0.001	2.24 x 10 ⁻⁴	1.77 x 10 ⁻³
0.01	2.49 x 10 ⁻⁵	1.98 x 10 ⁻³
0.10	2.52 x 10 ⁻⁶	1.99 x 10 ⁻³
1.0	2.52 x 10 ⁻⁷	1.99 x 10 ⁻³

It is clear that at $[\text{HClO}_4] > 0.01\text{M}$ most of the reaction proceeds via the protonated form of DAN.

At the pH at which DAN is used as an analytical agent i.e. in biological systems, calculated k_o for the nitrosation is slower as can be seen in table (2.7).

Table 2.7

k_o for the nitrosation of DAN at pH values used in typical biological systems.

$$[\text{DAN}] = 2.0 \times 10^{-3} \text{ mol dm}^{-3}$$

<u>pH</u>	<u>k_o/s^{-1}</u>
6.5	1.89×10^{-2}
7.0	1.89×10^{-2}
7.5	1.89×10^{-1}

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CHAPTER 3

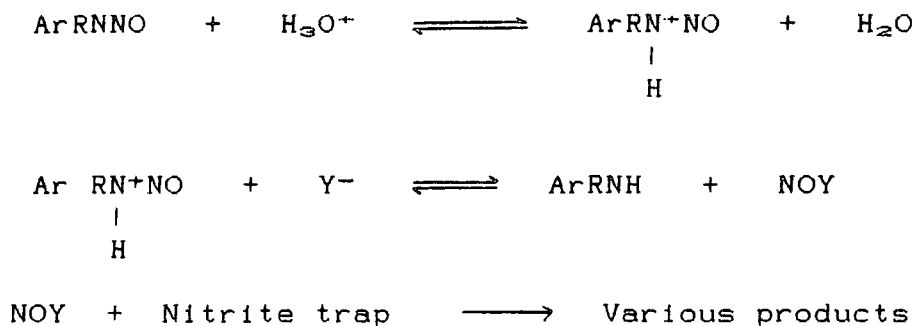
Denitrosation reactions

3.1 Introduction

Denitrosation of N-nitroso compounds is usually carried out for the purpose of analysis and characterisation of the compounds on the destruction of potentially dangerous materials including the detoxification of residues before disposal. This is an important feature in some areas of the chemical industry where quantities of nitrosamines are sometimes produced as by products. Health and safety requirements necessitate that potentially carcinogenic materials of this kind are not exposed to the general public.

The requirements of the denitrosation process include: firstly, the completion of the process within a reasonable time period; and secondly, the recovery of the preferably non-toxic product should be as complete as possible. Mild conditions are favoured so that all products may be disposed of readily. This will also minimise side reactions such as the acid-catalysed transnitrosation to another co-extracted amine receptor, for example, in the detection of the N-nitroso compounds in food products.

It is well known that aliphatic nitrosamines can be almost quantitatively converted into the corresponding secondary amines and nitrous acid (or nitrosyl chloride) by boiling with hydrochloric acid¹. Denitrosation of aromatic N-nitrosamines in aqueous acid solution has been examined mechanistically and has been shown to occur by the rate-determining attack of a nucleophile (for example Cl^- , Br^- , I^- , SCN^- and $\text{CS}(\text{NH}_2)_2$) at the nitroso-nitrogen atom as shown in scheme (3.1) below^{2, 10}.



Scheme (3.1)

Denitrosation is usually reversible but the reverse reaction can be prevented by the addition of sufficient nitrite trap such as sulphamic acid, sodium azide and hydrazoic acid¹¹.

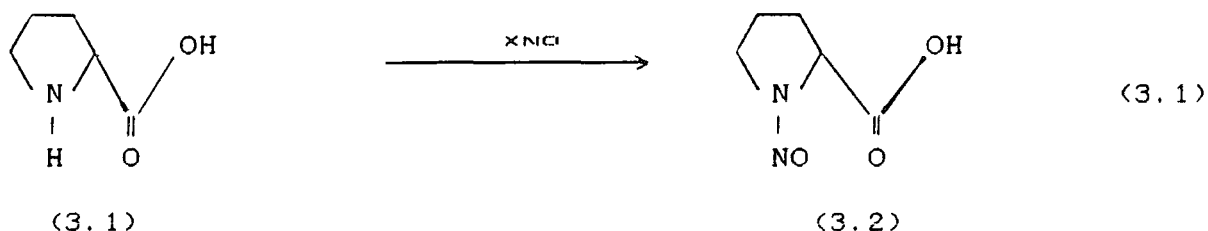
The denitrosation of heterocyclic nitrosamines however, has not been studied extensively³. The common amino acid, proline, was the heterocycle chosen in this case. Although N-nitrosoproline (NNP) does not seem to be carcinogenic⁴, there has been an interest in nitrosamines formed from amino acids in general, with nitrite under mildly acidic conditions⁵ for example, in the stomach⁶, because of their carcinogenic properties.

NNP denitrosation was carried out in various solvents including water, ethanol, acetic acid and acetonitrile, using various catalysts and nitrite traps.

The denitrosation of NNP was contrasted to that of N-nitrososarcosine, an acyclic amine which also contains a β -carbonyl group, N-nitrosopyrrolidine, a heterocycle without a β -carboxyl group, as well as dimethylnitrosamine, a typical aliphatic amine.

3.2 Denitrosation of N-nitrosoproline in various solvents

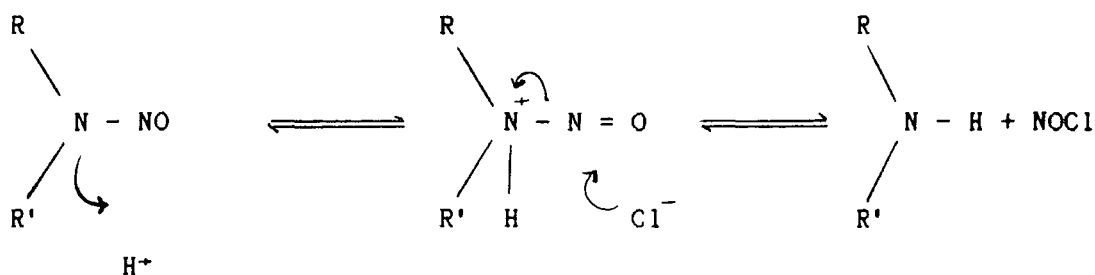
Proline (P) (3.1) is an amino acid which occurs in most proteins, and as such, is a model compound for nitrosation studies of peptides and proteins. As a secondary amine, when nitrosated⁷, it gives rise to stable N-nitrosoproline (equation (3.1)) (3.2).



The nitrosation of proline has been studied in many cases^{7,8}. The denitrosation of NNP however, has not been studied kinetically.

Classically, the nitrosation of a secondary amine by nitrous acid has been considered to be reversible. Hence, in the presence of a strong acid, protonation of the nitrogen atom from the parent secondary amine should occur, with the elimination of the nitrosyl group, in the reverse denitrosation process.

Scheme (3.2) illustrates this process, with the nitrosyl group combining with the chloride anion.

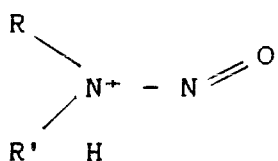


R, R' = alkyl groups

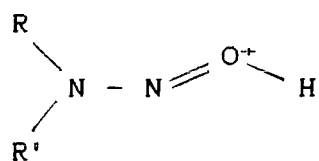
Scheme (3.2)

In general, the equilibrium lies well over to the nitrosamine side so that denitrosation does not occur readily, unless the nitrous acid is removed from the solution by the use of nitrite traps, such as sulphamic acid and sodium azide.

As the reactions are acid catalysed, the protonated form of the nitrosamine is clearly the reactive species. There is some uncertainty as to which of two protonated species (3.3) and (3.4) due to protonation at the amino nitrogen atom and at the nitroso oxygen atom respectively, is dominant.

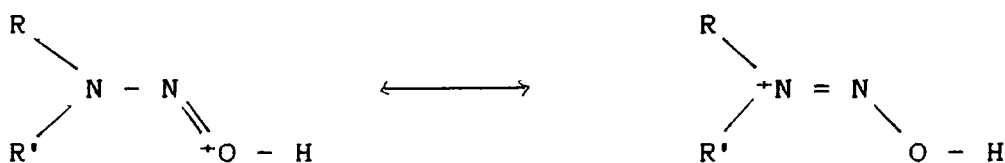


(3.3)



(3.4)

Physical evidence favours the O-protonation species. N.m.r. studies have shown that in strong acids such as 'magic acid', the two methyl groups of nitrosodimethylamine are non-equivalent, which implies no free rotation about the N-N bond¹². Calculations also favour the O-protonated form as the more stable¹³. Furthermore, there is resonance stabilisation in the O-protonated form (3.5).



(3.5)

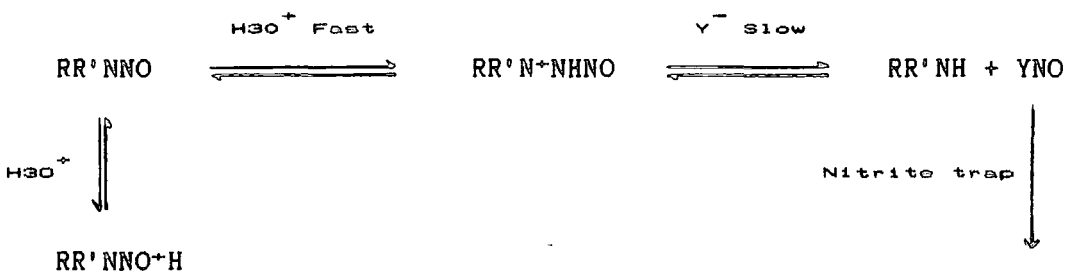
Thus, the N-protonated form will exist in lower concentrations. However, as the principle of microscopic reversibility requires that the same mechanism occurs in the reverse reaction, denitrosation is believed to occur via the N-protonated form.

Denitrosation is also found to occur more readily in the presence of added nucleophiles (for example Cl^- , Br^- , I^- , SCN^- and $\text{SC}(\text{NH}_2)_2$).

Because this reaction does not proceed rapidly in water, the use of other solvents has been investigated. The first successful denitrosation of a N-nitrosamine in a solvent other than water, was accomplished by Eisenbrand and Preussmann in glacial acetic acid¹⁴. Since then, many other solvents have been used, such as ethanol¹⁵. In this case, the denitrosation of N-methyl-N-nitrosoaniline (NMNA) was carried out, in ethanol-containing hydrogen chloride, in the absence of a nitrite trap. The solvents we have used also include ethanol, 80% aqueous acetic acid and acetonitrile.

3.2.1 Denitrosation of NNP in water

The denitrosation of several compounds have been studied in aqueous acid solution^{9, 10, 16}. In the presence of nitrite traps (with concentrations exceeding a minimum threshold so that the reaction is zero order with respect to the trap) and with added nucleophiles, all the kinetic results, including a kinetic isotope effect $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} \sim 0.3$, are consistent with the mechanism set out in scheme (3.3).



Scheme (3.3)

NNP was prepared by the method used by Lijinsky *et al*¹⁷ (see chapter 7). The reactions were carried out at 25°C, by following the decrease in absorbance at 350nm due to NNP, using a conventional spectrophotometer. At this wavelength, the absorbance due to other components was negligible. Only the most powerful nucleophile, thiourea, was found to be effective as a catalyst. Very little detectable reaction occurred even in the presence of quite high concentrations of Br⁻ and SCN⁻. The concentration of the sulphuric acid used ranged from 1M - 6M. The reaction did not proceed without a trap: sulphamic acid was used.

The result of varying the acid concentration is shown in table (3.1). The reactions showed good first-order behaviour and each value of k_o , the observed rate constant, is an average of four runs. Because such high acid concentrations are used, k_o is plotted against the acidity function, and not the acid concentration. The plot of k_o vs $h_o/(K_m + h_o)$ is shown in fig.(3.1) (see scheme (3.4), where K_a is the acid dissociation constant for protonated thiourea and H_o is the acidity function based on Hammett indicators¹⁸).

Table 3.1

Acidity dependence for the denitrosation of NNP

[Thiourea] = 0.1M
 [NNP] = 0.01M
 [Sulphamic Acid] = 0.2M

<u>[H₂SO₄]/M</u>	<u>h₀^{1.8}</u>	<u>10⁵ k₀/s⁻¹</u>
0.8	1.6	9.34
1.6	4.0	27.9
2.4	11.8	62.1
3.2	30.0	92.2
4.0	69.0	119.0
4.8	160.0	138.0

Table 3.2

[SC (NH₂)₂] dependence for the denitrosation of NNP

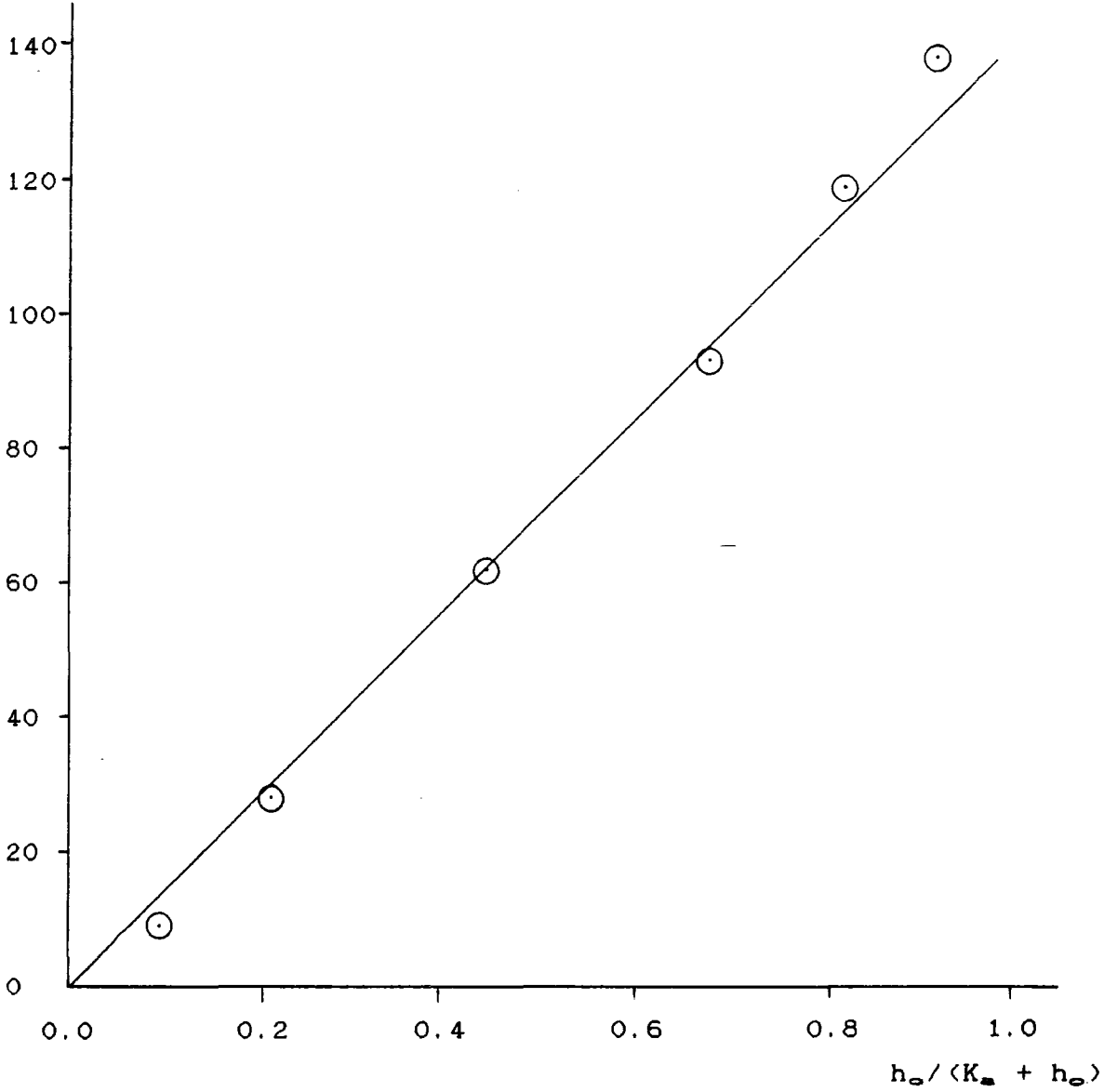
[H₂SO₄] = 3.2M
 [NNP] = 0.01M
 [Sulphamic Acid] = 0.1

<u>[SC (NH₂)₂]/M</u>	<u>10³ k₀/s⁻¹</u>
0.05	0.342
0.10	0.869
0.15	1.44
0.20	1.67
0.25	2.01
0.30	2.43
0.35	3.13
0.40	3.80
0.45	4.07

Figure 3.1

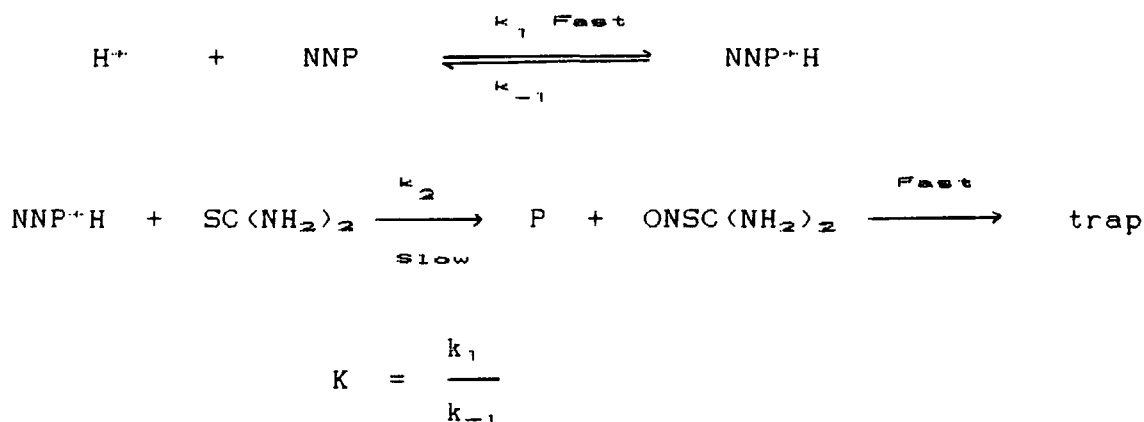
Acidity dependence for the denitrosation of NNP.

$10^5 k_o/s^{-1}$



Varying the concentration of thiourea also yields a straight line when k_o is plotted against $[SC(NH_2)_2]$ (see table (3.2)).

The expected outline mechanism is set out in scheme (3.4):



Scheme (3.4)

The results are consistent with the rate equation (3.3):

$$\text{Rate} = k_2 [NNP^+H] [SC(NH_2)_2] \quad (3.2)$$

$$= k_2 K [NNP] h_o [SC(NH_2)_2] \quad (3.3)$$

Remembering that $[SC(NH_2)_2]_{\text{Total}} = [SC(NH_2)_2] + [HSC^+(NH_2)_2]$ and substituting acidity functions for acid concentrations yields equation (3.4).

$$\text{Rate} = k_2 K [NNP] h_o [SC(NH_2)_2]_{\text{Total}} \frac{K_a}{(K_a + h_o)} \quad (3.4)$$

The expression for k_o is then given in equation (3.5):

$$k_o = k_2 K h_o [\text{SC}(\text{NH}_2)_2]_{\text{T}} \frac{K_a}{(K_a + h_o)} \quad (3.5)$$

The slope of the plot of k_o vs $h_o/(K_a + h_o)$ gives a value of $1.47 \times 10^{-3} \pm 7.46 \times 10^{-5} = k_2 K K_a [\text{Thio}]_{\text{Total}}$. Thus the value for $k_2 K K_a$ obtained from this plot is 0.014. The slope of the plot of k_o vs $[\text{CS}(\text{NH}_2)_2]$ gives a value of $8.75 \times 10^{-3} \pm 5.15 \times 10^{-5} = k_2 K K_a h_o / (K_a + h_o)$ so that the value for $k_2 K K_a$ obtained from this plot = 0.013 which is in reasonable agreement with the above value.

The denitrosation of NNP therefore appears to follow a similar mechanism to that of aromatic N-nitrosamines^{9,10}: a rate-determining nucleophilic attack by thiourea on the protonated form of the nitrosamine. This behaviour has also been observed for the N-nitroso derivative of the amino acid tryptophan, N-acetyl-N-nitrosotryptophan, at low acidities¹⁴.

3.2.2 Denitrosation in ethanol

Ethanol is a less polar solvent than water, with a dielectric constant of 24.3 compared to that of water, 78.4. The kinetics of denitrosation of N-nitrosamines in ethanol have been studied previously. The mechanism seems to be different from the denitrosation reaction in water. There is now an absence of kinetic nucleophilic dependence. This lack of nucleophile dependence has also been found for the denitrosation of nitrosamides¹⁹, a nitrososulphonamide⁹ and nitrosoureas^{20,21}.

An explanation which covers these observations²⁰ retains the outline mechanism given in scheme (3.3) but the rate-limiting step now, is the protonation of the nitrosamine, rather than the attack of the nucleophile Y⁻. This is of course more likely in less polar solvents and for nitrosamines containing electron withdrawing groups.

The reaction conditions used were similar to those used with water as solvent. Ethanol was dried using the magnesium method. Thiourea was the nucleophile used in this experiment once again. The trap used in this case was ascorbic acid, at concentrations > 0.01M when the reaction was zero-order with respect to ascorbic acid. Good first-order behaviour was once again observed, with the first-order rate constants reproducible to ± 4%. The result of varying the concentration of sulphuric acid used is shown in table (3.3).

Table 3.3

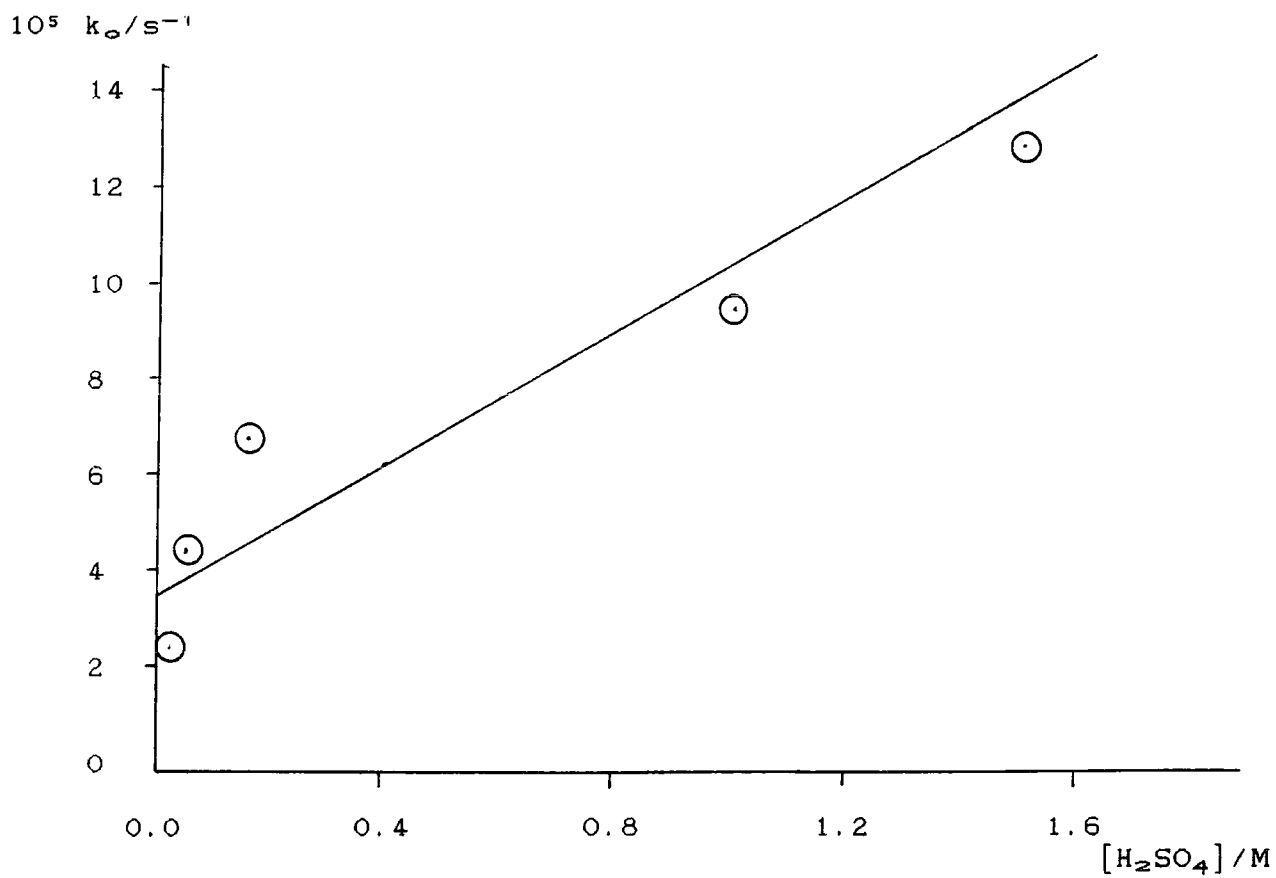
Acidity dependence for the denitrosation of NNP in ethanol

[Thiourea]	=	.025M
[NNP]	=	.005M
[Ascorbic Acid]	=	.02M

<u>[H₂SO₄]/M</u>	<u>10⁵ k_o/s⁻¹</u>
0.25	2.36
0.50	4.60
0.75	6.90
1.0	9.54
1.5	13.0

Figure 3.3

Acidity dependence for the denitrosation of NNP in ethanol



The plot of the observed rate constant, k_o vs acid concentration gives a straight line.

The dependence on thiourea concentration is shown in table (3.4). Here, we observe no nucleophile dependence.

Table 3.4

Nucleophile dependence for the denitrosation of NNP in ethanol

[H₂SO₄] = 1M
 [NNP] = .005M
 [Ascorbic Acid] = .02M

<u>[Thiourea]/M</u>	<u>10⁵ k_o/s⁻¹</u>
.01	7.90
.025	8.85
.04	9.08
.05	8.26

The lack of dependence on the concentration of the nucleophile suggests either that the loss of NO⁺ is a unimolecular unassisted process, or that the rate-determining step now precedes nucleophilic attack. It is thought that the latter is the more likely explanation. The mechanism differs from scheme (3.4) in k₁ being the slow step and k₂ being the fast step. The rate constant derived from such a mechanism is shown in equation (3.6).

$$k_o = k_1 [H^+] \quad (3.6)$$

As mentioned, a similar mechanism was observed for nitrosamides and nitrososulphonamides. This is due to the presence of electron-withdrawing substituents >=O and -SO_2 which increases the value of k_2 . Also, in a less polar solvent, a large increase in k_2 (where charged species are involved) occurs, whereas only a small increase in k_{-1} occurs. Hence the limit $k_2[\text{Y}^-] \gg k_{-1}$ can be achieved in ethanol, with a powerful nucleophile such as thiourea, resulting in zero-order dependence on $[\text{Y}^-]$.

Further evidence for this mechanism comes from kinetic isotope effects. The results, using EtOD instead of EtOH as solvent are given in table (3.5)

Table 3.5

Kinetic isotope effect for the denitrosation of NNP in ethanol

[Ascorbic Acid] = .05M
 [NNP] = .005M
 $[\text{CS}(\text{NH}_2)_2]$ = .025M

<u>[Sulphuric Acid]</u>	<u>$10^5 k_{\text{EtOH}}$</u>	<u>$10^5 k_{\text{EtOD}}$</u>	<u>$k_{\text{EtOH}}/k_{\text{EtOD}}$</u>
0.5	4.60	2.61	1.76
1.0	9.54	6.04	1.58
1.5	13.0	8.07	1.61

Values of k_{E+OH}/k_{E+OD} lie in the range 1.5 - 1.7, supporting the rate-limiting proton transfer. This is in contrast to the kinetic isotope effect (ca 0.3) which has been observed for the denitrosation of aromatic nitrosamines in water when nucleophilic attack at the protonated nitrosamine is rate-limiting.

3.2.3 Denitrosation in 80% acetic acid

As this solvent was found to be effective in nitrosation²² reactions, it was thought appropriate to investigate the denitrosation process in this medium.

Once again the reaction was studied by following the disappearance of the absorbance at 340nm due to NNP. At concentrations greater than 0.05M, the reaction was zero order with respect to added trap, which was sodium azide in this case. Both Br^- and $SC(NH_2)_2$ were used as catalyst. Reaction occurred quite readily in both cases. Good first-order behaviour was found for all reactions and the observed duplicate rate constant values agreed to within $\pm 4\%$. The dependence on nucleophile concentrations is shown in table (3.6).

Table 3.6

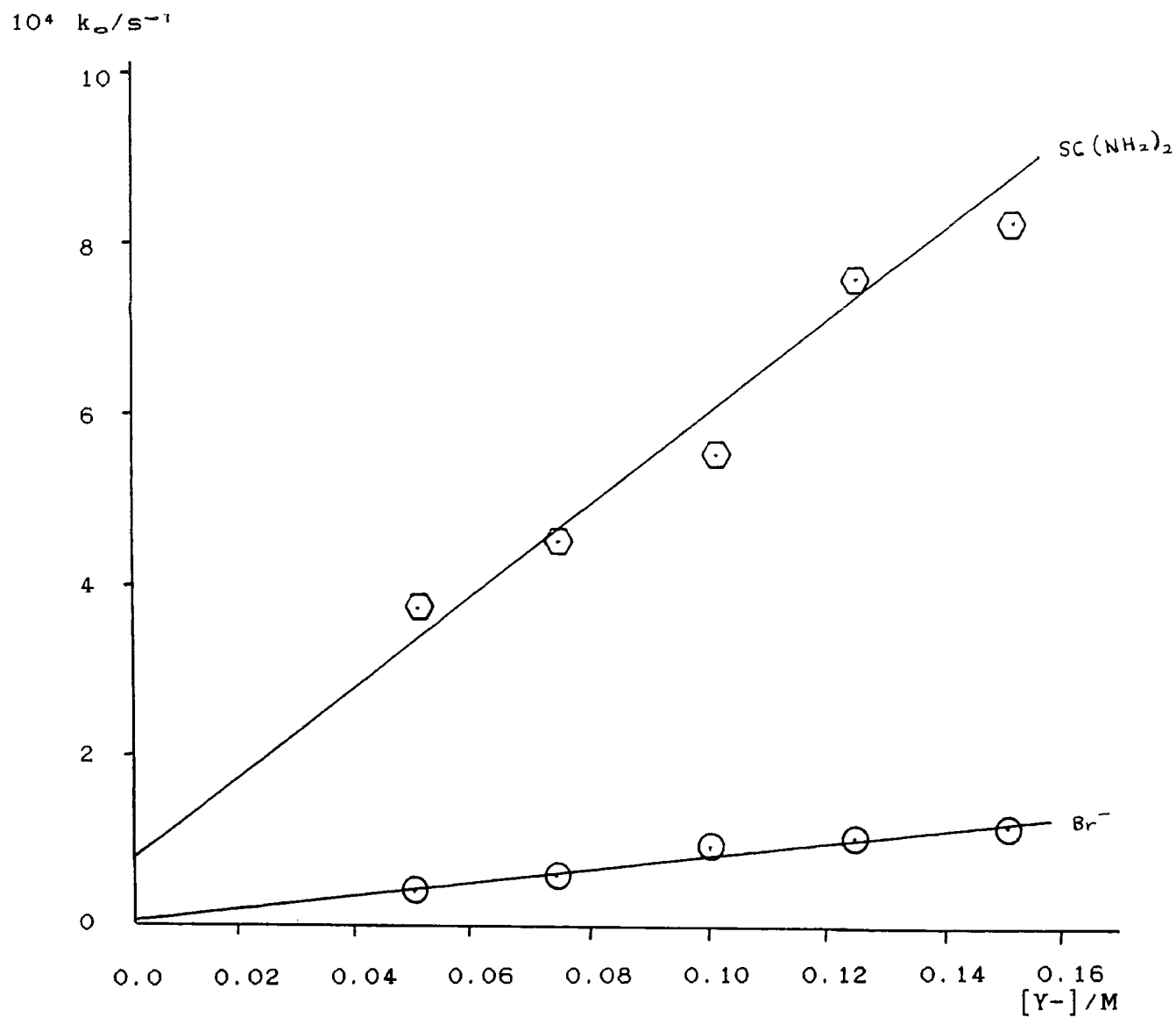
Nucleophile dependence for the denitrosation of NNP in 80% acetic acid

[NNP] = 0.005M
[N₃⁻] = 0.01M
[H₂SO₄] = 1M

<u>[Y⁻]</u>	<u>Br⁻</u> <u>10⁵ k_o/s⁻¹</u>	<u>SC(NH₂)₂</u> <u>10⁴ k_o/s⁻¹</u>
0.05	4.52	3.70
0.075	6.45	4.52
0.10	9.59	5.53
0.125	10.80	7.63
0.15	11.90	8.38

Figure 3.4

Nucleophile dependence for the denitrosation of NNP in 80% acetic acid



For the acidity dependence of this reaction, the results are given in table (3.7).

Table 3.7

Acid dependence of the denitrosation of NNP in 80% acetic acid.

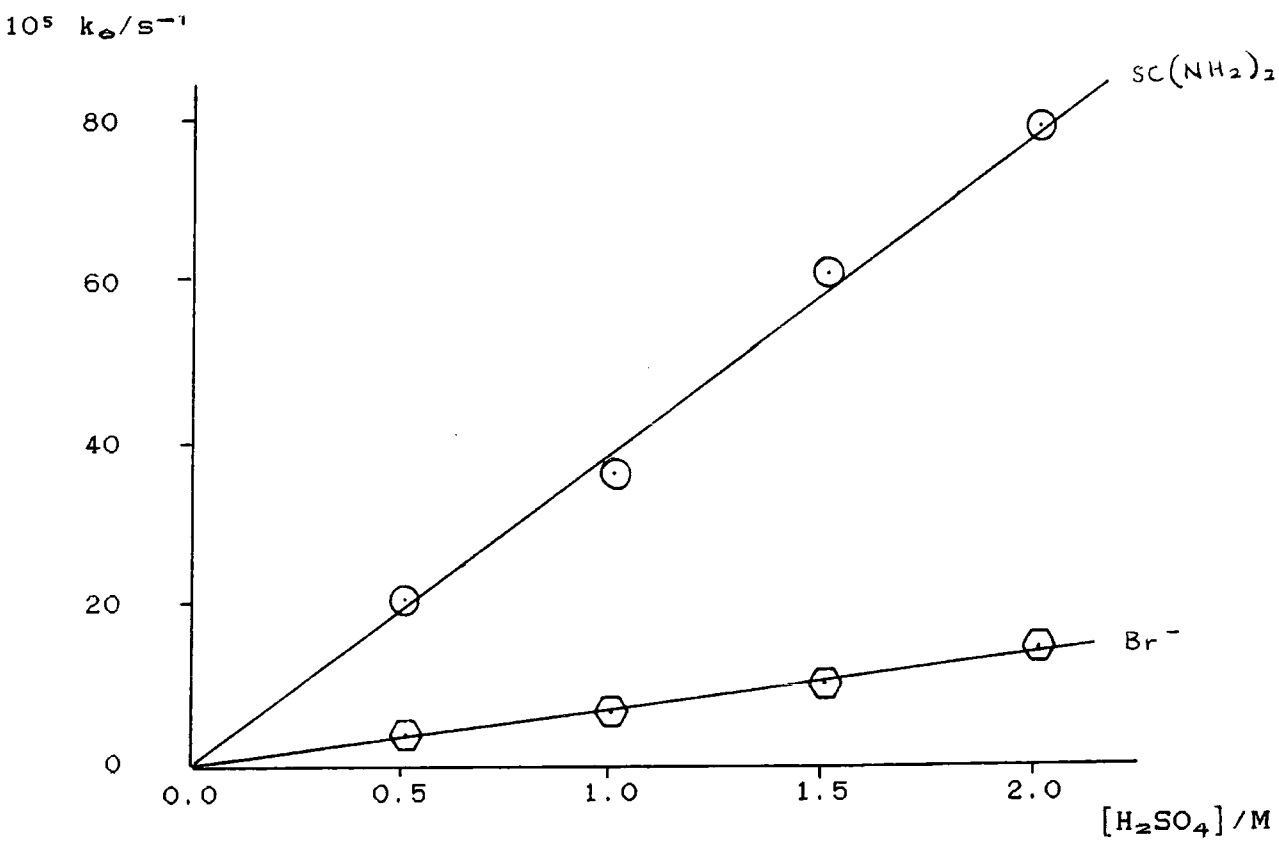
<u>[H₂SO₄]/M</u>	<u>[Br⁻]/M</u>	<u>[Thiourea]/M</u>	<u>10⁵ k_o/s⁻¹</u>
0.5	0.05	-	3.63
1.0	0.05	-	7.52
1.5	0.05	-	11.0
2.0	0.05	-	15.3
0.5	-	0.05	20.1
1.0	-	0.05	37.0
1.5	-	0.05	60.1
2.0	-	0.05	79.3

Plots of observed rate constants vs acid concentration and vs nucleophile concentrations are linear. Hence the expression $k_o = k_2 K h_o^{\times} [Y^-]$ given by the mechanistic scheme (3.3) applies here. NNP behaves in this solvent as it does in water. h_o^{\times} is not known, so absolute values of k_1, K cannot be calculated. However, a comparison of the values of k_2 can be made if we approximate $h_o^{\times} \approx h_o$.

For example for Br⁻, k_2 from the plot of k_o vs [Br⁻] is 7.21×10^{-9} ; k_2 from the plot of k_o vs [H₂SO₄] is $7.16 \times 10^{-9} \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$.

Figure 3.5

Acid dependence of the denitrosation of NNP in 80% acetic acid



It is worth pointing out that, as in water solvent, the proton transfer written in scheme (3.3) as occurring in one stage may be an oversimplification. It is unusual for proton transfers of this type to be rate-determining; it could well be that protonation occurs at the nitroso oxygen atom followed by a rearrangement to nitrogen and it is also possible that other intermediates may be present. Protonated nitrosamine species have never been positively identified, although there are indications that several structures survive at different acidities.

Acetic acid/water has proved to be slightly more effective solvent for denitrosation than water and ethanol.

3.2.4 Denitrosation in Acetonitrile

Acetonitrile has been found to be a very effective solvent for nitrosation using alkyl nitrites^{2,3}. Its use as a solvent for denitrosation is investigated here.

The reaction was followed at 360nm due to the disappearance of the reactant. The reaction occurred readily in the presence of thioglycolic acid (TGA) as a nucleophile but not ethanol. No added catalyst was required here. Direct nitrosation of nucleophiles by nitrosamines has been established^{1,6}, but is usually limited to fairly powerful nucleophiles and also to non-basic nucleophiles because of the fairly high acid concentrations required.

Good first-order behaviour was observed for all the reactions and the observed first-order rate constant for each reaction is an average of four runs. The acidity dependence of the reaction is shown in table (3.8).

Table 3.8

Acidity dependence for the denitrosation of NNP in acetonitrile.

[NNP] = 0.0025M

[TGA] = 0.1M

<u>[H₂SO₄]/M</u>	<u>10⁵k_o/s⁻¹</u>
0.10	2.95
0.125	3.54
0.20	6.59
0.30	8.88
0.40	10.8
0.55	17.1
0.60	18.7
0.85	24.1
0.90	27.5

A good straight line is obtained when k_o is plotted against [H₂SO₄]. Nucleophile dependence for this reaction is shown in table (3.9).

Figure 3.6

Acidity dependence for the denitrosation of NNP in acetonitrile.

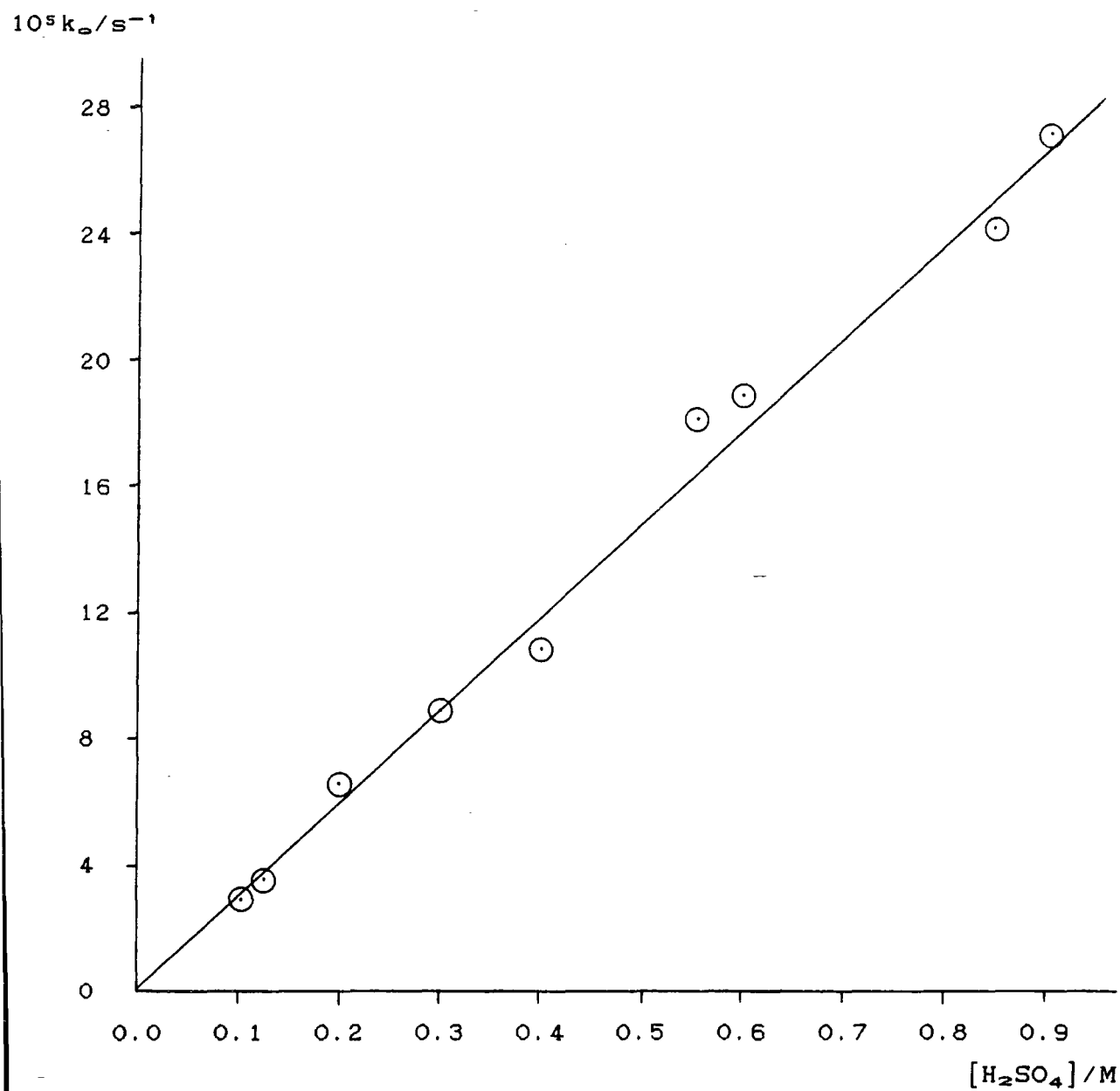


Table 3.9

Nucleophile dependence for denitrosation of NNP in acetonitrile

$$[\text{H}_2\text{SO}_4] = 0.2\text{M}$$

<u>[TGA]/M</u>	<u>$10^5 k_o / \text{s}^{-1}$</u>
0.005	0.69
0.025	3.74
0.050	6.00
0.075	6.05
0.10	6.03
0.15	6.19
0.20	6.15

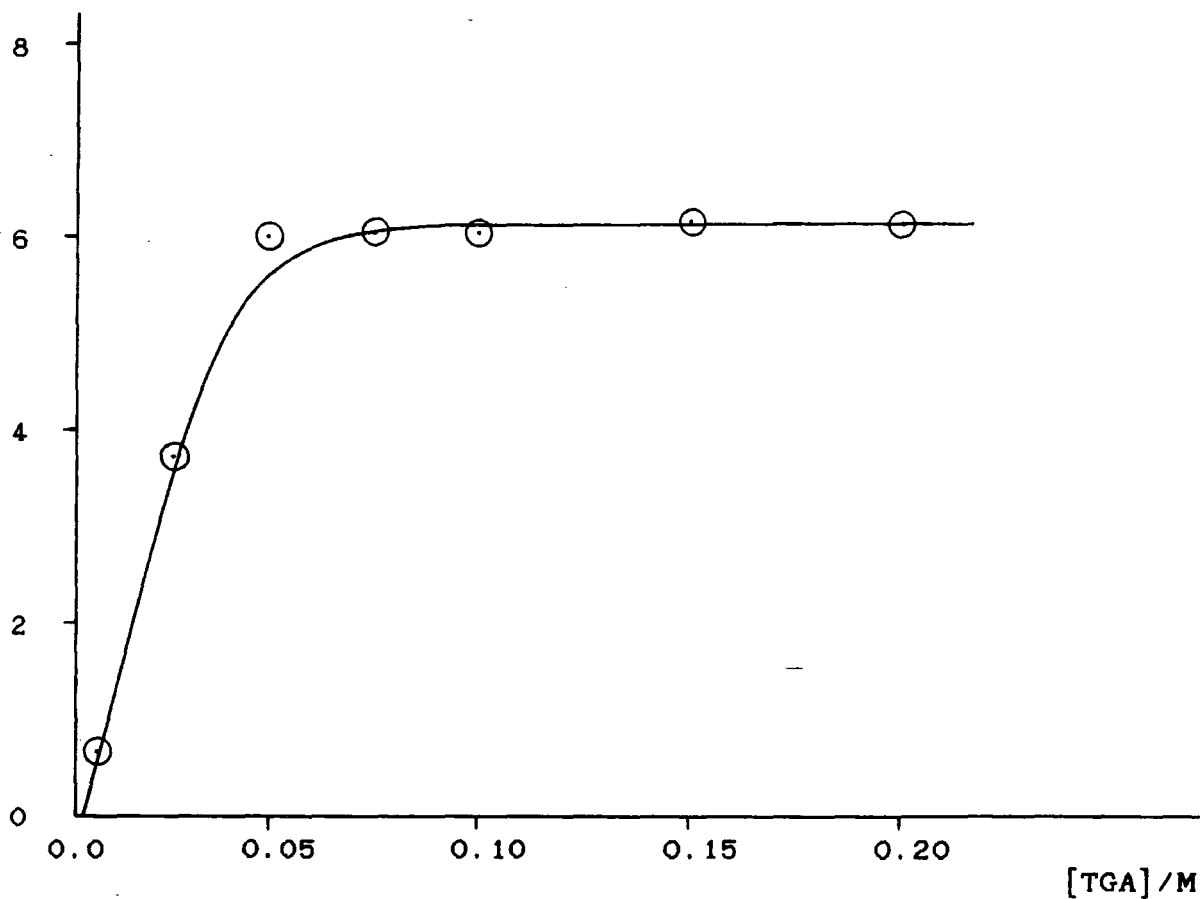
Hence, at low concentrations of TGA, the reaction is first-order with respect to TGA, but at high TGA concentrations, the reaction is zero-order with respect to TGA. This is an intermediate situation between that in water and that in ethanol. In terms of scheme (3.4), this situation occurs when $k_2[Y^-] \approx k_{-1}$.

k_2 calculated from the slope of the plot of k_o vs $[\text{H}_2\text{SO}_4]$ is 2.98×10^{-9} .

Figure 3.7

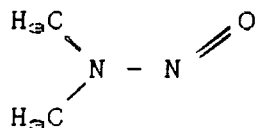
Dependence on [TGA] for the denitrosation of NNP in acetonitrile

$10^5 k_o/s^{-1}$



3.3 Denitrosation of Dimethylnitrosamine (DMN)

Dimethylnitrosamine (3.3) was chosen as a simple aliphatic nitrosamine so that its denitrosation may be compared to that of the heterocycle NNP and also of aromatic nitrosamines..



(3.3)

The solvents chosen for the denitrosation of DMN include ethanol, a less polar solvent than water, and 80% acetic acid, where the denitrosation of NNP was slightly more efficient than water, as the reaction was too slow to measure in water. With ethanol, two strong catalysts SCN^- and $\text{SC}(\text{NH}_2)_2$, in the presence of sulphamic acid as trap, failed to denitrosate DMN. In 80% acetic acid however, first-order kinetics was observed, with $\text{SC}(\text{NH}_2)_2$ as catalyst, in the presence of azide trap.

The nucleophile dependence of the reaction is shown in table (3.10).

Table 3.10

Nucleophile dependence for the denitrosation of DMN in 80% acetic acid.

[DMN] = 0.005M
 [H₂SO₄] = 1M
 [N₃⁻] = 0.01M

<u>[Thiourea] / M</u>	<u>10⁵ k_o / s⁻¹</u>
0.05	1.49
0.075	2.50
0.10	3.50
0.15	4.78
0.20	6.06

The acidity dependence of this reaction is given in table 3.11.

Table 3.11

Acidity dependence for the denitrosation of DMN in 80% acetic acid

[DMN] = 0.005M
 [N₃⁻] = 0.01
 [SC(NH₂)₂] = 0.05M

<u>[H₂SO₄] / M</u>	<u>10⁵ k_o / s⁻¹</u>
0.5	1.90
1.0	4.05
1.5	5.47
2.0	7.25

Figure 3.8

Nucleophile dependence for the denitrosation of DMN in 80% acetic acid

$10^5 k_o/s^{-1}$

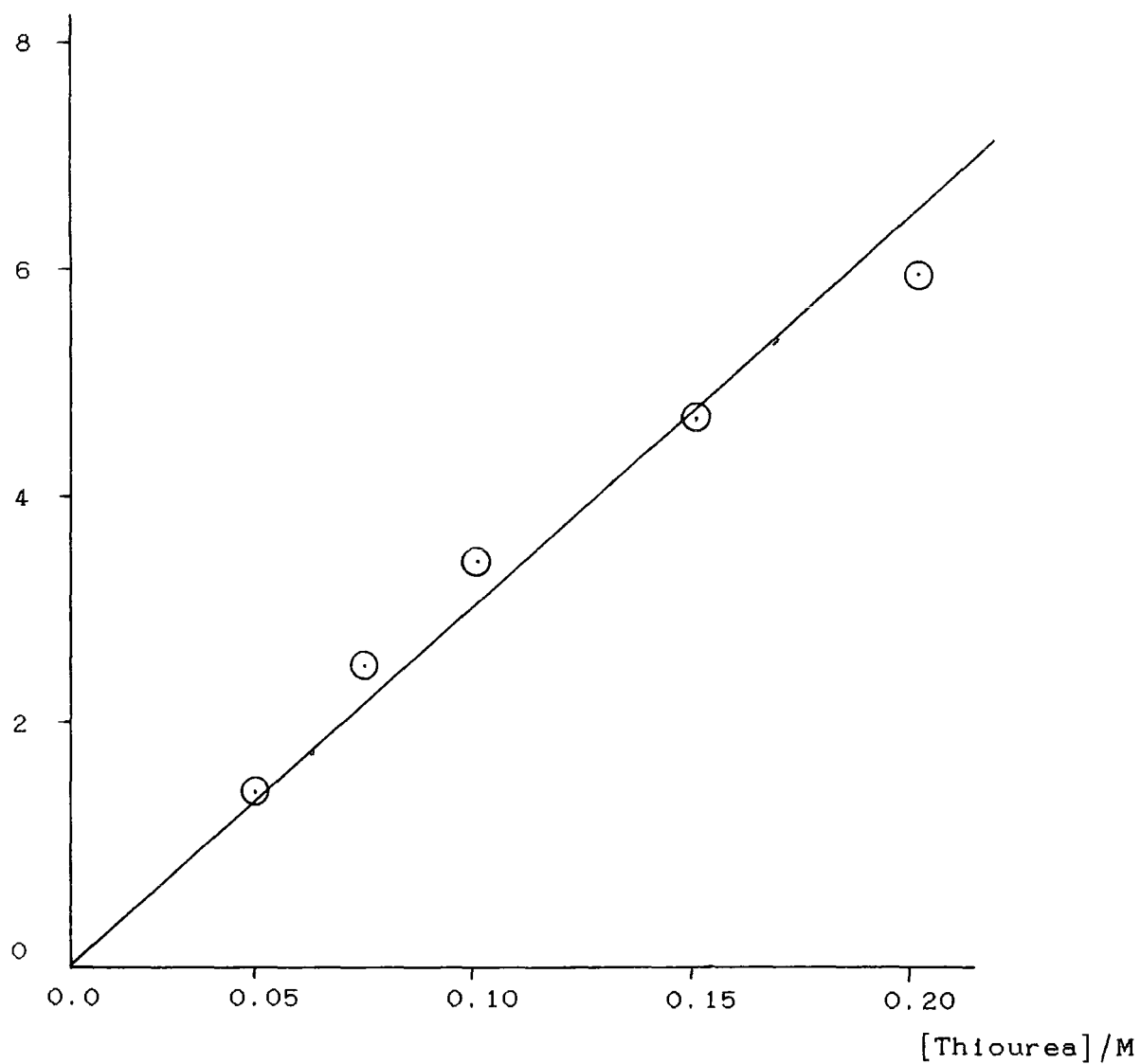
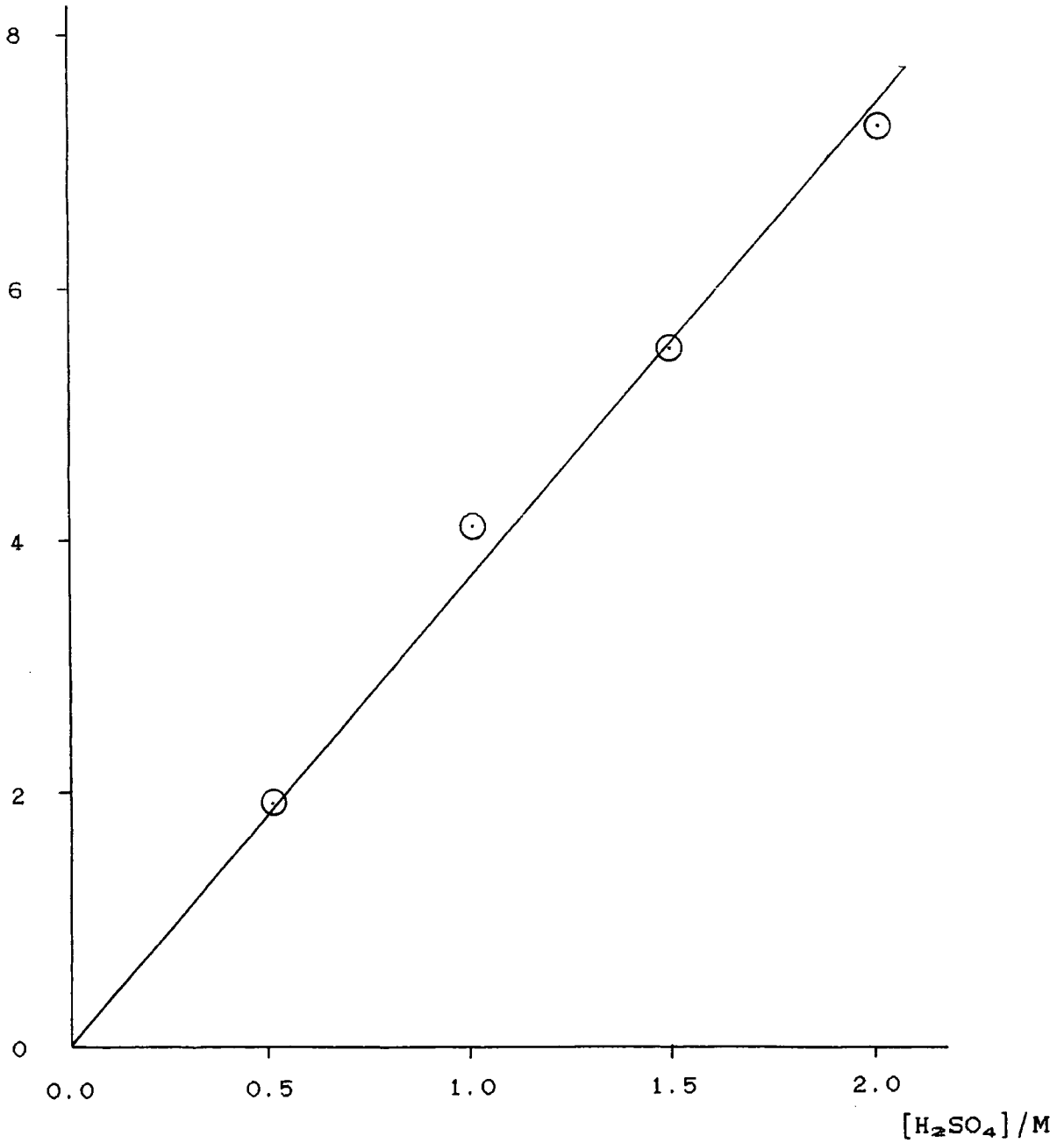


Figure 3.8

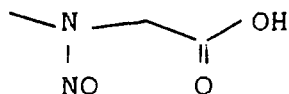
Acidity dependence for the denitrosation of DMN in 80% acetic acid

$10^5 k_o/s^{-1}$



3.4 Denitrosation of N-nitrososarcosine

Nitrososarcosine (NS) (3.4) was chosen as a comparison to NNP because it also contains the β -carboxyl group functionality but does not have the cyclic feature.



The denitrosation of NS was investigated in water as solvent, at 25°C, following the disappearance of the absorbance at 340nm due to NS. The trap used in this case was sulphamic acid, at concentrations greater than .01M, when the reaction is zero order with respect to the trap. As the concentrations of sulphuric acid required were quite high, acidity functions were once again used. The results of varying the acid concentration are shown in table (3.12).

The result of varying the thiourea concentration is shown in table (3.13).

The results are consistent with the mechanism outlined in scheme (3.3). From the plot of k_o vs $h_o/(K_a + h_o)$, the value for the slope obtained is 0.00216 ($= k_2 K K_a [CS(NH_2)_2]_{total}$), so that $k_2 K K_a = 0.0108 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ (scheme (3.4)). From the plot of k_o vs $[CS(NH_2)_2]$, the value of the slope is 0.00128, giving $k_2 K K_a = 0.0088 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$. Comparing these values to those obtained for the denitrosation of NNP in water ($0.0147 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ and $0.0131 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ respectively), it is clear that the rates for denitrosation of NNP and NS are similar. This suggests that the β -electron withdrawing group in compounds is the dominant factor controlling their reactivity in their reactions.

Table 3.12

Acidity dependence for the denitrosation of NS in water.

$$[\text{NS}] = 0.002\text{M}$$

$$[\text{Thiourea}] = 0.2\text{M}$$

$$[\text{Sulphamic Acid}] = 0.02\text{M}$$

<u>$[\text{H}_2\text{SO}_4]$</u>	<u>h_o</u>	<u>$10^4 k_o/\text{s}^{-1}$</u>
0.4	0.40	1.31
0.8	1.57	2.07
1.0	1.78	2.24
1.2	2.54	2.90
1.6	3.98	4.35
2.0	7.08	6.53

Table 3.13

Nucleophile dependence for the denitrosation of NS in water

$$[\text{NS}] = 0.002\text{M}$$

$$[\text{H}_2\text{SO}_4] = 1.2\text{M} \quad (h_o = 2.54)$$

$$[\text{Sulphamic Acid}] = 0.02\text{M}$$

<u>$[\text{Thiourea}]/\text{M}$</u>	<u>$10^5 k_o/\text{s}^{-1}$</u>
0.01	1.08
0.02	2.88
0.03	3.96
0.04	4.48
0.05	6.68

Figure 3.9

Acidity dependence for the denitrosation of NS in water

$10^4 k_{\text{d}}/\text{s}^{-1}$

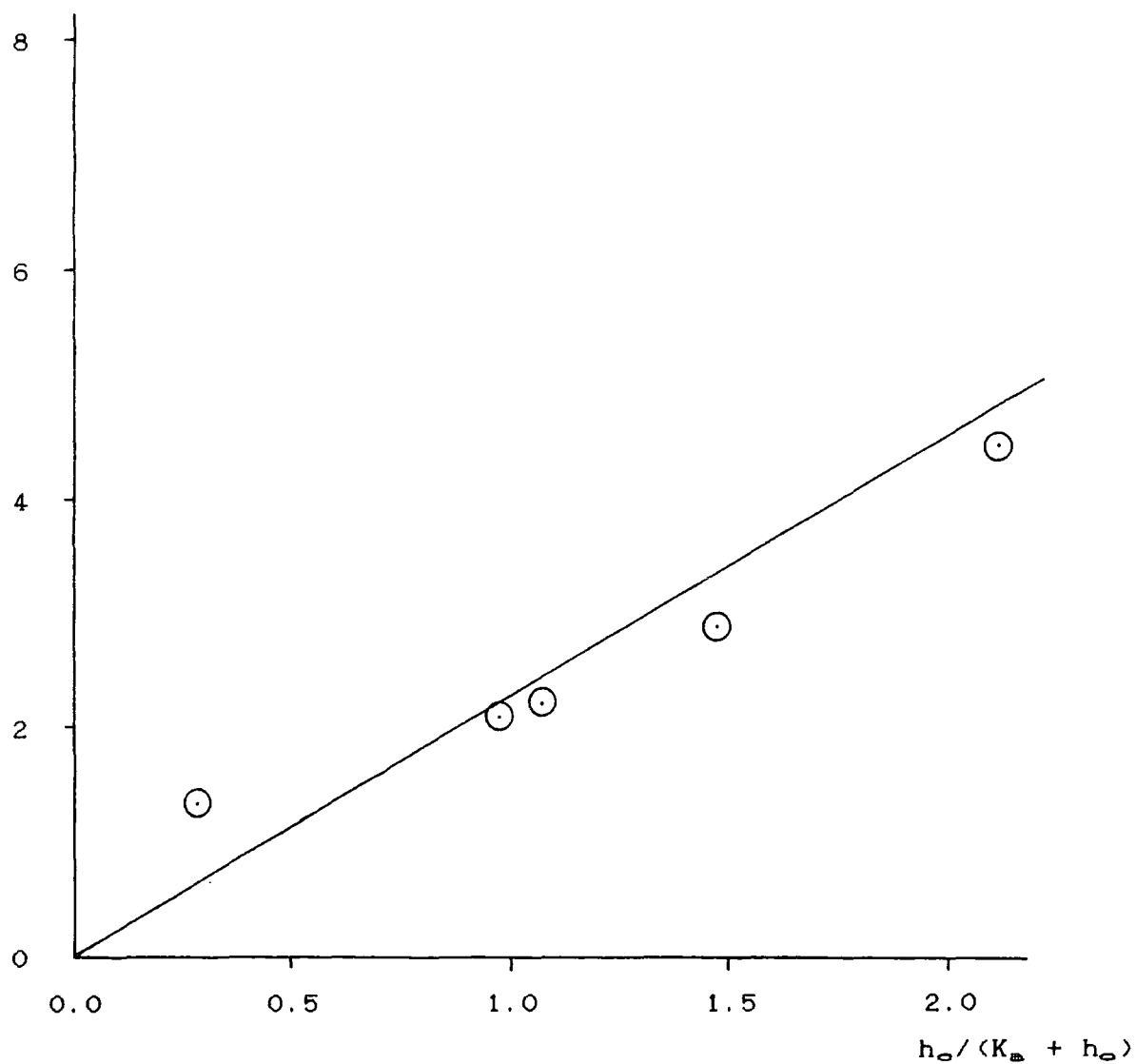
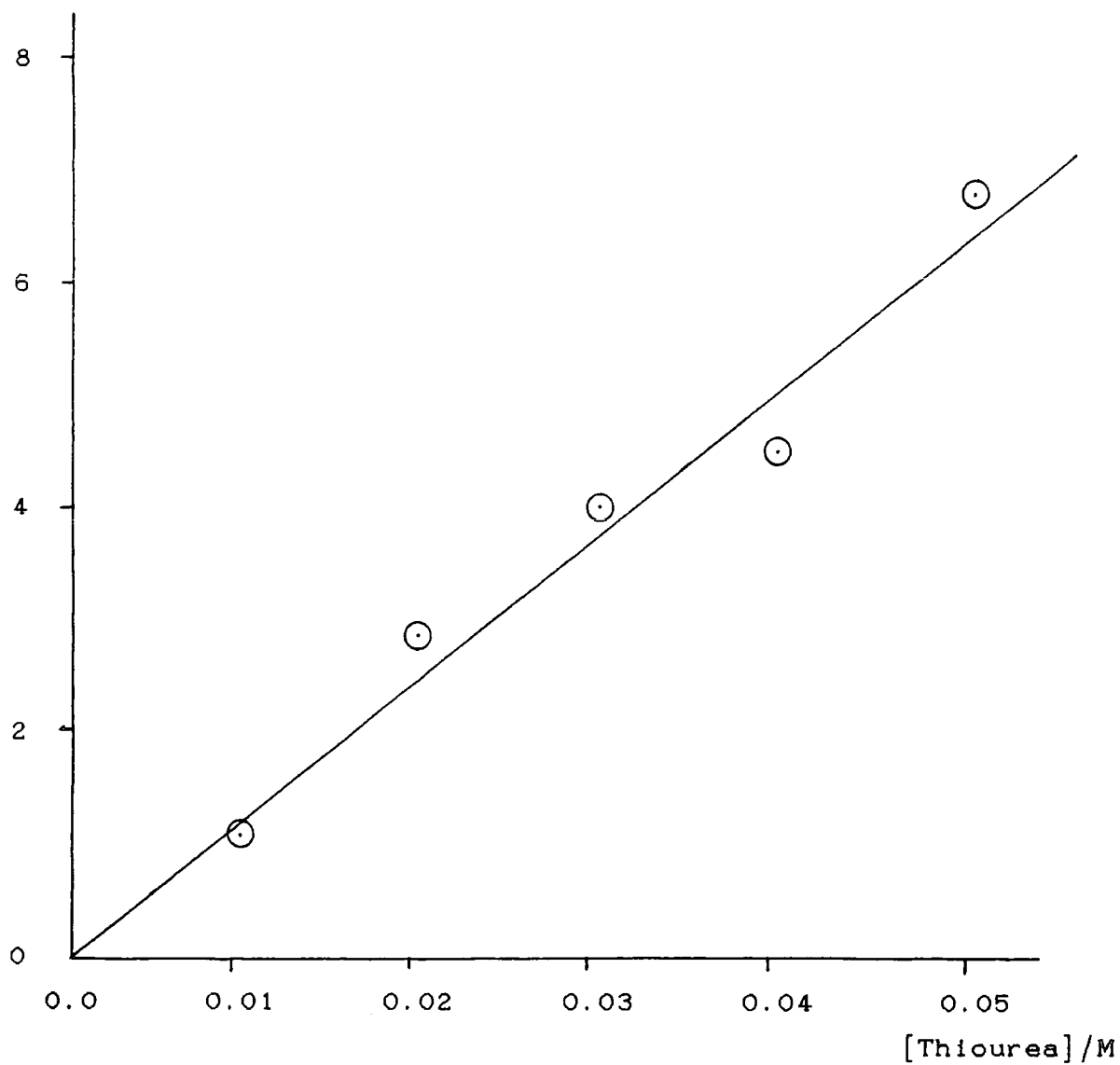
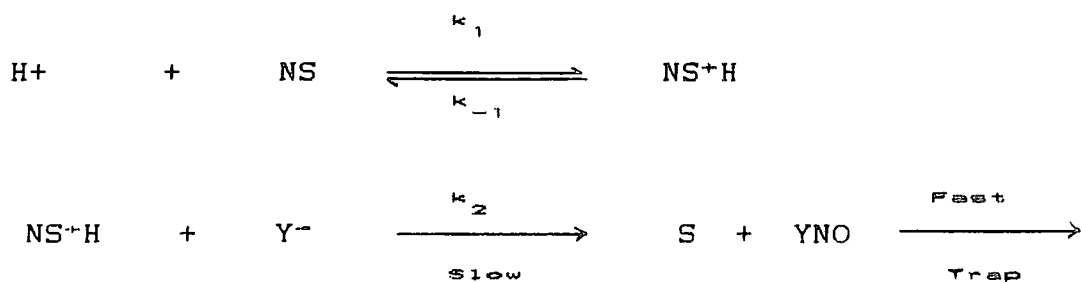


Figure 3.10

Nucleophile dependence for the denitrosation of NS in water

$10^5 k_{\text{cat}}/\text{s}^{-1}$



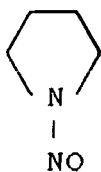


S = sarcosine

Scheme (3.4)

3.5 Denitrosation of N-nitrosopyrrolidine

The denitrosation reaction of N-nitrosopyrrolidine (NP) (3.5) was compared to that of NNP because it also contains the cyclic feature but not the β -carboxyl group.



(3.5)

The reactions were carried out in water and ethanol as solvents. No change in the spectrum was observed when repeated scans of the reaction mixture were made from 190nm - 500nm at thirty-minute intervals for 24 hours.

The denitrosation of NP then does not occur readily, once again suggesting that it is the β -electron withdrawing group in NNP, rather than the cyclic structure of the heterocycle, which is the dominant feature in this process.

3.6 Discussion

Denitrosation of some aliphatic heterocyclic nitrosamines has been shown to occur and have been studied mechanistically. One such reaction is that of nitrosopiperazine derivatives where denitrosation occurs at 50°C with nitrite trap in 3M hydrochloric acid²⁴. Similarly, it was found necessary to resort to fairly strong acidic conditions to denitrosate NNP, even in the presence of the most powerful catalyst in the solvents used.

Table (3.14) compares the rate constants between NNP, NS or NNP, DMN in different solvents, for the denitrosation process. It is evident that NNP and NS behave similarly in water, DMN and NP proved to be much more difficult to denitrosate.

Table 3.14

Comparing rate constants for the denitrosation of NNP, NS and DMN

	<u>Values compared</u>		<u>Solvent</u>
	<u>$k_2 K K_a \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$</u>		
NNP	0.015	0.013	Water
NS	0.011	0.0088	
NNP	0.039	0.047	80% Acetic Acid
DMN	0.0030	0.0030	

The lack of reactivity of NP towards denitrosation in water suggests that it is not the cyclic structure but rather the β -electron withdrawing group in NNP and NS that is the dominant factor influencing the nucleophilic attack in this denitrosation process.

The pKa values of the corresponding amines may give an indication of the extent of protonation of their nitroso counterparts: proline 10.8²⁵; sarcosine 9.7²⁶; pyrrolidine 11.3²⁷; dimethylamine 10.7²⁸ at 25°C. There are relatively small differences in pKa values, which if translated to the N-nitroso compounds, suggests that the reactivity towards denitrosation in a given solvent is governed by factors affecting the nucleophilic attack, i.e. including the electronic properties of the substituents.

Also 80% acetic acid proved to be a more efficient solvent than water, with the less polar acetonitrile being the most efficient. This could arise because the nature of the nucleophiles changes with the solvent. The less polarisable strongly solvated ions become more reactive as the solvent polarity decreases. Hydroxylic solvents solvate ions strongly because of hydrogen bonding. Thus the reactivity sequence in water, $I^- < Br^- < Cl^-$ changes in non-polar solvents so that $Cl^- < Br^- < I^-$.

Hence, the β -carboxyl, electron-withdrawing function and solvents play the significant roles in the denitrosation process.

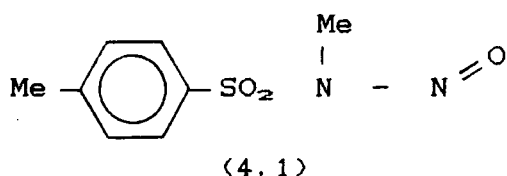
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CHAPTER 4

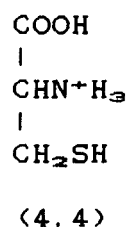
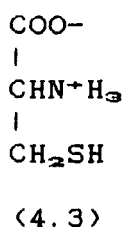
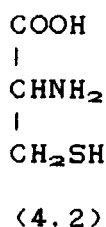
Nitrosation using N-methyl-N-nitrosotoluene-p-sulphonamide

4.2 Mechanism of S-nitrosation of cysteine using MNTS

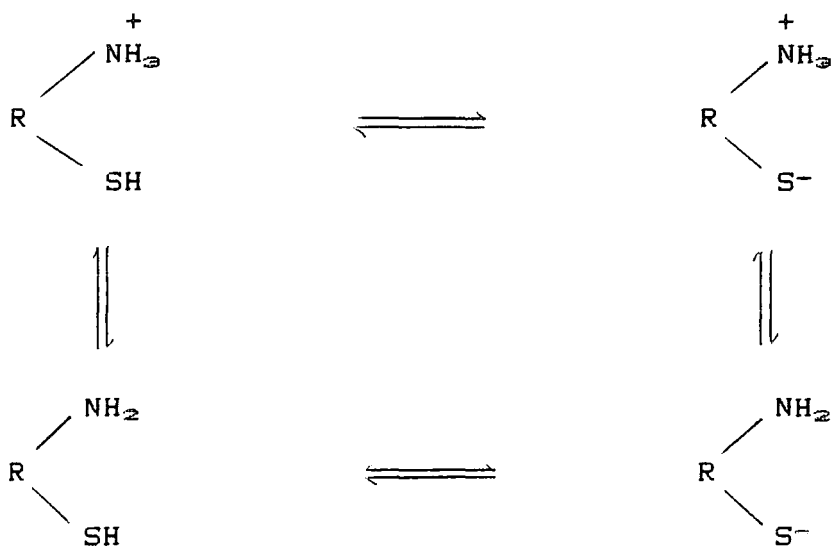
In dilute aqueous acid solution, it is known² that MNTS (4.1) undergoes hydrolysis to give nitrous acid in an essentially irreversible reaction. This reaction is fairly general for nitrosamides. In neutral and basic solutions, MNTS was more stable. It is known that MNTS and cysteine, a powerful nucleophilic substrate, react readily at pH 7 in 25% aqueous ethanol to yield finally the disulphide cystine and N-methyl-toluene-p-sulphonamide, both quantitatively¹¹. The reaction mixture initially turned light red probably due to the formation of S-nitrosocysteine which decomposed on standing to give cystine and nitric oxide.



Cysteine (4.2) is a naturally occurring amino acid and can exist in many forms depending on the pH of the solution. In mildly acidic conditions, cysteine exists partly as the zwitterion (4.3) and partly in the N-protonated form (4.4).



The pK_a of the carboxyl group pK_1 is 1.9 ± 0.1 and in neutral and basic conditions will be fully ionised. The species and equilibria which have to be considered now are shown in scheme (4.2).



Scheme (4.2)

The nitrosation of cysteine and its derivatives by MNTS was studied in the pH range 6-12. The buffer solutions used were suitable aliquots of the range of buffers listed in table (7.1) (see chapter 7, section 7.2).

MNTS and the cysteine derivatives used were of the highest purity grade available (Aldrich Chemicals). On mixing solutions of cysteine and MNTS, a red coloured solution was formed, typical of S-nitroso species in solution¹². After some hours a white precipitate formed, which was identified by elemental analysis and by its i.r. spectrum, as cystine. Kinetic measurements were carried out at 25°C by noting the increasing absorbance at 330nm due to S-nitrosocysteine. Most of the faster reactions were carried out by stopped-flow spectrophotometry and the remainder (typically at pH < 6) by conventional recording spectrophotometry. The solvent was 25% ethanol-water (v/v) and the cysteine concentration was always in a greater than twenty-fold excess over that of MNTS. Good first-order behaviour was found and all first order rate constants (k_{obs}) were mean values of four separate measurements, with a standard error of $\pm 4\%$. A typical run is shown in table (4.1).

Table 4.1

Typical run for the reaction of MNTS with Cysteine at pH = 5.86

$$[\text{MNTS}] = 2 \times 10^{-4} \text{ mol dm}^{-3}$$

$$[\text{Cysteine}] = 4.05 \times 10^{-3} \text{ mol dm}^{-3}$$

t/s	0	600	1200	1800	2400	3000	3600	4200
Abs	.094	.115	.135	.150	.161	.172	.183	.188
$10^4 k_o/s^{-1}$	-	2.91	3.13	3.10	2.98	3.02	3.16	3.01

t/s	4800	5400	6000	6600	7200	∞
Abs	.194	.199	.203	.206	.209	0.225
$10^4 k_o/s^{-1}$	3.00	2.99	2.97	2.93	2.92	-

$$\text{Average value of } k_o = 3.01 \times 10^{-4} \text{ s}^{-1}$$

Although S-nitrosocysteine and other thionitrites have been isolated¹³, most are very unstable in their pure forms and also decompose in solution. All of the S-nitroso species examined in the present work were, however, sufficiently stable to allow the kinetic studies to be carried out without interference from the decomposition. Thionitrites generally have broad absorptions in the u.v.-visible region centred at around 330nm and ¹⁵N n.m.r. measurements have characterised such species in solution¹⁴.

The measured first-order rate constants were determined at each of five different cysteine concentrations at each pH value. Plots of k_o vs [cysteine] were all linear, passing through the origin, thus establishing rate equation (4.1), where k_2 is the derived second-order rate constant.

One set of results for reactions at pH 5.86 are shown in table (4.2) and, in fig. (4.1) the plot of k_o vs [cysteine].

$$\text{Rate} = k_2 [\text{RSH}] [\text{MNTS}] \quad (4.1)$$

Table 4.2

Values of k_o obtained at different [cysteine], all at pH 5.86

<u>10^3 [Cysteine]/mol dm⁻³</u>	<u>10^4 k_o/s⁻¹</u>
4.05	3.01
6.08	4.51
8.11	6.18
10.1	7.83
12.1	9.37

The values of k_2 at different pH values in table (4.3). A plot of k_2 vs pH is shown in figure (4.2) and is the typical S-shaped curve expected for a system involving reaction with an anionic form of the substrate.

Figure 4.1

Plot of k_o vs different [Cysteine]

$10^4 k_o / s^{-1}$

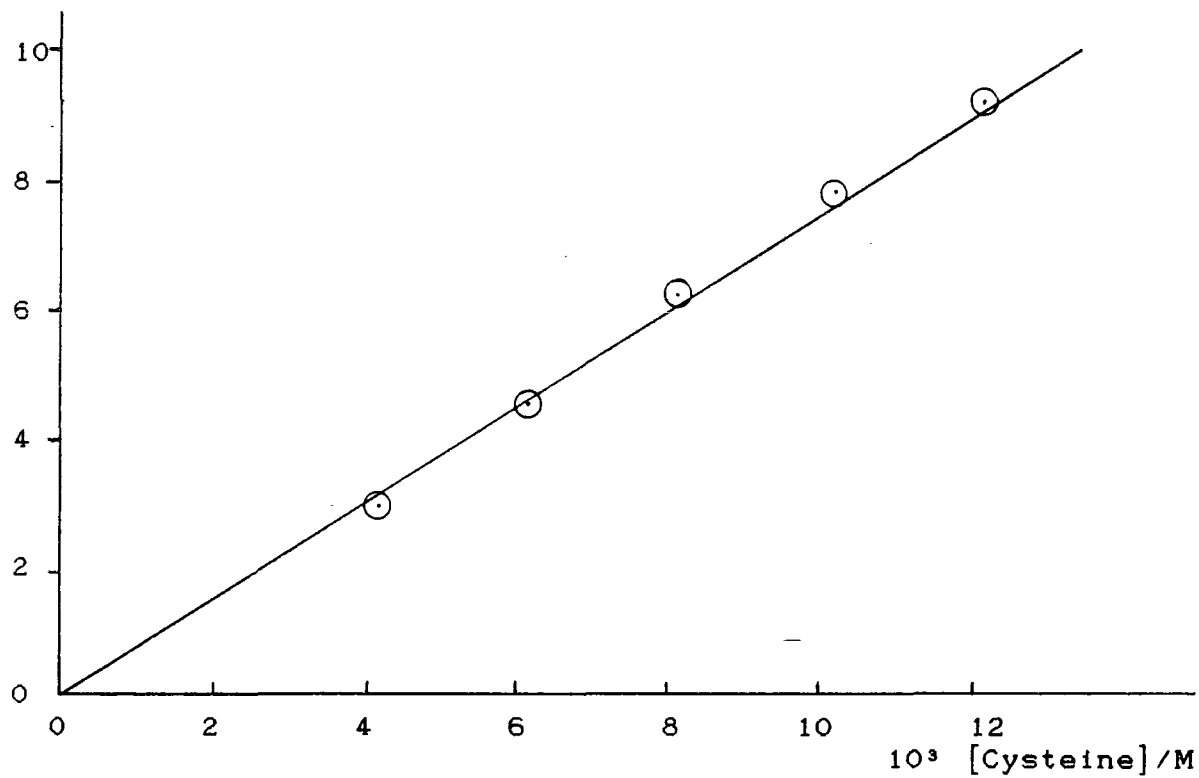
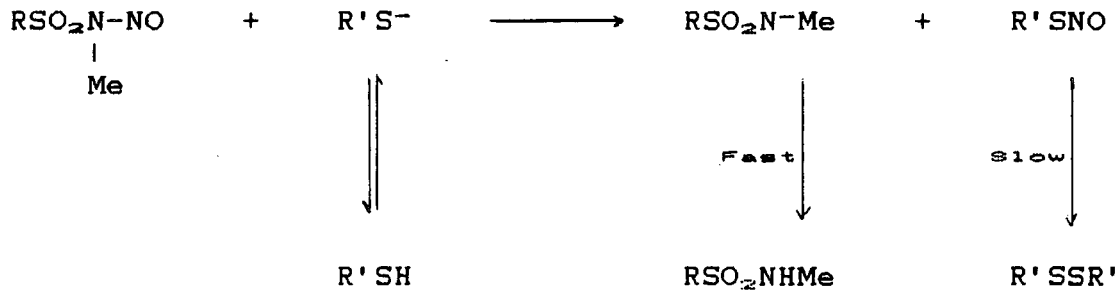


Table 4.3

Values of k_2 as a function of pH for the reaction of MNTS with cysteine.

pH	$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$	pH	$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$
5.12	0.012	8.18	9.72
5.67	0.045	8.26	11.0
5.86	0.077	8.74	17.7
5.92	0.092	9.12	21.0
7.43	2.60	10.5	23.9
7.82	5.59	12.6	25.0
7.95	6.82		

The most likely acid-base equilibrium involved here is the ionisation of the thiol group to give the thiolate anion, as S-nitrosation is in the process being investigated here. The outline mechanism is given in scheme (4.3).



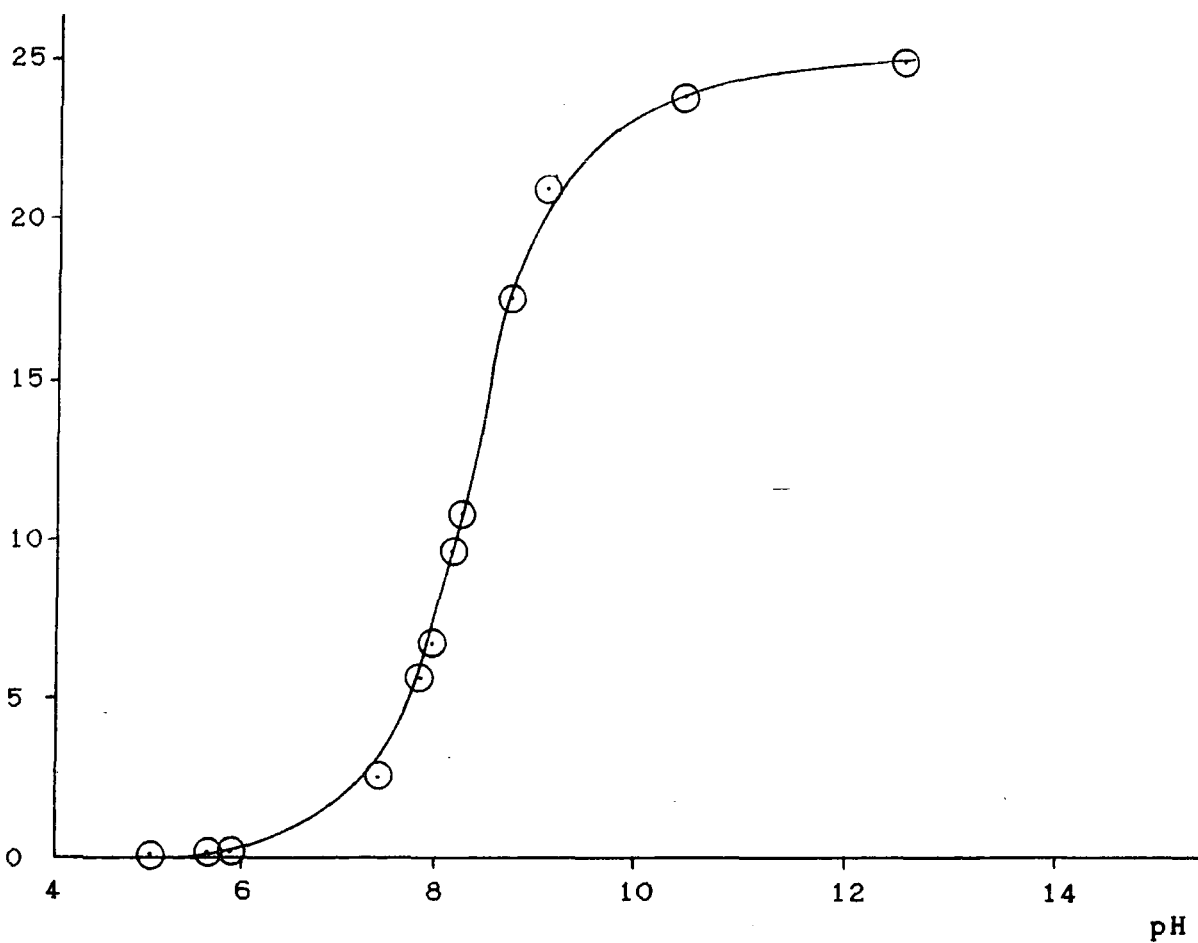
R = 4 - MeC₆H₄
R' = COOHCH(NH₂)CH₂

Scheme (4.3)

Figure 4.2

Plot of k_2 against pH for the nitrosation of cysteine by MNTS. The solid line is the calculated curve and the points are the experimental ones.

k_2/dm^3
 $\text{mol}^{-1} \text{s}^{-1}$



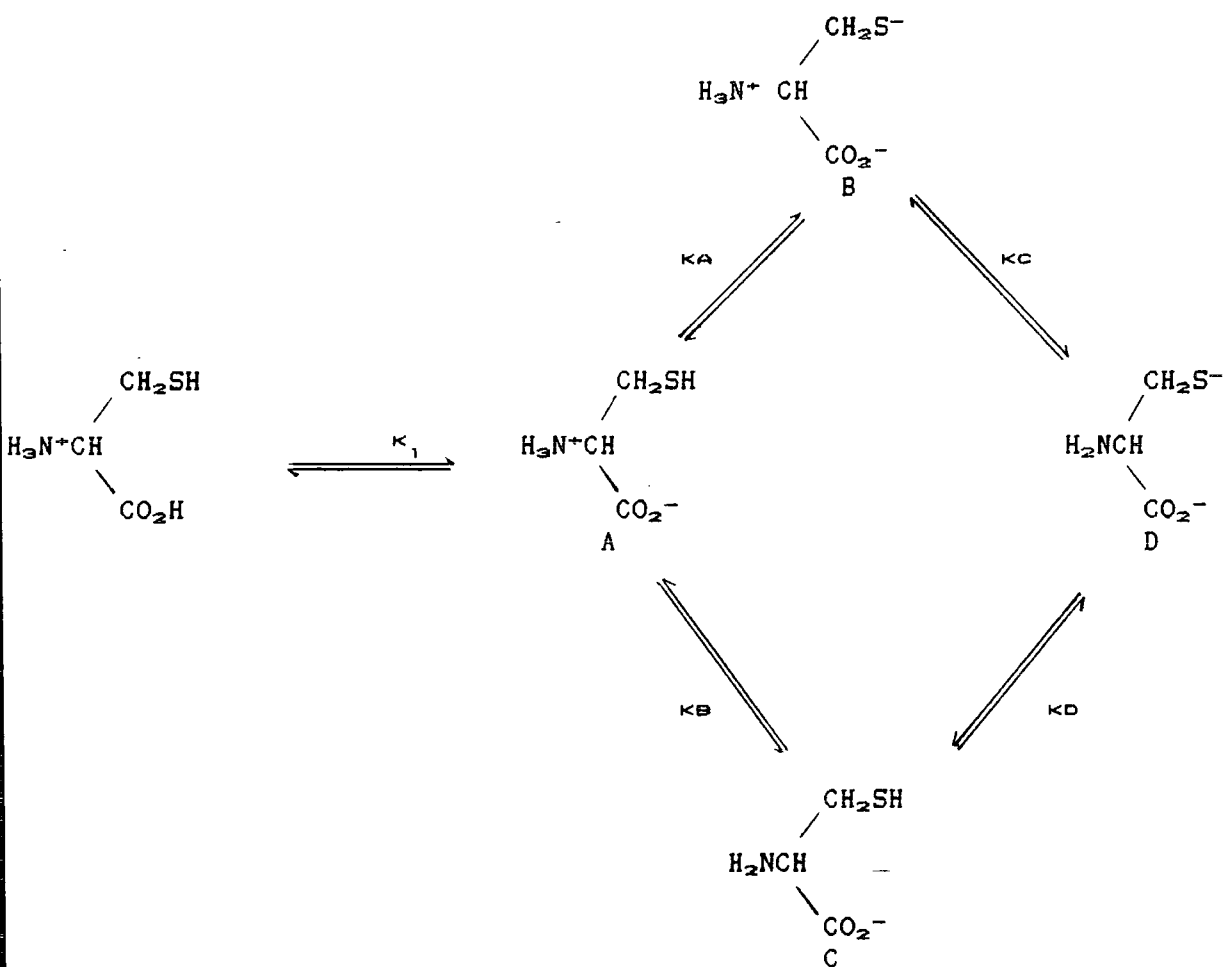
The expression for k_2 expected from scheme (4.3) is given in equation (4.2) where k is the second-order rate constant for reaction between MNTS and $R'S^-$, and is given by the limiting value of k_2 at high pH; K_a is the acid dissociation constant for $R'SH$ ionisation.

$$k_2 = \frac{k K_a}{K_a + [H^+]} \quad (4.4)$$

There has been considerable confusion in the earlier literature regarding the identification and magnitude of the various ionisation modes in cysteine. The situation is now resolved, and is set out in scheme (4.4).

The ionisation of the carboxylate group is virtually complete in the pH range studied. The microscopic constants (K_A), (K_B), (K_C), (K_D) refer to the various ionisations shown for which the macroscopic pK_a value is usually quoted at ~ 8.37 ¹⁵. More recently the individual microscopic constants have been determined and it is apparent that the $-N^+H_3$ and $-SH$ ionisations in cysteine are close together; four sets of values of the microscopic constants have been determined¹⁶ as follows: (pK_a)_A at 8.21, 8.53, 8.50, 8.64; (pK_a)_B at 8.65, 8.86, 8.85, 8.62; (pK_a)_C at 10.0, 10.36, 10.35, 10.47 and (pK_a)_D at 9.56, 10.03, 10.00, 10.49. If reaction occurs at S^- then the involvement of B and D must be considered. The fraction of B and D can be expressed in terms of the various microscopic K values and $[H^+]$, given in equation (4.3).

$$\frac{[B] + [D]}{[RS^-]_{max}} = \frac{\frac{K_A}{K_B} + \frac{K_D}{[H^+]}}{\frac{[H^+]}{K_B} + \frac{K_A}{K_B} + 1 + \frac{K_A}{[H^+]}} \quad (4.3)$$



Scheme (4.4)

This analysis was used for the nitrosation of cysteine and its derivatives by alkyl nitrites²⁰. However, nitrosation with MNTS followed a profile between that of total thiolate and the $^+NH_3RS^-$ form suggesting some degree of preference for reaction with the latter, which could arise from some intramolecular interaction in the transition state which favours this pathway.

The quoted macroscopic value of 8.37 is reasonably close to the microscopic constant for SH ionisation to $^+NH_3RS^-$ and is used, together with that of $25.0 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ for k , to calculate the k_2 -pH plot. The solid line in figure (4.2) is indeed the calculated curve, and the points are the experimentally determined ones, the agreement is good. Small changes in the value of pK_a do not change the position of the calculated curve very much. An alternative procedure is to determine K_a from the experimental results. Rearrangement of equation (4.2) to equation (4.4) shows that K_a can be obtained from the intercept of a plot of the left-hand side of equation (4.4) vs pH.

$$\log (k_2^{-1} - k^{-1}) = -\text{pH} - \log k K_a \quad (4.4)$$

This is shown in figure (4.3); the measured slope is -1.01 and pK_a determined as 8.47 which is in good agreement with the microscopic $(pK_a)_A$ values for SH ionisation in cysteine.

Figure (4.2) shows further that k_2 tends to zero at low pH, so that there is no evidence of reaction of MNTS with the thiol form of cysteine. This contrasts markedly with the corresponding reactions of nitrous acid with thiols in acid solution, where often reaction occurs with rate constants within a power of ten or so of the encounter value. As expected MNTS is a less powerful nitrosating species than that generated in acid solutions of nitrous acid.

Other cysteine derivatives behave in a similar way to cysteine itself. The same pattern of kinetic behaviour is observed in the reactions of MNTS with N-acetyl cysteine, cysteine methyl carboxylate, and cysteine ethyl carboxylate. The experimental results for these substrates are given in tables (4.4), (4.5) and (4.6) respectively.

Table 4.4

Values of k_{22} as a function of pH for the reaction of MNTS with N-acetylcysteine.

<u>pH</u>	<u>$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>	<u>pH</u>	<u>$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>
7.67	0.190	9.70	11.0
8.71	1.92	9.91	13.7
9.04	3.81	10.04	15.4
9.24	5.37	10.38	19.0
9.41	7.97	11.86	23.3

Table 4.5

Values of k_2 as a function of pH for the reaction of MNTS with cysteine methyl ester.

<u>pH</u>	<u>$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>	<u>pH</u>	<u>$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>
6.40	0.034	9.48	13.4
7.65	0.708	9.89	16.5
8.42	3.35	10.29	18.0
9.00	8.49	10.71	18.7
9.30	11.8	11.10	19.1

Table 4.6

Values of k_2 as a function of pH for the reaction of MNTS with cysteine ethyl ester.

<u>pH</u>	<u>$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>	<u>pH</u>	<u>$k_2/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$</u>
7.15	0.190	8.88	6.86
7.50	0.410	9.08	9.13
8.11	1.59	9.25	11.2
8.30	2.37	10.28	18.5
8.61	4.60	11.46	19.6

As can be seen from the limiting values of k_{cat} at high pH, the reactivity of the $\text{R}'\text{S}^-$ species is not significantly affected by N-acetyl substitution or by esterification. All the cysteine derivatives give excellent straight line plots for equation (4.3) with slopes very close to -1. The derived pK_{m} values are given in table (4.8) together with a selection of literature values.

Table 4.8

Values of $(\text{pK}_{\text{a}})_{\text{A}}$ determined from equation (4.4).

<u>Thiol</u>	<u>$(\text{pK}_{\text{a}})_{\text{A}}$</u>	<u>Literature values ^a</u>
Cysteine	8.47	8.21, 8.53, 8.50, 8.64
N-Acetylcysteine	9.76	9.76 ^b
Cysteine methyl ester	9.15	8.99 ^b , 9.1
Cysteine ethyl ester	9.24	9.17 ^b , 9.09, 8.87

^a All are microscopic values except ^b which are macroscopic, and are taken from reference 17, CRC Handbook of Biochemistry and Molecular Biology 3rd edition, vol. 1, J. Inczedy and J. Marothy, Acta Chim. Acad. Sci. Hung., 1975, 86, 1, and R.K. Boggess, J. R. Absher, S. Morelen, L.T. Taylor and J.W. Hughes, Inorg. Chem., 1983, 22, 1273.

For all there is good agreement between our values and those in the literature.

MNTS is itself decomposed in alkaline solution, in the familiar reaction to generate diazomethane in solution. We have measured the rate constants for this reaction over a range of pH values, by noting the disappearance of the absorbance at 260nm. This reaction is very much slower than the nitrosation reaction under all our experimental conditions, and there is no need to correct our nitrosation rate constants.

The possibility of thiolate anion nitrosation by other N-nitroso species has also been examined. Each of the following, however, failed to yield any detectable S-nitrosocysteine, even at pH 11: dimethylnitrosamine, nitrosoproline, and nitrososarcosine. It is clear that reaction will not occur unless there is a powerful electron-withdrawing group close to the amino nitrogen atom. An obvious family of compounds to test is therefore the nitrosamides, which would be expected to behave in a similar fashion to the nitrososulphonamides. Literature reports^{11,17} that N-Methyl-N-nitrosourea yields no cystine when treated with cysteine, and that there is also no colour evidence for the formation of S-nitrosocysteine, have been confirmed using conventional spectrophotometry. Use of nitrosamides in this way is complicated, particularly at high pH by the concurrent hydrolysis reaction (i.e. nucleophilic attack at the carbonyl carbon atom). It is reported that¹⁸ N-methyl-N-nitrosourethane gives many products when treated with cysteine, none of which can be rationalised in terms of a nitrosation reaction. However, N-methyl-N-nitro-N-nitrosoguanidine does produce some cystine¹¹ and the reaction is thought to involve concurrent nucleophilic attack at the nitroso group and at the imino carbon atom.

In the course of this study, the reaction of MNTS with a number of other substrates was investigated by some other workers (see section (4.3) for discussion).

4.3 Discussion

The ability of MNTS to nitrosate amines in neutral and alkaline conditions has also been investigated¹⁹. In the presence of ammonia, hydroxylamine, hydrazine or primary, secondary or tertiary amines, a transnitrosation reaction occurred in which the nucleophiles attacked the nitrogen atom of the MNTS N=O group, rather than the -SO₂ group, which contrasts with their behaviour with other nitrosamides. The reactivity of secondary amines correlates well with their pK_a values and the tertiary amines investigated had reactivities similar to those of secondary amines of similar pK_a values, but primary amines and NH₃ react considerably more slowly. The observed rate constants are ~ 10² times slower than those observed for the reactions between thiolate anions and MNTS. The more nucleophilic substrates are therefore more effective in this nitrosation process.

It has also recently been shown that alkyl nitrites react under mildly alkaline conditions with thiols via the thiolate ions to give thionitrites²⁰. Clearly the reactions have strong similarities to those described in this chapter. The following thiols reacted with a range of alkyl nitrites in water at 25°C in the pH range 6-13 to give the corresponding thionitrites in solution; L-cysteine, N-acetyl-L-cysteine, L-cysteine methyl ester, L-cysteine ethyl ester, glutathione and thioglycolic acid. From the pH-dependence of the rate constant, it was shown that reaction occurs only via the thiolate anions RS⁻. For N-acetyl-L-cysteine and thioglycolic acid, only one RS⁻ species is possible and quantitative kinetic analysis yields pK_a values for RSH ionisation in good agreement with the literature values.

For each of the remaining thiols, two thiolate ions (NH_2RS^- and $^-\text{NH}_3\text{RS}^-$) are possible and the measured rate constants for all alkyl nitrites generally followed the total thiolate ion concentration (as a function of pH) obtained from the published microscopic pK_a values. An alternative approach of fitting the kinetic results to the concentration curve by computer yields microscopic pK_a values generally in good agreement with the literature values.

With simple alkyl nitrites (ethyl, isopentyl, isopropyl and t-butyl) steric effects appear to be the major influences in reactivity, whereas electron-withdrawing substituents in the 2-position greatly increase the rate constants. The reactions of 2,2,2-trichloroethyl nitrite were too fast to measure at all pH values, and the reaction of 2,2,-dichloroethyl nitrite could only be followed kinetically in the pH range 6-8.25, even by stopped-flow spectrophotometry.

Thus, the breaking of the stronger N-N bond, in the case of MNTS requires the presence of a very strongly electron-withdrawing $-\text{SO}_2$ group, whereas alkyl nitrites without electron-withdrawing substituents were capable of taking part in O-N bond breaking reactions. The results in the present analysis show that N-acetyl-L-cysteine reacts via the thiolate anion and the reaction with L-cysteine occurs via the total thiolate concentration, but surprisingly the reactions of both carboxylic esters follow a profile which is between that of total thiolate and the NH_2RS^- form. This suggests that there is some degree of preference for reaction with the latter, which could arise from some intramolecular interaction in the transition state which favours this pathway.

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CHAPTER 5

The nitrosation of phenols using alkyl nitrite and nitrosothiol

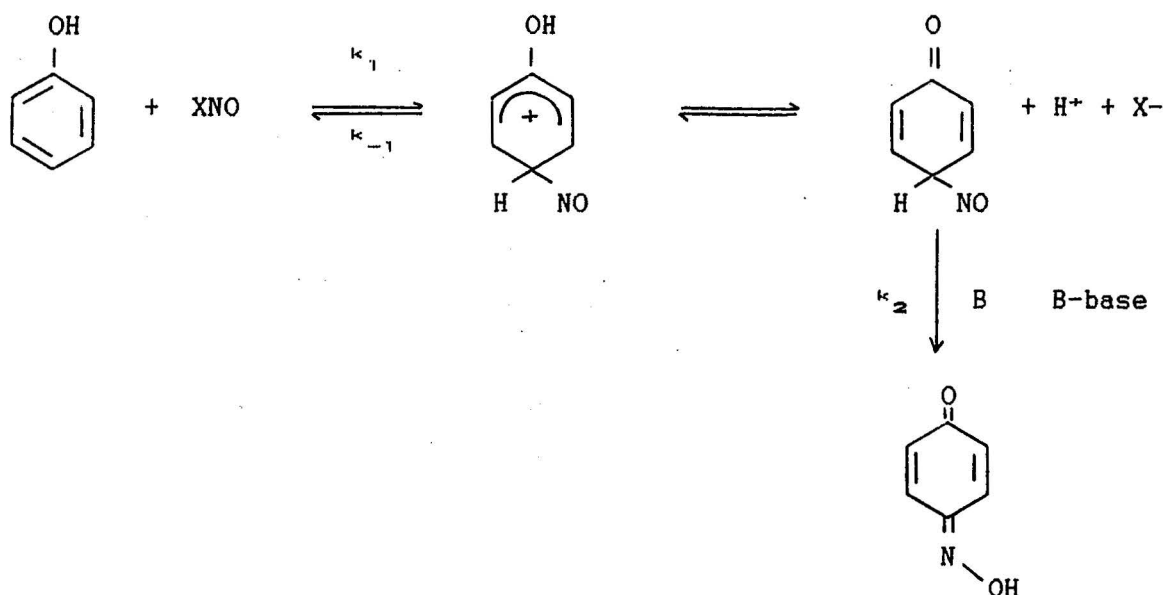
5.1 Introduction

The nitrosation of phenols and naphthols by aqueous acidic nitrite has been widely studied in aromatic C-nitrosation reactions. These reactions occur less readily than the nitration reactions because electron-releasing groups are necessary to activate the aromatic ring and the nitrosonium ion has been estimated¹ to be $\sim 10^4$ times less reactive than the nitronium ion.

The C-nitroso compounds formed, exist primarily as the tautomeric benzo (or naphtho) quinone monooximes; the nitroso compounds from phenols, phenyl ethers and from the photochemical reaction of phenol with aqueous nitrite have been discussed previously (see chapter 1, section 1.5.1).

The mechanism of C-nitrosation of phenol and its derivatives has largely been studied by Challis and co-workers, who propose a mechanism involving the reversible formation of an intermediate, followed by a proton transfer to the medium, the A-S_E2 mechanism well-known in other aromatic electrophilic substitution reactions, including nitration² and halogenation³. The proton transfer to the medium from the aromatic ring has been proposed as the rate-determining step from kinetic isotope experiments (typically $k_H/k_D \sim 3.5$) with phenol substituted with deuterium in the 4-position^{24, 25}. This rate-limiting proton transfer is unusual in aromatic electrophilic substitution, e.g. in nitration and halogenation where the rate-determining step is generally the attack of the electrophile. The intermediate in the case of a phenol or naphthol is thought to be a neutral dienone species (see chapter 1, scheme (1.26)), although it has not been detected spectroscopically, possibly due to its high reactivity. The mechanism has been fully discussed in chapter 1, section 1.5.1. The expected general rate equation from the mechanism outlined in scheme (1.26) is given in equation (1.37).

The ionisation of nitrous acid to nitrite ion has not been included, for simplicity, although this has to be accounted for in $\text{pH} > 2$ solutions. (Scheme (1.26) and equation (1.37) are reprinted below).



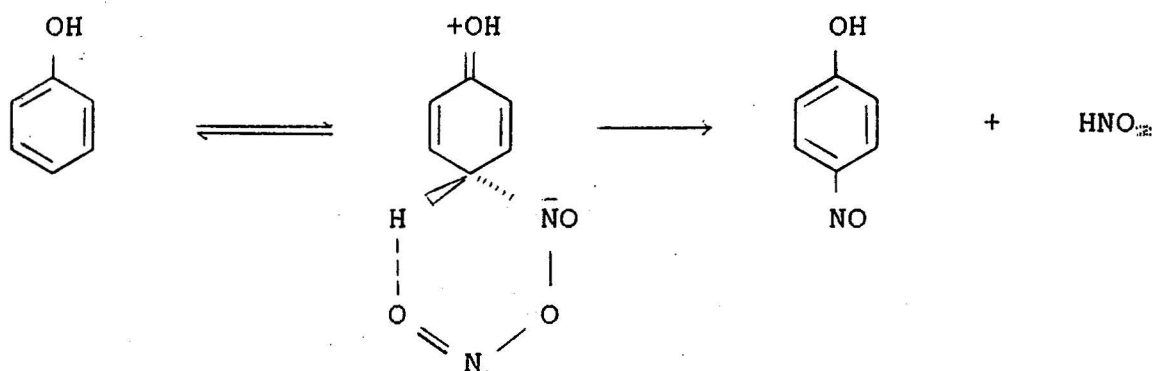
$$\text{Rate} = \frac{k_1 k_2 [\text{PhOH}] [\text{H}^+] [\text{HNO}_2] [\text{B}] [\text{X}^-] K_{\text{XNO}}}{k_{-1} [\text{H}^+] [\text{X}^-] + k_2 [\text{B}]} \quad (1.37)$$

X = nucleophile.

When $\text{X}^- = \text{H}_2\text{O}$ i.e. in the absence of added nucleophile, there are two limiting conditions: when $k_{-1} [\text{H}^+] \gg k_2 [\text{B}]$, a primary kinetic isotope effect, independence of acidities and general base catalysis is predicted; when $k_{-1} [\text{H}^+] \ll k_2 [\text{B}]$, the reaction would be acid-catalysed with no kinetic isotope effect.

The effect of added nucleophiles has also been studied⁴ for the C-nitrosation of 2 naphthol: Cl^- , Br^- and SCN^- were all found to catalyse the reaction via the formation of the corresponding nitrosyl compounds, which attack naphthol to form a steady-state intermediate, eventually yielding 1-nitroso-2-naphthol.

The nitrosation of phenol in aprotic solvents such as benzene, cyclohexane and chloroform has also been investigated⁵ and the following mechanism proposed (see scheme (5.1)), following the absence of an observed kinetic isotope effect, the slower rates of reaction and the absences of the ortho-nitroso-phenol in the product.



Scheme (5.1)

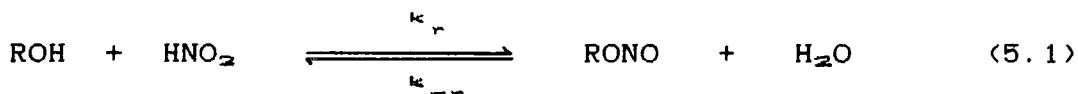
p-Nitrosophenol, prepared from phenol and aqueous nitrous acid is an intermediate in some of the large scale industrial productions of the common drug paracetamol. Interest in C-nitrosocompounds has increased since the discovery that they can catalyse⁶ or inhibit⁷ the formation of N-nitrosamines. A wide variety of monophenolic compounds ingested with foods, drugs and food additives may interact with nitrite under the condition existing in the stomach. These monophenolics may consume nitrite that would be available for nitrosamine formation and thus inhibit nitrosamine formation, but nitrosophenols may also activate nitrite to stimulate nitrosamine formation. The inhibiting or catalytic effect depends on the structure of these phenols, on the pH of the reaction mixtures, on the relative concentrations of nitrite and phenols and on the nature of the nitrosatable substrates.

As the interaction of phenols with acidified nitrite ion has been extensively studied, a study of its interaction with O- and S-nitroso compounds has now been undertaken. The reaction has been studied in the pH range 2.55 to 9.5, the O-nitroso compound chosen being iso-amyl nitrite (IAN), while the S-nitroso compound chosen is S-nitroso-N-acetyl penicillamine (SNAP). This study was undertaken in part to compare the reactivities of alkyl nitrites and thionitrites.

5.2 Nitrosation of phenols using iso-amyl nitrite

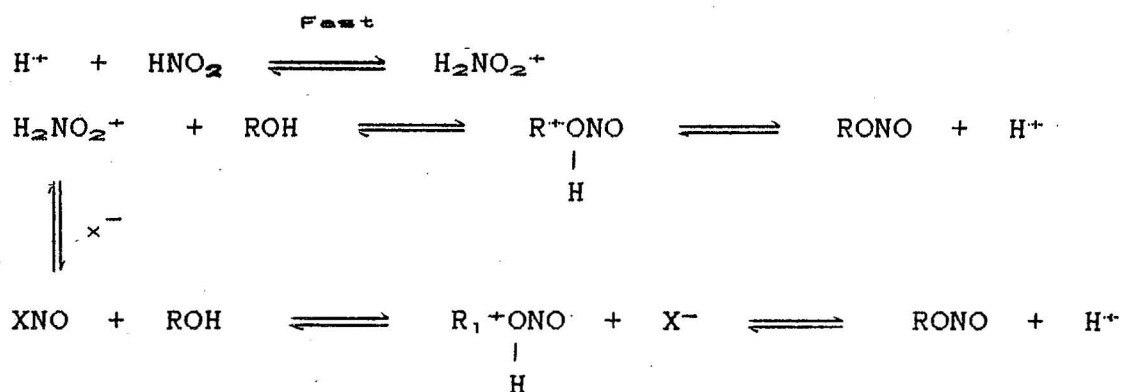
Alkyl nitrites have been and are much as nitrosating agents, both in aqueous solution, in acidic and alkaline conditions, and in non-aqueous solvents. (See chapter 1, section 1.5).

In aqueous acid solution, alkyl nitrites readily undergo hydrolysis to give equilibrium concentrations of nitrous acid and the corresponding alcohol. Hydrolysis has been studied mechanistically by several workers: Allen⁸ studied the reaction in a dioxane-water mixture and more recently⁹ kinetic studies have been made in the reactions of alcohols and nitrous acid, allowing values of the rate constant for the forward and reverse reactions (equation (5.1)) to be determined.



The equilibrium constants, K, for alkyl nitrite formation have also been determined directly by spectrophotometry for a range of aliphatic alcohols¹⁰.

Both forward and reverse reactions are acid-catalysed and are also catalysed by nucleophiles X⁻ such as halide ion and thiocyanate ion⁹ (see scheme (5.2)).



Scheme (5.2)

Nitrosation by alkyl nitrites can in principle then occur directly or via the nitrous acid formed on hydrolysis, in aqueous acidic solution. A study¹¹ of the nitrosation of sulphanilamide with cyclohexyl nitrite and with nitrous acid concludes that a common nitrosating agent is involved. Further, rate reductions of amine nitrosations by added¹² alcohols, indicate the formation of equilibrium concentrations of alkyl nitrites. Reactions¹³ of isopropyl and t-butyl nitrite with sulphamic acid, hydrazoic acid thioglycolic acid, cysteine, N-methylaniline and thiourea also indicate that it is the free nitrous acid (or its protonated form) which reacts with the substrates rather than a mechanism involving a direct attack by the alkyl nitrites.

At high [substrate] for the more reactive species, the rate-limiting step becomes the hydrolysis of RONO, particularly for t-butyl nitrite, where it is easier for the nitrosation of the substrate by nitrous acid to compete with the re-nitrosation of the alcohol, since the rate of the latter reaction is much smaller for a tertiary alcohol than it is for a secondary alcohol.

Under different experimental conditions, however, alkyl nitrites apparently do act directly e.g. in aqueous alkali¹⁴ and in alcoholic solvents¹⁵. Interestingly, rate-limiting NO⁺ formation has been found in the acid-catalysed nitrosation of methanol, thioglycolic acid, and water in acetonitrile, using alkyl nitrites or nitrous acid¹⁶.

The reaction between alkyl nitrite and phenol was investigated in the pH range 2.5 to 9. The alkyl nitrite used in this case was iso-amyl nitrite, purified by distillation. Fresh solutions of iso-amyl nitrite were made up frequently in the appropriate buffer solutions. Kinetic measurements were obtained by following the formation of the nitrosophenol: at 380nm for the phenol reaction and for the reaction of 2,4,6-trimethylphenol. In all reactions there was an excess (> 20) of [phenol] with respect to [iso-amyl nitrite], resulting in pseudo first-order reaction conditions. All experiments were duplicated, and in no case did the results of the duplicates differ by more than 3%. All experiments were carried out at 25°C. d₆-Phenol was used to investigate kinetic isotope effects.

The nitrosation of phenol using nitrous acid has been reported to yield at least 95% p-nitrosophenol, with the remainder being σ -nitrosophenol¹⁷. In the present investigation, analysis of the reaction solutions after at least ten half-lives, by t.l.c. confirmed that these were the only two products formed and the product ratio appeared to be pH independent: comparative u.v. assay with authentic material showed a yield of p-nitrosophenol $> 90\%$ throughout the pH range examined kinetically. The product of nitrosation of 2,4,6-trimethylphenol is the 3-nitroso derivative, the only product observed on t.l.c., analysis. N.m.r. analysis was also carried out on the p-nitrosophenol formed.

The influence of the concentration of phenol on the rate of formation of p-nitrosophenol was studied at pH 4.3 and pH 7.2 by varying the concentration of phenol over the range 0.02 - 0.06 mol l⁻¹ while maintaining the concentration of iso-amyl nitrite at 1 x 10⁻³ mol l⁻¹ (see table (5.1)). Good first-order behaviour was observed.

Table 5.1

Dependence on concentration of phenol for the reaction with iso-amyl nitrite at pH 4.3 and pH 7.2 at 25°C

$$[\text{iso-amyl nitrite}] = 0.001\text{M}$$

<u>[Ph OH]/M</u>	<u>k_o/s⁻¹</u>	
	<u>pH = 4.3</u>	<u>pH = 7.2</u>
.02	1.38 x 10 ⁻⁴	1.09 x 10 ⁻⁴
.03	2.67 x 10 ⁻⁴	1.76 x 10 ⁻⁴
.04	3.28 x 10 ⁻⁴	2.37 x 10 ⁻⁴
.05	4.25 x 10 ⁻⁴	2.81 x 10 ⁻⁴
.06	5.24 x 10 ⁻⁴	3.53 x 10 ⁻⁴

Graph 5.1

Dependence on phenol concentration for reaction with iso-amyl nitrite at pH 4.3 and pH 7.2 at 25°C

$10^4 k_o / s^{-1}$

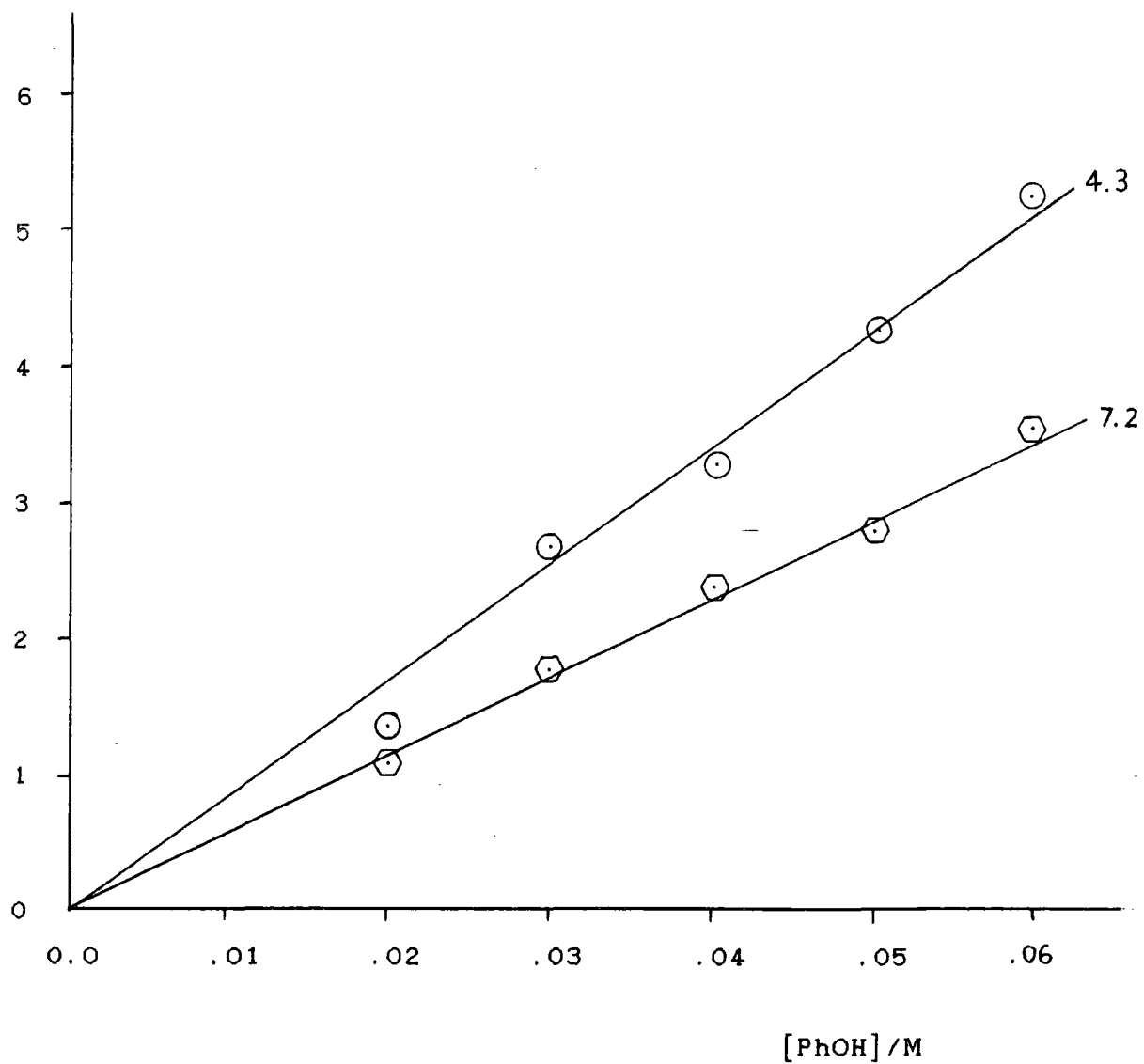


Table 5.2

Typical kinetic run of the reaction of phenol with iso-amyl nitrite at pH 3.98 at 25°C.

$$[\text{PhOH}] = 0.03\text{M}$$

$$[\text{iAN}] = 0.001\text{M}$$

t/s	0	75	150	225	300	375	450
Abs _t	0.065	0.087	0.11	0.13	0.15	0.18	0.20
10 ⁴ k _o /s ⁻¹	-	4.42	4.60	4.50	4.49	4.98	4.96
t/s	525	600	657	750	825	900	∞
Abs _t	0.22	0.24	0.25	0.27	0.29	0.30	0.74
10 ⁻⁴ k _o /s ⁻¹	4.96	5.00	4.74	4.83	4.92	4.76	-

$$k_o = 4.76 (\pm 0.34) \times 10^{-4} \text{ s}^{-1}$$

The variation of the observed rate constant, k_o, with pH at constant phenol and iso-amyl nitrite concentrations at 25°C is shown in table (5.3). If reaction occurs by prior hydrolysis of the alkyl nitrite, then the rate equations are those given by equations (5.2) and (5.3).

$$\text{Rate} = k_2 [\text{HNO}_2] [\text{PhOH}] \quad (5.2)$$

$$= \frac{k_2 [\text{PhOH}] [\text{RONO}]_{\tau}}{1 + K[\text{ROH}]} \quad (5.3)$$

$$\text{where } K = \frac{k_r}{k_{-r}} \quad (\text{see equation (5.1)})$$

$$= k_o [\text{RONO}]_{\tau}$$

$$k_o = \frac{k_2 [\text{PhOH}]}{1 + K[\text{ROH}]} \quad (5.4)$$

In any one run with $K = 0.77$ (c.f. EtONO)^{9,10} and the maximum $[\text{ROH}] = 0.001\text{M}$ the inequality $1 \gg K[\text{ROH}]$ applies. Taking the pK_a of nitrous acid ($\text{pK}_N = 3.15$) into account, the acidity dependence of the second-order rate constant, k_2 , is given in table (5.3).

Table 5.3

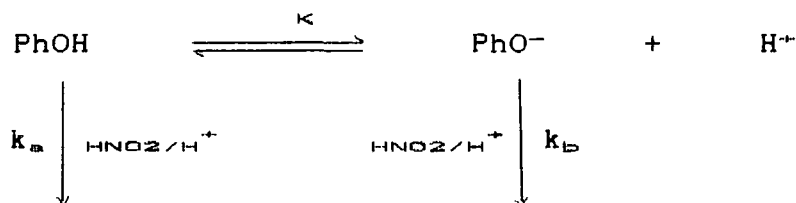
Acidity dependence of k_2 for the nitrosation of phenol with iso-amyl nitrite at 25° ($\text{pH} < 5.25$)

$$\begin{aligned} [\text{PhOH}] &= 0.03\text{M} \\ [\text{IAN}] &= 0.001\text{M} \end{aligned}$$

pH	$k_2 / \text{l mol}^{-1} \text{s}^{-1}$
2.55	0.357
3.02	0.249
3.62	0.146
3.98	0.124
4.30	0.134
5.25	0.130

As can be seen, there is no acid dependence in the pH range 3.62 to 5.25. This is consistent with the mechanism involving rate-limiting proton expulsion from the dienone intermediate when nitrous acid is the nitrosating agent used. At higher acid concentrations, the reaction shows acid catalysis. This observation is in agreement with work by Challis²⁴, who proposes that onset of another pathway for the decomposition of the dienone intermediate which is acid-catalysed, and which competes with the spontaneous decomposition. This additional pathway probably involves protonation of the nitroso oxygen atom.

At higher pH values, the pK_m of phenol must be taken into account, as well as a possible reaction pathway via the phenolate anion, (see scheme 5.3).



Scheme (5.3)

The rate equation is given in equation (5.5) and the expression for the observed rate constant, k_D is given in equation (5.6).

$$\text{Rate} = k_m [\text{PhOH}] [\text{RONO}] + k_D [\text{PhO}^-] [\text{RONO}] \quad (5.5)$$

$$k_D = \frac{k_m [\text{H}^+] + k_D K_D [\text{PhOH}]_T}{[\text{H}^+] + K_D} \quad (5.6)$$

where $[\text{PhOH}]_T$ = total phenol concentration

and $K_D = K_m$ of Phenol = $10^{-9.99} \text{ M}$

The results for the nitrosation of phenol in alkaline/neutral conditions are shown in table (5.4). k_o' is k_o with correction for the dissociation constant of nitrous acid, K_N . k_o' is equal to $k_o([H^+] + K_N)/[H^+]$.

Table 5.4

Acidity dependence of k_o for the nitrosation of phenol with iso-amylnitrite at 25°C (pH > 5.25).

[PhOH] = 0.03M
[iAN] = 0.001M

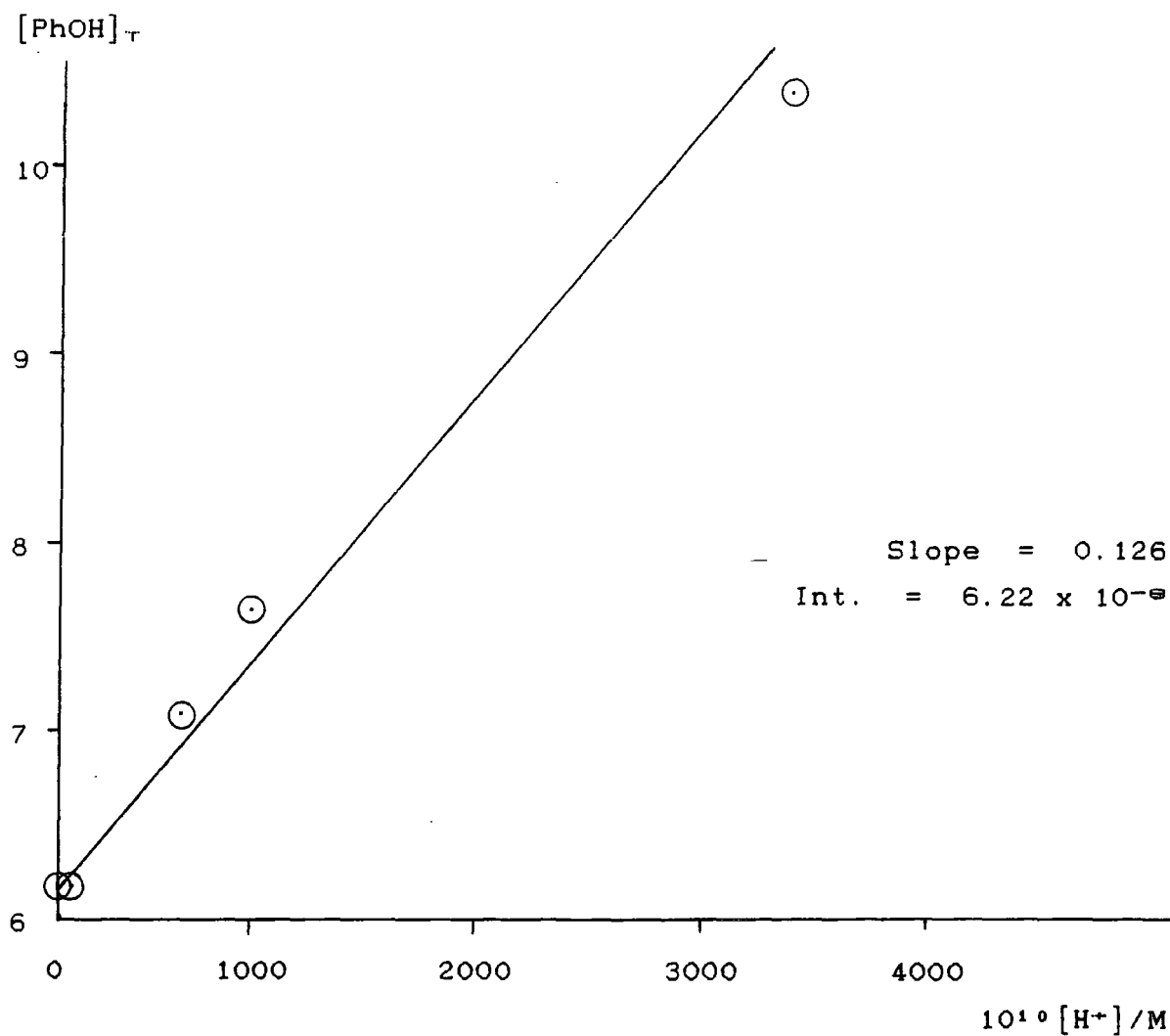
pH	$10^6 k_o / s^{-1}$	k_o' / s^{-1}	$10^8 k_o' ([H^+] + K_P) / [PhOH]_T$
6.47	4.37	9.16×10^{-3}	10.4
7.00	3.19	0.023	7.67
7.20	2.99	0.34	7.09
8.54	2.51	0.62	6.15
9.09	2.30	2.00	6.13
9.54	1.93	4.75	6.18

A plot of $k_o' ([H^+] + K_P) / [PhOH]_T$ vs $[H^+]$ would give a slope = k_m and an intercept = $k_o K_P$ (see graph (5.2)).

Graph 5.2

Acidity dependence of k_o for the nitrosation of phenol with iso-
amyl nitrite at 25°C at pH > 5.25.

$$\frac{10^8 k_o' ([H^+] + K_a)}{[PhOH]_T}$$



From graph (5.2), $k_a = 0.13 \pm 0.05 \text{ s}^{-1}$ and $k_b K_a = (6.22 \pm 0.05) \times 10^{-9} \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$. k_b , the rate constant for the reaction of the phenolate ion is then $\sim 610 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$. It is more reactive than the phenol, as expected for reactions with electrophilic species.

In the acid range then, reaction occurs via the neutral phenol species, whereas at higher pH values reaction occurs via both the neutral phenol and via the phenolate anion.

The effect of added Br^- and SCN^- has also been investigated and the results are shown in table (5.5) below.

Table 5.5

Br^- catalysis for the nitrosation of phenol using iso-amyl nitrite at 25°C at pH 3.02.

<u>$[\text{Br}^-]/\text{M}$</u>	<u>$10^3 k_o/\text{s}^{-1}$</u>
0.03	4.2
0.05	4.87
0.07	4.09
0.10	4.05
0.20	4.52

The complete lack of catalysis supports the mechanism involving rate-limiting proton expulsion from the dienone intermediate. In other words, $k_1 [\text{H}^+] [\text{X}^-] \gg k_2 [\text{B}]$ in equation (1.37) so that no nucleophile catalysis is expected. Once again similar results are obtained with the work by Challis & co-workers using nitrous acid.

Primary hydrogen isotope effects were also observed and are shown in table (5.6).

Table 5.6

Kinetic deuterium isotope effects for the nitrosation of phenol using iso-amyl nitrite at different pH values at 25°C.

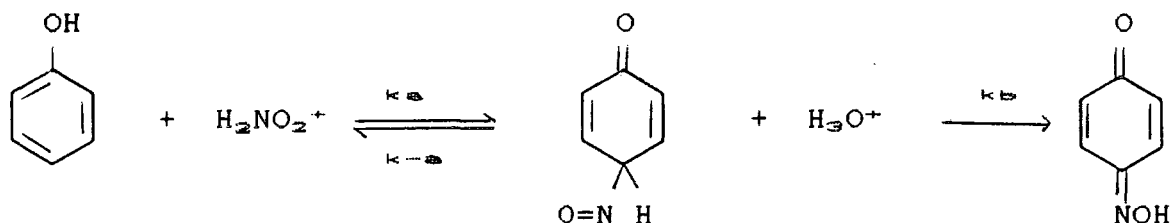
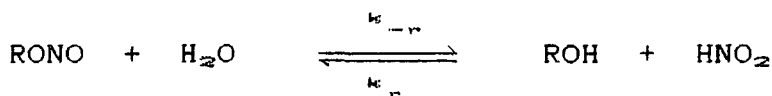
$$\begin{aligned} [\text{PhOH}] &= 0.03\text{M} \\ [\text{iAN}] &= 0.001\text{M} \end{aligned}$$

<u>pH</u>	<u>$k_2^{\text{H}}/\text{s}^{-1}$</u>	<u>$k_2^{\text{D}}/\text{s}^{-1}$</u>	<u>$k_2^{\text{H}}/k_2^{\text{D}}$</u>
2.55	0.357	0.089	4.0
4.30	0.134	0.041	3.3
7.20	0.034	0.023	1.5

In the acid range, substantial hydrogen isotope effects are observed. This would be expected for rate-limiting proton transfer. At pH 7.20 however, $k_2^{\text{H}}/k_2^{\text{D}}$ ratio is small which is expected in equation (5.8).

The alkyl nitrite is initially hydrolysed to nitrous acid, which then effects the nitrosation of phenol. Further evidence comes from the retardation of the reaction when a large excess of alcohol, iso-amyl alcohol, was added. At pH 4.0, $[\text{PhOH}] = 0.03\text{M}$, $[\text{iAN}] = 0.001\text{M}$, k_2 when 0.1M iso-amyl alcohol was added was observed to be $1.71 \times 10^{-5} \text{ s}^{-1}$ compared to the value of $4.78 \times 10^{-4} \text{ s}^{-1}$ in the absence of added alcohol.

The data then, fit the A-S_E2 mechanism, proposed by Challis^{1,8} for the nitrosation of phenol using acidified nitrite. The reaction scheme is shown in scheme (5.4).



Scheme (5.4)

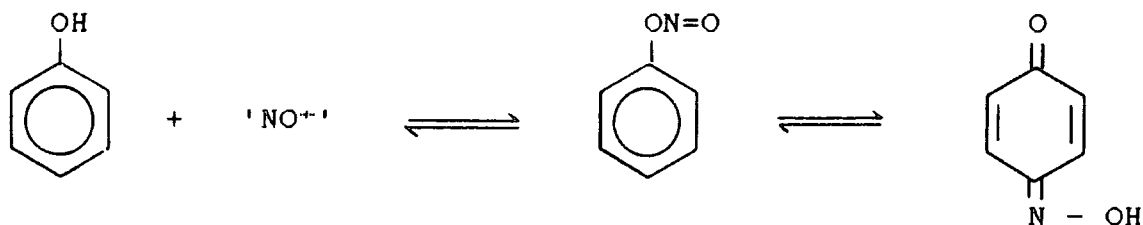
The collapse of the intermediate neutral dienone to reactants (step k_{-m}) is acid-catalysed, whereas formation of products is catalysed by a basic species including water. In the acid range $k_{-m}[\text{H}^+] \gg k_B$ so that the rate equation becomes (5.7).

$$\text{Rate} = \frac{k_m k_b [\text{PhOH}] [\text{RONO}]_T}{k_{-m} (1 + K [\text{ROH}])} \quad (5.7)$$

At neutral and alkaline pH values, $k_B \gg k_{-m}[\text{H}^+]$ so that the rate equation is now given by equation (5.8)

$$\text{Rate} = \frac{k_1 [\text{PhOH}] [\text{H}^+] [\text{RONO}]_T}{1 + K [\text{ROH}]} \quad (5.8)$$

The reaction via an initial O-nitrosation followed by a rearrangement resulting in the C-nitroso product (see scheme (5.5)) cannot be ruled out. This type of rearrangement is found for aromatic N-nitrosamines, first discovered in 1886 by Fisher and Hepp¹⁹. This mechanism cannot be distinguished kinetically from the direct C-nitrosation reaction.



Scheme (5.5)

The possibility of reaction via a radical pathway was investigated by e.s.r. spectroscopy. Because the phenoxy radical has a short half-life, the 2,4,6-trimethyl (TMP) derivative was used as the latter has a short-life long enough to detect its presence (see chapter 7, section 7.1.3). There was no evidence of formation of the 2,4,6-trimethyl phenoxy radical however. Further, the addition of a radical trap, 2-methyl-2-nitroso propane dimer did not affect the reaction rate.

The nitrosation of 2,4,6-trimethyl phenol proceeded less rapidly than phenol, possibly due to steric hindrance. Apart from this steric factor, the behaviour of 2,4,6-trimethyl phenol towards nitrosation is similar to that of phenol, as can be seen from tables (5.7) and (5.8).

Table 5.7

Dependence on concentration of TMP for the reaction with iso-amyl nitrite at pH 2.33 at 25°C.

$$[\text{IAN}] = 0.001\text{M}$$

<u>[TMP]/M</u>	<u>$10^5 k_{\text{e}}/\text{s}^{-1}$</u>
.03	5.36
.05	9.01
.07	12.9

Table 5.8

Acidity dependence of k_2 for the nitrosation of TMP with iso-amyl nitrite at 25°C

$$\begin{aligned} [\text{TMP}] &= 0.03\text{M} \\ [\text{IAN}] &= 0.001\text{M} \end{aligned}$$

<u>pH</u>	<u>$10^3 k_2 / \text{l mol}^{-1} \text{s}^{-1}$</u>
2.33	2.06
3.02	1.91
4.00	1.81
4.30	1.82

5.3 Nitrosation of phenols using S-nitroso-N-acetyl-penicillamine

The reactions of phenols with S-nitroso-N-acetyl penicillamine (SNAP) show a quite different pattern of experimental behaviour than do the corresponding reactions of iso-amyl nitrite.

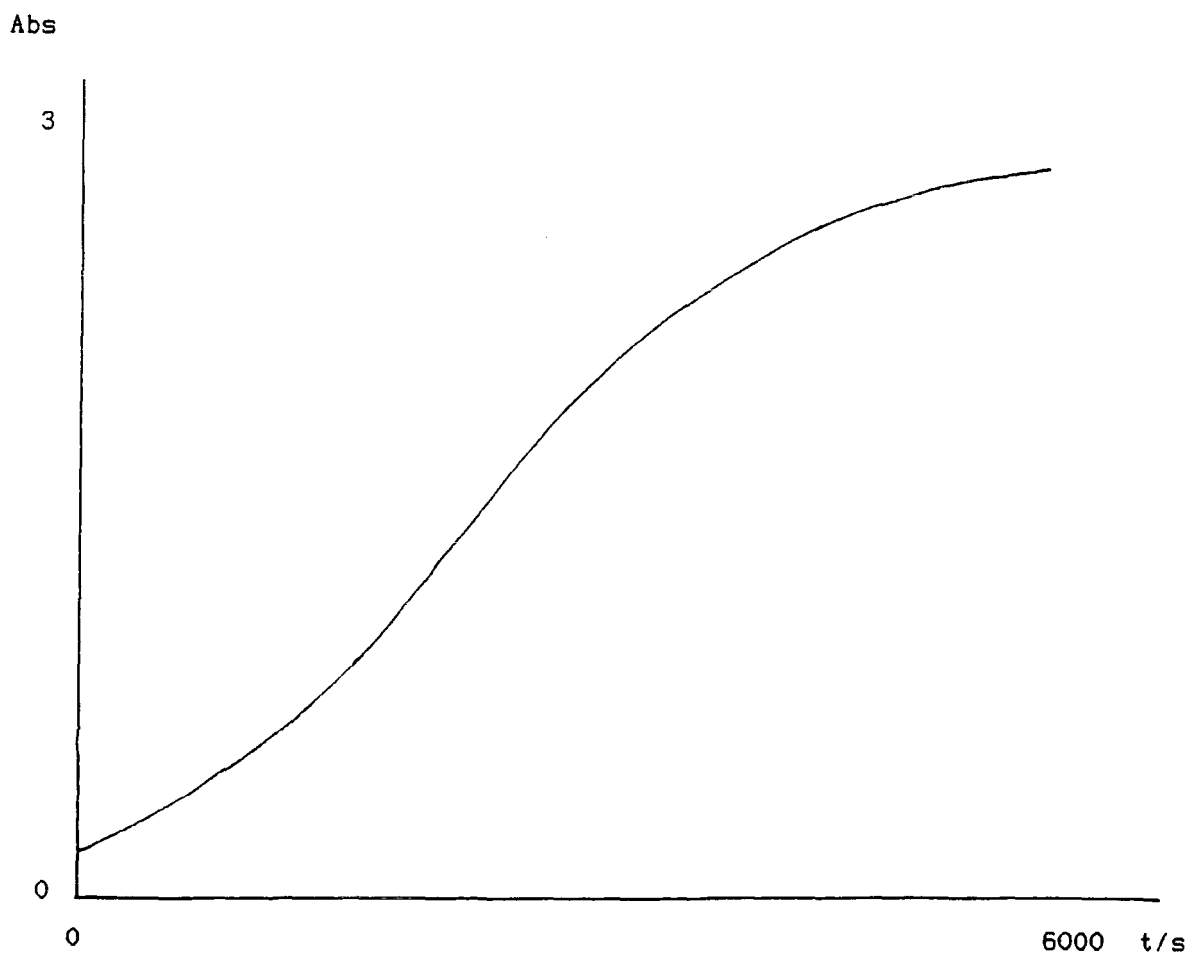
Under the same experimental conditions, first-order behaviour was not observed; the reaction profile is shown in graph (5.4).

The reaction is of mixed order and the formation of an intermediate is implicated i.e. an $A \rightarrow B \rightarrow C$ type reaction.

Anisole was found to be totally unreactive in the same pH range studied for iso-amyl nitrite.

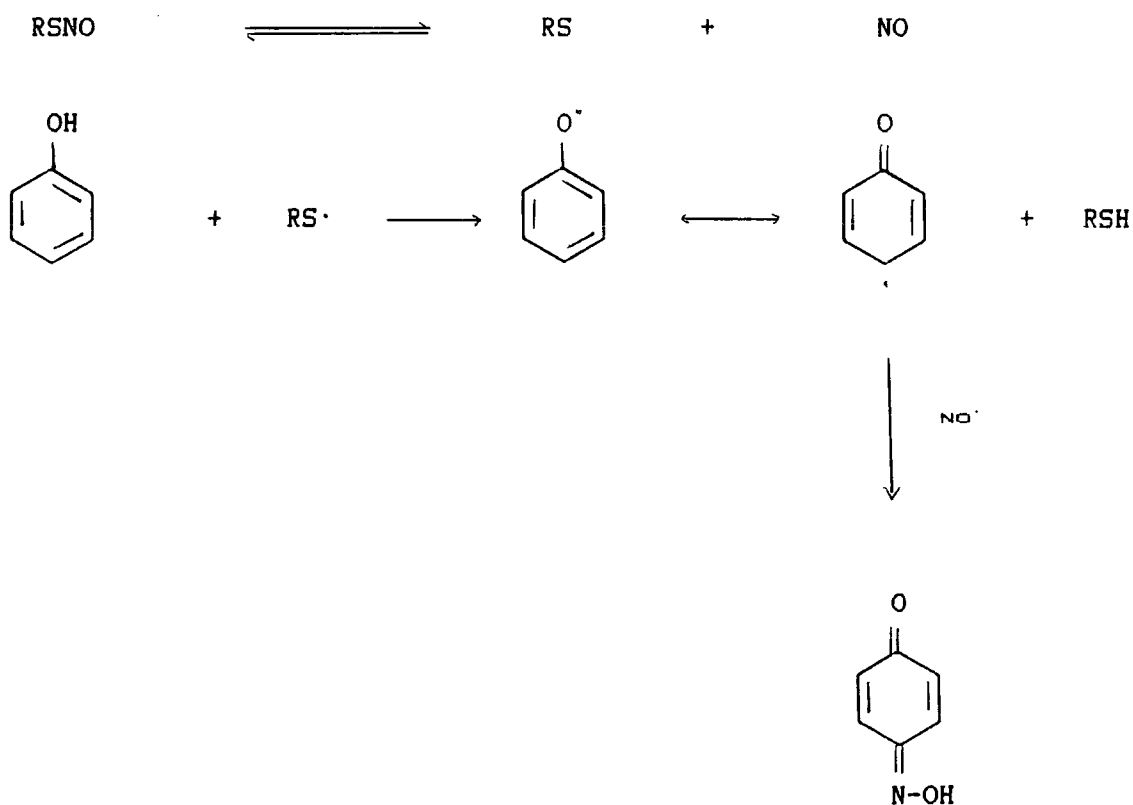
Graph 5.4

Reaction profile for the reaction of SNAP with phenol at pH 7 at 25°C.



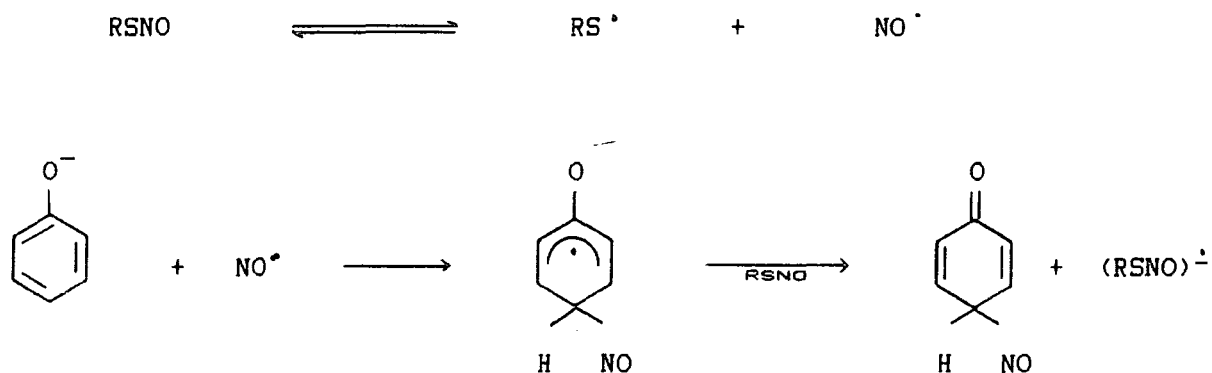
E.s.r. studies with 2,4,6-trimethylphenol showed the formation of the 2,4,6-trimethylphenoxy radical, when TMP was reacted with SNAP at pH 7.0 at 25°C. Further, the decomposition of SNAP to the thiol radical and NO radical was detected by irradiating a solution of SNAP in buffer 7.0 with a 100kW high-pressure mercury lamp in a photo-reaction vessel, because of the short half-life of thiol radicals. The spectra of both the TMP and thiol radicals are shown in chapter 7, section 7.1.3.

Thus a radical pathway seems to be implicated for the reaction of SNAP with phenols. A possible scenario is illustrated in scheme (5.6).



Scheme (5.6)

In alkaline conditions, another radical pathway is also conceivable, which involves the radical anion of phenol (see scheme (5.7)).



Scheme (5.7)

The decomposition of thionitrites thermally and chemically is well-known²⁰. Further evidence for the radical pathway comes from the fact that the reaction at pH 4.0 was considerably slower than that at pH 7.0, corresponding to the slower rate of decomposition of thionitrites at pH 4.0 than at pH 7.0, which has been observed but for which no mechanistic explanation has been found. The radical chain $S_{RN}1$ mechanism proposed in scheme (5.7) was first proposed by Bunnett, Rossi and Kornblum for the reaction of aromatic halides with various anions²¹.

One electron transfer reactions of phenol with nitrite ion²² and with N-nitrosamines²³ have been found to occur photochemically.

Further, the addition of the radical scavenger, 2-methyl-2-nitroso propane dimer accelerated the decomposition of SNAP indicating a radical reaction with SNAP.

It is possible that the two electron transfer mechanism (as with iso-amyl nitrite) occurs simultaneously, but the two pathways cannot be separated.

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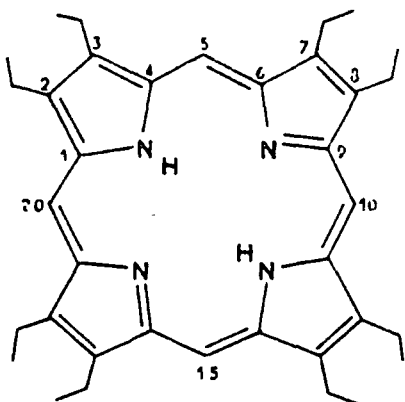
CHAPTER 6

Nitrosation of haems -

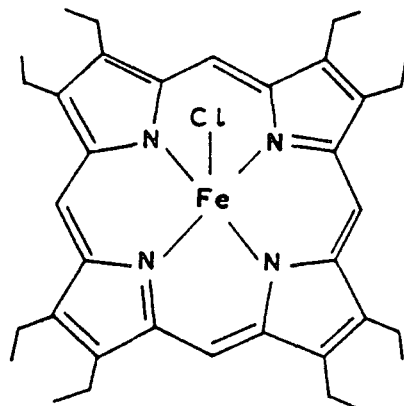
6.1 Introduction

The formation of nitrosylhaems has been much studied because they feature widely in biological systems. The basic structure of haems is shown in structures (6.1), (6.1a), (6.2), (6.3), (6.3a) and (6.3b) below: a porphyrin ring surrounds the metal at the centre. The metal may exist in the +2 or +3 oxidation states. The porphyrin ring may contain vinyl and/or methyl ester groups. In haemoglobin, myoglobin and other haemo proteins, protein molecules surround this haem moiety.

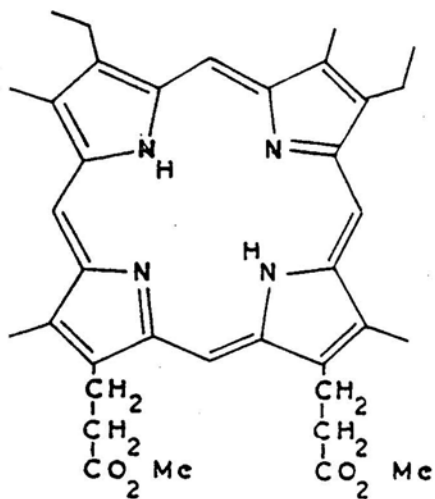
Octaethylporphyrin (6.1) and octaethylhaemin (6.1a) are model compounds, which contain eight identical saturated β -substituents, so simplifying product identification; protohaemin dimethyl ester (6.3a) contains the arrangement of β -substituents found in the haem moiety of myoglobin.



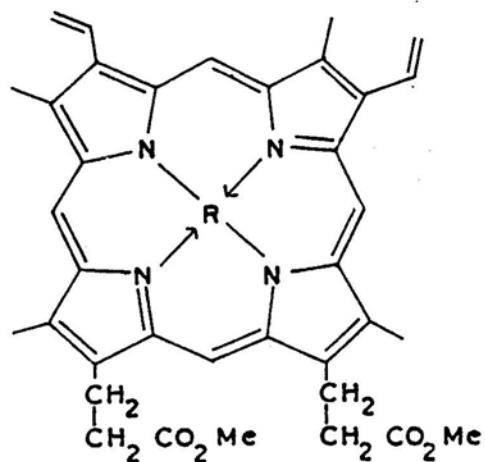
(6.1) Octaethylporphyrin



(6.1a) Octaethylhaemin



(6.2) Mesoporphyrin
dimethyl ester

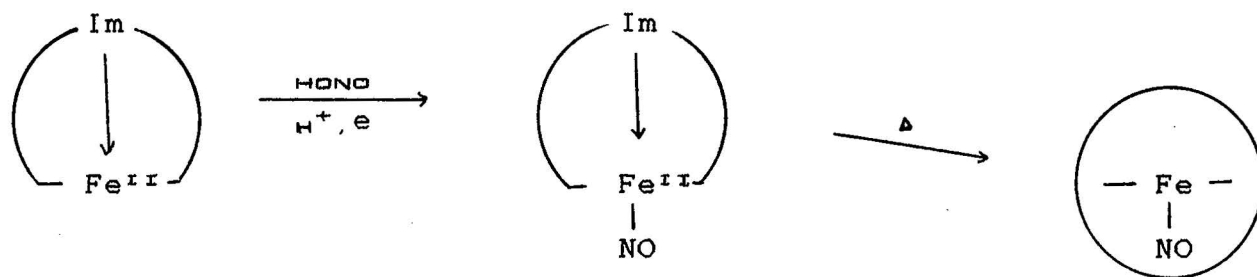


(6.3) R=2H: Protoporphyrin dimethyl ester

(6.3a) R=Fe^{III} Cl: Protohaemin dimethyl ester

(6.3b) R=FeNO: Nitrosyl protohaem dimethyl ester

Nitrosylhaem has been identified as the pink pigment found in cured meat. This was first recognised by Haldane in 1901¹ and is currently viewed as a denatured nitrosylmyoglobin (see scheme (6.1)).



Myoglobin (Fe²⁺P)

Nitrosylmyoglobin (NOFe²⁺P)

Denatured nitrosyl
myoglobin

Scheme (6.1). The horizontal line represents protoporphyrin seen edge on and the curved line represents the globin (protein), containing an amino acid which provides the imidazole (Im) ligand. P stands for porphyrin dianion throughout this chapter.

This pink pigment forms when meat products are treated with sodium nitrite, which is employed in regulated amounts in the curing processing of meat products. Sodium nitrite is used firstly as an antibacterial agent, in preventing the growth of the bacterium *Clostridium botulinum*; secondly, for its palability; and thirdly as a cosmetic agent, generating the attractive pink colour.

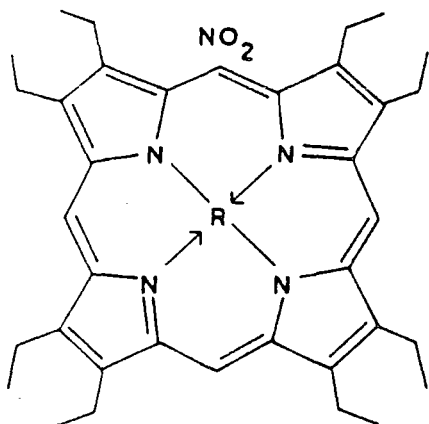
Nitrosylhaems probably also feature in the control of blood pressure. Recently, it has been proposed that the group of "nitrovasodilators" which includes alkyl nitrates, alkyl nitrites, nitrosothiols, furoxans, sydnonimines and nitroprusside, exert their effect via a common mechanism i.e. the generation of nitric oxide (NO)². These compounds mimic the physiological process of endothelium-derived relaxing factor (EDRF) release, responsible for endothelial controlled vasodilation³. The biosynthetic precursor of EDRF has been found, to be L-arginine⁴.

Alkyl nitrates⁵, alkyl nitrites and furoxans require the presence of thiols, such as cysteine, to be active, while sydnomimines, nitrosothiols and nitroprusside do not. This is believed to correspond to the NO-releasing properties of these nitrovasodilators. Alkyl nitrates, alkyl nitrites and furoxans require a mercapto group to release NO whereas sydnonimines, nitrosothiols and nitroprusside spontaneously release NO⁶.

The pharmacodynamic action of these compounds is mediated by an activation of the soluble isoenzyme of guanylate cyclase^{7,8}. The chemical nature of the active metabolite is still the subject of controversy: there is a school of thought which supports S-nitrosocysteine, rather than NO, as the EDRF⁹. Whether EDRF is NO or S-nitrosocysteine, the enzyme guanylate cyclase, which binds a haem prosthetic group, is activated when NO binds to the Fe at the haem site, displacing the Fe out of the plane of the porphyrin ring, leaving a protoporphyrin IX-like structure. Protoporphyrin-IX is known to be an activator of soluble guanylate cyclase¹⁰. In light of this, it was thought interesting to investigate the direct reaction between alkyl nitrite and nitrosothiols with haems.

Bonnett and co-workers have studied the reaction of haemins with acidified NaNO₂ and have found reactions at both the porphyrin ring and at the metal¹¹⁻¹⁵.

Treatment of octaethylporphyrin with an excess of NaNO₂ in aqueous acetic acid-sulphuric acid at room temperature yields the meso-nitration product: 5-nitro-octaethylporphyrin^{12,13}. Similar results were obtained for the octaethylhaemin and porphyrin dimethyl ester (see structure (6.4)).

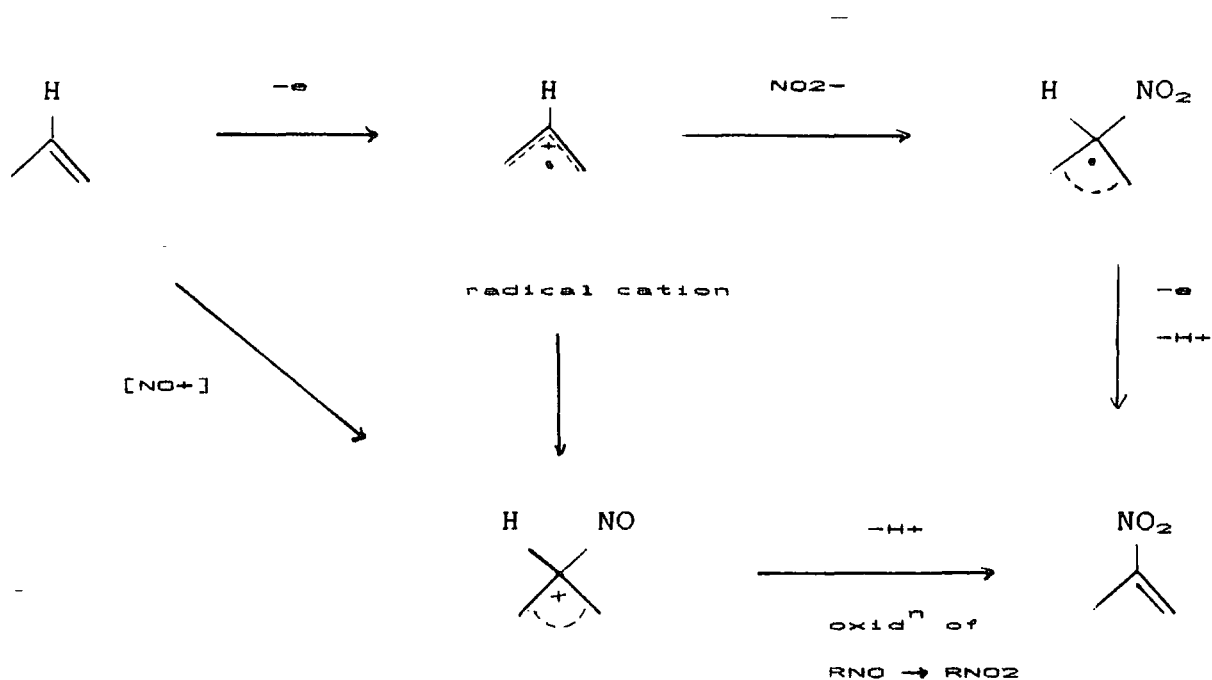


R = 2H: 5-Nitrooctaethyl-
porphyrin

R = FeCl: 5-Nitrooctaethyl-
haemin

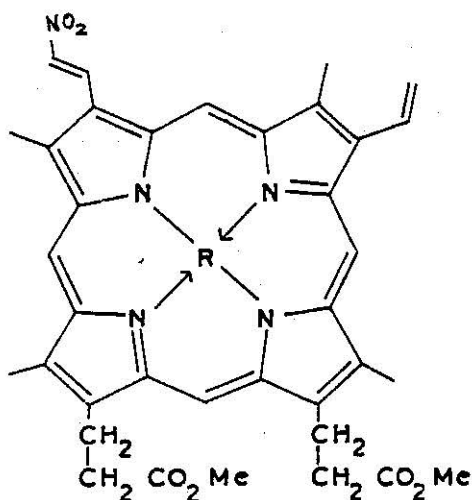
(6.4)

The possible mechanism for the formation of the meso-nitration product is outlined in scheme (6.2). The heteroaromatic system is first oxidised to an intermediate radical cation, which is then attacked either by the nitrite ion or by NO. Alternatively, electrophilic C-nitrosation is followed by oxidation of the nitroso compound to the nitro compound.

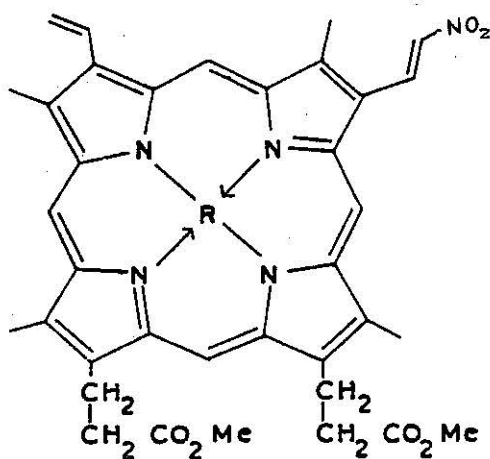


Scheme (6.2)

With protoporphyrin dimethyl ester and protohaemin, further green products were observed¹⁴, due to attack at the vinyl group yielding the nitrosites (see (6.5) and (6.6)) together with numerous other minor products. Because of this complication, we have avoided the use of derivatives containing vinyl groups in our investigations.



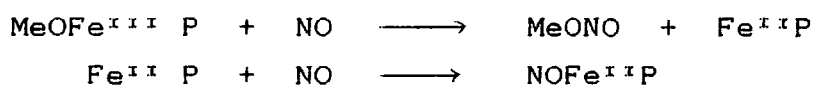
(6.5)



(6.6)

Under certain conditions, reaction occurred at the metal itself. Nitrosylation of Fe resulted in the formation of the nitrosylhaem. The two reaction conditions used by Bonnett were $D(g)$ in THF and acidified NO_2^- in THF.

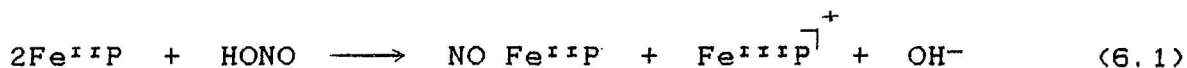
The reactions of NO (and other diatomic molecules such as O₂, CO and CS) with metalloporphyrins have been intensively investigated¹⁶, because of their relevance to biologically significant haemoproteins. With the haemoproteins, myoglobin and haemoglobin only one stable product is observed¹⁷, that corresponding to a {FENO}⁷ species¹⁸, irrespective of whether the protein was in the Fe^{II} or Fe^{III} form. The reaction of NO with Fe^{II} and Fe^{III} porphyrinates yields five and six-coordinate {FENO}⁷ species¹⁹⁻²². The ferric reactant is reduced via reductive nitrosylation in the presence of NO¹³ (see scheme (6.3)).



Scheme (6.3)

This is further supported by magnetic and spectroscopic work by Wayland and Olson²⁰, who found evidence for the Fe^{II} complex. The complex was formulated as Fe^{II}TPP(Cl⁻)(NO⁺) produced when Fe^{III}TPP Cl reacts with NO (TPP = tetraphenyl porphyrin).

Under the second reaction condition i.e. with acidified nitrite, the reaction was slow and complex. The stoichiometric formation of the nitrosyl using acidified nitrite requires a reductant²³: this may either be one of a number of naturally occurring reducing agents in meats, in the meat curing process, or ascorbic acid, often added to the cure to effect more efficient formation of nitrosylhaem. In the absence of other reductants the iron (II) porphyrin may fulfil this role, with the consequent formation of the nitrosyl complex and the Fe^{III} complex in a 1:1 ratio^{23,24} (see equation (6.1)).



It is evident that NO produced from the reaction of the reducing agent with HONO reacts more efficiently with the metalloporphyrins. Before looking at the reaction between alkyl nitrites and nitrosothiols with haems therefore, the reaction between these reagents with reducing agents such as ascorbic acid must be investigated.

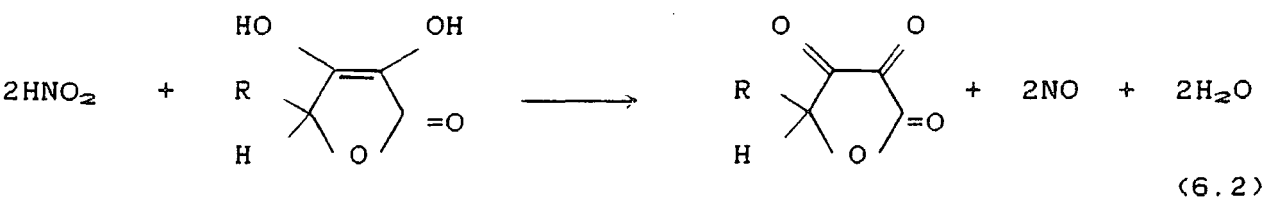
The reaction between ascorbic acid and haems must also be investigated as the reducing agent added will be expected to react with Fe^{III} in the system, in addition to its reaction with the nitrosating agent.

Finally, the direct interaction between the nitrosating agents: alkyl nitrites and nitrosothiols with haems has also been looked at.

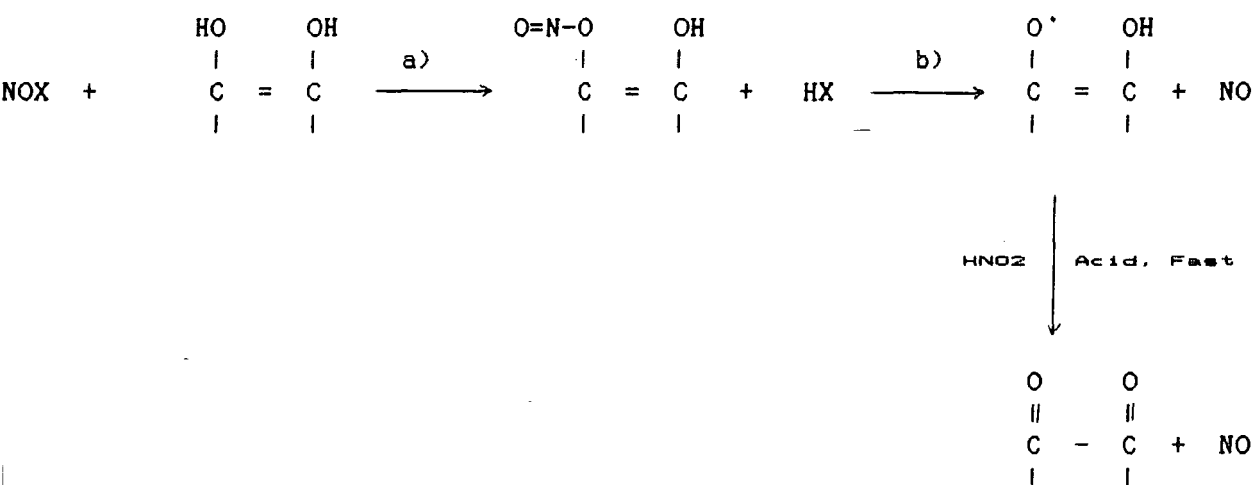
All experiments that have been carried out constitute a preliminary investigation of the reactivities of the different compounds involved in the complex reaction system.

6.2 Reaction of ascorbic acid with alkyl nitrites and nitrosothiols

The reaction between ascorbic acid and nitrous acid has been studied by Bunton and Dahn²⁵. Their reactions were carried out at 0°C in dioxan because the reactions were too fast in water. The stoichiometry of the reaction is given in equation (6.2).



The mechanism proposed is shown in scheme (6.4). The nitrosating agent is responsible for electrophilic attack on the ascorbic acid, with the formation of nitrite, which then decomposes, releasing NO and an ascorbate radical. It is possible for steps a) and b) to be synchronous; the process would then be considered as a transfer of a single electron to the nitrosating agent. In either event it was concluded that the first step of the reaction between a nitrosating agent and ascorbic acid giving the intermediate is followed by a rapid reaction with nitrous acid.



Scheme (6.4)

The nitrosating agent is the nitrous acidium ion or the nitrosonium ion at $\text{pH} < 1$ and dinitrogen trioxide at higher pH values²⁵. At pH 3-5, a significant fraction of the ascorbic acid ($\text{pK}_{\text{m}} = 4.17$) exists as the presumably more reactive monoanion, so that the formation of dinitrogen trioxide becomes rate-limiting, rather than the reaction of ascorbate with dinitrogen trioxide. The reaction is catalysed by added nucleophiles: chloride ion, bromide ion and thiocyanate ion, as expected, at $\text{pH} < 1$.

Nitrosation reactions using alkyl nitrites in aqueous acidic solutions is discussed in chapter 5 in the reaction of iso-amyl nitrite with phenol. All the results are in agreement with the initial reversible hydrolysis of the alkyl nitrite, giving free nitrous acid, which then effects nitrosation.

The reaction of S-nitroso-N-acetyl penicillamine (SNAP) was also studied, as an example of a reaction of a nitrosothiol with ascorbic acid.

The reactions were carried out under pseudo-first order conditions with $[\text{RONO}]$ and $[\text{SNAP}] \ll [\text{Ascorbic acid}]$. All runs were carried out in buffer solutions at 25°C . Because of the low pK_{a} of ascorbic acid (4.17), solutions of ascorbic acid were made up to the appropriate pH for each reaction. The reaction was followed by monitoring the decrease of the absorbance at 360nm due to the disappearance of the alkyl nitrite and at 330nm due to the disappearance of SNAP. The alkyl nitrite used in this case was once again, iso-amyl nitrite (IAN). The formation of NO was detected by mass spectrometry (see chapter 7, section 7.1.6).

In the case of iAN, good first-order behaviour was observed. The effect of variation of ascorbic acid and acid concentration on the observed first-order rate constant, k_o , was investigated and the results are shown in tables (6.1) and (6.2) respectively. The effect of adding a nucleophile, Br^- was also investigated and the results shown in table (6.3).

Table 6.1

Dependence of k_o on [AA] at 25°C at pH 4.0

$$[RONO] = 0.0025M$$

<u>[AA]</u>	<u>k_o/s^{-1}</u>
.02	.0083
.03	.0085
.04	.0116
.05	.0140
.06	.0090

Table 6.2

Dependence of k_o on pH at 25°C

$$[AA] = 0.03M$$

$$[RONO] = 0.0025M$$

<u>pH</u>	<u>k_o/s^{-1}</u>	<u>$k_o/[H^+]$</u>
3.40	.0328	82.4
3.71	.0189	96.9
3.77	.0147	86.6
3.89	.0116	90.0
4.60	.00244	97.1

Table 6.3

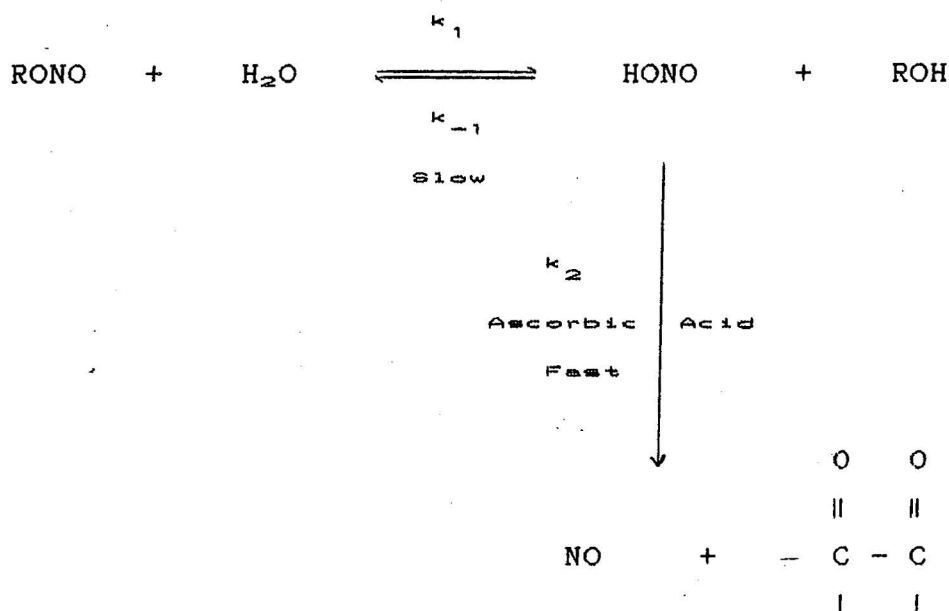
Dependence of k_o on $[Br^-]$ at pH 4.0

$$[AA] = 0.03M$$

$$[RONO] = 0.0025M$$

<u>$[Br^-]$</u>	<u>k_o/s^{-1}</u>
.01	.0076
.02	.0087
.05	.0124
.07	.0145
.09	.0169

The measured reaction rate constant is independent of the [AA]. k_o is dependent on both $[H^+]$ and $[Br^-]$. The indications are that the reaction occurs via rate-limiting hydrolysis of the alkyl nitrite, followed by a rapid reaction between the nitrous acid with the ascorbate ion. The reaction scheme is shown below (see scheme (6.5)).



Scheme (6.5)

When $k_2[AA] \gg k_{-1}[\text{ROH}]$ the rate-equation becomes equation (6.2), and the observed rate constant is k_1 .

$$\text{Rate} = k_1[\text{RONO}] \quad (6.2)$$

$$k_o = k_1 \quad (6.3)$$

The value of k_1 will be acid dependent and will be subject to nucleophile catalysis, as expected for the hydrolysis of alkyl nitrites. Allen³⁹ established the rate law (equation (6.4)) for the hydrolysis of propyl nitrite and t-butyl nitrite.

$$\text{Rate} = k[H^+][\text{RONO}] \quad (6.4)$$

This enables a value of $90.5 \pm 6.6 \text{ l mol}^{-1} \text{ s}^{-1}$ to be obtained from the values of k_2 shown in table (6.2) (c.f. $282 \text{ l mol}^{-1} \text{ s}^{-1}$ for the hydrolysis of EtONO^{40}).

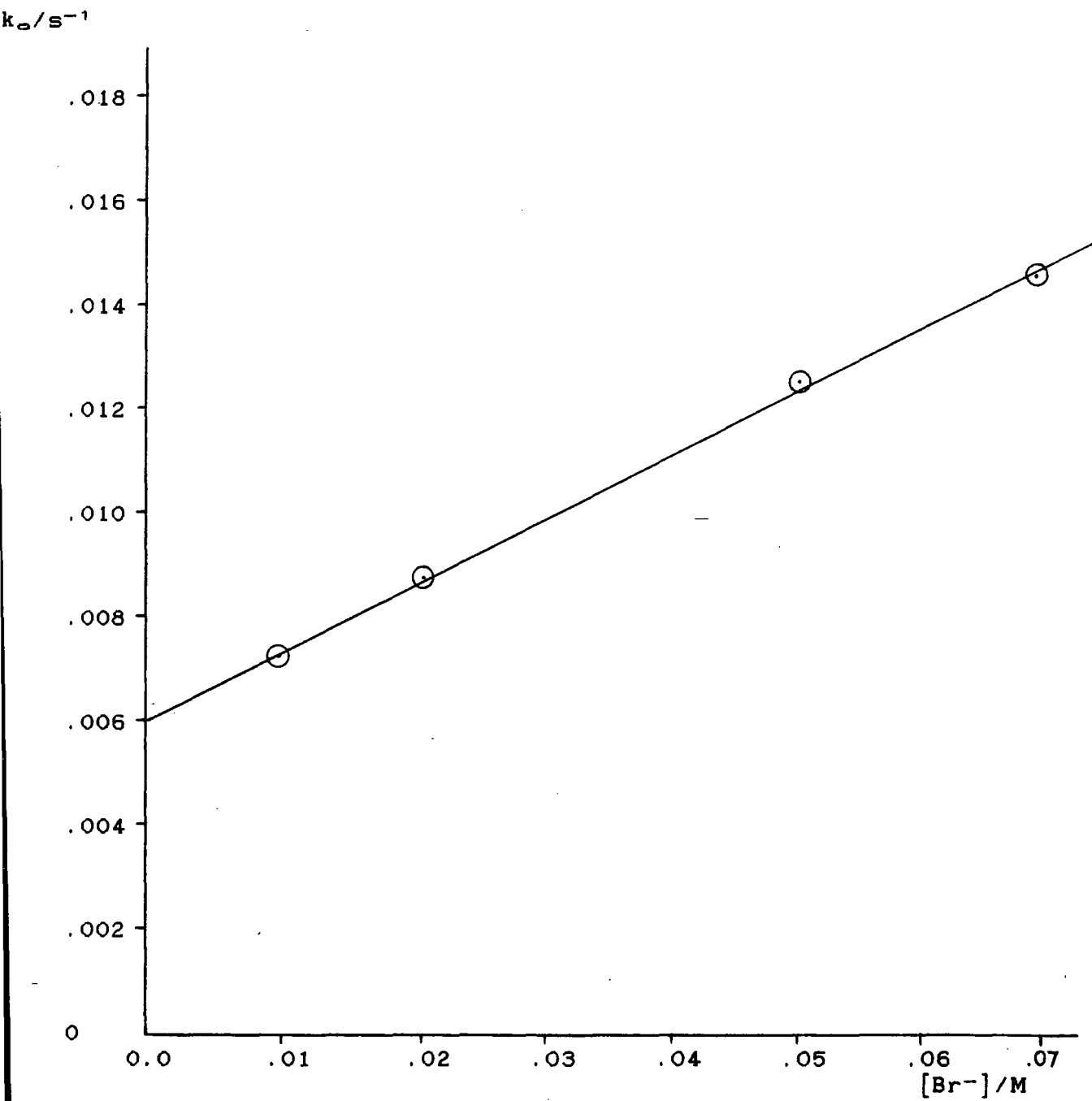
For the Br^- catalysed reaction the plot of k_1 vs $[\text{Br}^-]$ gives a slope = $k_3[\text{H}^+]$, where k_3 is the third-order rate constant for the Br^- catalysed hydrolysis of alkyl nitrite (see graph (6.1)), k_3 from the slope is $1160 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$ (c.f. $1050 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$ for the third-order rate constant for the bromide-catalysed hydrolysis of methanol⁴⁰).

The reaction of nitrous acid with the ascorbate anion, which is predominant in the pH range studied is very rapid. This is comparable to the work by Dahn²⁵ *et al* where the formation of dinitrogen trioxide becomes rate-limiting in the nitrosation of ascorbic acid by acidified nitrite in the pH range 3-5.

In the case of SNAP, the reaction profile showed mixed order kinetics (see graph (6.2)). Because of the ease with which SNAP decomposes to yield the thiol radical and the NO radical, and the ready formation of ascorbate radicals in solution, it is possible that a reaction pathway involving radicals occurs. NO was detected, once again by mass spectrometry, in the gaseous products formed from the reaction mixtures

Graph 6.1

Br^- dependence of k_o in the nitrosation of ascorbic acid by iso-
amyl nitrite.



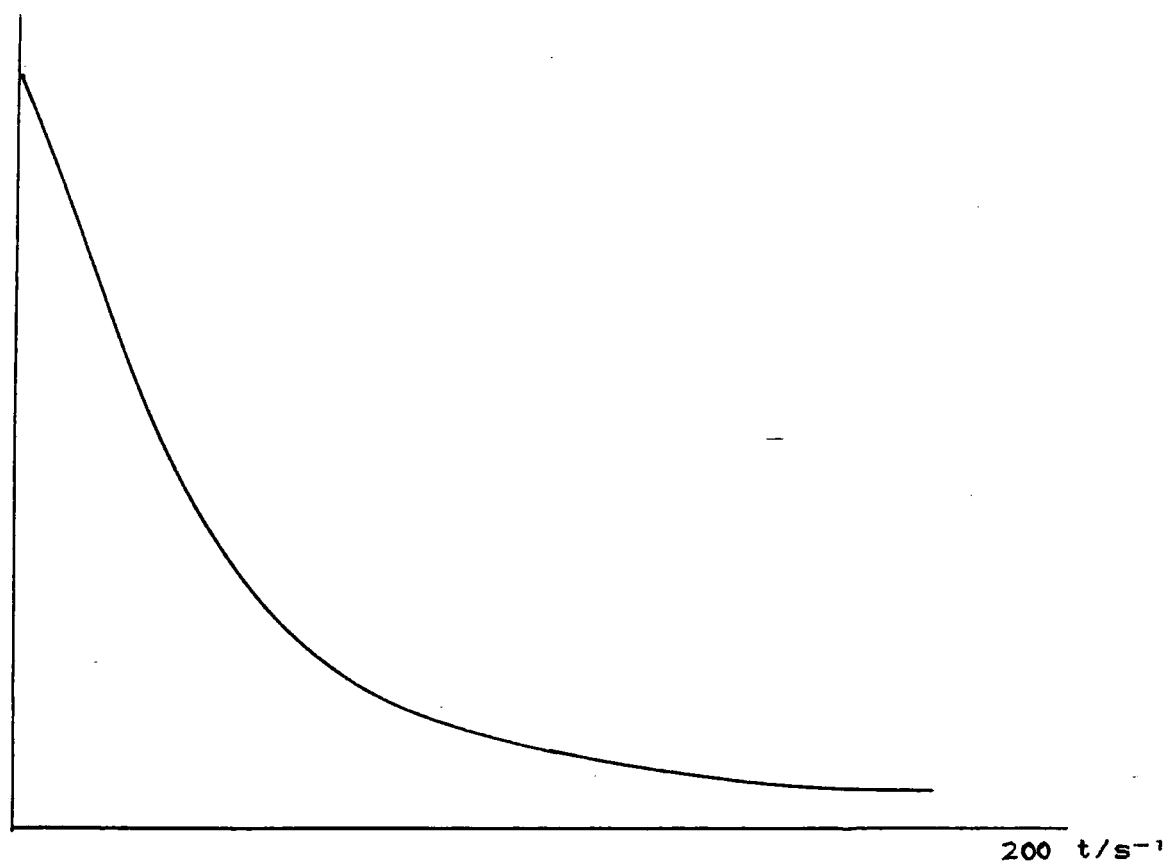
Graph 6.2

Reaction between SNAP and ascorbic acid at pH 4.0

[AA] = 0.1M

[SNAP] = 0.0005M

Abs



6.3 Reaction of ascorbic acid with haems

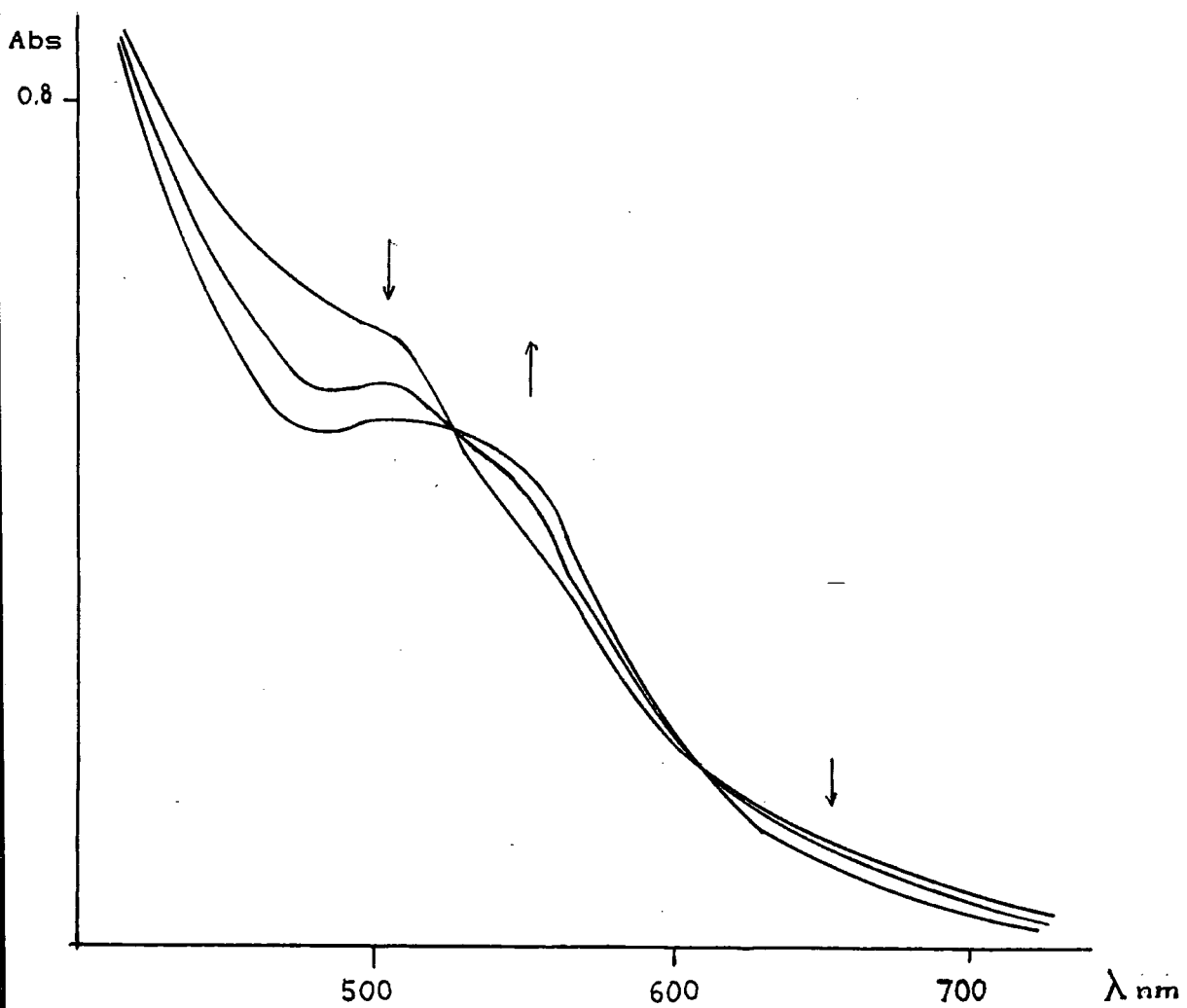
In order to prepare the pigment formed in meat-curing, reductants such as sodium dithionite²⁶ or ascorbic acid^{27,28} are required, in the reaction of NO with iron (III) porphyrin. It is likely that iron (III) porphyrin is reduced to iron (II) porphyrin which then reacts with NO. Further, evidence points to the formation of iron (II) nitrosyls whether iron (III) or iron (II) is nitrosylated under anaerobic conditions (see discussion in section 6.1). The reduction of iron (III) porphyrins by ascorbic acid is investigated, as ascorbic acid is present in the systems we have been considering; the reaction of iron (II) porphyrins with alkyl nitrites and nitrosothiols.

The reduction of metmyoglobin²⁹⁻³³ and cytochrome c^{34,35} have been extensively studied. Both the monoanion and dianion are involved in the reduction of Fe³⁺ in the blood pigments. Ascorbic acid is thought to form a complex in the first stage by binding to the globin of the molecule; electron transfer from the ascorbic acid to the Fe³⁺ then occurs either via the porphyrin ring or the imidazol co-ordinating the haem group in the case of myoglobin or by quantum mechanical tunnelling in the case of cytochrome c.

The repeated scans at 5 minute intervals of the reaction between ascorbic acid and haemin are shown in graph (6.3). The reaction was carried out at pH 7 with the concentrations of haemin and ascorbic acid as shown. Haemin (bovine) was purchased from Aldrich Chemicals and was initially dissolved in tetrahydrofuran. The resulting aqueous solution contained 10% tetrahydrofuran. The solutions were bubbled with N₂ overnight to prevent aerial oxidation of Fe²⁺ porphyrins. The reaction was carried out at 25°C. As can be seen, there is an increase in absorbance at 522nm³⁵ and decrease at 610nm³² due to the formation of Fe²⁺ porphyrin.

Graph 6.3

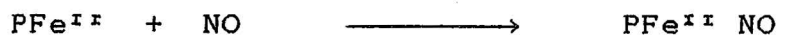
Reaction of $5 \times 10^{-4}M$ ascorbic acid with 1×10^{-4} haemin at pH 7.0 at $25^{\circ}C$



It is clear than that iron (III) porphyrin is reduced to iron (II) porphyrin in the presence of ascorbic acid. We can now use this to investigate the reaction between the haems and alkyl nitrites and nitrosothiols.

6.4 Reaction between haems and alkyl nitrites and nitrosothiols

The reaction of nitrous acid and alkyl nitrites with haemoglobin and myoglobin have been studied by Doyle et al^{37,38}. Iron (II) porphyrins are oxidised by both nitrous acid and alkyl nitrites to the iron (III) porphyrins with the resulting formation of NO. The NO formed can then combine rapidly with the iron (II) porphyrin (see scheme (6.6)).



Scheme (6.6)

The rate of reaction is dependent on the conformational states of haemoglobin, reflecting the oxidative susceptibility of the conformers. To avoid this complication, the iron porphyrin moiety (without the protein molecules) is used in this investigation.

Haemin was reduced by ascorbic acid by incubating an excess concentration of ascorbic acid to haemin in a water bath with a 25°C thermostat for three hours to ensure that iron (III) has been reduced to the iron (II) porphyrin (see section 6.3). The solutions were bubbled with N₂ and sealed in air-tight flasks under nitrogen. Iso-amyl nitrite was distilled before use and the S-nitroso-N-acetyl penicillamine was synthesised (see chapter 7, section 7.4). Reactions were followed at 600nm by stopped-flow spectrophotometry. The results are shown in graphs (6.4) and (6.5).

Both reactions with iso-amyl nitrite and with SNAP show a reaction profile which are more complex than a simple first-order behaviour. It is possible that the reactions involve one-electron transfer, resulting in the formation of Fe (III) porphyrin and NO, as in the case of the myoglobins and haemoglobins^{37, 38}. The reaction of haemoglobin with nitrous acid (or ethyl nitrite) results in the concurrent formation of nitrosylhaemoglobin and methaemoglobin due to the reductive release of nitric oxide from nitrous acid (or ethyl nitrite)³⁷. The composite experimental results are interpreted as the nitrous acid (or ethyl nitrite) association with haemoglobin followed by rate-limiting electron transfer resulting in nitric oxide and hydroxide (or alkoxide) production. Nitric oxide is proposed to exist in equilibrium with its dimer, and oxidation of haemoglobin by (NO)₂ is competitive with nitric oxide association with haemoglobin.

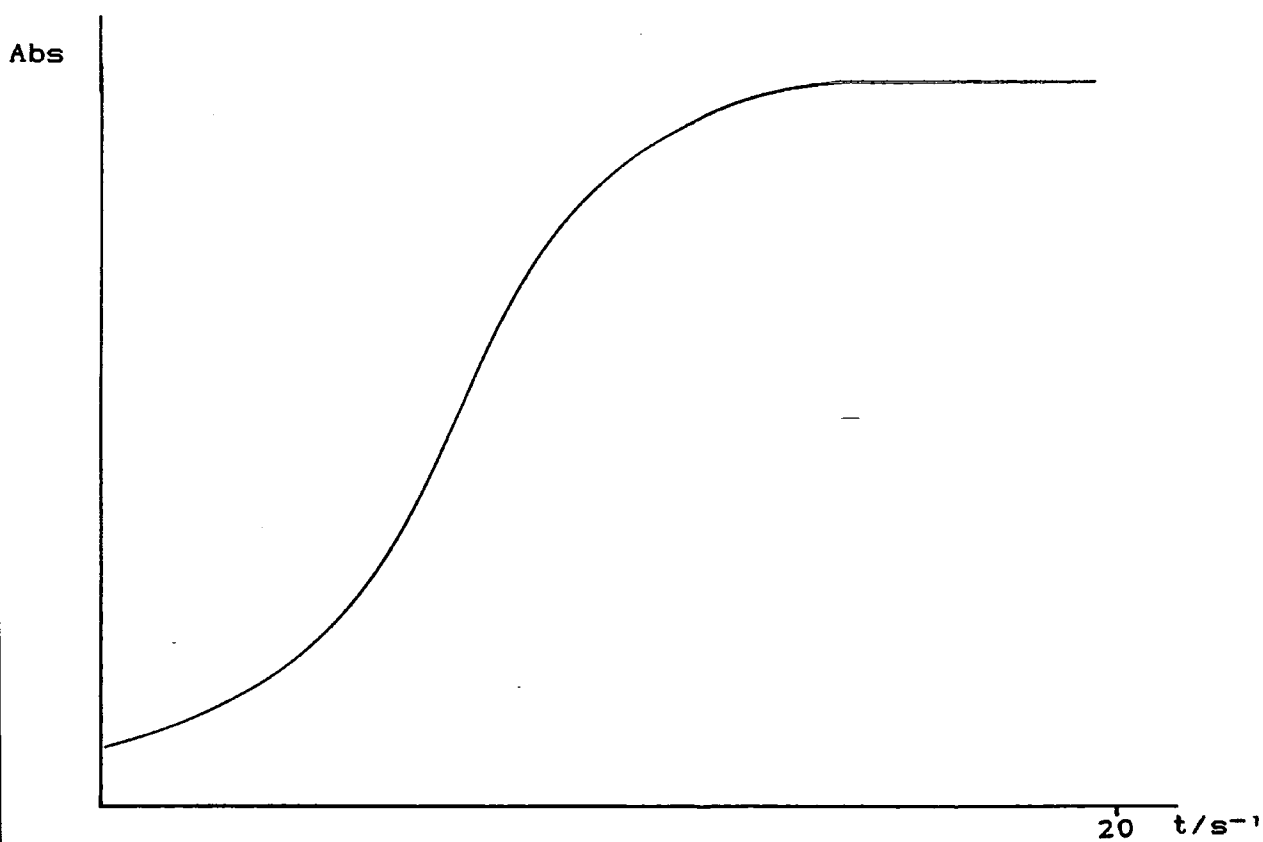
Graph 6.4

Reaction of haemin with iso-amyl nitrite after reduction by ascorbic acid at pH = 7.1 at 25°C.

$$[\text{Haemin}] = 2 \times 10^{-4} \text{ M}$$

$$[\text{IAN}] = 0.005 \text{ M}$$

$$[\text{AA}] = 0.01 \text{ M}$$



Graph 6.5

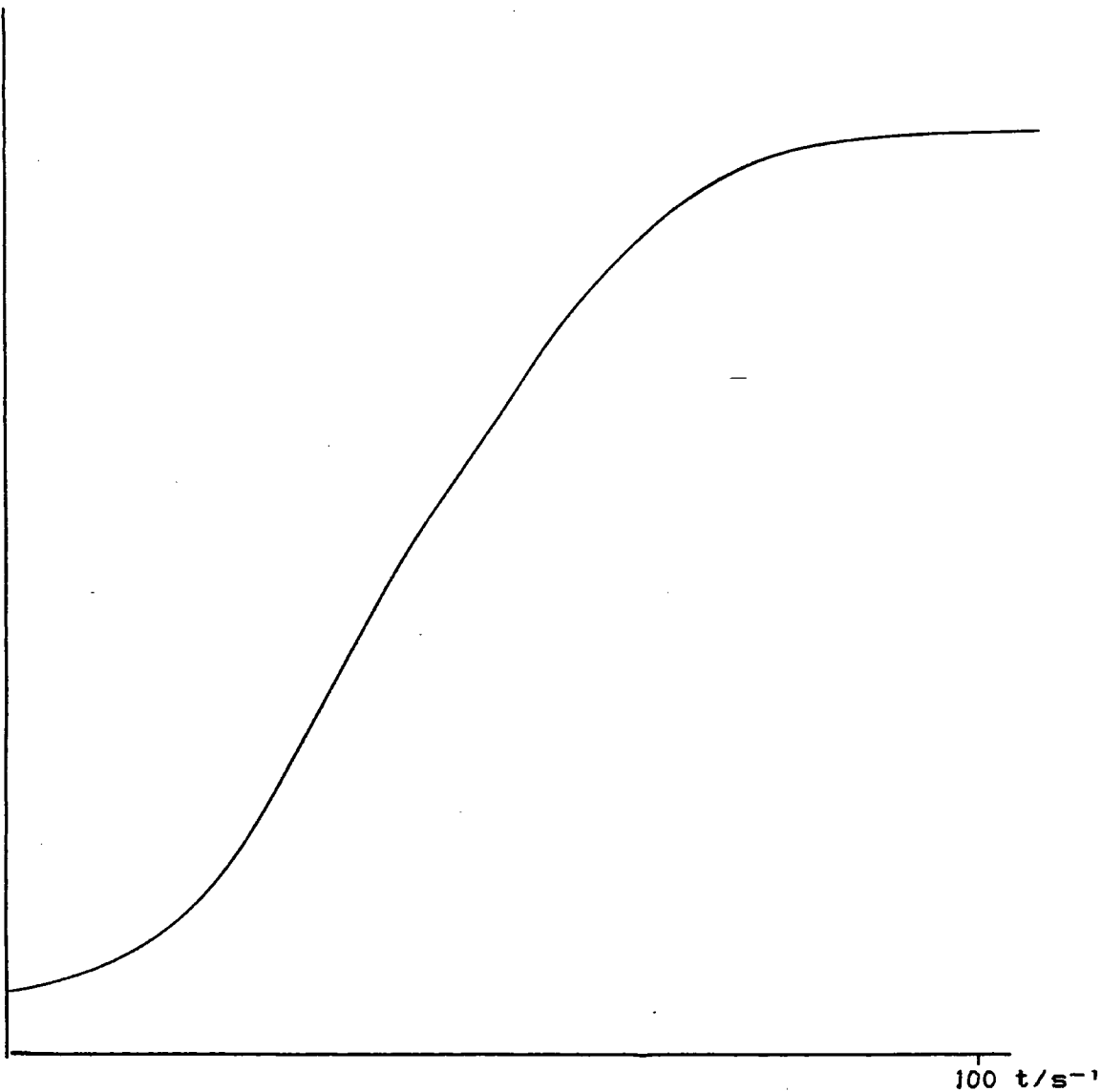
Reaction of haemin with SNAP after reduction by ascorbic and at pH 7.1 at 25°C.

[Haemin] = 2×10^{-4} M

[SNAP] = 0.005M

[AA] = 0.01M

Abs



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CHAPTER 7

Experimental details

7.1 Experimental techniques used

The kinetic measurements for all the experiments were made using conventional u.v./visible spectrophotometers or a stopped-flow spectrophotometer. Qualitative results were obtained with n.m.r. and e.s.r. spectroscopy, mass spectroscopy and H.P.L.C. in the identification of products.

7.1.1 U.v./visible spectrophotometry

The spectrophotometers used include the Perkin Elmer Lambda 2, Lambda 3 and the Philips PU8720.

Rate measurements for the nitrosation of 2,3-diaminonaphthalene, the S-nitrosation of cysteine and its derivatives, the denitrosation of nitrosamines, the nitrosations of phenol, ascorbic acid and haems with O- and S- nitroso compounds were made by mixing thermostated (in a water bath at 25°C) stock solutions of the substrate and the appropriate nitrosating agents, and then transferring this reaction mixture to a 1cm quartz cell. An identical cell containing the solvent was used as the reference. Scans of the spectra of starting materials and the reaction mixture were recorded, so that an appropriate wavelength could be chosen to follow the difference in absorbance between the sample and reference cell, monitored as a function of time. The changes would be due either to the formation of the product or the disappearance of the starting material. Reactions can also be followed at a fixed wavelength; kinetic data can then be obtained from the change of absorbance with time.

7.1.2 Stopped-flow spectrophotometry

For reactions with half-lives less than 50s, the HI-TECH Scientific SF-3 series stopped-flow spectrophotometer was used. This is illustrated in fig.(7.1). The two solutions, A and B (usually the substrate and the nitrosating agent) are contained in two separate reservoirs each leading to a syringe, identical with each other. A single piston drives the two syringes so that equal volumes of each solution are mixed. On mixing, the reaction solution flows into a third syringe, with a plunger attached at one end. The plunger is forced against a stop as the syringe is filled, and stops the flow, simultaneously triggering the recording of the reaction.

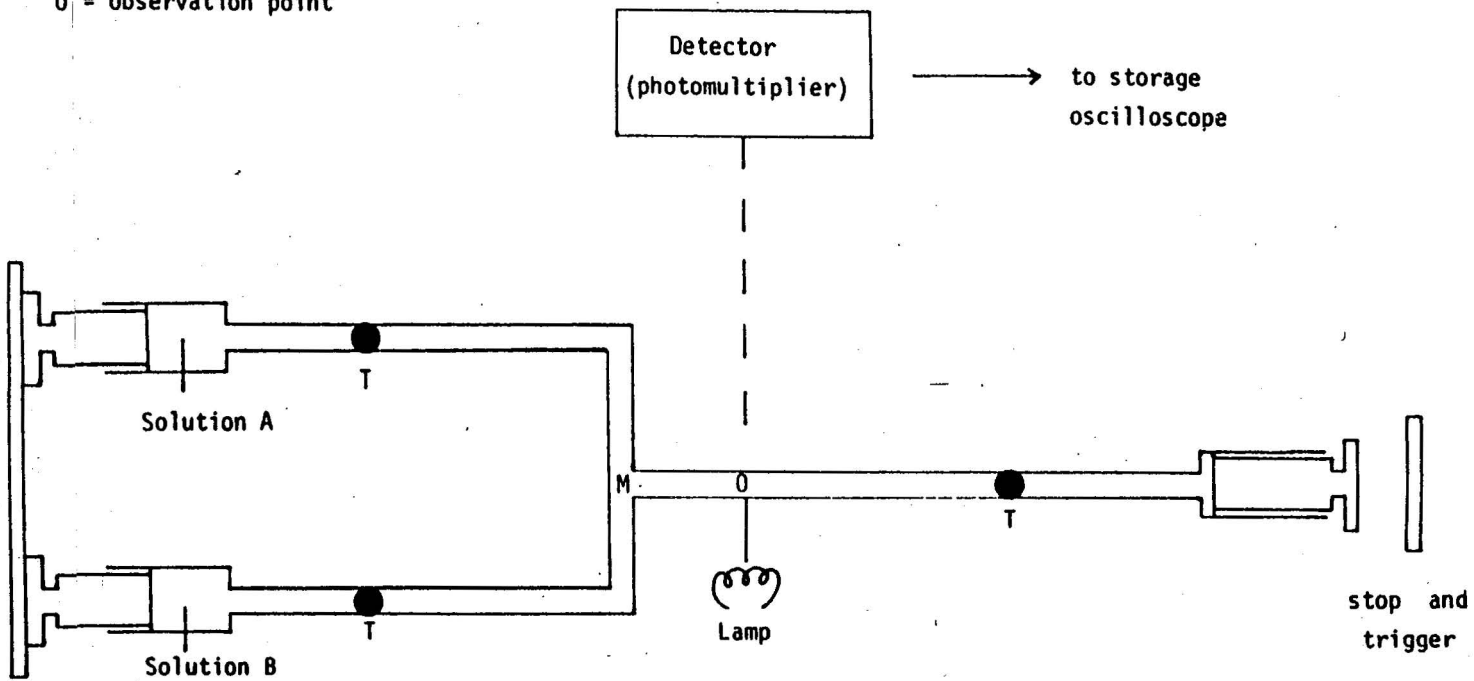
The reaction is followed by using a beam of monochromatic light which passes through the cell. The intensity of the beam is converted to an electrical signal and amplified by a photomultiplier, which has a voltage of approximately -6 volts across it. If this signal were to be used, the change in voltage due to the reaction proceeding would appear as a very small voltage change superimposed on the photomultiplier's standing output voltage, so an equal but opposite voltage is added to the standing voltage (biasing) allowing amplification by the recording equipment of the voltage change only. Therefore with just a non-absorbing solution at the observation point the final voltage is zero and any voltage change observed results from the progression of the reaction. The voltage changes were recorded and analysed to give the rate constant, by an Apple IIe microcomputer by either running a kinetic analysis program supplied by HI-TECH (via a fast analogue to digital converter) or by using the First-Order Rate Constant Evaluation (FORCE) program written by Mr. C. Greenhalgh.

The determination of rate constants using the stopped-flow spectrophotometer was carried out for the nitrosation of 2,3-diaminonaphthalene, the S-nitrosation of cysteine and its derivatives in basic media, the nitrosation of ascorbic acid using nitroso thiols and the nitrosation of haems.

Figure 7.1

Schematic diagram of a Stopped-Flow Spectrophotometer

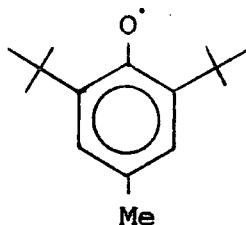
- T = three way taps
- M = mixing point
- O = observation point



7.1.3 E.s.r. Spectroscopy

E.s.r. measurements were made on a Bruker ESP300 X-band spectrometer with 100kHz modulation using an aqueous sample cell.

E.s.r. was successfully used to investigate the reactions between phenols and alkyl nitrites or nitrosothiols. Because the half-life of the phenoxyl radical is short, ($2k_t = 1.3 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$)³ the radical of 2,6-di-tertiary butyl, 4-methyl phenol (7.1) was used instead ($2k_t = 1.5 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$)⁴



(7.1)

Splitting for (7.1) : $a(\text{Me})$ 11.50G; $a(2\text{H})$ 1.63G, $g = 2.0045$.
The spectrum can be seen below (figure (7.2)).

The thiol radical resulting from the decomposition of S-nitroso-N-acetyl penicillamine (SNAP) could also be detected when a solution of SNAP in pH 7.0 was irradiated continuously using a 100 kW mercury lamp. The spectrum can be seen below. (figure (7.3)).

Figure 7.2

E.s.r. spectrum of 2,6-ditertiary butyl, 4-methyl phenol radical

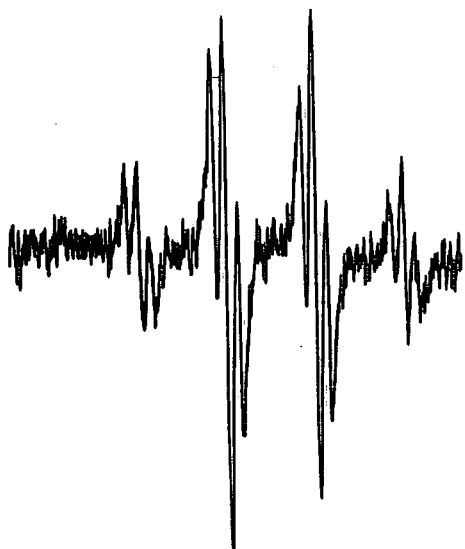
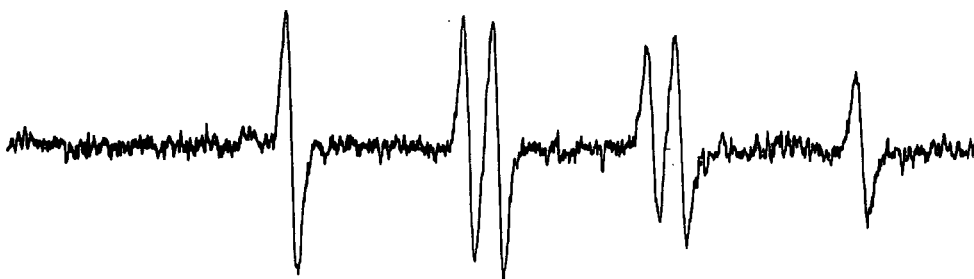


Figure 7.3

E.s.r. spectrum of the N-acetyl-penicillamine radical



1G \approx 2mm

7.1.4 N.m.r. spectroscopy

N.m.r. spectra were recorded on either a Hitachi/Perkin Elmer R-24B (60 MHz) or a Bruker AC250 (250 MHz) instrument. Shift measurements are quoted as ' δ ' values relative to tetramethylsilane.

The products of nitrosation of phenol using iso-amyl nitrite, and SNAP were both subject to n.m.r. analysis after extraction (see section 7.1.5) to prove that p-nitrosophenol was the main product of both nitrosation reactions.

The spectral data for the isolated product were as follows:-
(in CDCl_3 , internal standard TMS)

δ ppm: 6.48 (2H,d, H_2 and H_6 , $J_{2,3-5,6} = 10\text{Hz}$), 7.18 (1H,dd, H_3 or H_5 , $J_{2,3} = 10\text{Hz}$, $J_{3,5} = 3\text{Hz}$), 7.72 (1H,dd, H_5 or H_3 , $J_{6,5} = 10\text{Hz}$, $J_{5,3} = 3\text{Hz}$), 9.02 (1H, br s, OH).

7.1.5 T.L.C. and H.P.L.C.

T.L.C. and H.P.L.C. were used to isolate the product of nitrosation of phenol.

The reaction mixture was extracted with ether (100ml x 2) under acidic conditions (pH 4). The main product in the ether extract was separated preparatively on pre-coated thin-layer chromatography (TLC) plates, polyamide 11F254 (solvent $\text{CHCl}_3/\text{MeOH}$, 8/2 and Silica gel 60F254 (solvent benzene/MeOH, 9/1). The fractionated product was finally purified by high performance liquid chromatography (H.P.L.C.) with a reverse phase Anachem ODS2 columns (solvent benzene/MeOH, 9/1), on the Varian Vista 5500.

7.1.6 Mass spectrometry

Mass spectrometric measurements were made using a 7070E instrument supplied by V.G. Analytical Ltd. Electron Impact and Chemical Ionisation were both used.

Mass spectrometry was used to determine m/e values of molecular ions of products of synthesis or reaction and their corresponding fragments.

Mass spectrometry was also used to detect the presence of NO gas liberated, in the reaction between ascorbic acid and alkyl nitrites or nitrosothiols (see chapter 6, section 6.2). A Schlenk tube was flushed with nitrogen and the solutions of ascorbic acid, alkyl nitrite or nitrosothiol were introduced into the tube, after bubbling N₂ through the solutions overnight in a nitrogen atmosphere. A sample of the gaseous product formed from the reaction mixture was obtained from the Schlenk tube using a syringe.

Only Electron Impact was used in this case. As can be seen from the spectrum below, NO was detected in the mixture of gases extracted from the surface of the reaction mixture in the Schlenk tube.

Figure 7.4

Spectrum of NO detected in the reaction between ascorbic acid and alkyl nitrite.

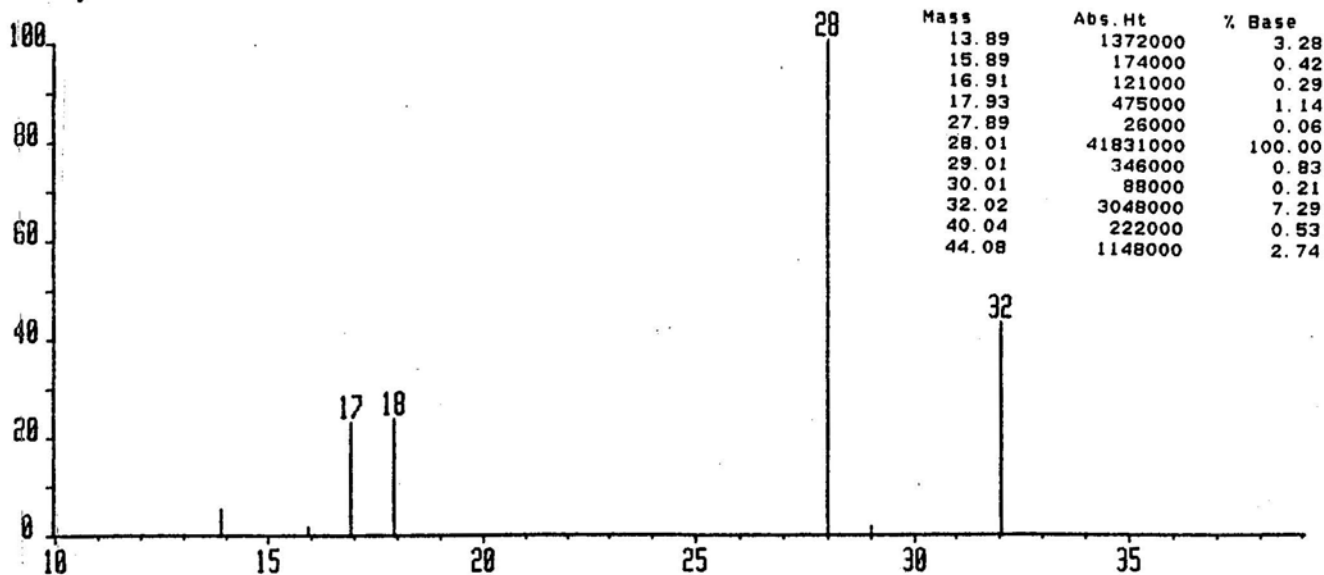
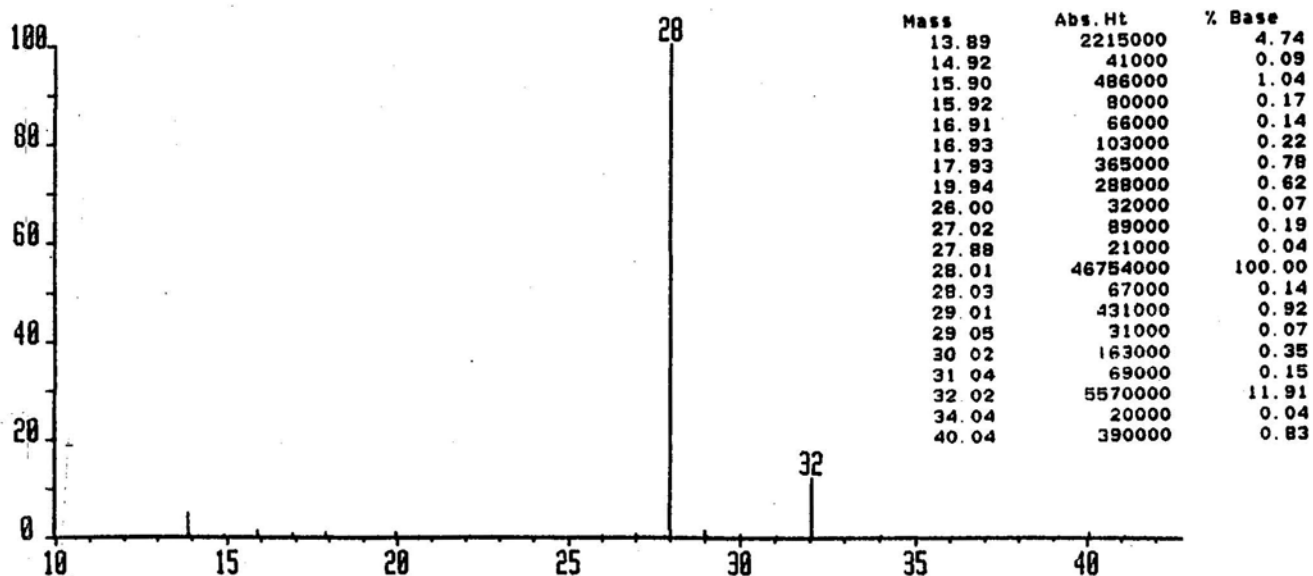


Figure 7.5

Spectrum of NO detected in the reaction between ascorbic acid and SNAP.



7.1.7 pH meter

All pH measurements were carried out using a PTI-6 Universal digital pH meter (accurate to ± 0.02 pH units).

7.2 Buffers

The buffer solutions used for the pH range studied are listed in table (7.1).

Table 7.1

<u>pH range</u>	<u>Buffer System</u>
2.2 - 4.0	0.1M Potassium hydrogen phthalate + x ml of 0.1M HClO_4
4.1 - 5.9	0.1M Potassium hydrogen phthalate + x ml of 0.1M NaOH.
5.8 - 8.0	0.1M Potassium dihydrogen phosphate + x ml of 0.1M NaOH.
8.0 - 9.1	0.025M Borax + x ml of 0.1M HClO_4
9.2 - 10.8	0.025M Borax + x ml of 0.1M NaOH.
10.9 - 12.0	0.05M Disodium hydrogen phosphate + x ml of 0.1M NaOH.
12.0 - 13.0	0.2M KCl + x ml of 0.2M NaOH

7.3 Determination of the observed rate constant

All the reactions studied were carried out under pseudo-first order conditions, with the concentration of the substrate at least 20 times that of the nitrosating reagent.

For the first-order reaction $R \rightarrow P$ (where R is the reactant and P is the product), the rate of formation of the product or the rate of disappearance of the reactant can be expressed by equation (7.1).

$$-\frac{dR}{dt} = \frac{dP}{dt} = k[R] \quad (7.1)$$

Equation (7.1) on integration, yields the expression for the observed first-order rate constant (equation (7.2)).

$$k_o = \frac{1}{t} \ln \frac{[R]_o}{[R]_t} \quad (7.2)$$

where $[R]_o$ and $[R]_t$ are the concentrations of the reactants at time $t = 0$ and $t = t$ respectively.

Using Beer-Lamberts law $A = \epsilon cl$ (where A is the absorbance, ϵ is the molar extinction coefficient, c is the concentration and l is the pathlength) and assuming that the pathlength of the cell is 1 cm the expression of the absorbance at time $t = 0$ and $t = t$ can be derived (equations (7.3) and (7.4)).

$$A_0 = \epsilon_R [R]_0 \quad (7.3)$$

$$A_t = \epsilon_R [R]_t + \epsilon_P [P]_t \quad (7.4)$$

Since $[P]_t = [R]_0 - [R]_t$, substituting for $[P]_t$ into equation (7.4) gives:

$$A_t = \epsilon_R [R]_t + \epsilon_P ([R]_0 - [R]_t) \quad (7.5)$$

$$A_t = \epsilon_R [R]_t + \epsilon_P [R]_0 - \epsilon_P [R]_t \quad (7.6)$$

But $A^\infty = \epsilon_P [R]_0 = \epsilon_P [P]^\infty$ since $[P]^\infty = [R]_0$

Thus $(A_t - A^\infty) = \epsilon_R [R]_t - \epsilon_P [R]_t$

$$[R]_t = \frac{(A_t - A^\infty)}{\epsilon_R - \epsilon_P} \quad (7.7)$$

Similarly $A_0 = \epsilon_R [R]_0$

And $A^\infty = \epsilon_P [P]^\infty = \epsilon_P [R]_0$

$$(A_0 - A^\infty) = \epsilon_R [R]_0 - \epsilon_P [R]_0$$

$$[R]_0 = \frac{(A_0 - A^\infty)}{(\epsilon_R - \epsilon_P)} \quad (7.8)$$

Substituting equation (7.7) and (7.8) into equation (7.2) gives:

$$k_0 = \frac{1}{t} \ln \frac{(A_0 - A^\infty)}{(A_t - A^\infty)} \quad (7.9)$$

Rearranging equation (7.9) gives:

$$\ln (A_t - A^\infty) = -k_0 t + \ln (A_0 - A^\infty) \quad (7.10)$$

Therefore a plot of $\ln (A_t - A^\infty)$ or $\ln (A^\infty - A_t)$ versus t should be linear with a slope of $-k_0$. The infinity values, A^∞ was determined after a period of ten half-lives and the appearance of absorbance was followed for at least two half-lives.

For experiments carried out using the stopped-flow technique the value of the rate constant was determined using the "FORCE" or HI-TECH kinetics programme. The "FORCE" programme calculates the value of k_o from the slope of a plot of $\ln (V^\infty - V_t)$ versus t , where V is the output signal voltage, and under the experimental conditions used the voltage is proportional to the absorbance. In this case, a linear regression combined with least squares fit method is used to calculate the value of k_o . However, the HI-TECH program initially calculates the value of k_o from the slope of a calculated plot of $\ln (V^\infty - V_t)$ versus time, then optimises this value iteratively, using non-linear regression analysis, thus removing some of the errors inherent in using linear regression methods. Due to the errors in measuring fast reactions, the value of k_o quoted for these reactions is the mean of at least five separate determinations and the error quoted is the standard deviation between the individual k_o values.

7.4 Chemical reagents

2,3-Diaminonaphthalene, sodium nitrite, dimethylnitrosamine, diazald, phenol, 2,4,6-trimethylphenol, anisole, ascorbic acid, haemin and octaethylhaemin were all purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset, England. These reagents were of the highest grade available and used without further purification.

L-cysteine, L-cysteine-methylester-hydrochloride, L-cysteine-ethyl ester-hydrochloride, N-acetyl-L-cysteine, and perchloric acid (>70%) were all purchased from Fluka AG and used without further purification.

Iso-amyl nitrite was purchased from Aldrich Chemical Co. Ltd and distilled (b.p. 99°C) before use.

All solvents used including ethanol, acetic acid, acetonitrile, tetrahydrofuran and dioxan were purchased from May & Baker Ltd, Dagenham, England.

N-Nitrosoproline and nitrososarcosine were synthesised using the method of Lijinsky et al¹.

To prepare N-nitroso-L-proline, 10g proline was dissolved in 6.7ml HCl and 100ml water cooled in ice. 8g NaNO₂ was slowly added. After one hour, the water was evaporated from the reaction mixture using a rotary evaporator, and the nitrosoproline was extracted with pure acetone. The acetone was removed by evaporation in the cold in a stream of N₂. The first crop of crystals which appeared weighed 5.2g and was crystallised from chloroform, m.p. 99-100°C. The second crop weighed 0.8g, m.p. 98.5-99°C. Both were colourless. Recrystallisation from benzene gave pale yellow crystals m.p. 100-101°C. This i.r. spectrum in chloroform showed ν_{max} at 1430 cm⁻¹ (NO) and 1730 cm⁻¹ (CO). Found C, 41.67; H, 5.58; N, 19.43; Calc. C, 41.55; H, 5.55; N, 19.45%. Mass spec.: parent peak at 144.053 a.m.u. C₅H₈N₂O₃ = 144.0535. Fragments at m/e 99 (loss of COOH) and 69 (loss of COOH, NO).

To prepare N-nitrososarcosine, 11.0g of NaNO₂ was added over a period of 40 minutes to an ice cold, magnetically stirred solution of 13.4 ml conc. HCl and 7.1g sarcosine in 20ml water. The mixture was stirred in the cold for one hour after the addition was complete. The water was evaporated under reduced pressure and the residue was extracted with 80ml acetone.

The acetone solution was filtered and evaporated and the oily residue (7g) was allowed to crystallise. Repeated recrystallisation from chloroform gave a pale yellow sample, m.p. 66-67°. The i.r. in chloroform showed ν_{\max} at 1455 cm^{-1} (NO) and 1735 cm^{-1} (CO). Found C, 30.37; H, 5.08; N, 23.79; calc. C, 30.51; H, 5.12; N, 23.72%. Mass spec. parent peak at m/e 118, fragments at m/e 88 (loss of NO) and m/e 73 (loss of COOH).

S-nitroso-N-acetyl penicillamine, (SNAP) was prepared in the way described by Field et al². 20 mmol NaNO_2 in 20ml of water was added to N-acetyl-D,L-penicillamine (10 mmol) dissolved in MeOH -1N HCl (20ml each) with 2ml of conc. H_2SO_4 during 20 minutes with vigorous stirring at 25°C. After 15 minutes, SNAP was separated off, washed well with water, and air-dried (63%), to give deep green crystals with red reflections, m.p. 152-154°C.

References

1. Lijinsky, W., Keefer, L. and Loo, J., Tetrahedron, 1970, 26, 5137.
2. Field, L., Dilts, R.V., Ramanathan, R., Lenhert, P.G. and Carnahan, G.E., J. Chem. Soc., Chem. Comm., 1978, 249.
3. Micic, O.I. and Nenaclovic, M.T., J. Phys. Chem., 1976, 80, 940.
4. Land, E.J. and Porter, G., Trans. Faraday Soc., 1963, 59, 2016.

APPENDIX
RESEARCH COLLOQUIA, SEMINARS,
LECTURES AND CONFERENCES

The Board of Studies in Chemistry requires that each postgraduate research thesis contains an appendix listing:-

- A) All research colloquia, seminars and lectures arranged by the Department of Chemistry and by the Durham University Chemical Society during the period of the author's residence as a postgraduate student.

- B) All research conferences attended and papers presented by the author during the period when research for the thesis was carried out.

- C) Details of the postgraduate induction course.

COLLOQUIA, LECTURES AND SEMINARS GIVEN BY

INVITED SPEAKERS

1st AUGUST 1987 to 31st JULY 1988

(* Denotes lectures attended)

* BIRCHALL, Prof. D. (I.C.I. Advanced Materials) Environmental Chemistry of Aluminium	25th April 1988
* BORER, Dr. K. (University of Durham Industrial Research Labs.) The Brighton Bomb - A Forensic Science View.	18th February 1988
BOSSONS, L. (Durham Chemistry Teachers' Centre) GCSE Practical Assessment.	16th March 1988
* BUTLER, Dr. A.R. (University of St. Andrews) Chinese Alchemy.	5th November 1987
* CAIRNS-SMITH, Dr. A. (Glasgow University) Clay Minerals and the Origin of Life.	28th January 1988
DAVIDSON, Dr. J. (Herriot-Watt University) Metal Promoted Oligomerisation Reactions of Alkynes.	November 1987
* GRADUATE CHEMISTS (Northeast Polytechnics and Universities R.S.C. Graduate Symposium	19th April 1988
GRAHAM, Prof W.A.G. (University of Alberta, Canada) Rhodium and Iridium Complexes in the Activation of Carbon-Hydrogen Bonds.	3rd March 1988
GRAY, Prof. G.W. (University of Hull) Liquid Crystals and their Applications.	22nd October 1987
* HARTSHORN, Prof, M.P. (University of Canterbury, New Zealand) Aspects of Ipso-Nitration.	7th April 1988
* HOWARD, Dr. J. (I.C.I. Wilton) Chemistry of Non-Equilibrium Processes.	3rd December 1987
JONES, Dr. M.E. (Durham Chemistry Teachers' Centre) GCSE Chemistry Post-mortem.	29th June 1988
JONES, Dr. M.E. (Durham Chemistry Teachers' Centre) GCE Chemistry A Level Post-mortem.	6th July 1988

- KOCH, Prof. H.F. (Ithaca College, U.S.A.) 7th March 1988
Does the E2 Mechanism Occur in Solution?
- LACEY, Mr. (Durham Chemistry Teachers' Centre) 9th February 1988
Double Award Science.
- * LUDMAN, Dr. C.J. (Durham University) 10th December 1987
Explosives.
- * McDONALD, Dr. W.A. (I.C.I. Wilton) 11th May 1988
Liquid Crystal Polymers
- MAJORAL, Prof. J.P. (Université Paul Sabatier) 8th June 1988
Stabilisation by Complexation of Short-Lived Phosphorus Species.
- MAPLETOFT, Mrs. M. (Durham Chemistry Teachers' Centre) 4th November 1987
Salters' Chemistry.
- NIETO DE CASTRO, Prof. C.A. (University of Lisbon and Imperial College) 18th April 1988
Transport Properties of Non-Polar Fluids.
- * OLAH, Prof. G.A. (University of Southern California) 29th June 1988
New Aspects of Hydrocarbon Chemistry
- * PALMER, Dr. F. (University of Nottingham) 21st January 1988
Luminescence (Demonstration Lecture).
- * PINES, Prof. A. (University of California) 28th April 1988
Berkeley, U.S.A. - Some Magnetic Moments
- RICHARDSON, Dr. R. (University of Bristol) 27th April 1988
X-Ray Diffraction from Spread Monolayers
- ROBERTS, Mrs. E. (SATRO Officer for Sunderland) 13th April 1988
Talk - Durham Chemistry Teachers' Centre - "Links Between Industry and Schools".
- ROBINSON, Dr. J.A. (University of Southampton) 27th April 1988
Aspects of Antibiotic Biosynthesis.
- ROSE van Mrs. S. (Geological Museum) 29th October 1987
Chemistry of Volcanoes.
- SAMMES, Prof. P.G. (Smith, Kline and French) 19th December 1987
Chemical Aspects of Drug Development.
- * SEEBACH, Prof. D. (E.T.H. Zurich) 12th November 1987
From Synthetic Methods to Mechanistic Insight.

- SODEAU, Dr. J.** (University of East Anglia) 11th May 1988
 Durham Chemistry Teachers' Centre Lecture:
 Spray Cans, Smog and Society.
- SWART, Mr. R.M.** (I.C.I.) 16th December 1987
 The Interaction of Chemicals with Lipid Bilayers.
- * **TURNER, Prof. J.J.** (University of Nottingham) 11th February 1988
 Catching Organometallic Intermediates.
- * **UNDERHILL, Prof. A.** (University of Bangor) 25th February 1988
 Molecular Electronics.
- WILLIAMS, Dr. D.H.** (University of Cambridge) 26th November 1987
 Molecular Recognition.
- * **WINTER, Dr. M.J.** (University of Sheffield) 15th October 1987
 Pyrotechnics (Demonstration Lecture).

COLLOQUIA, LECTURES AND SEMINARS GIVEN BY

INVITED SPEAKERS

1st AUGUST 1988 to 31st JULY 1989

- ASHMAN, Mr. A. (Durham Chemistry Teachers' Centre) 3rd May 1989
The Chemical Aspects of the National Curriculum.
- AVEYARD, Dr. R. (University of Hull) 15th March 1989
Surfactants at your Surface.
- * AYLETT, Prof. B.J. (Queen Mary College, London) 16th February 1989
Silicon-Based Chips:- The Chemist's Contribution.
- * BALDWIN, Prof. J.E. (Oxford University) 9th February 1989
Recent Advances in the Bioorganic Chemistry of Penicillin Biosynthesis.
- * BALDWIN & WALKER, Drs. R.R. & R.W. (Hull University) 24th November 1988
Combustion: Some Burning Problems.
- BOLLEN, Mr. F. (Durham Chemistry Teachers' Centre) 18th October 1988
Lecture about the use of SATIS in the classroom.
- * BUTLER, Dr. A.R. (St. Andrews University) 15th February 1989
Cancer in Linxiam: The Chemical Dimension.
- * CADOGAN, Prof. J.I.G. (British Petroleum) 10th November 1988
From Pure Science to Profit.
- CASEY, Dr. M. (University of Salford) 20th April 1989
Sulphoxides in Stereoselective Synthesis
- CRESSEY & WATERS, Mr. D. & T. 1st February 1989
(Durham Chemistry Teachers' Centre)
GCSE Chemistry 1988: "A Coroner's Report".
- * CRICH, Dr. D. (University College London) 27th April 1989
Some Novel Uses of Free Radicals in Organic Synthesis.
- * DINGWELL, Dr. J. (Ciba Geigy) 18th October 1988
Phosphorus-containing Amino Acids: Biologically Active Natural and Unnatural Products.
- ERRINGTON, Dr. R.J. (University of Newcastle upon Tyne) 1st March 1989
Polymetalate Assembly in Organic Solvents.

- FREY, Dr. J.** (Southampton University) 11th May 1989
Spectroscopy of the Reaction Path: Photodissociation
Raman Spectra of NOCl.
- GRADUATE CHEMISTS**, (Polytechs and Universities in
North East England) - R.S.C. Symposium for presentation
of papers by postgraduate students. 12th April 1989
- * **HALL, Prof. L.D.** (Addenbrooke's Hospital, Cambridge) 2nd February 1989
NMR - A Window to the Human Body
- HARDGROVE, Dr. G.** (St. Olaf College, U.S.A.) December 1988
Polymers in the Physical Chemistry Laboratory
- * **HARWOOD, Dr. L.** (Oxford University) 23rd January 1988
Synthetic Approaches to Phorbois Via Intramolecular
Furan Diels-Alder Reactions: Chemistry under Pressure
- JAGER, Dr. C.** (Friedrich-Schiller University GDR) 9th December 1988
NMR Investigations of Fast Ion Conductors of the
NASICON Type.
- JENNINGS, Prof. R.R.** (Warwick University) 26th January 1989
Chemistry of the Masses
- * **JOHNSON, Dr. B.F.G.** (Cambridge University) 23rd February 1989
The Binary Carbonyls
- JONES, Dr. M.E.** (Durham Chemistry Teachers' Centre) 14th June 1989
Discussion Session on the National Curriculum
- JONES, Dr. M.E.** (Durham Chemistry Teachers' Centre) 28th June 1989
GCSE and A Level Chemistry 1989
- LUDMAN, Dr. C.J.** (Durham University) 18th October 1988
The Energetics of Explosives.
- MACDOUGALL, Dr. G.** (Edinburgh University) 22nd February 1989
Vibrational Spectroscopy of Model Catalytic Systems
- MAKRO, Dr. I.** (Sheffield University) 9th March 1989
Catalytic Asymmetric Osmylation of Olefins
- McLAUCHLAN, Dr. K.A.** (University of Oxford) 16th November 1988
The Effect of Magnetic Fields on Chemical Reactions
- * **MOODY, Dr. C.J.** (Imperial College) 17th May 1989
Reactive Intermediates in Hetrocyclic Synthesis.
- * **MORTIMER, Dr. C.** (Durham Chemistry Teachers' Centre) 14th December 1988
The Hindenberg Disaster - an Excuse for Some Experiments

- * **NICHOLLS**, Dr. D. (Durham Chemistry Teachers' Centre) 11th July 1989
Demo. "Liquid Air".
- PAETZOLD**, Prof P. (Aachen) 23rd May 1989
Iminoboranes $\text{XB}\equiv\text{NR}$: Inorganic Acetylenes?
- PAGE**, Dr. P.C.B. (University of Liverpool) 3rd May 1989
Stereocontrol of Organic Reactions Using 1,3-dithiane-1-oxides.
- POLA**, Prof. J. (Czechoslovak Academy of Sciences) 15th June, 1989
Carbon Dioxide Laser Induced Chemical Reactions - New Pathways in Gas-Phase Chemistry
- * **REES**, Prof. C.W. (Imperial College London) 27th October 1988
Some Very Heterocyclic Compounds
- REVELL**, Mr. P. (Durham Chemistry Teachers' Centre) 14th March 1989
Implementing Broad and Balanced Science 11-16
- * **SCHMUTZLER**, Prof, R. (Technische Universitat Braunschweig) 6th October 1988
Fluorophosphines Revisited - New Contributions to an Old Theme.
- * **SCHROCK** Prof. R.R. (M.I.T.) 13th February 1989
Recent Advances in Living Metathesis
- SINGH**, Dr. G. (Teesside Polytechnic) 9th November 1988
Towards Third Generation Anti-Leukaemics.
- * **SNAITH**, Dr. R. (Cambridge University) 1st December 1988
Egyptian Mummies: What, Where, Why and How?
- STIBR**, Dr. R. (Czechoslovak Academy of Sciences) 16th May 1989
Recent Developments in the Chemistry of Intermediate-Sited Carboranes.
- * **VON RAGUE SCHLEYER**, Prof. P. (Universitat Erlangen Nurnberg) 21st October 1988
The Fruitful Interplay Between Computational and Experimental Chemistry.
- WELLS**, Prof. P.B. (Hull University) 10th May 1989
Catalyst Characterisation and Activity.

COLLOQUIA, LECTURES AND SEMINARS GIVEN BY

INVITED SPEAKERS

1st AUGUST 1988 to 31st JULY 1990

ASHMAN, Mr. A. (Durham Chemistry Teachers' Centre) The National Curriculum - an update.	11th October 1989
* BADYAL, Dr. J.P.S. (Durham University) Breakthroughs in Heterogeneous Catalysis	1st November 1989
* BECHER, Dr. J. (Odense University) Synthesis of New Macrocyclic Systems using Heterocyclic Building Blocks.	13th November 1989
BERCAW, Prof. J.E. (California Institute of Technology) Synthetic and Mechanistic Approaches to Ziegler-natta Polymerization of Olefins.	10th November 1989
BLEASDALE, Dr. C. (Newcastle University) The Mode of Action of some Anti-tumour Agents.	21st February 1990
BOLLEN, Mr. F. (Formerly Science Advisor, Newcastle LEA) What's New in Satis, 16-19	27th March 1990
BOWMAN, Prof. J.M. (Emory University) Fitting Experiment with Theory in Ar-OH	23rd March 1990
* BUTLER, Dr. A. (St. Andrews University) The Discovery of Penicillin: Facts and Fancies.	7th December 1989
CAMPBELL, Mr. W.A. (Durham Chemistry Teachers' Centre) Industrial catalysis - some ideas for the National Curriculum.	12th September 1989
CHADWICK, Dr. P. (Dept. of Physics, Durham University) Recent Theories of the Universe (with Reference to National Curriculum Attainment Target 16).	24th January 1990
* CHEETHAM, Dr. A.K. (Oxford University) Chemistry of Zeolite Cages.	8th March 1990
* CLARK, Prof, D.T. (ICI Wilton) Spatially Resolved Chemistry (using Nature's Paradigm in the Advanced Materials Arena)	22nd February 1990
COLE-HAMILTON, Prof. D.J. (St. Andrews University) New Polymers from Homogeneous Catalysis.	29th November 1989

- * **CROMBIE**, Prof. L. (Nottingham University) 15th February 1990
The Chemistry of Cannabis and Khat.
- DYER**, Dr. U. (Glaxo) 31st January 1990
Synthesis and Conformation of C-Glycosides.
- * **FLORIANI**, Prof. C. (University of Lausanne, Switzerland) 25th October 1989
Molecular Aggregates - A Bridge between homogeneous and Heterogeneous Systems.
- GERMAN**, Prof. L.S. (USSR Academy of Sciences - Moscow) 9th July 1990
New Syntheses in Fluoroaliphatic Chemistry:
Recent Advances in the Chemistry of Fluorinated Oxdiranes.
- GRAHAM**, Dr. D. (B.P. Research Centre) 4th December 1989
How Proteins Absorb to Interfaces.
- * **GREENWOOD**, Prof. N.N. (University of Leeds) 9th November 1989
Novel Cluster Geometries in Metalloborane Chemistry.
- * **HOLLOWAY**, Prof. J.H. (University of Leicester) 1st February 1990
Noble Gas Chemistry
- * **HUGHES**, Dr. M.N. (King's College London) 30th November 1989
A Bug's Eye View of the Periodic Table
- HUISGEN**, Prof. R. (Universitat Munchen) 15th December 1989
Recent Mechanistic Studies of [2+2] Additions.
- IDDON**, Dr. B. (University of Salford) 15th December 1989
Schools' Christmas Lecture - The Magic of Chemistry.
- JONES**, Dr. M.E. (Durham Chemistry Teachers' Centre) 3rd July 1990
The Chemistry A Level 1990
- JONES**, Dr. M.E. (Durham Chemistry Teachers' Centre) 21st November 1989
GCSE and Dual Award Science as a starting point for A level Chemistry - how suitable are they?
- JOHNSON**, Dr. G.A.L. (Durham Chemistry Teachers' Centre) 8th February 1990
Some aspects of local Geology in the National Science Curriculum (attainment target 9)
- KLINOWSKI**, Dr. J. (Cambridge University) 13th December 1989
Solid State NMR Studies of Zeolite Catalysts.
- * **LANCASTER** Rev. R. (Kimbolton Fireworks) 8th February 1990
Fireworks - Principles and Practice.

- LUNAZZI, Prof. L.** (University of Bologna) 12th February 1990
Application of Dynamic NMR to the Study of
Conformational Enantiomerism.
- * **PALMER, Dr. F.** (Nottingham University) 17th October 1989
Thunder and Lightning.
- * **PARKER, Dr. D.** (Durham University) 16th November 1989
Macrocycles, Drugs and Rock 'n' roll.
- * **PERUTZ, Dr. R.N.** (York University) 24th January 1990
Plotting the Course of C-H Activations with
Organometallics.
- PLATONOV, Prof. V.E.** (USSR Academy of Sciences - 9th July 1990
Novosibirsk)
Polyfluoroindanes: Synthesis and Transformation.
- POWELL, Prof. R.L.** (ICI) 6th December 1989
The Development of CFC Replacements.
- POWIS, Dr. I.** (Nottingham University) 21st March 1990
Spinning off in a huff: Photodissociation of
Methyl Iodide.
- RICHARDS, Mr. C.** (Health and Safety Executive, 28th February 1990
Newcastle)
Safety in School Science Laboratories and COSHH
- ROZHKOV, Prof. I.N.** (USSR Academy of Sciences - 9th July 1990
Moscow)
Reactivity of Perfluoroalkyl Bromides.
- * **STODDART, Dr. J.F.** (Sheffield University) 1st March 1990
Molecular Lego.
- * **SUTTON, Prof. D.** (Simon Fraser University, 14th February 1990
Vancouver B.C.)
Synthesis and Applications of Dinitrogen and Diazo
Compounds of Rhenium and Iridium.
- THOMAS, Dr. R.K.** (Oxford University) 28th February 1990
Neutron Reflectometry from Surfaces.
- THOMPSON, Dr. D.P.** (Newcastle University) 7th February 1990
The role of Nitrogen in Extending Silicate
Crystal Chemistry.

(B) CONFERENCES ATTENDED.

European Symposium on Organic Reactivity II, University of Padova, Italy, 27th August - 1st September 1989. Poster presented: 'S-nitrosation under mild conditions using alkyl nitrites and a nitrososulphonamide.'

Conference on The Current State of the Chemistry of the Nitrous Acid and its derivatives, Santiago de Compostela, 26th-29th March 1990. Paper presented: 'Nitrosation of Haems'.

(C) FIRST YEAR INDUCTION COURSE, OCTOBER 1987

This course consists of a series of one hour lectures on the services available in the department.

- 1) Departmental organisation.
- 2) Safety matters.
- 3) Electrical appliances and infra-red spectroscopy.
- 4) Chromatography and microanalysis.
- 5) Atomic absorptiometry and inorganic analysis.
- 6) Library facilities.
- 7) Mass spectroscopy.
- 8) Nuclear magnetic resonance spectroscopy.
- 9) Glassblowing technique.

