Kinetic and product studies involving thionitrites

Morris, Philippa Ann

KINETIC AND PRODUCT STUDIES INVOLVING THIONITRITES

BY

PHILIPPA ANNE MORRIS, B.SC. (NEWCASTLE)

(GRADUATE SOCIETY)

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TO

MY

FAMILY

MEMORANDUM

The work for this thesis has been carried out in the Department of Chemistry at the University of Durham between October 1983 and August 1986. 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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PHILIPPA A. MORRIS

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ABSTRACT

The kinetics of nitrosation of cysteine, cysteine methyl ester, N-acetylcysteine, penicillamine, N-acetylpenicillamine, glutathione and thioglycolic acid was undertaken. These thiols exhibited identical rate laws which are interpreted as nitrosation at sulphur by $H_2NO_2^+/NO^+$. The rate constants determined show the high reactivity of thiols towards the nitrosating agent. The nucleophile catalyzed reactions were also investigated and the order of reactivity NOCl > NOBr > NOSCN was observed. Normally in these nucleophile catalyzed reactions there is a first order dependence on [thiol]. However, for N-acetylcysteine and thioglycolic acid at high [thiol] the rate of formation of NOX tends to become the rate determining stage. The difference in rate constants between cysteine and penicillamine and their N-acetyl derivatives is explained in terms of internal stabilization.

The decomposition of S-nitrosocysteine (S-NOCys) at pH 5.5, 7 and 9.8 in the presence and absence of Cl^-, Br^- and SCN^-, and also alanine and sodium bicarbonate at pH 7, and S-nitrosoglutathione (GS-NO) at pH 7 in the presence and absence of alanine, Cl^-, and sodium bicarbonate was studied. The decomposition profiles were complex, but showed that S-NOCys was least stable at pH 7, and that GS-NO was more stable than S-NOCys. The addition of the aforementioned species did not significantly affect the rate of decomposition of the thionitrites.

Finally the potential of S-NOCys, GS-NO and S-nitroso-N-acetyl-penicillamine as nitrosating agents towards amines was investigated at pH 7 and pH 8. These thionitrites nitrosated morpholine to give approximately the same yield of N-nitrosomorpholine (ca 17%) at pH 7, and less at pH 8 for S-NOCys and GS-NO. The addition of sodium acetate, sodium chloride, sodium bicarbonate, alanine and glucose, compounds liable to be present *in vivo*, did not significantly affect the yield of N-nitrosomorpholine. The transnitrosation reaction was complete before total decomposition of the thionitrite and a direct reaction between the thionitrite and morpholine is proposed.

CONTENTS

CHAPTER ONE

PRINCIPLES OF NITROSATION

1.1 Introduction

The reaction of nitrous acid with primary and secondary amines is one which is well established and has been the subject of several reviews.[1]

$$RNH_2 + HNO_2 \longrightarrow RN_2^+$$

$$R_2NH + HNO_2 \longrightarrow R_2NNO$$

One of the first nitrosation reactions carried out was in 1846 when Piria converted aspartic acid to malic acid.[2] This was closely followed by the isolation of diazonium salts by Griess,[1a] from the reaction of primary aromatic amines with nitrous acid. Since then studies of the reaction of nitrous acid with amines have been expanded to include nitrosation of secondary and tertiary amines. The mechanisms of these reactions are now well understood, and are thought to involve the same pathway, starting with electrophilic addition of NO^+ to the nitrogen atom, the product undergoing either a few or several subsequent steps, depending on its stability.

For secondary and tertiary amines, the reaction stops with formation of the nitrosamine, that is after proton or R group loss from the initial product.

$$R_2R^1N \xrightarrow{HNO_2} R_2NNO$$

R^1 = H for secondary amines

For primary amines the product of proton loss undergoes migration of the remaining proton, and then rapid formation of the diazonium ion.

$$RNHNO \longrightarrow RNNOH \longrightarrow RN_2^+$$

Diazonium ions of primary aromatic amines are stabilized by resonance structures involving the benzene ring and undergo

many well-known reactions, for example azo coupling.

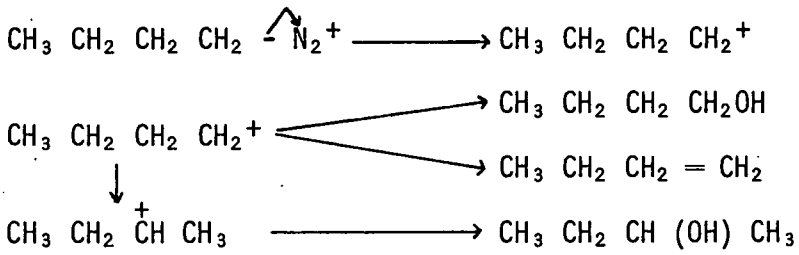
$$Ar\ N_2^+ + Ar^1H \longrightarrow Ar - N = N - Ar^1 + H^+$$

Primary aliphatic diazonium ions are unstable and rapidly evolve nitrogen to form the cation R^+ which can undergo a variety of reactions to yield the observed decomposition products.[3]

$$RN_2^+ \longrightarrow N_2 + R^+$$

$$R^+ \longrightarrow \text{alcohols and alkenes}$$

For example:



scheme 1.1

The rate determining stage of these reactions is generally attack on the amine by the nitrosating agent.[4] This and the fact that the amines react by the same mechanism are shown by the applicability of the rate equations to the nitrosation of primary aliphatic, primary aromatic and secondary amines, even though a variety of products are observed.[5,6]

Since the findings of Magee and Barnes[7] that nitrosamines are carcinogenic, there has been an increase in interest in the nitrosation of secondary amines, and the identification of nitrosating agents, catalysts and inhibitors. The nitrite ion is used as a curing agent due to its anti-microbial activity[8] and nitrate in foods and water supplies can be reduced to nitrite by bacteria in vivo[9], leading to an elevation of the salivary nitrite concentration.[10] Therefore there is much

interest in in vivo as well as in vitro formation of nitrosamines.[11] Nitrosamines are not direct-acting carcinogens, like nitrosamides, but require enzymatic activation to form an alkylating agent.[8] There are a number of substrates which can be alkylated, especially the N^7 position in guanine, but alkylation at the O^6 position in guanine appears to be the most important regarding their mutagenic action as this position is involved in base pairing.[8] The biochemical properties of nitrosamines have been reviewed.[12]

More recently the study of nitrosation reactions has been widened to encompass nitrosation at other sites, including sulphur, oxygen and carbon.

The remainder of this chapter will centre on N-nitrosation, reflecting the emphasis in the literature.

1.2 Diazotization and N-Nitrosation

Aqueous acidic solutions of sodium nitrite contain a variety of potential nitrosating species depending on the acid used and the presence of buffers.[1] The majority of the reactions discussed in this section were carried out using perchloric acid, since nitrosyl perchlorate is ionic[13] and so does not interfere in the reaction.[14] The effect of certain anions and buffers on nitrosation reactions will be discussed in subsequent sections.

1.2.1 Nitrous Anhydride Mechanism

The first kinetic investigations of nitrosation reactions gave apparently conflicting results. Hantzsch and Schumann[15] studied the diazotization of several amines in dilute acid using equal

concentrations of nitrous acid and amine. They observed a second order reaction, and assumed that this meant that the reaction was first order in each reactant, giving the rate equation:

$$\text{rate} = k\ [Ar\overset{+}{N}H_3]\ [HNO_2] \qquad (1)$$

They also found that the rate of reaction was similar for all the amines studied.

Taylor[5,16] studied the nitrosation of a variety of primary aliphatic amines and dimethylamine and found that the nitrosation of all these amines followed a third order rate equation:

$$\text{rate} = k\ [RNH_2]\ [HNO_2]^2 \qquad (2)$$

It was Hammett[17] who suggested that rate equation (2) could be explained in terms of a pre-equilibrium involving nitrous anhydride formation, nitrous anhydride then nitrosating the amine in the rate determining step, as shown in scheme 1.2

$$2HNO_2 \overset{\text{fast}}{\rightleftharpoons} N_2O_3 + H_2O$$

$$RNH_2 + N_2O_3 \xrightarrow{\text{slow}} \overset{+}{R}NH_2NO + NO_2^-$$

$$\overset{+}{R}NH_2NO \longrightarrow RN_2^+$$

scheme 1.2

Later work has shown this to be the case. Hughes, Ingold and Ridd[18] have studied the reaction by using a reactive amine and low acid concentrations so that the amine is predominantly in the unprotonated form. If the mechanism outlined in scheme 1.2 is correct, it sould be possible, in principle, to make the rate of nitrosation faster than the rate of nitrous anhydride formation, that is to make the formation of nitrous

anhydride the rate determining step. Using aniline as the amine, the rate equation at sufficiently low acid becomes zero order in aniline, that is

$$\text{rate} = k\,[HNO_2]^2 \qquad (3)$$

Hence the rate of formation of nitrous anhydride can be determined. Similar work using sodium azide[19] has also been carried out under conditions such that the formation of nitrous anhydride is rate limiting, and the observed rate constant is in good agreement with the previous determination.

Further evidence that the rate observed is in fact the rate of nitrous anhydride formation comes from ^{18}O experiments.[20] Bunton, Llewellyn and Stedman studied the rate of oxygen exchange between nitrous acid and water. They found that the rate was proportional to $[HNO_2]^2$, and exchange was occurring via nitrous anhydride formation. The rate constant is similar to that previously obtained.

$$2HNO_2 \rightleftharpoons H_2NO_2^+ + NO_2^-$$

$$H_2NO_2^+ + NO_2^- \rightleftharpoons N_2O_3 + H_2O$$

scheme 1.3

This achievement of rate limiting nitrous anhydride formation also explains the apparent discrepancy between the findings of Hantzsch and Schumann, and Taylor. Hantzsch and Schumann's studies were carried out at low acidities, and so in all probability the second order reaction they observed was rate limiting nitrous anhydride formation.

This mechanism involves electrophilic attack of the nitrosating agent on the nitrogen atom, and so it would be expected that the

higher the basicity of the amine, the higher its reactivity towards nitrous anhydride. Larkworthy[21] studied the rate of nitrosation of various aromatic amines and, indeed, found such a relationship.

The rate constants for nitrosation by this mechanism (k in equation 2) necessarily include the equilibrium constant for the formation of nitrous anhydride. However, there has been some uncertainty regarding this value. It has been determined as 0.2 dm^3mol^{-1}at 20°C [22], and this value has been used for some time. Recently it has been redetermined as 3.03×10^{-3} dm^3 mol^{-1} at 22°C [23], which is in much better agreement with the value of 9×10^{-3} dm^3mol^{-1} at 25°C calculated from thermodynamic cycles.[24] Markovits et al explain the difference between this value and the earlier value in terms of the high acid concentrations used in the previous determinations. This means that the rate constants previously calculated are approximately 10^2 smaller than if the new value is used, and so nitrous anhydride may be more reactive than previously thought. In fact recently it has been claimed that the rate of nitrosation of the azide ion and m-chlorophenyl hydrazine is close to the diffusion controlled limit.[25]

1.2.2 Acid Catalyzed Nitrosation

During their studies of rate limiting nitrous anhydride formation, Hughes, Ingold and Ridd[26] found that the rate of diazotization was faster than would be expected by equation (3). The difference was found to be more pronounced when the amine was a weaker base than aniline, and at very low concentrations of nitrous acid. They attributed the effect as being due to

the presence of another mechanism of diazotization. Investigation under conditions which favoured this mechanism and not diazotization by nitrous anhydride revealed that the rate equation had the form:

$$\text{rate} = k_3 [Ar\ NH_2] [HNO_2] [H^+] \qquad (4)$$

The nitrosating agent in this case is either the nitrous acidium ($H_2NO_2^+$) or nitrosonium (NO^+) ion, which are not kinetically distinguishable. These reagents are expected to be more reactive than nitrous anhydride, which is why they react more readily with weakly basic amines. The similarity of the rate constants for the nitrosation of a variety of substrates has led to the suggestion[27] that the reaction by the nitrous acidium or nitrosonium ion is occurring for reactive substrates at the diffusion controlled limit.

Attempts[28] to make the rate of formation of the nitrosating agent rate limiting, as achieved for nitrosation by nitrous anhydride, proved unsuccessful for diazotization reactions, but it has been claimed for the nitrosation of hydrogen peroxide.[29] Benton and Moore repeated the earlier work of Anbar and Taube, but in the absence of buffers and at higher acidities and again observed a tendency towards zero order dependence on the substrate at high peroxide concentrations, which they interpreted in terms of rate limiting nitrosonium ion formation. However peroxide concentrations were of the order of 1M to achieve this, and it could be that this levelling off is in fact a medium effect.

The same effect has been noted for the nitrosation of alcohols[30] but this was interpreted as a medium effect as for a variety of alcohols the limiting values were not the same, as expected

if the rate determining step was now the formation of the nitrosating agent.

Further arguments against the involvement of the nitrosonium ion as the nitrosating agent under these conditions are again from ^{18}O experiments. The nitrosation of sodium azide was carried out in ^{18}O water.[31] The two possibilities for nitrosation by the reagents are depicted in scheme 1.4.

$$H_2NO_2^+ \underset{}{\overset{\text{fast}}{\rightleftharpoons}} NO^+ + H_2O$$

$$NO^+ + N_3^- \xrightarrow{\text{slow}} N_3NO \xrightarrow{\text{fast}} N_2 + N_2O$$

or

$$H_2NO_2^+ + N_3^- \xrightarrow{\text{slow}} N_3NO$$

$$N_3NO \xrightarrow{\text{fast}} N_2 + N_2O$$

scheme 1.4

If the reaction was taking place by the first mechanism, then rapid ^{18}O exchange between the water and nitrous acid would occur, so it would be expected that the percentage of $N_2{}^{18}O$ would be similar to the ^{18}O percentage of the water. The results showed that there was much less ^{18}O in the product, and so it was concluded that the reaction was occurring by the second mechanism.

Spectroscopic investigations of the species present with nitrous acid in different acid concentrations have been carried out. The presence of the nitrous acidium ion in acid solutions of between 40 and 70% (by weight) has been reported.[32] However, Bayliss et al[33] later revised their methods and, in accord with the findings of Singer and Vamplew[34], found no spectroscopic evidence for the existence of the nitrous acidium ion, rather the spectrum at low acid concentrations was that of nitrous acid

and, starting at 45% perchloric acid, a band appeared which was assigned to the nitrosonium ion. The summation of the two species accounted for the total "nitrous acid" concentration. Using the spectroscopic data and thermodynamic cycles, the equilibrium constant for the formation of the nitrosonium ion

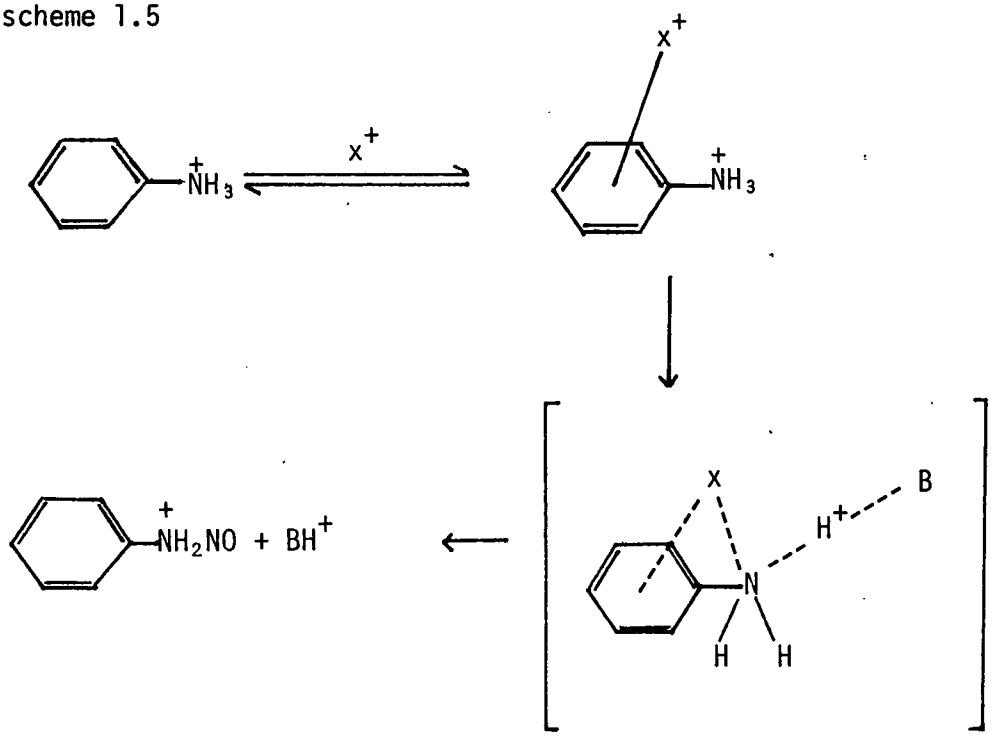
$$HONO + H_3O^+ \rightleftharpoons NO^+ + H_2O$$

has been calculated to be of the order of 10^{-7}.

With the most basic amines in the acidity range of 0.5 to 3M acid, the presence of an additional kinetic term

$$\text{rate} = k_3 \, [Ar\overset{+}{N}H_3] \, [HNO_2] \, h_0 \qquad (5)$$

has been detected.[35] This has been interpreted as being due to attack of the protonated amine by the nitrous acidium or nitrosonium ion, and a mechanism by which the nitrosating agent first interacts with the π electrons of the protonated amine, followed by proton loss from the amino group and migration of the NO group has been proposed, as shown in scheme 1.5



scheme 1.5

This mechanism is substantiated by substituent effects on the rate of nitrosation of aniline derivatives[36], the rate being increased by electron releasing substituents.

An analogous mechanism has been proposed for the nitrosation of hydroxylamine[37] and alkylhydroxylamines[38]. In this case nitrosation occurs at the oxygen of the protonated form ($R\overset{+}{N}H_2OH$) followed by migration of the -NO group to the nitrogen.

1.2.3 Nitrosation at High Acidity

As the acid concentration is increased above 6M, the rate of nitrosation of basic amines increases and then decreases rapidly,[39] following the rate equation

$$\text{rate} = k\,[Ar\overset{+}{N}H_3]\,[HNO_2]\,h_0^{-2} \qquad (6)$$

This has been interpreted in terms of a change in the rate determining step. Instead of the nitrosation of the amine being rate limiting, the loss of the proton to the medium is the rate determining process. This is consistent with the significant deuterium kinetic isotope effect observed.

1.3 Nitrosation at other sites

As mentioned earlier, the field of nitrosation reactions now includes nitrosation at a number of other sites, including oxygen, sulphur and carbon.

Studies on O- and S- nitrosation have shown that acid catalyzed nitrosation, giving the rate equation

$$\text{rate} = k_3[S][HNO_2][H^+] \qquad (7)$$

applies to these substrates as well as to nitrosation at

nitrogen. Again this rate equation has been interpreted as rate limiting electrophilic attack by the nitrous acidium or nitrosonium ion.

1.3.1 O-Nitrosation

Although the existence of alkyl nitrites has been known for some time[40], and they have been used as nitrosating agents, it is only recently that kinetic studies on the nitrosation of alcohols has been undertaken.

The first direct measurements were carried out in non-aqueous solvents[41], and Aldred, Williams and Garley[42] undertook the first direct measurements of the nitrosation of a number of alcohols in aqueous solution, and showed that equation (7) applies to O-nitrosation.

Evidence that the reaction is indeed electrophilic attack on the oxygen atom of the alcohol by the nitrous acidium or nitrosonium ion comes from the studies of acid hydrolysis of alkyl nitrites. Allen[43] studied the hydrolysis of an optically active alkyl nitrite and showed that complete retention of configuration occurred, implying fission of the nitrogen - oxygen bond and not the carbon - oxygen bond.

$$R^*ONO + H_2O \rightleftharpoons R^*OH + HNO_2$$

This implies that nitrosation is occurring by addition of NO^+ onto the oxygen of the alcohol.

O-nitrosation is a reversible reaction, with equilibrium constants of the order of 1[42]. This reversibility has been used to account for the observed reduction in the overall rate constant for the nitrosation of morpholine in the presence of alcohols.[44]

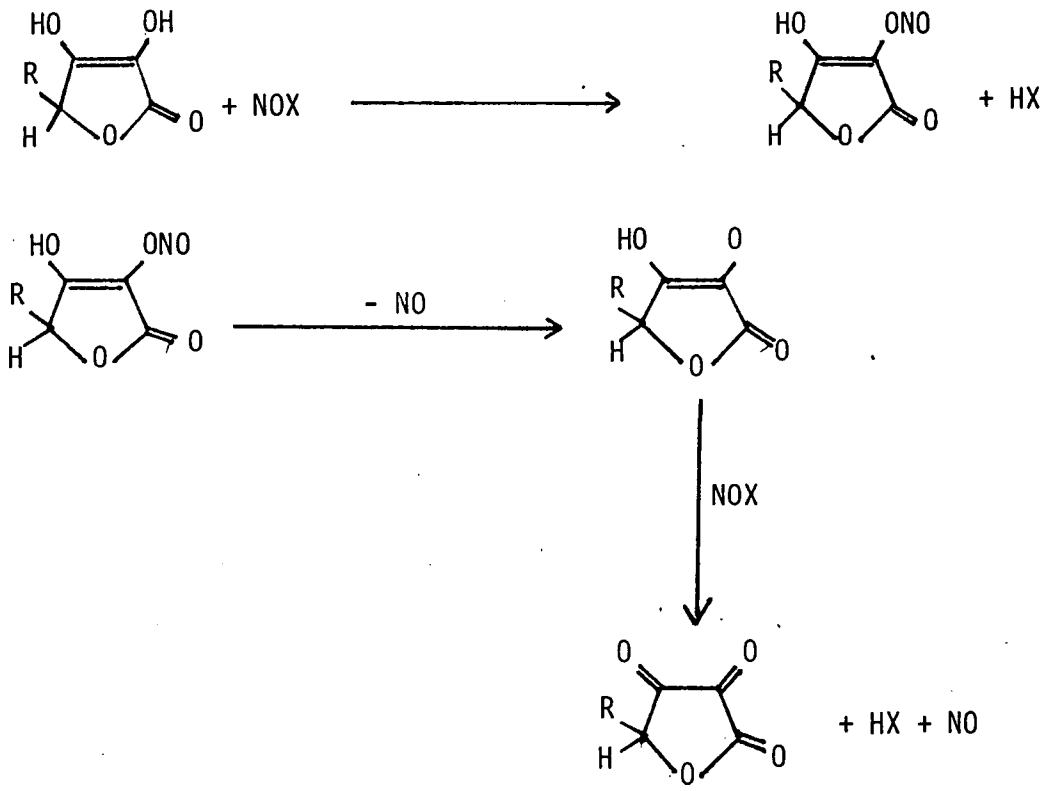
$$HNO_2 + R_2NH \longrightarrow R_2NNO + H_2O$$

$$HNO_2 + ROH \rightleftharpoons RONO + H_2O$$

scheme 1.6

In fact this decrease in the rate of nitrosation of amines by the addition of alcohols has been used to determine the equilibrium constant for the formation of the alkyl nitrite.[45]

One exception to the reversibility of O- nitrosation is the nitrosation of ascorbic acid[46], which reacts irreversibly with nitrous acid to form dehydroxyascorbic acid, via formation of the nitrite ester at the 3-hydroxy position, as in scheme 1.7. The kinetics of this reaction have been investigated[47], and it has been shown that the usual nitrosation rate equations apply here.

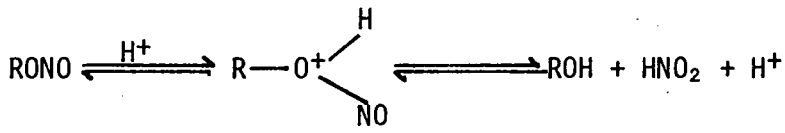


scheme 1.7

The amount of ascorbate necessary to inhibit nitrosation of other substrates has been shown to depend on whether the reaction is carried out under aerobic or anaerobic conditions.[48] Under anaerobic conditions, equal concentrations of nitrite and ascorbate led to oxidation of only half the ascorbate, as expected by the above mechanism. In the presence of air, however, equal concentrations of nitrite and ascorbate led to complete oxidation of the ascorbate. This difference can be explained either in terms of oxidation of nitric oxide (which is an ineffectual nitrosating agent[49]) to nitrogen dioxide, or oxidation of the semiquinone intermediate by oxygen.

1.3.2 S- Nitrosation

Studies on the nitrosation of thiols, to give the thionitrite, have shown that the usual rate equation for acid catalyzed nitrosation applies here.[50] S- nitrosation is a more rapid process than O- nitrosation, but whereas O- nitrosation is a reversible reaction, S- nitrosation, under the typical conditions used, is essentially irreversible. This difference has been explained in terms of the difference in basicity and nucleophilicity between sulphur and oxygen.[42] Sulphur is more nucleophilic than oxygen, due to the fact that sulphur is the more polarizable of the two, and so S- nitrosation is more rapid than O- nitrosation. The reverse reaction, hydrolysis, is an acid catalyzed process and is thought to involve protonation of the thionitrite[51] or alkyl nitrite[42] at sulphur and oxygen respectively as the first step, as shown in scheme 1.8 for alkyl nitrites.



scheme 1.8

Basicity is dominated by the strength of the bond formed with the proton[52] and due to the higher O-H bond energy compared to the S-H bond energy[53] oxygen is more basic than sulphur.

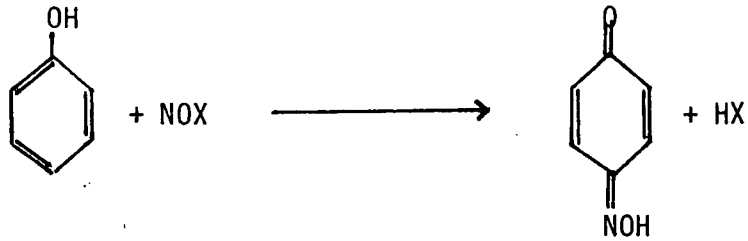
O—H	464.4 kj/mol
S—H	368.2 kj/mol

This means that alkyl nitrites are more susceptible to protonation, and hence hydrolysis, than thionitrites.

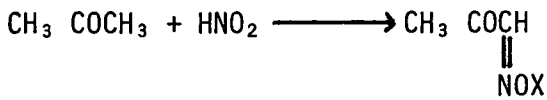
S-nitrosation reactions will be discussed in more detail in section 2.1.

1.3.3 C-Nitrosation

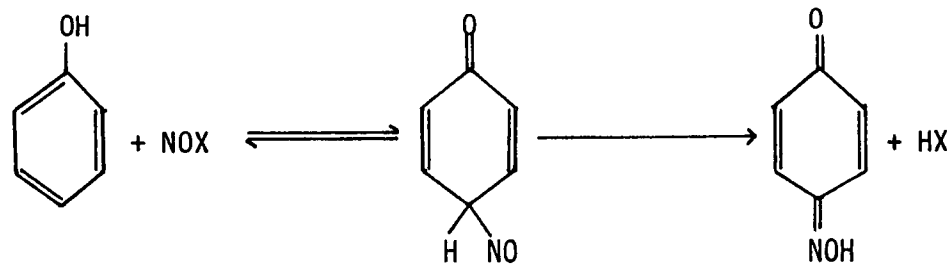
C-nitrosation reactions include the nitrosation of aromatic substrates[54], for example:



and ketones[55], for example



The C-nitrosation of phenol, anisole[54], and a number of p - substituted phenols[56] has been studied and found to go by an A-S_E2 reaction mechanism.



scheme 1.9

In these reactions proton loss from the intermediate can be rate limiting. However for certain substrates the rate of nitrosation is rate limiting, and both the nitrosonium or nitrous acidium ion[57,58] and nitrous anhydride[59] have been identified as the nitrosating agent.

1.4 Halide Ion Catalysis

The catalysis of nitrosation reactions by the addition of halide ions is now well established.

An early report of this catalysis was by Schoutissen[60] who observed an increase in the rate of diazotization of several aromatic amines with the addition of hydrochloric acid. Schmid[61] undertook a kinetic investigation of this reaction and determined that the rate of diazotization in the presence of chloride and bromide ions could be explained by the addition of an extra term

$$\text{rate} = k\,[C_6H_5\overset{+}{N}H_3]\,[HNO_2]\,[X^-] \qquad (8)$$

$X^- = Cl^-$ or Br^-

It was Hammett[17], however, who noticed that this term could be rearranged to give

$$\text{rate} = k_4\,[C_6H_5NH_2]\,[H^+]\,[HNO_2]\,[X^-] \qquad (9)$$

and that the product $[H^+]\ [HNO_2]\ [X^-]$ could be explained in terms of a pre-equilibrium involving the formation of NOX as shown in scheme 1.10

$$H^+ + HNO_2 + X^- \rightleftharpoons NOX + H_2O$$

$$NOX + C_6H_5NH_2 \longrightarrow C_6H_5\overset{+}{N}H_2NO + Cl^-$$

$$C_6H_5\overset{+}{N}H_2NO \longrightarrow C_6H_5N_2^+ + H_2O$$

scheme 1.10

The full rate equation, therefore, is

$$\text{rate} = k_3[C_6H_5NH_2]\,[HNO_2]^2 + k_4[C_6H_5NH_2]\,[HNO_2]\,[H^+]\,[X^-] \qquad (10)$$

It can be seen, therefore, that catalysis by X^- becomes significant at high concentrations of X^- and at low nitrous acid concentrations (since the rate of uncatalyzed diazotization is proportional to the square of the nitrous acid concentration.)

Hughes and Ridd[62] studied the diazotization of aniline and o- chloroaniline in an attempt to make the rate of formation of NOX rate limiting. They found that it was not possible for chloride ion catalysis, but succeeded for bromide and iodide ion catalysis. In accordance with the above mechanism, they determined the rate equation:

$$\text{rate} = k\ [H^+]\ [HNO_2]\ [X^-] \qquad (11)$$

k_4 in equation (9) necessarily contains the equilibrium constant for the formation of NOX (K_{NOX}). These have now been determined as 1.1×10^{-3} $dm^6\ mol^{-2}$ for NOCl[63] and 5.1×10^{-2} $dm^6\ mol^{-2}$ for NOBr[64] at 25°C and so the rate constants for nitrosation by NOX can be determined. From these rate constants it appears that for reactive substrates (e.g. anilines[65]), the reaction is occurring close to the diffusion controlled limit.[27]

Studies show that for less reactive substrates, nitrosyl chloride is more reactive than nitrosyl bromide, as expected due to the difference in electronegativity between chloride and bromide.[16,27] The catalytic effect is dominated by the size of K_{NOX} and hence the bromide ion shows a higher catalytic activity compared to the chloride ion.

Catalysis by halide ions is not restricted to N-nitrosation. It has now been shown to apply as well for O-[42], S-[50] and C-nitrosation.[58]

1.5 Catalysis by Thiocyanate and Thiourea

In addition to halide ion catalysis, other well known catalysts include the thiocyanate ion, thiourea and alkyl thiourea derivatives.

The effect of thiocyanate has been known for some time. Bunton, Llewellyn and Stedman[20] noted an increase in the rate of oxygen exchange between nitrous acid and water in the presence of thiocyanate. Due to the high concentration of thiocyanate used in these experiments, they studied its effect on the diazotization of aniline. Thiocyanate increased the rate of diazotization and the rate was independent of the amine concentration. This study showed the rate equation:

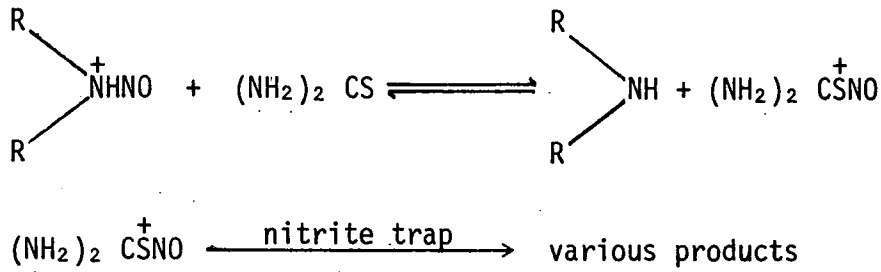
$$\text{rate} = k_3[H^+]\ [HNO_2]\ [SCN^-] \qquad (12)$$

consistent with the formation of nitrosyl thiocyanate applied, analogous with the situation for nitrosyl halides.

The effectiveness of thiocyanate as a catalyst and its presence in human saliva, means that this reaction has received attention due to the possibility of its catalyzing *in vivo* nitrosation.[66]

There are two possible sites in thiocyanate where nitrosation can occur, that is at the sulphur or nitrogen. That the nitrosation in fact occurs on the sulphur atom is in accordance with the HSAB theory,[67] with nitrosation occurring on the "soft" sulphur rather than the "hard" nitrogen.

The involvement of thiourea in nitrosation reactions was shown in the denitrosation of nitrosamines.[68]



scheme 1.11

The reversibility of this reaction, that is that S-nitroso thiourea can act as a nitrosating agent, was demonstrated by a decrease in the rate of the reaction by the addition of an amine.

The catalysis of nitrosation reactions by thiourea and alkylthioureas was shown directly by Masui et al[69] for the nitrosation of dimethylamine. Kinetic studies of the catalysis of the nitrosation of morpholine by thiourea[70] demonstrated that this catalytic activity could be interpreted in terms of formation of S-nitrosothiourea which then acted as the nitrosating agent. The same mechanism applies for the catalytic activity of the alkylthioureas.[71]

As for the reactions involving nitrosyl halides, the equilibrium constants for the formation of nitrosyl thiocyanate and S-nitrosothiourea need to be known in order to calculate

k_2, the rate constant for the nitrosation of the substrate by these species, where

$$\text{rate} = k_2 [S] [NOX]$$

These K_{NOX} values have been determined as 32 $dm^6\ mol^{-2}$ [72] for nitrosyl thiocyanate and 5000 $dm^6\ mol^{-2}$ for [73] S-nitrosothiourea, and k_2 values have been calculated for a number of amines. Comparison of the k_2 values for these nucleophilic catalysts in nitrosation reveals the general trend NOCl > NOBr > NOSCN > $NO\overset{+}{S}C(NH_2)_2$ for N^{-1}, O^{-42}, and S-nitrosation[50] reactions. The K_{NOX} values principally determine the amount of catalysis observed, increasing along the above series in accordance with Pearsons nucleophilicity parameters.[74] Comparision of the order of catalytic activity of these nucleophilic catalysts, therefore, shows the general trend $(NH_2)_2\ C\overset{+}{S}NO$ > SCN^- > Br^- > Cl^-.

The size of the equilibrium constants for nitrosyl thiocyanate and S-nitrosothiourea means that they are powerful catalysts of nitrosation reactions. This is illustrated by the work of Meyer and Williams[70] who obtained the ratio 4,200 : 240 : 1 for catalysis of the nitrosation of morpholine by thiourea, thiocyanate, and bromide respectively. The reactivity of these nitrosyl species is, however, the reverse of the above sequence.

1.6 Other Species Involved in Nitrosation Reactions

Perhaps one of the most frequently encountered species outside of those already described is nitrosyl acetate which is formed when reactions are carried out in acetate buffers. Hughes and Ridd[75] studied the effect of acetate buffer on the

diazotization of aniline where the rate of formation of the nitrosating agent was rate limiting. They found that the reaction was catalyzed according to the equation

$$\text{rate} = k\,[HNO_2]^2\,[AcO^-] \qquad (14)$$

which they concluded was due to the formation of nitrosyl acetate in a rapid pre-equilibrium, which then formed nitrous anhydride in a rate determining step.

$$AcO^- + H_2NO_2^+ \rightleftharpoons AcONO + H_2O$$

$$AcONO + NO_2^- \longrightarrow N_2O_3 + AcO^-$$

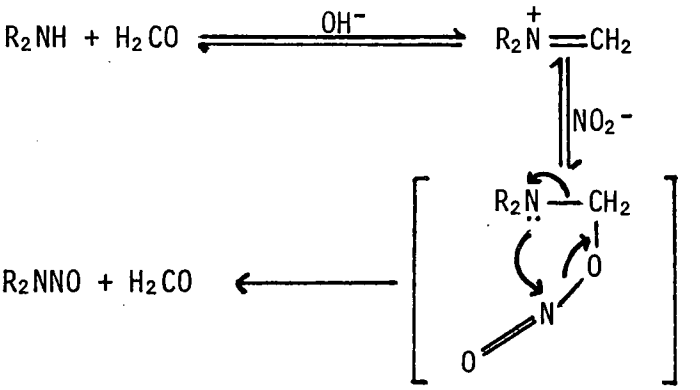
scheme 1.12

In this case nitrosyl acetate itself did not become the main nitrosating agent otherwise the reaction would become first order with respect to nitrous acid, instead of the observed order of 1.8. The same order with respect to nitrite has been observed for the nitrosation of dimethylamine[76] in the presence of acetate buffer. Consistent with this is the recent study on the nitrosation of morpholine,[77] where catalysis by the acetate ion was only observed when the formation of nitrous anhydride was the rate determining stage. However nitrosyl acetate has been shown to be an important nitrosating agent for the nitrosation of azide,[78] N- methyl-aniline and piperidine.[79]

Studies on the pH dependence of the nitrosation of amines by nitrous anhydride have shown that the pH maximum for this reaction for simple amines is pH 3.4 [11,80] close to the pKa for nitrous acid. Mirvish's studies on the pH dependence of the nitrosation of dimethylamine[81] have again shown this pH maximum, and he reports a negligible rate of nitrosation

above approximately pH 5.5. However nitrosation at neutral to alkaline pH can occur.

In the studies on nitrosation reactions the nitrite ion is normally considered not to be a nitrosating agent.[82] However Keefer and Roller[83] have reported the nitrosation of several secondary amines by the nitrite ion in the presence of aldehydes in alkaline solution. They proposed that the reaction occurred via an imminium ion, as in scheme 1.13.



scheme 1.13

Kinetic studies in the presence of formaldehyde of the nitrosation of dimethylamine in both acid and alkaline solutions[84], and of morpholine and diethylamine in alkaline solution [85] are consistent with this mechanism.

The nitroprusside ion can also effect the nitrosation of amines in alkaline solutions up to pH 12. Maltz, Grant and Navoroli[86] investigated the products of the reactions of amines with nitroprusside and identified them as being the products of nitrosation, and they proposed the mechanism in scheme 1.14.

$$RNH_2 + Fe(CN)_5NO^{2-} \rightleftharpoons RNH_2\,NO\,Fe(CN)_5^{2-}$$

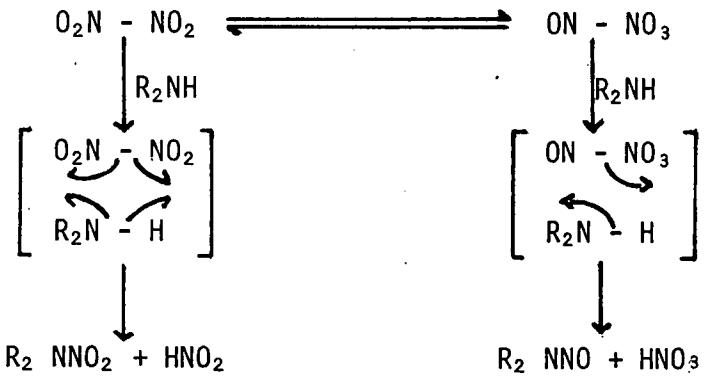
$$RNH_2\,NO\,Fe(CN)_5^{2-} + RNH_2 \longrightarrow RNHNO + H^+ + RNH_2\,Fe(CN)_5^{2-}$$

$$RNHNO \longrightarrow \text{decomposition products}$$

scheme 1.14

Casado et al[87] studied the kinetics of this reaction, and obtained results in agreement with this proposed mechanism.

The reaction of gaseous dinitrogen tetroxide with amines is well known in organic solvents[88] to form a mixture of nitrosamines and nitroamines. Challis and Kyrtopoulos[89] have shown rapid nitrosation of a variety of amines occurs in aqueous alkaline solution by gaseous dinitrogen trioxide and dinitrogen tetroxide. Although dinitrogen trioxide and dinitrogen tetroxide are fairly dissociated in the gaseous phase, the dissociation should be less in solution.[90] That the reaction by dinitrogen tetroxide leads to nitration and nitrosation, they explain in terms of reaction by the $O_2N - NO_2$ and $ON - NO_3$ isomers respectively[91], as shown in scheme 1.15.



scheme 1.15

The reaction by dinitrogen trioxide is by the ON - ONO isomer, by an analogous mechanism to nitrosation by the ON - NO_3 isomer of dinitrogen tetroxide.

More recently studies using nitrogen dioxide as the nitrosating agent have been reported[92] in a variety of solvents. Again nitrosation and nitration was found to occur with N-methylaniline as the amine leading to a variety of products.

Nitrosation by nitrogen dioxide was explained in terms of dimerization to form dinitrogen tetroxide which then reacts as in the above scheme, and the reaction was inhibited by the addition of sodium azide. Nitration, in contrast, occurs by reaction with nitrogen dioxide itself, reacting via a radical mechanism.

Studies using nitric oxide itself as a nitrosating agent under anaerobic conditions have shown that this reaction is extremely slow ($t_{\frac{1}{2}}$ ~ 8 days)[89], but if air is let into the reaction vessel, rapid nitrosation occurs, presumably by oxidation of nitric oxide to nitrogen dioxide, leading to reaction by dinitrogen trioxide or dinitrogen tetroxide. Under anaerobic conditions the rate is independent of the amine concentration and amine basicity. This has been interpreted as being due to leakage of air into the reaction vessel, and that the rate of oxidation of nitric oxide is the rate limiting process. However a number of species can act as a catalyst causing nitrosation to occur in organic solvents. A number of metal salts, e.g. Cu^{I} and Ag^{I}[49] cause rapid nitrosation to occur. The mechanism for Ag^{I} catalysis of the nitrosation of piperidine has been proposed as[93]

$$2\ Ag^{I} + 4\ R_2NH \equiv\equiv\equiv Ag^{0} + Ag^{II}\ [R_2NH]_4$$

$$Ag^{II}\ [R_2NH]_4 \longrightarrow Ag^{I}\ [R_2\overset{+\cdot}{N}H]\ [R_2NH]_3$$

$$Ag^{I}\ [R_2\overset{+\cdot}{N}H]\ [R_2NH]_3 \xrightarrow{NO} R_2NNO + 2R_2NH + R_2\overset{+}{N}H_2 + Ag^{I}$$

scheme 1.16

The greatest acceleration of the rate of nitrosation of amines by nitric oxide is observed by the addition of iodine[49]. Kinetic studies[94] are consistent with the reaction proceeding via

formation of nitrosyl iodide in the rate determining step which then acts as the effective nitrosating agent.

$$2NO + I_2 \rightleftharpoons 2\ NOI$$

$$NOI + R_2NH \longrightarrow R_2NNO + HI$$

scheme 1.17

More recently the involvement of a number of iodides[95] has been shown, the reaction again proceeding via formation of nitrosyl iodide which reacts as shown in the above scheme.

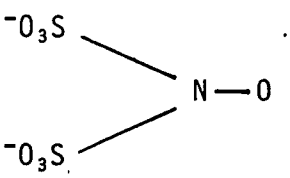
$$HI + NO \rightleftharpoons NOH + \tfrac{1}{2}\ I_2$$

$$NO + \tfrac{1}{2}\ I_2 \rightleftharpoons NOI$$

$$NOI + R_2NH \longrightarrow R_2NNO + HI$$

scheme 1.18

Other species which can effect nitrosation under alkaline conditions include Fremy's salt (potassium nitrosodisulphonate)[96]



and alkyl nitrites bearing β-electron withdrawing substituents[97] Recently nitrosothiosulphate has been shown to be a nitrosating agent under acid conditions.[98]

In summary, nitrosation can occur at a number of sites, including nitrogen, oxygen, sulphur and carbon. The involvement of a number of species in nitrosation reactions has been identified, so nitrosation can be brought about under a wide range of conditions.

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CHAPTER TWO

S-NITROSATION

2.1 Introduction

The existence of thionitrites (RSNO) has been known for some time. In fact there are reports in the literature as early as 1839 that thiols react with nitrous acid to give red coloured species.[1]

$$RSH + HNO_2 \longrightarrow RSNO + H_2O$$

red/green

However, it has not been until relatively recently that this reaction has been studied mechanistically. The area of S-nitrosation has been recently reviewed.[2]

Tasker and Jones[3] observed that when nitrosyl chloride was bubbled through a solution of benzene thiol, the solution turned port-wine red and hydrogen chloride was evolved. The solution then rapidly evolved nitric oxide and the disulphide was formed, the colour of the solution fading at the same time. They postulated that the nitrosyl chloride was nitrosating the thiol to form the red coloured thionitrite and hydrogen chloride. They accounted for the formation of the disulphide in terms of the instability of this thionitrite which decomposed generating the disulphide and nitric oxide.

$$C_6H_5SH + NOCl \longrightarrow C_6H_5SNO + HCl$$

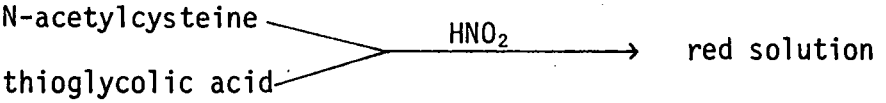
$$2C_6H_5\ SNO \longrightarrow (C_6H_5S)_2 + 2NO$$

scheme 2.1

Rheinboldt[4] studied the nitrosation of several primary and secondary thiols but again only obtained the disulphide as the product due to the instability of the thionitrites. However,

he and Vorländer and Mittag[5] managed to isolate triphenylmethyl thionitrite as a green solid. Later Lecher and Siefken[1] achieved the isolation of ethylthionitrite as the product of the nitrosation of ethane thiol under conditions where air was excluded. Since then several thionitrites have been isolated, for example t-butylthionitrite[6], which is stable as a solid for a few days at room temperature, and S-nitroso-N-acetylpenicillamine[7], which is indefinitely stable as a solid and which decomposes slowly in solution.

Bonnett et al[8] observed coloured solutions when acidified nitrite solutions reacted with thiols.



^{15}N N.M.R. studies on these products gave results consistent with thionitrite formation. E.S.R. studies[9] have also been carried out on the nitrosation of thiols and again results in accordance with thionitrite formation were obtained.

That thionitrites are coloured species has led to this property being used for the identification of thiols in solution[10], and is the basis of a quantitative test for nitrosyl sulphuric acid using thioglycolic acid as the reagent.[11] The physical properties and reactions of thionitrites have been reviewed.[12]

It has now been shown that the usual N-nitrosation and diazotization reagents can effect the nitrosation of thiols, for example, nitrous acid,[13] dinitrogen tetroxide,[14] nitrosyl-chloride,[15] and alkyl nitrites.[1] The easiest methods for

generating thionitrites are dinitrogen tetroxide[16] and equimolar amounts of the thiol in an inert solvent at -10°C or t-butylnitrite in chloroform.[17] Both methods lead to rapid quantitative generation of the thionitrite.

As mentioned in Chapter One, S-nitrosation is a rapid process. Under the normal conditions used to study this reaction, it is essentially irreversible,[18] in contrast to O-nitrosation where the equilibrium constants are of the order of 1.[19] The reason for this was given in Chapter One. The high reactivity of thiols towards nitrosating agents and the irreversibility of S-nitrosation means that thiols are potential nitrite traps. The denitrosation of a nitrosamine has been carried out in the presence of 3-mercaptopropanoic acid[18] and this thiol was found to be efficient in suppressing the reversibility of the denitrosation reaction.

One of the first kinetic investigations of the nitrosation of thiols was by Kresze and Winkler[20] who studied the nitrosation of t-butyl thiol by nitrous acid. They established the rate equation

$$\text{rate} = k_3[HNO_2][H^+][RSH] \qquad (1)$$

This is the familiar rate equation for acid catalyzed nitrosation[21] observed for a large range of substrates. As for nitrosation at other centres, it is interpreted as rate determining electrophilic attack by the nitrous acidium ion or nitrosonium ion on the sulphur atom followed by rapid proton loss from the protonated thionitrite as shown in scheme 2.2 using the nitrous acidium ion as the nitrosating agent.

$$HNO_2 + H^+ \underset{}{\overset{\text{fast}}{\rightleftharpoons}} H_2NO_2^+$$

$$H_2NO_2^+ + RSH \xrightarrow{\text{slow}} R\overset{+}{S}HNO + H_2O$$

$$R\overset{+}{S}HNO \xrightarrow{\text{fast}} RSNO + H^+$$

scheme 2.2

The arguments for and against the involvement of the nitrous acidium ion or nitrosonium ion as the effective nitrosating agent were raised in Chapter One and will not be repeated here.

Rate equation (1) has now been established for several other thiols.[18,19] Under the typical conditions used to study this reaction, the thiol group is not significantly protonated and so the complications that occur in N-nitrosation reactions due to protonation of the amine do not arise in S-nitrosation reactions.

The study of the nitrosation of thiols has now been extended to include catalysis by nucleophiles such as chloride, bromide and thiocyanate ions which are established catalysts for N-nitrosation, diazotization[21] and O-nitrosation[19] reactions. As in the aforementioned cases, it has been shown that the catalysis of S-nitrosation reactions by these ions can be interpreted by the addition of the extra kinetic term to the rate equation

$$\text{rate} = k_4 [HNO_2][H^+][RSH][X^-] \qquad (2)$$

$$X^- = Cl^-, Br^-, SCN^-$$

This is due to the formation of the NOX species in a pre-equilibrium step, these species then effecting nitrosation of the substrate.

$$HNO_2 + H^+ + x^- \underset{}{\overset{K_{NOX}}{\rightleftharpoons}} NOX + H_2O$$

$$NOX + RSH \xrightarrow{k_2} \overset{+}{R}SHNO + x^-$$

$$\overset{+}{R}SHNO \longrightarrow RSNO + H^+$$

scheme 2.3

Re-writing equation (2) as:

$$\text{rate} = k_2 \, K_{NOX} \, [HNO_2] \, [H^+] \, [RSH] \, [x^-]$$

The K_{NOX} values have been independently determined as 1.14×10^{-3} l^2 mol^{-2} (NOCl),[22] 5.1×10^{-2} l^2 mol^{-2} (NOBr)[23] and 32 l^2 mol^{-2} (NOSCN)[24] at 25°C and so the k_2 values, the actual second order rate constant for the nitrosation of the thiols by the NOX species where

$$\text{rate} = k_2 \, [NOX] \, [RSH]$$

can be determined. Some of these k_2 values are shown in Table 2.1.

Table 2.1: k_2 values

	NOCl	NOBr	NOSCN		Reference
N-acetyl-penicillamine	2.6×10^6	1.4×10^5	3.0×10^3	31°C	19
cysteine	1.2×10^6	5.8×10^4	6.9×10^2	25°C	this work
thioglycolic acid	1.4×10^7	1.1×10^6	2.5×10^4	25°C	this work

These values show that k_2 decreases along the series NOCl > NOBr > NOSCN, as expected due to the electronegativities of the x^- species. The order of catalytic activity of these ions is, however, $SCN^- > Br^- > Cl^-$ due to the dominating effect of the K_{NOX} values.

Al-Mallah, Collings and Stedman[25] studied the nitrosation of thiourea, investigating an earlier observation by Werner[26] that the action of nitrous acid on thiourea gave different products depending on the acidity at which the reaction was carried out. At low acidities, the following reaction occurred:

$$HNO_2 + CS(NH_2)_2 \longrightarrow H^+ + SCN^- + N_2 + 2H_2O$$

whereas at high acidities the CC'-dithio formamidinium ion was formed.

$$2H^+ + 2HNO_2 + 2CS(NH_2)_2 \downarrow$$

$$(NH_2)_2 \overset{+}{C} SS \overset{+}{C} (NH_2)_2 + 2NO + 2H_2O$$

The structure of this dication has been confirmed by x-ray crystallographic analysis of its salts.[27]

Al-Mallah et al showed that the reaction path at high acidity involved the formation of an equilibrium concentration of S-nitrosothiourea, and that the formation of this species followed the rate equation:

$$\text{rate} = k_3 [HNO_2][H^+][CS(NH_2)_2]$$

The products at low acidity are consistent with the products of the N-nitroso species which Al-Mallah et al interpreted in terms of an intramolecular S- to N- rearrangement of the nitroso group to form N-nitrosothiourea.

$$H^+ + HNO_2 + CS(NH_2)_2 \rightleftharpoons (NH_2)_2 \overset{+}{C}SNO$$

$$(NH_2)_2 \overset{+}{C}SNO \rightleftharpoons H^+ + (NH_2)(\bar{N}H)\overset{+}{C}SNO$$

$$(NH_2)(\bar{N}H)\overset{+}{C}SNO \longrightarrow NH_2\ CSNHNO$$

$$NH_2\ CSNHNO \longrightarrow NH_2 - CS - N = N - OH$$

$$NH_2\ CS - N = N - OH \longrightarrow H^+ + HNCS + N_2 + OH^-$$

scheme 2.4

However, ^{15}N N.M.R. studies[28] have shown that direct N-nitrosation occurs at low acidity, and S-nitrosation at high acidity.

Nitrosamines[29] and alkyl nitrites,[30] as well as other conventional nitrosating agents, can effect the nitrosation of thiourea. That nitrosamines can nitrosate thiourea by a direct reaction was shown by Williams[29] who demonstrated that the nitrosation of thiourea occurred in the presence of a nitrite trap which excludes the possibility of reaction by the liberated nitrous acid.

The nitrosation of a number of alkylthioureas has been studied[31] and the rate constants determined. These rate constants are shown in Table 2.2.

Table 2.2: k_3 values for thiourea and alkylthioureas.

	$k_3/l^2mol^{-2}s^{-1}$
$CS(NH_2)_2$	6,960
$Me\ NH\ CS\ NH_2$	5,620
$CS\ (NHMe)_2$	6,610
$Me_2NCS\ NH_2$	5,790
$CS\ (NMe_2)_2$	4,340

The diffusion controlled limit[32] for the nitrosation of a neutral substrate by the nitrous acidium or nitrosonium ion is thought to be around 7,000 $l^2mol^{-2}s^{-1}$ based on the similarity

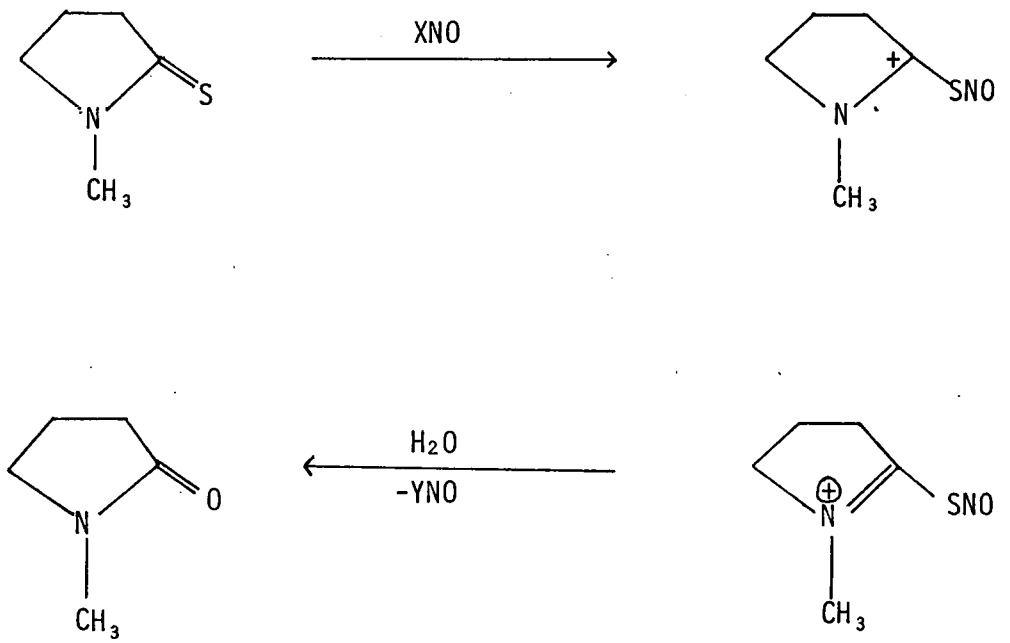
of rate constants for a variety of reactive substrates. This means that the nitrosation of thioureas and alkylthioureas is occurring at, or close to, the diffusion controlled limit.

In this discussion of the nitrosation of thiourea the product of S-nitrosation is given as the CC'-dithioformamidinium ion. However, urea can be the product of the reaction under certain conditions.[33]

$$(NH_2)_2\ CS \xrightarrow{HNO_2/H^+} (NH_2)_2\ CO$$

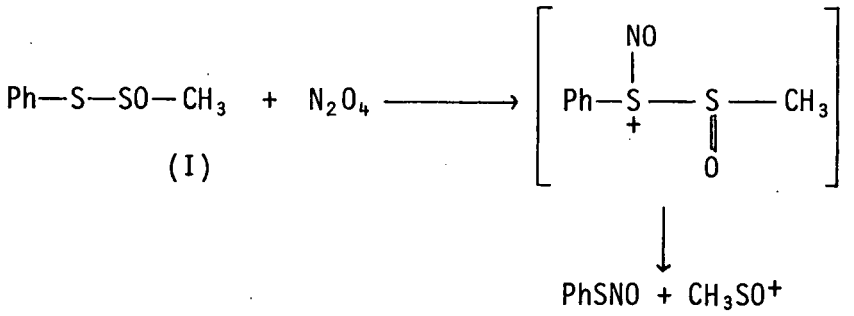
This reaction may be occurring by nucleophilic attack of water on S-nitrosothiourea, or by loss of HSNO and subsequent hydration of the carbodiimide.

Jørgensen and Lawesson[34] studied the >=S to >=O transformation of N-methyl-2-thiopyrrolidine using aqueous acidic sodium nitrite and again proposed that the reaction involves the formation of the S-nitroso species followed by a hydrolysis reaction.



scheme 2.5

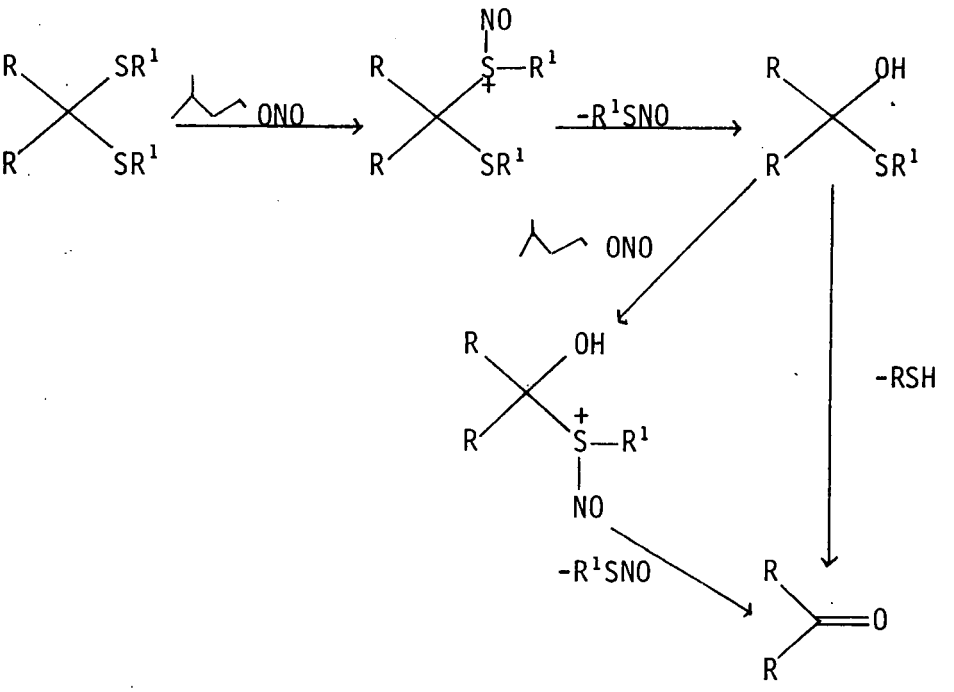
Thionitrites have also been shown to be intermediates in the reaction of thiosulphinates and disulphides with dinitrogen tetroxide.[35] For example



scheme 2.6

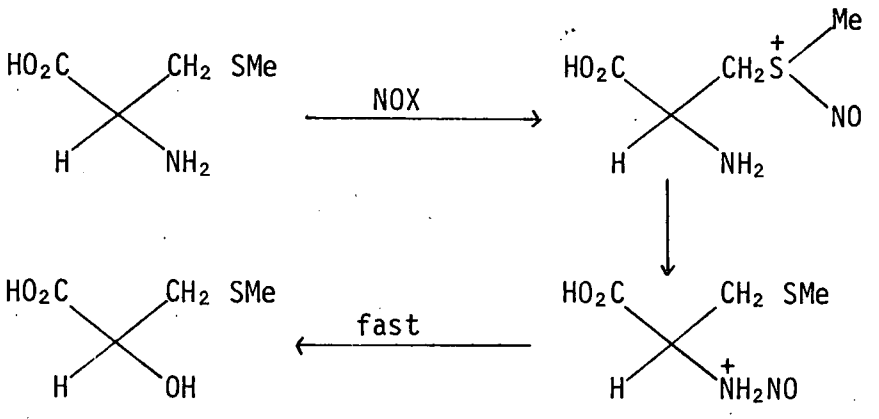
The u.v. spectrum of the product of the reaction of thiosulphinate (I) with dinitrogen tetroxide was identical to that of S-phenylthionitrite.

Dethioacetalization by i-amyl nitrite has also been proposed to go via the intermediate formation of thionitrites.[36] Reaction of dithioacetal with i-amyl nitrite gave a red coloured solution and at the end of the reaction the disulphide was obtained in 70 to 80% yield.



scheme 2.7

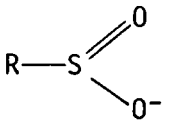
Meyer and Williams[37] studied the deamination of the amino acids methionine, S-methylcysteine and alanine, alanine being structurally similar to the other two amino acids, but without the presence of the sulphur containing group. The results showed that the deamination of methionine and S-methylcysteine was significantly faster than the deamination of alanine, which was interpreted in terms of an intramolecular S- to N-rearrangement occurring in methionine and S-methylcysteine as shown in scheme 2.8 for S-methylcysteine.



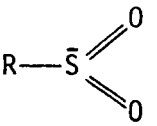
scheme 2.8

The absence of sulphur in alanine means that such an intramolecular rearrangement cannot occur and hence the rate of deamination of alanine is slower than that for methionine and S-methylcysteine.

The reaction of sulphinic acid with nitrous acid has been known for a long time,[2] the reaction going to give the hydroxylamine derivative. In fact this method has been used to synthesize hydroxylamines.[38] Structures (II) and (III) are thought to contribute to the overall structure of the sulphinate ion,[2] and so it is not surprising that sulphinic acids undergo S-nitrosation.

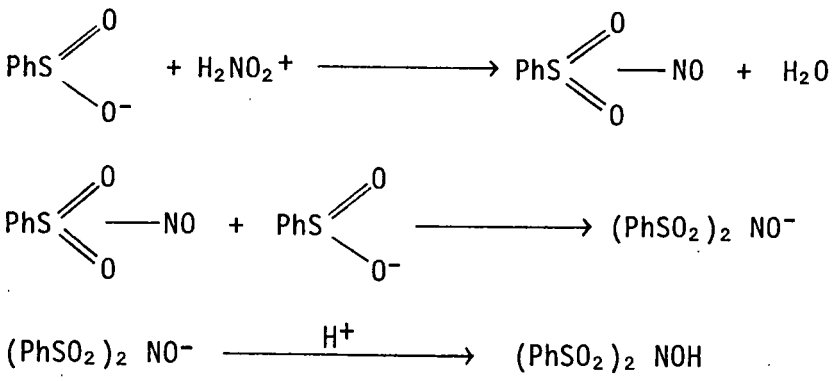


(II)



(III)

What is unusual about this reaction is firstly that the hydroxylamine is the product and secondly that two moles of sulphinic acid react per mole of nitrous acid. Recently this reaction has been studied mechanistically.[39] The reaction was shown to be first order in substrate, indicating that the nitrosation of the sulphinic acid is the rate determining step. Bryant and Williams also demonstrated that both the acid and the anion were reactive towards nitrous acid since a plot of the first order rate constant against the acid concentration was linear at acidities above ~0.06M, but with a significant positive intercept and the plot was curved at lower acidities. The rate constant for the nitrosation of benzene sulphinic acid was determined at 820 l^2 mol^{-2} s^{-1} and for the anion 11,800 l^2 mol^{-2} s^{-1}. These rate constants show the high reactivity of both the acid and the anion towards nitrous acid, the anion being more reactive. Bryant and Williams accounted for the stoichiometry of the reaction and the formation of the hydroxylamine in terms of the nitroso-sulphinate ion reacting with another molecule of acid or its anion which finally forms the hydroxylamine on the addition of a proton from the solvent, as shown in scheme 2.9 for the anion.



scheme 2.9

The reaction of the thiosulphate ion with nitrous acid gives a yellow coloured species, which is thought to be the S-nitroso species $[O_3SSNO]^-$. Early kinetic studies on this reaction[40] yielded a rate equation which was not integral in thiosulphate concentration. Garley and Stedman[41] studied this reaction and showed that the rate equation for the nitrosation of the thiosulphate ion was:

$$\text{rate} = k_1 [H^+][HNO_2][S_2O_3^{2-}] + k_2 [HNO_2]^2$$

The first term of this rate equation is easily recognizable as the rate equation for nitrosation by the nitrous acidium ion or nitrosonium ion, and the second term represents rate determining formation of nitrous anhydride, as observed previously, for example the nitrosation of reactive amines[42] and the azide ion.[43] k_1 was determined as 18,000 $l^2 mol^{-2} s^{-1}$. The diffusion controlled limit[32] for nitrosation by the nitrous acidium or nitrosonium ion is thought to be about 7,000 $l^2\ mol^{-2}\ s^{-1}$ for a neutral molecule, and approximately 11,000 $l^2\ mol^{-2}\ s^{-1}$ for a singly negatively charged species, and it would be expected that the diffusion controlled limit would be higher for a doubly negatively charged species and this value of 18,000 $l^2\ mol^{-2}\ s^{-1}$ is thought to represent this

diffusion controlled limit.

Thionitrites have also been implicated in the mechanism of vasodilators[44] such as organic nitrites and nitrates and nitroprusside. The mechanism is thought to involve the nitrosation of tissue-bound thiols, such as cysteine and glutathione, by these compounds, the thionitrite then activating the enzyme guanylate cyclase, which catalyses the conversion of GTP (guanosine triphosphate) to cGMP (cyclic guanosine monophosphate), cGMP mediating the relaxation of vascular smooth muscle.

This chapter describes the nitrosation of several thiols, firstly a series of cysteine derivatives and structurally related compounds and secondly a relatively simple thiol, thioglycolic acid.

2.2 Nitrosation of Cysteine

Cysteine is a naturally occurring amino acid of structure

$$HS\ CH_2\ CH\ (NH_2)\ CO_2H$$

Cysteine can exist in a number of forms, the dominant form depending on the pH of the solution.

At strongly alkaline pH (pH 11) it should be possible to get the form of cysteine

$$^{-}S\ CH_2\ CHNH_2\ CO_2^{-}$$

As the pH of the solution is lowered, the species

$$HS\ CH_2\ CH\ NH_2\ CO_2^{-}$$

will become dominant, and at pHs around neutrality the zwitterion

$$HS\ CH_2\ CH\ \overset{+}{N}H_3\ CO_2^-$$

will be the dominant form in solution. The pKa value for the carboxyl group of cysteine has been determined as 1.92,[45] and therefore in acid solution (about 0.1M acid) the N-protonated form of cysteine

$$HS\ CH_2\ CH\ \overset{+}{N}H_3\ CO_2H$$

will be dominant.

The acid catalyzed nitrosation of cysteine has been previously investigated[18,31], and the established rate equation for acid catalyzed nitrosation was found to apply.

$$\text{rate} = k_3[HNO_2][H^+][\text{cysteine}]$$

Dix and Williams[18] studied the nucleophile catalyzed nitrosation of cysteine, and found that the results could be interpreted by the addition of an extra kinetic term

$$\text{rate} = k_2\ K_{NOX}\ [HNO_2][H^+][\text{cysteine}][x^-]$$

which is the familiar rate equation for nitrosation by the NOX species.

Recently Casado et al[46] have studied the nitrosation of cysteine and determined that the two forms of the thiol present under their experimental conditions (namely the zwitterion (iv) and the N-protonated form (v)) are reactive towards the nitrous acidium ion or nitrosonium ion.

$$HS\ CH_2\ CH\ (\overset{+}{N}H_3)\ CO_2^- \xrightarrow{HNO_2/H^+} ON\ SCH_2\ CH\ (\overset{+}{N}H_3)\ CO_2^-$$

(iv)

$\uparrow\downarrow$ Ka

$$HS\ CH_2\ CH\ (\overset{+}{N}H_3)\ CO_2H \xrightarrow{HNO_2/H^+} ON\ S\ CH_2\ CH\ (\overset{+}{N}H_3)\ CO_2H$$

(v)

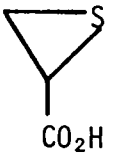
Casado et al determined the rate constants for nitrosation of both forms assuming that the nitrosonium ion is the effective nitrosating agent.

$$NO^+ + (iv) \xrightarrow{k_1} NOCys$$

$$NO^+ + (v) \xrightarrow{k_2} NOCys$$

and found that the zwitterion (iv) is more reactive than the N-protonated form (v) ($k_1 = 6.4 \times 10^9$ l mol^{-1} s^{-1} and $k_2 = 1.7 \times 10^9$ l mol^{-1} s^{-1}). Casado et al proposed that this is due to the fact that (iv) is a formally neutral molecule whereas (v) is a positively charged species.

In these reactions the product is assigned as the S-nitroso derivative, which has been isolated as a red solid,[47] and not the product resulting from N-nitrosation. However, with an excess of sodium nitrite over the cysteine concentration, the product (vi) has been isolated,[48] presumably the result of N-nitrosation, deamination and then ring closure.



(vi)

The mechanism of this reaction was not investigated, and it could be that S-nitrosation is happening initially, followed by an S- to N- rearrangement of the nitroso group similar to that proposed by Meyer and Williams.[37]

The nitrosation of cysteine and catalysis of this reaction by chloride, bromide and thiocyanate ions were undertaken. The effect of alanine and glucose on both the rate constant and yield of this reaction was investigated. Studies using

S-nitrosocysteine as a nitrosating agent were conducted and the effect of alanine and glucose, both nitrosatable species, on this reaction was investigated (see Chapter Four) and so these experiments were done to ensure that these compounds were not interfering with the nitrosation of cysteine itself. This section describes the results of these experiments.

2.2.1 Acid Catalyzed Nitrosation

The effect of the acid and cysteine concentrations on the rate constant of the reaction was studied by variation of these concentrations separately.

As mentioned already, cysteine exists in both the zwitterionic and N-protonated forms, therefore the substrate dependence experiments were carried out using an excess of acid over the cysteine concentration so that the loss of acid due to protonation of the carboxyl group of the zwitterion to form the N-protonated species is negligible. For the lower acid concentrations used for studying the dependence of the rate constant on the acid concentration, the acid and cysteine concentrations are comparable. Therefore the acid concentrations were corrected for protonation of the zwitterion. The zwitterionic and N-protonated forms are related by the pKa of the carboxyl group, which has been determined as 1.92.[45]

$$\underset{\text{(v)}}{HS\ CH_2\ CH\ (\overset{+}{N}H_3)\ CO_2H} \underset{}{\overset{Ka}{\rightleftharpoons}} \underset{\text{(iv)}}{HS\ CH_2\ CH\ (\overset{\pm}{N}H_3)\ CO_2^-} + H^+$$

The concentration of (v) can then be calculated by solving the quadratic equation:

$$[v]^2 + [v]\ (Ka + [\text{cysteine}]_T \text{ and } [H^+]_T) - [\text{cysteine}]_T[H^+]_T = 0$$

where $[\text{cysteine}]_T$ and $[H^+]_T$ refer to the added concentration of cysteine and acid respectively. $[H^+]c$, the corrected acid concentration, can then be calculated from

$$[H^+]c = [H^+]_T - [v]$$

Table 2.3: Dependence of k_0 on [cysteine]

$[HClO_4] = 0.19$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[\text{cysteine}]$/M	k_0/s^{-1}
0.5	0.357 ± 0.007
1	0.69 ± 0.006
2	1.27 ± 0.046

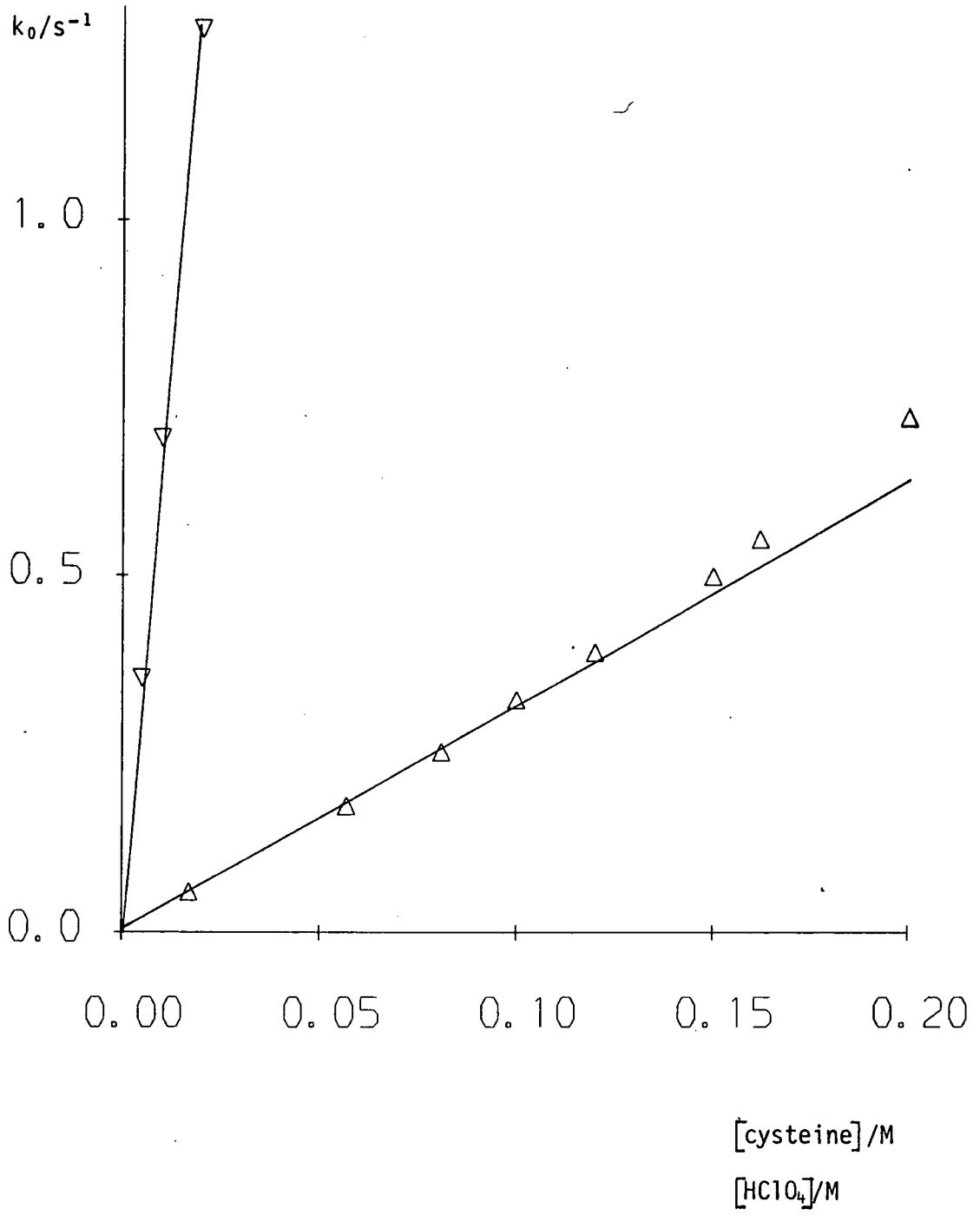
Table 2.4: Dependence of k_0 on acid concentration

$[\text{Cysteine}] = 1 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[H^+]c$/M	k_0/s^{-1}
1.29	0.057 ± 0.001
5.02	0.178 ± 0.003
7.36	0.253 ± 0.002
9.23	0.326 ± 0.002
11.3	0.394 ± 0.005
14.2	0.499 ± 0.004
15.3	0.552 ± 0.004
19.1	0.721 ± 0.005

Figure 2.1 : Dependence of k_0 on [cysteine] and [acid]



▽ = [cysteine] dependence

△ = $[HClO_4]$ dependence

As can be seen from Tables 2.3 and 2.4 and Figure 2.1, the expected first order dependence on cysteine and acid is observed, consistent with the rate equation

$$\text{rate} = k_3\,[HNO_2]\,[H^+]\,[\text{cysteine}]$$

k_0 for all the acid catalyzed nitrosation reactions is the observed first order rate constant defined by

$$\text{rate} = k_0\,[HNO_2] \qquad (3)$$

where

$$k_0 = k_3\,[H^+]\,[\text{cysteine}] \qquad (4)$$

From equation (4) it can be seen that k_3 can then be calculated from the slope of the lines in Figure 2.1 and was determined as 343 $l^2mol^{-2}s^{-1}$ from the cysteine dependence results, and 340 $l^2mol^{-2}s^{-1}$ from the acid dependence results.

The dependence of k_0 on the acidity shows a very slight positive intercept which can be attributed to reaction by both the zwitterion and N-protonated forms of cysteine, as shown by Casado et al.[46] The reason for this positive intercept can be seen by re-examining the mechanism and accounting for reaction by both forms of cysteine.

$$\underset{\text{(v)}}{HS\,CH_2\,CH\,(\overset{+}{N}H_3)\,CO_2H} \overset{Ka}{\rightleftharpoons} \underset{\text{(iv)}}{HS\,CH_2\,CH\,(\overset{+}{N}H_3)\,CO_2^-} + H^+$$

$$HNO_2 + H^+ \overset{K_N}{\rightleftharpoons} H_2NO_2^+$$

$$\text{(v)} + H_2NO_2^+ \xrightarrow{k_1} \text{NOCys}$$

$$\text{(iv)} + H_2NO_2^+ \xrightarrow{k_2} \text{NOCys}$$

defining

$$k_1' = k_1 K_N$$

$$k_2' = k_2 K_N$$

then the full rate equation becomes:

$$\text{rate} = k_1'[HNO_2][H^+][v] + k_2'[HNO_2][H^+][iv] \qquad (5)$$

$$K_A = \frac{[iv][H^+]}{[v]}$$

therefore $$[iv] = \frac{K_A [v]}{[H^+]}$$

substituting into equation (5)

$$\text{rate} = k_1'[HNO_2][H^+][v] + \frac{k_2' \; Ka \; [HNO_2][H^+][v]}{[H^+]}$$

$$[\text{cysteine}]_T = [iv] + [v]$$

$$[\text{cysteine}]_T = [v] + \frac{K_A[v]}{[H^+]}$$

$$[\text{cysteine}]_T = [v]\left(1 + \frac{K_A}{[H^+]}\right)$$

$$\therefore [v] = \frac{[\text{cysteine}]_T \; [H^+]}{K_A + [H^+]}$$

$$\therefore \text{rate} = \frac{k_1'[HNO_2][H^+]^2[\text{cysteine}]_T}{K_A + [H^+]} + \frac{k_2' \; K_A[HNO_2][H^+]^2[\text{cysteine}]_T}{(K_A + [H^+])\,[H^+]}$$

$$[H^+] >> K_A$$

$$\text{rate} = \frac{k_1' [HNO_2][H^+]^2[\text{cysteine}]_T}{[H^+]} + \frac{k_2' K_A[HNO_2][H^+]^2[\text{cysteine}]_T}{[H^+]^2}$$

$$\therefore \text{rate} = k_1' [HNO_2][H^+][\text{cysteine}]_T + k_2' K_A [HNO_2][\text{cysteine}]_T$$

$$\text{but rate} = k_0 [HNO_2]$$

where $k_0 = k_1' [H^+][\text{cysteine}]_T + k_2' K_A [\text{cysteine}]_T$

Therefore a graph of k_0 against $[H^+]$ should give a linear plot with

$$\text{gradient} = k_1' [\text{cysteine}]_T$$

and a positive intercept given by $k_2' Ka [\text{cysteine}]_T$

2.2.2 Nucleophile Catalysis

The effect of added nucleophiles on the rate of nitrosation of cysteine was studied and the results are presented in Tables 2.3 to 2.5

Table 2.5: Chloride ion catalysis

$[\text{cysteine}] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.194$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Cl^-]$/M	k_0/s^{-1}
0.096	0.899 ± 0.015
0.24	1.24 ± 0.028
0.48	1.93 ± 0.057

Table 2.6: Bromide ion catalysis

$[cysteine] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.194$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Br^-]$/M	k_0/s^{-1}
0.1	1.15 ± 0.052
0.25	1.87 ± 0.076
0.5	3.43 ± 0.203

Table 2.7: Thiocyanate ion catalysis

$[cysteine] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.194$ M

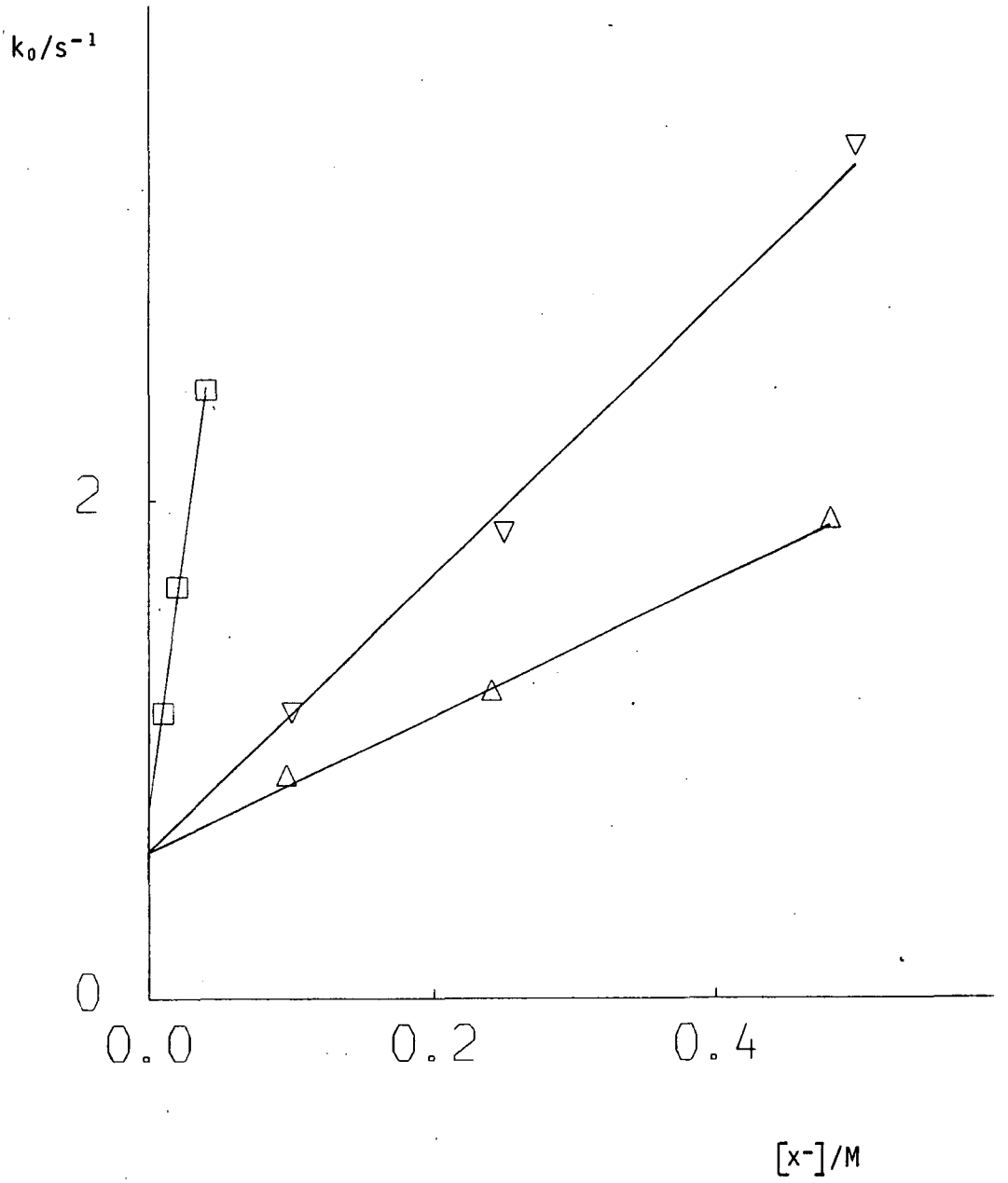
$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[SCN^-]$/M	k_0/s^{-1}
1	1.15 ± 0.037
2	1.65 ± 0.042
4	2.45 ± 0.098

As can be seen from Figure 2.2 nucleophile catalysis occurs and the reaction is first order with respect to the nucleophile.

Catalysis by these species is due to the formation of the NOX species in a rapid pre-equilibrium.

Figure 2.2 : Dependence of k_0 on nucleophile concentration



$$HNO_2 + H^+ + X^- \overset{K_{NOX}}{\rightleftharpoons} NOX + H_2O$$

$$NOX + RSH \xrightarrow{k_2} \overset{+}{R}SHNO + X^-$$

$$\overset{+}{R}SHNO \longrightarrow RSNO + H^+$$

scheme 2.10

The full rate equation for the S-nitrosation is

$$\text{rate} = k_3[H^+][HNO_2][RSH] + k_2 K_{NOX} [HNO_2][H^+][RSH][X^-]$$

In the above tables k_0 is the first order rate constant given by

$$\text{rate} = k_0 [HNO_2]$$

where $k_0 = k_3 [RSH][H^+] + k_2 K_{NOX} [RSH][H^+][X^-]$ (6)

This definition of k_0 for nucleophile catalyzed nitrosation is used throughout the Chapter.

The intercept value in Figure 2.2 therefore corresponds to the non-nucleophile catalyzed rate of reaction. k_2, the actual rate constant for nitrosation of the thiol by the NOX species, can then be determined from the graph of k_0 against nucleophile concentration since the K_{NOX} values are all known. These k_2 values are shown in Table 2.8.

Table 2.8: k_2 values

Nucleophile	k_2/l mol^{-1} s^{-1}
Cl^-	1.22×10^6
Br^-	5.82×10^4
SCN^-	690

As can be seen from these results, the established order of catalysis by the NOX species NOCl > NOBr > NOSCN is observed here.

Finally the cysteine concentration was varied in the presence of chloride, bromide and thiocyanate ions and the results are shown in the following Tables and Figure 2.3.

Table 2.9: [Cysteine] variation in the presence of the chloride ion.

$[Cl^-]$ = 0.5 M

$[HClO_4]$ = 0.21 M

$[NaNO_2]$ = 1 x 10^{-4} M

10^3[cysteine]/M	k_0/s^{-1}
2.5	0.505 ± 0.012
5	1.07 ± 0.038
10	2.21 ± 0.1

Table 2.10: [Cysteine] variation in the presence of the bromide ion

$[Br^-]$ = 0.5 M

$[HClO_4]$ = 0.21 M

$[NaNO_2]$ = 1 x 10^{-4} M

10^3[cysteine]/M	k_0/s^{-1}
2.5	0.973 ± 0.033
5	1.89 ± 0.032
10	3.78 ± 0.16

Table 2.11: [Cysteine] variation in the presence of the thiocyanate ion.

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

$[HClO_4] = 0.19$ M

$[NaNO_2] = 1 \times 10^{-4}$M

10^2[cysteine]/M	k_0/s^{-1}
0.5	1.26 ± 0.043
1	2.41 ± 0.11
2	4.48 ± 0.13

Figure 2.3 shows that in the presence of these nucleophiles the catalyzed reaction is first order with respect to the thiol so that rate limiting NOX formation does not occur here. From equation (6) it can be seen that k_2 can be calculated from these results, since the gradient of these lines is given by

$$\text{slope} = k_3 [H^+] + k_2 K_{NOX} [H^+][X^-]$$

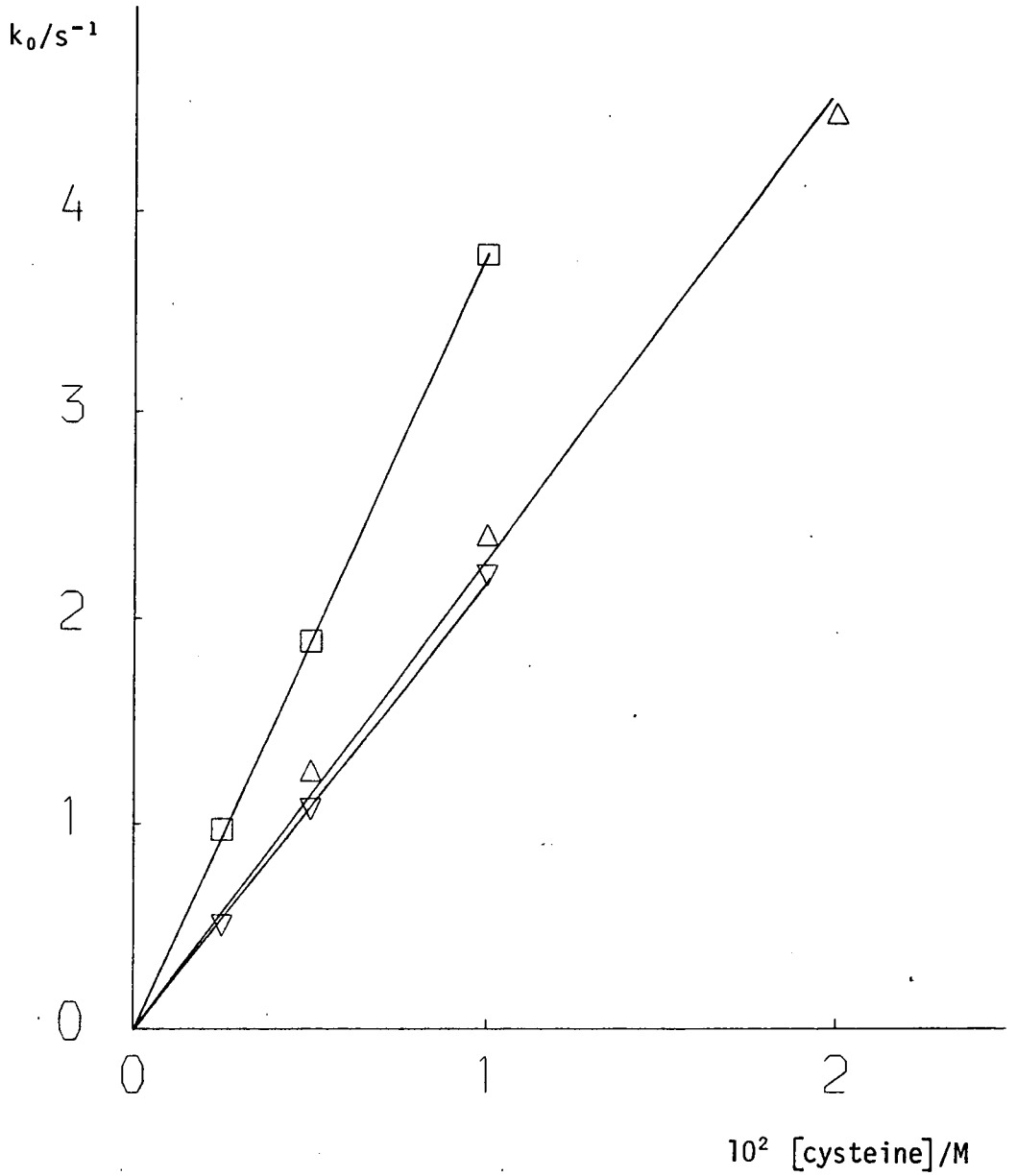
using the value of 340 l^2 mol^{-2} s^{-1} for k_3 , these k_2 values were determined and are given in Table 2.12.

Table 2.12: k_2 values

Nucleophile	k_2/l mol^{-1} s^{-1}
Cl^-	1.22×10^6
Br^-	5.8×10^4
SCN^-	649

If these k_2 values are compared with those in Table 2.8, it can be seen that there is good agreement between these two

Figure 2.3 : Dependence of k_0 on [cysteine] in the presence of nucleophiles



□ in the presence of bromide

▽ in the presence of chloride

△ in the presence of thiocyanate

methods of evaluating k_2.

2.2.3 Nitrosation of Cysteine in the Presence of Alanine and Glucose

In view of the studies described in Chapter Four, the effect of alanine and glucose on the rate constant for the nitrosation of cysteine and the yield of the thionitrite, that is competitive nitrosation, was studied. Similar studies have been carried out by observing N-nitrosation in the presence of alcohols[49,50] thiols[50,51,52] and p-cresol[52] at acid pH. These species caused a reduction in the rate constant and yield of N-nitrosamine due to effective competition for the available nitrosating agent. Williams and Aldred[50] have shown that at high enough thiol concentration, N-nitrosation could be completely inhibited.

Table 2.13 shows the effect of glucose and alanine on k_0, the first order rate constant for the nitrosation of cysteine as define by equation (3).

Table 2.13: Effect of glucose and alanine on k_0

$[\text{cysteine}] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.19$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

10^2[glucose]/M	10^2[alanine]/M	k_0/s^{-1}
0	0	0.655 ± 0.009
1	0	0.654 ± 0.005
2	0	0.658 ± 0.007
4	0	0.636 ± 0.013
0	1	0.606 ± 0.011
0	2	0.59 ± 0.005
0	4	0.531 ± 0.01

As can be seen from these results, glucose had no effect on the rate of nitrosation of cysteine whereas alanine causes an apparent decrease in the rate of nitrosation of cysteine. However, alanine can exist in either the zwitterionic or N-protonated form.

$$CH_3\ CH\ (\overset{+}{N}H_3)\ CO_2H \underset{}{\overset{Ka}{\rightleftharpoons}} CH_3\ CH\ (\overset{+}{N}H_3)\ CO_2^- + H^+$$

(vii) (viii)

The pKa value for the carboxyl group of alanine is 2.35. Therefore the concentration of alanine in the N-protonated form(vii) can be calculated by solving the quadratic equation.

$$[vii]^2 + [vii]\ (Ka + [ala]_T + [H^+]_T) - [ala]_T[H^+]_T = 0$$

where $[ala]_T$ and $[H^+]_T$ are the total concentrations of alanine and acid respectively.

The actual acid concentration, $[H^+]_C$, can then be calculated from

$$[H^+]_C = [H^+]_T - [vii]$$

Calculating the individual values of k_3 at each alanine concentration gives the values shown in Table 2.14.

Table 2.14: k_3 values

$10^2[alanine]/M$	$k_3/l^2mol^{-2}s^{-1}$
0	344
1	336
2	345
4	352

By comparing these values, it can be seen that the apparent affect of alanine can be interpreted purely in terms of reduction of the acid concentration.

The results of the product yield investigations are presented in Table 2.15.

Table 2.15: Product yields

$[cysteine] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.19$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[alanine]/M$	$10^2[glucose]/M$	Abs 330 nm
0	0	0.303
1	0	0.315
2	0	0.289
4	0	0.309
0	1	0.31
0	2	0.313
0	4	0.313

It can be seen from all these results that the addition of alanine and glucose had no effect on either the rate constant for the nitrosation of cysteine or the yield of the thionitrite. The rate constant for the nitrosation of glucose has been determined as 124 $l^2mol^{-2}s^{-1}$ at 0^0C[19] and for alanine as 0.0013 l $mol^{-1}s^{-1}$ at 25^0C for the bromide ion catalyzed reaction[37] (the non catalyzed nitrosation of alanine being inconveniently slow to measure). The difference in rate constants for the nitrosation of cysteine and alanine means

that the lack of effect of alanine is quite expected. The rate constant determined for the nitrosation of glucose means that it should compete with cysteine for the available nitrite. However k_0 is the first order rate constant defined by

$$\text{rate} = k_0 [HNO_2]$$

Therefore the presence of glucose should have no effect on k_0, which is shown by the results in Table 2.13. In considering the effect of glucose on the yield of S-nitroso-cysteine, it has to be borne in mind that the nitrosation of glucose is a reversible reaction with an equilibrium constant of 1.5[19], whereas the nitrosation of cysteine under these conditions is essentially irreversible.

$$ROH + HNO_2 \underset{\longleftarrow}{\overset{H^+}{\longrightarrow}} RONO + H_2O$$

$$RSH + HNO_2 \xrightarrow{H^+} RSNO + H_2O$$

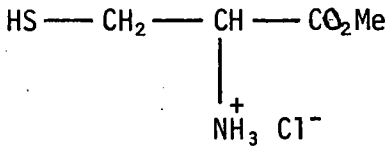
scheme 2.11

Therefore even though an equilibrium concentration of the alkyl nitrite may well be formed, the thiol reacting irreversibly with the nitrous acid will have the effect of moving the equilibrium for the formation of the alkyl nitrite to the left, and hence increase the concentration of S-nitrosocysteine, which may account for the same absorbance value at 330 nm being observed in the presence of glucose.

2.3 Nitrosation of the Methyl Ester of Cysteine Hydrochloride

This section and the following one show the effect of substitution at the carboxyl group and amino group of cysteine on the rate constant for the nitrosation of the substrate.

Firstly the nitrosation of the methyl ester of cysteine hydrochloride (MeCys) was studied.



The initial product was considered to be the S-nitroso derivative. However a final product of N-nitrosation (ix) has been isolated[48] when the reaction was carried out with an excess of sodium nitrite over the methyl ester of cysteine concentration at 0^0C.

(ix)

However, as for cysteine, it is likely that S-nitrosation occurs first, followed by an S- to N- rearrangement of the nitroso group resulting in the formation of (ix) after ring closure, similar to the work of Meyer and Williams.[37]

2.3.1 Acid Catalyzed Nitrosation

Since the hydrochloride salt of the methyl ester of cysteine was used only the dependence of the rate constant on the acid concentration was studied. Since the methyl ester of cysteine cannot exist in the zwitterionic form, there is no complication due to some of the acid being involved in protonation of the zwitterion. The result of this investigation is shown in Table 2.16.

Table 2.16: Dependence of k_0 on acid concentration

$[MeCys] = 1 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[H^+]$/M	k_0/s^{-1}
4.95	0.087 ± 0.001
8.1	0.163 ± 0.005
9.9	0.215 ± 0.009
19.8	0.47 ± 0.02

These results, also presented in Figure 2.4, show that the expected first order dependence on the acid is observed, consistent with the acid catalyzed rate equation.

$$\text{rate} = k_3 [HNO_2][H^+][MeCys]$$

The same definition of k_0 is used here as for the acid catalyzed nitrosation of cysteine. That is

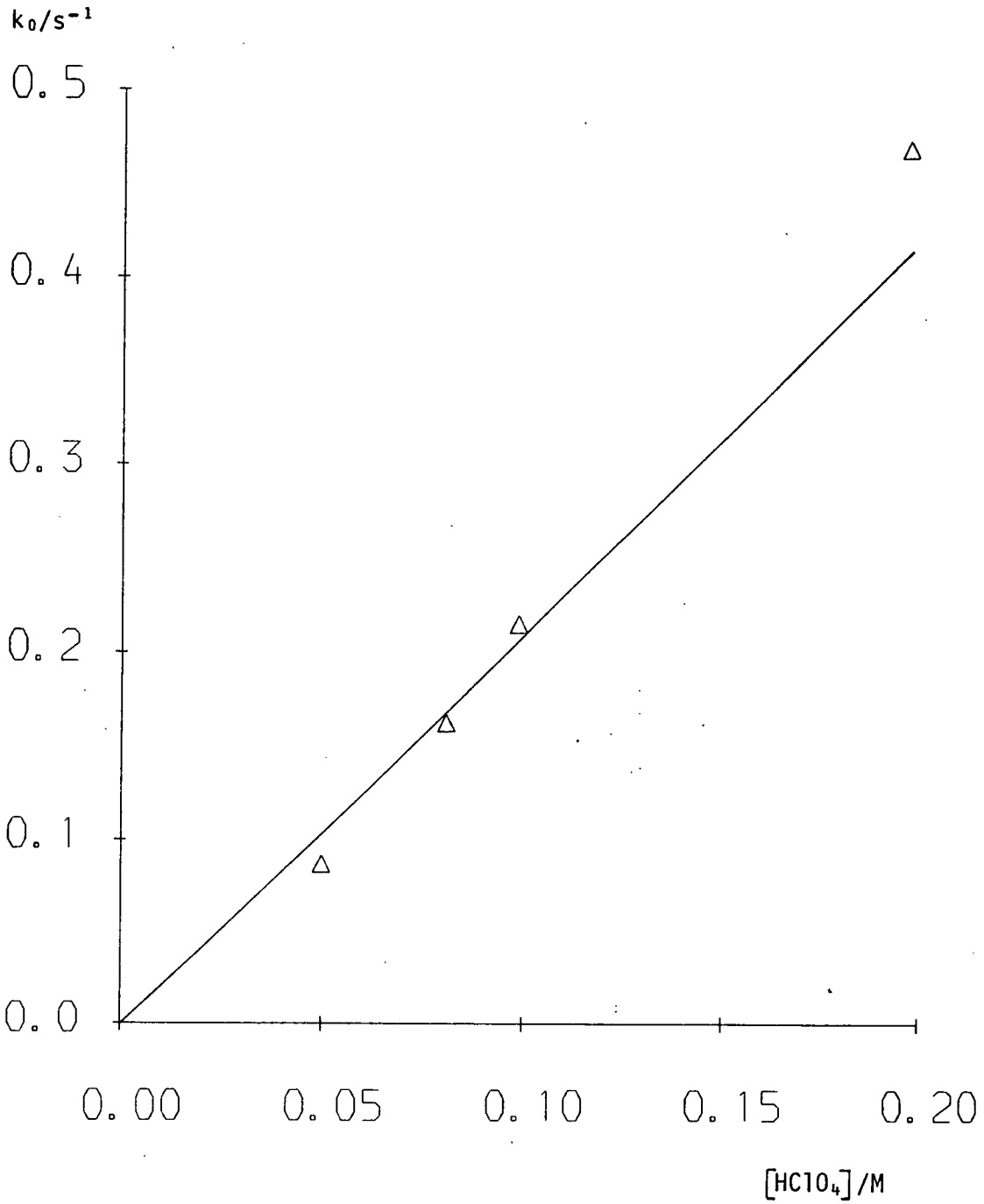
$$k_0 = k_3 [H^+][MeCys]$$

k_3 can then be evaluated from the gradient of the line in Figure 2.4, and was calculated as 213 $l^2mol^{-2}s^{-1}$. The graph of k_0 against acid concentration shows no intercept value, consistent with the fact that the methyl ester of cysteine cannot exist in the zwitterionic form, unlike cysteine where a slight positive intercept was observed.

2.3.2 Nucleophile Catalysis

Catalysis of the nitrosation of the methyl ester of cysteine hydrochloride by chloride, bromide and thiocyanate ions was studied by variation of the nucleophile concentration. The

Figure 2.4 : Dependence of k_0 on acid concentration



results are shown in Tables 2.17 to 2.19

Table 2.17: Chloride ion catalysis

$[MeCys] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.198$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Cl^-]$/M	k_0/s^{-1}
0.1	0.738 ± 0.005
0.25	1.05 ± 0.017
0.5	1.66 ± 0.039

Table 2.18: Bromide ion catalysis

$[MeCys] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.198$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Br^-]$/M	k_0/s^{-1}
0.1	0.945 ± 0.009
0.25	1.57 ± 0.008
0.5	2.9 ± 0.018

Table 2.19: Thiocyanate ion catalysis

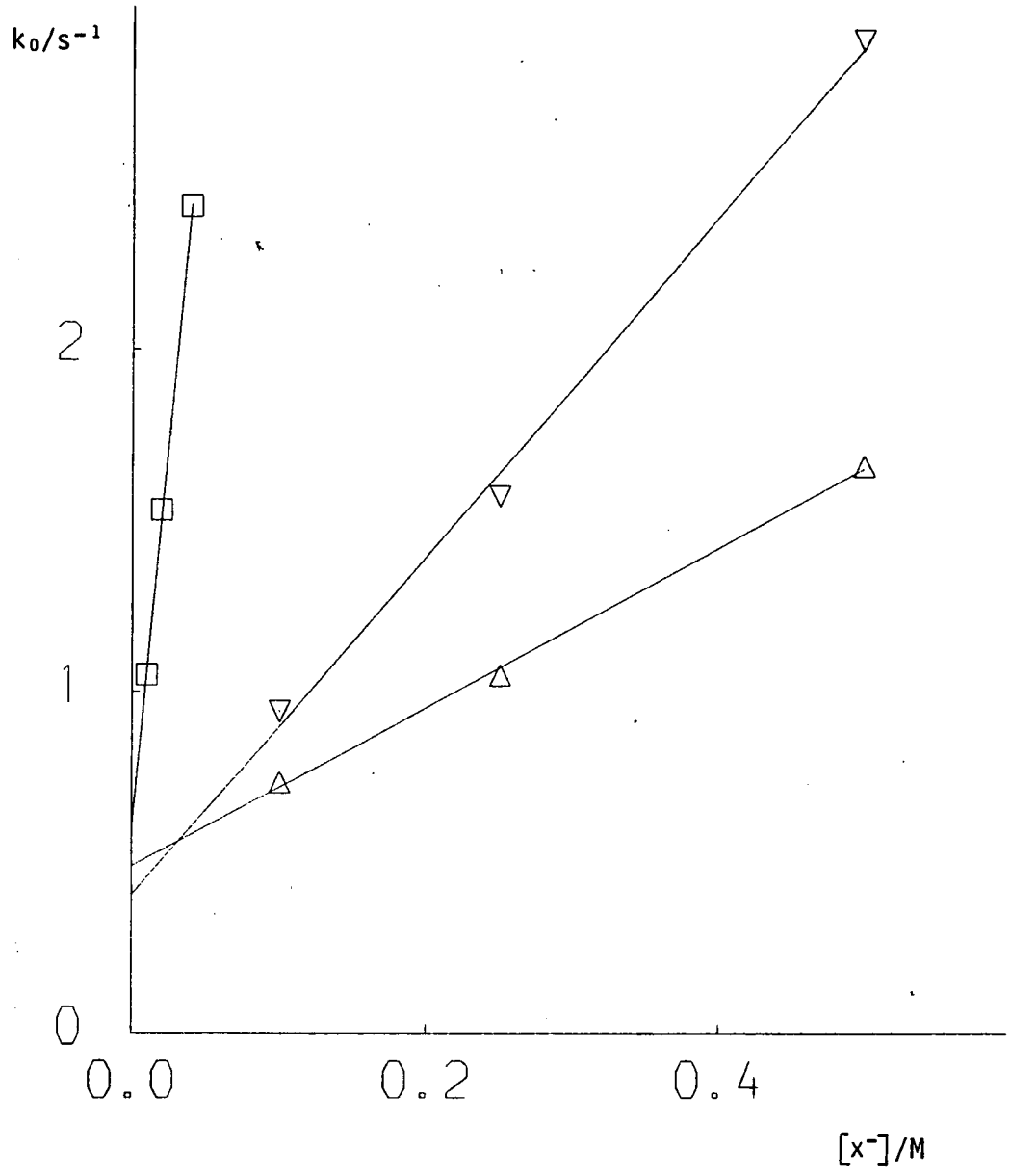
$[MeCys] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.198$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[SCN^-]$/M	k_0/s^{-1}
1	1.05 ± 0.03
2	1.53 ± 0.011
4	2.41 ± 0.022

Figure 2.5 : Dependence of k_0 on nucleophile concentration



□ x^- = thiocyanate

▽ x^- = bromide

△ x^- = chloride

As can be seen from Figure 2.5, the expected first order dependence on the added nucleophile is observed. By comparing the results in Tables 2.17 to 2.19, the order of catalytic activity of the ions can be seen to be $SCN^- > Br^- > Cl^-$. By the same reasoning used for the nitrosation of cysteine, the intercept value corresponds to the non nucleophile catalyzed rate of nitrosation. k_2 can be calculated from the gradient of the lines in Figure 2.5. These k_2 values are shown in Table 2.20.

Table 2.20: k_2 values

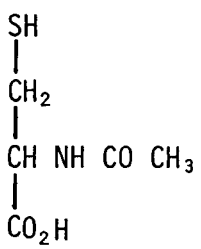
Nucleophile	k_2/l mol^{-1}s^{-1}
Cl^-	1.03×10^6
Br^-	4.88×10^4
SCN^-	712

These results show the now familiar order of catalysis NOCl > NOBr > NOSCN is observed here.

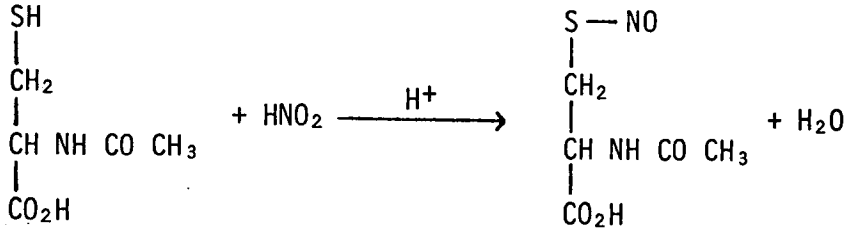
Since the hydrochloride salt of the methyl ester of cysteine was used, substrate variation studies in the presence of these nucleophiles was not investigated.

2.4 Nitrosation of N-Acetylcysteine

The effect of N-substitution on the rate of nitrosation of cysteine was investigated by studying the nitrosation of N-acetylcysteine.



Bonnett et al[8] observed a red coloured solution when N-acetylcysteine was reacted with acidified nitrite solutions. ^{15}N N.M.R. studies on the red solution yielded spectra consistent with the thionitrite derivative.



2.4.1 Acid Catalyzed Nitrosation

The acid catalyzed nitrosation of N-acetylcysteine (N-Ac Cys) was studied by investigating the dependence of the rate of nitrosation on the thiol and acid concentrations. Since the N-acetyl group will not undergo protonation under these conditions, there is no complication due to N-acetylcysteine existing in the two forms which cysteine itself can adopt. The results of these studies are shown in Table 2.21 and 2.22.

Table 2.21: Dependence of k_0 on [N-Ac Cys]

$[HClO_4] = 0.195$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

10^2[N-Ac Cys]/M	k_0/s^{-1}
0.5	1.67 ± 0.017
1	3.32 ± 0.047
2	6.38 ± 0.08

Table 2.22: Dependence of k_0 on acid concentration

$[N\text{-Ac Cys}] = 1 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[H^+]/M$	k_0/s^{-1}
4.86	0.68 ± 0.007
7.29	1.07 ± 0.015
9.73	1.44 ± 0.006

Figure 2.6 shows that there is no intercept on the acid dependence graph, as expected, since N-acetylcysteine cannot exist in two forms, the same as is observed for the methyl ester of cysteine hydrochloride.

These results show that a first order dependence on both N-acetylcysteine and acid is observed, in accordance with the rate equation for acid catalyzed nitrosation.

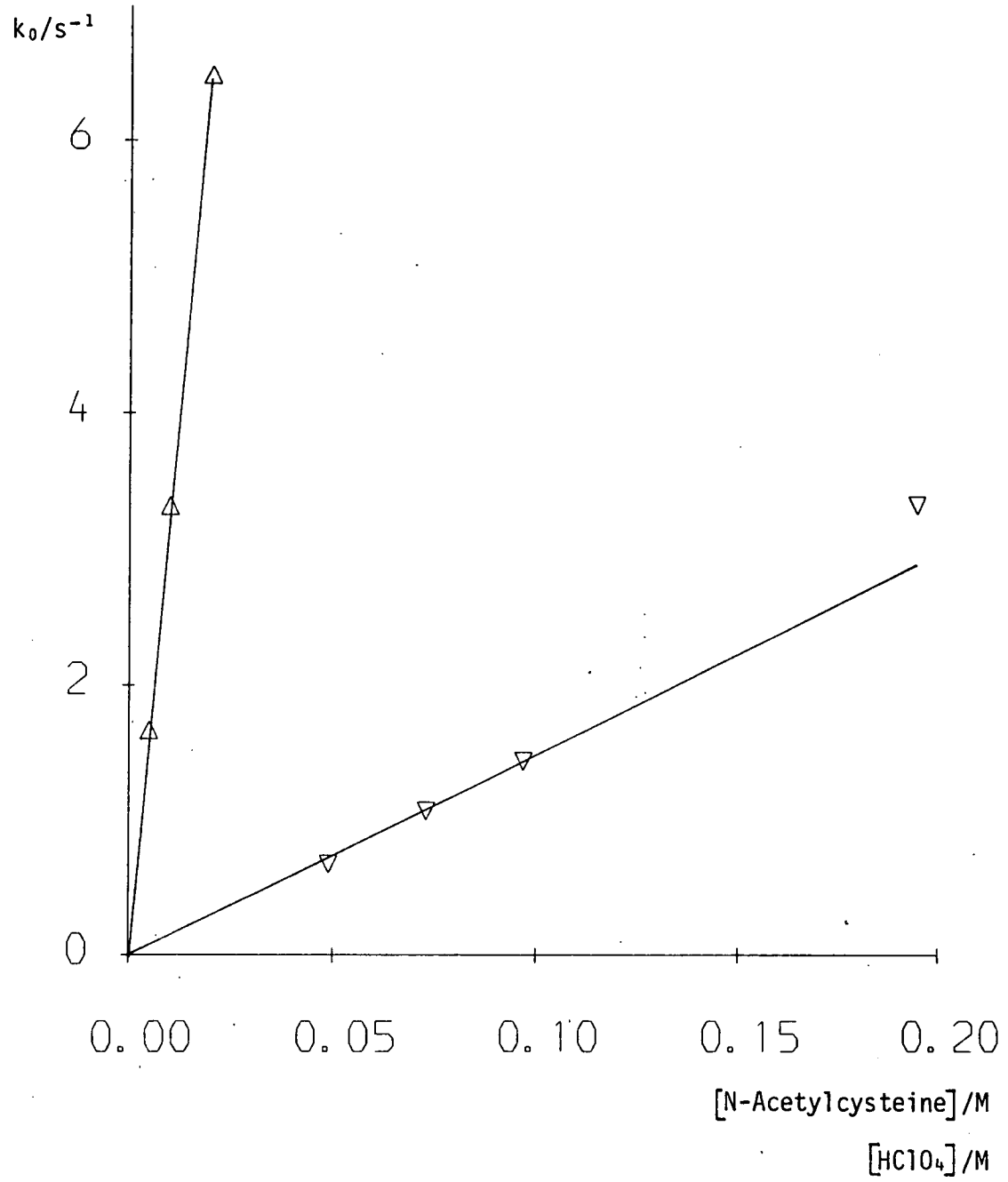
$$\text{rate} = k_3 [HNO_2][H^+][N\text{-Ac Cys}]$$

Calculation of k_3 from these results leads to the value of 1.71×10^3 l^2 mol^{-2} s^{-1} from the dependence of the rate constant on N-acetylcysteine concentration and 1.46×10^3 l^2 mol^{-2} s^{-1} from the dependence of the rate constant on the acid concentration. This represents an acceptable agreement between both procedures.

2.4.2 Nucleophile Catalysis

The catalysis of this reaction by chloride, bromide and thiocyanate ions was investigated by carrying out the nitrosation of N-acetylcysteine in the presence of different concentrations of nucleophile. Tables 2.23 to 2.25 present

Figure 2.6 : Dependence of k_0 on [N-acetylcysteine] and [acid]



△ = [N-acetylcysteine] dependence

▽ = $[HClO_4]$ dependence

the results of these experiments.

Table 2.23: Chloride ion catalysis

$[N\text{-Ac Cys}] = 1 \times 10^{-2}M$

$[HClO_4] = 0.19\ M$

$[NaNO_2] = 1 \times 10^{-4}\ M$

$[Cl^-]/M$	k_o/s^{-1}
0.1	5.05 ± 0.01
0.25	7.88 ± 0.067
0.5	13.8 ± 0.19

Table 2.24: Bromide ion catalysis

$[N\text{-Ac Cys}] = 1 \times 10^{-2}\ M$

$[HClO_4] = 0.19\ M$

$[NaNO_2] = 1 \times 10^{-4}\ M$

$[Br^-]/M$	k_o/s^{-1}
0.1	7.64 ± 0.081
0.25	13.9 ± 0.28
0.5	25.1 ± 1.27

Table 2.25: Thiocyanate ion catalysis

$[N\text{-Ac Cys}] = 1 \times 10^{-2}\ M$

$[HClO_4] = 0.19\ M$

$[NaNO_2] = 1 \times 10^{-4}\ M$

$10^2[SCN^-]/M$	k_o/s^{-1}
1	4.25 ± 0.15
2	5.24 ± 0.056
4	7.27 ± 0.13

Figure 2.7 shows that the expected first order dependence of the rate constant for the nitrosation of N-acetylcysteine on the nucleophile is observed here. The intercept value corresponds to the non nucleophile catalyzed rate of reaction. Again the k_2 values can be determined by using the rate equation for nucleophile catalyzed nitrosation.

$$\text{rate} = k_3[HNO_2][H^+][\text{N-Ac Cys}] + k_2 K_{NOX}[HNO_2][H^+][\text{N-AcCys}][X^-]$$

The results of these calculations are in Table 2.26.

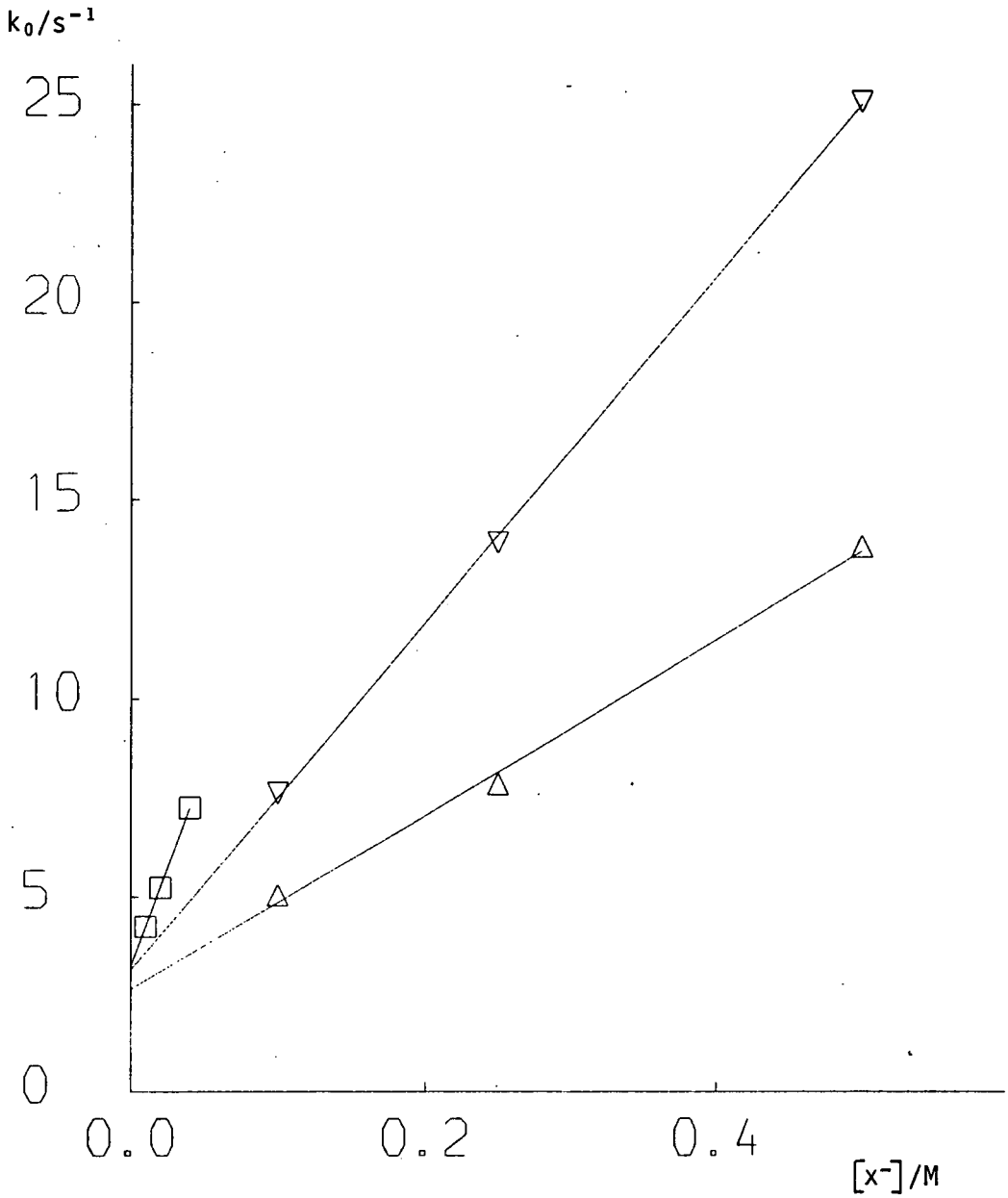
Table 2.26: k_2 values

Nucleophile	k_2/l mol^{-1} s^{-1}
Cl^-	1.01×10^7
Br^-	4.5×10^5
SCN^-	1.6×10^3

These show clearly the same order of catalysis
NOCl > NOBr > NOSCN

Lastly the N-acetylcysteine concentration was varied in the presence of these nucleophiles. The results are presented in Tables 2.27 to 2.29 and graphically in Figure 2.8.

Figure 2.7 : Dependence of k_0 on nucleophile concentration



□ x^- = thiocyanate

▽ x^- = bromide

△ x^- = chloride

Table 2.27: [N-Ac Cys] variation in the presence of the chloride ion

$[Cl^-]$ = 0.5 M

$[HClO_4]$ = 0.19 M

$[NaNO_2]$ = 1 x 10^{-4} M

10^2[N-Ac Cys]/M	k_0/s^{-1}
0.5	7.05 ± 0.13
1	13.8 ± 0.16
2.5	34.4 ± 0.6
5	66.4 ± 4.5
7.5	86 ± 3.4
10	112 ± 5.4

Table 2.28: [N-Ac Cys] variation in the presence of the bromide ion

$[Br^-]$ = 0.5 M

$[HClO_4]$ = 0.21 M

$[NaNO_2]$ = 1 x 10^{-4} M

10^2[N-Ac Cys]/M	k_0/s^{-1}
0.5	15.6 ± 0.29
1	30.2 ± 0.71
2.5	70.4 ± 1.4
5	126 ± 6.7
7.5	174 ± 5.2
10	225 ± 6

Table 2.29: [N-Ac Cys] variation in the presence of the thiocyanate ion

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

$[HClO_4] = 0.21$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[\text{N-Ac Cys}]/M$	k_0/s^{-1}
0.5	3.99 ± 0.045
1	7.8 ± 0.05
2.5	18.6 ± 0.32
5	32.4 ± 0.78
7.5	44.9 ± 2.1
10	64.1 ± 2.4

Figure 2.8 shows that the graphs of k_0 against N-acetylcysteine concentration have a tendency to curve off at high substrate concentration instead of exhibiting first order dependence throughout the range of N-acetylcysteine concentrations used. This can be explained by reconsidering the mechanism of the nucleophile catalyzed reaction.

$$H^+ + HNO_2 + X^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NOX + H_2O$$

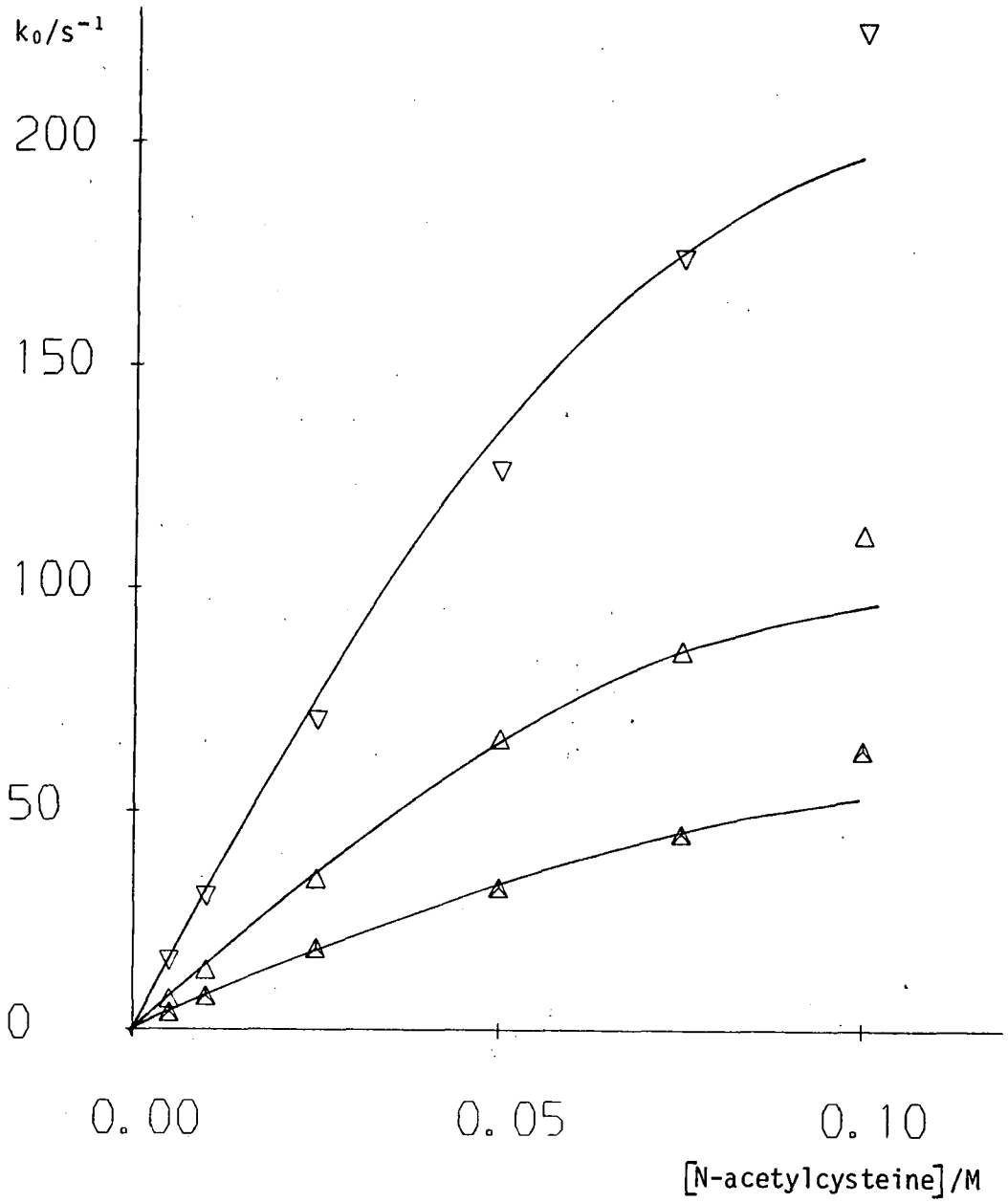
$$NOX + RSH \xrightarrow{k_2} RSNO + H^+ + X^-$$

$$\text{rate} = k_2 [NOX][RSH]$$

Using the steady state approximation on NOX gives

$$\text{rate} = \frac{k_1 k_2 [HNO_2][H^+][RSH][X^-]}{k_{-1} + k_2[RSH]}$$

Figure 2.8 : Dependence of k_0 on [N-acetylcysteine] in the presence of nucleophiles



△ in the presence of thiocyanate
▽ in the presence of bromide
△ in the presence of chloride

Under the conditions used

$$\text{rate} = k_0 \, [HNO_2]$$

where $$k_0 = \frac{k_1 \, k_2 \, [HNO_2] \, [H^+] \, [RSH] \, [X^-]}{k_{-1} + k_2 \, [RSH]}$$

When the concentration of the thiol is low, or the thiol is of low reactivity (that is small k_2) the inequality can be written

$$k_{-1} >> k_2 \, [RSH]$$

$\therefore$ k_0 can be written as

$$k_0 = \frac{k_1 \, k_2 \, [H^+] \, [RSH] \, [X^-]}{k_{-1}}$$

but $$\frac{k_1}{k_{-1}} = K_{NOX}$$

Therefore the full rate equation becomes

$$k_0 = k_3 \, [H^+] \, [RSH] + k_2 \, K_{NOX} [H^+] \, [X^-] \, [RSH]$$

which is the definition of k_0 previously given, which can be arrived at by considering the first step as a rapid pre-equilibrium.

At high thiol concentration, however, or if k_2 is very large, then it is possible that the other limit, that is

$$k_{-1} << k_2 [RSH]$$

could occur. This then leads to the rate equation

$$k_0 = k_1 \, [H^+] \, [X^-]$$

$\therefore$ $$\text{rate} = k_1 \, [HNO_2] \, [H^+] \, [X^-]$$

That is the rate of formation of NOX has become the rate limiting step.

Between these two extremes the rate equation is

$$\text{rate} = \frac{k_1\ k_2\ [HNO_2]\,[H^+]\,[X^-]\,[RSH]}{k_{-1} + k_2\ [RSH]}$$

and therefore the full rate equation is

$$k_0 = \frac{k_1\ k_2\ [H^+]\,[X^-]\,[RSH]}{k_{-1} + k_2\ [RSH]} + k_3[H^+]\,[RSH]$$

Inversion of this equation gives

$$\frac{1}{k_0 - k_3\ [H^+]\,[RSH]} = \frac{k_{-1}}{k_1\ k_2\ [H^+]\,[RSH]\,[X^-]} + \frac{1}{k_1\ [H^+]\,[X^-]}$$

Therefore a graph of $1/k_0 - k_3[H^+]\,[RSH]$ against $1/[RSH]$ should give a straight line graph with a gradient given by

$$\text{slope} = \frac{k_{-1}}{k_1\ k_2\ [H^+]\,[X^-]}$$

and an intercept given by

$$\text{intercept} = \frac{1}{k_1\ [H^+]\,[X^-]}$$

Tables 2.30 to 2.32 show the results of these calculations and Figure 2.9 shows that these double reciprocal plots are indeed linear. The values obtained for the gradients and intercepts can then be used to calculate k_{-1}, k_1 and k_2 values. These results are summarized in Table 2.33.

Figure 2.30 : Calculation of $\frac{1}{k_0 - k_3 [H^+][\text{N-AcCys}]}$ and $\frac{1}{[\text{N-AcCys}]}$ Reaction in the presence of the chloride ion.

$k_3 = 1.5 \times 10^3\ l^2\ mol^{-2}\ s^{-1}$ (from the acid dependence results)

$[\text{N-AcCys}]/M$	k_0/s^{-1}	$k_3[H^+][\text{N-AcCys}]/s^{-1}$	$k_0 - k_3[H^+][\text{N-AcCys}]/s^{-1}$	$\frac{1}{k_0 - k_3[H^+][\text{N-AcCys}]}/s$	$\frac{1}{[\text{N-AcCys}]}/M^{-1}$
5×10^{-3}	7.05	1.43	5.62	0.178	200
1×10^{-2}	13.7	2.86	10.8	0.0922	100
2.5×10^{-2}	34.4	7.14	27.3	0.0367	40
5×10^{-2}	66.4	14.3	52.1	0.0192	20
7.5×10^{-2}	86	21.4	64.6	0.0155	13.3
0.1	112	28.6	83.4	0.012	10

Figure 2.31 : Calculation of $\frac{1}{k_0 - k_3[H^+][\text{N-AcCys}]}$ and $\frac{1}{[\text{N-AcCys}]}$ Reaction in the presence of the bromide ion.

$k_3 = 1.5 \times 10^3\ l^2\ mol^{-2}\ s^{-1}$ (from the acid dependence results)

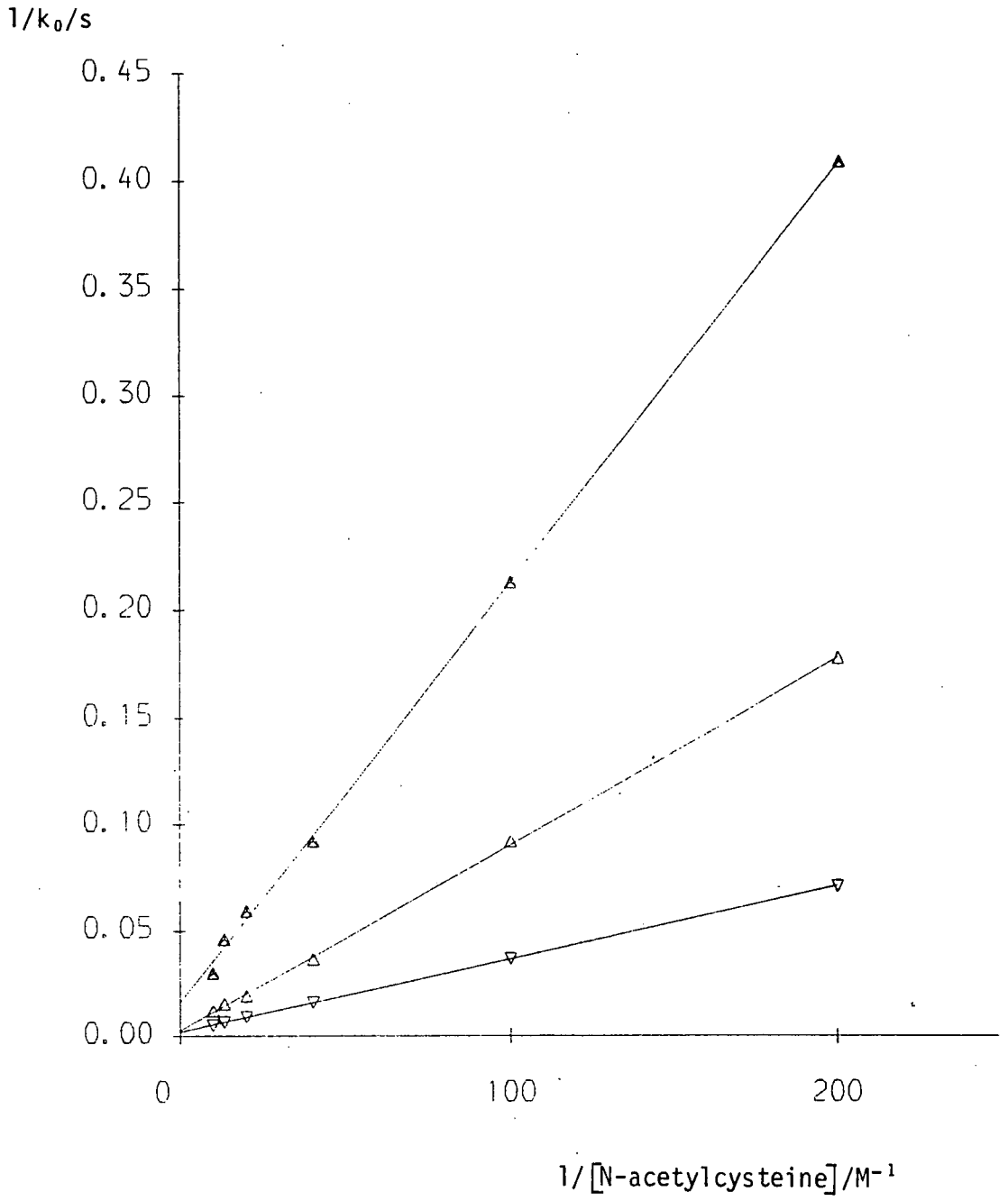
[N-AcCys]/M	k_0/s^{-1}	$k_3[H^+][\text{N-AcCys}]/s^{-1}$	$k_0 - k_3[H^+][\text{N-AcCys}]/s^{-1}$	$\frac{1}{k_0 - k_3[H^+][\text{N-AcCys}]}/s$	$\frac{1}{[\text{N-AcCys}]}/M^{-1}$
5×10^{-3}	15.6	1.55	14.1	0.0712	200
1×10^{-2}	30.2	3.11	27.1	0.0369	100
2.5×10^{-2}	70.4	7.76	62.6	0.016	40
5×10^{-2}	126	15.5	111	9.05×10^{-3}	20
7.5×10^{-2}	174	23.3	151	6.64×10^{-3}	13.3
0.1	225	31.1	194	5.16×10^{-3}	10

Figure 2.32 : Calculation of $\frac{1}{k_0 - k_3 [H^+] [N\text{-}AcCys]}$ and $\frac{1}{[N\text{-}AcCys]}$ Reaction in the presence of the thiocyanate ion.

$k_3 = 1.5 \times 10^3\ l^2\ mol^{-2}\ s^{-1}$ (from the acid dependence results)

$[N\text{-}AcCys]/M$	k_0/s^{-1}	$k_3[H^+][N\text{-}AcCys]/s^{-1}$	$k_0 - k_3[H^+][N\text{-}AcCys]/s^{-1}$	$\frac{1}{k_0 - k_3[H^+][N\text{-}AcCys]}/s$	$\frac{1}{[N\text{-}AcCys]}/M^{-1}$
5×10^{-3}	3.99	1.55	2.44	0.41	200
1×10^{-2}	7.8	3.11	4.7	0.213	100
2.5×10^{-2}	18.6	7.76	10.8	0.0923	40
5×10^{-2}	32.4	15.5	16.9	0.0593	20
7.5×10^{-2}	44.9	23.3	21.6	0.0463	13.3
0.1	64.1	31.1	33.1	0.0303	10

Figure 2.9 : Double reciprocal plots.



△ in the presence of thiocyanate

▽ in the presence of bromide

△ in the presence of chloride

Table 2.33: Determined rate constants

	Cl^-	Br^-	SCN^-
$k_1/l^2mol^{-2}s^{-1}$	3.5×10^3	4.03×10^3	6.71×10^3
k_{-1}/s^{-1}	3.1×10^6	7.89×10^4	210
$k_2/l\ mol^{-1}s^{-1}$	1.03×10^7	5.46×10^5	1.94×10^3

The k_2 values determined this way are in agreement with those calculated from the dependence of the rate constant on the nucleophile concentration (Table 2.26). Comparison of the k_1 values for the three nucleophiles shows that there is no major trend in these values, and the similarity of these values could be due to the diffusion controlled reaction

$$H_2NO_2^+ + X^- \longrightarrow NOX + H_2O$$

The k_{-1} values are the rate constants for the hydrolysis of NOX, or in other words O-nitrosation of water by the NOX species and show the expected trend NOCl > NOBr > NOSCN.

2.5 Nitrosation of Penicillamine and N-AcetylPenicillamine

Due to the difference in the rate constant for the nitrosation of cysteine and N-acetylcysteine (k_3 = 340 $l^2\ mol^{-2}\ s^{-1}$ and $1.46 \times 10^3\ l^2\ mol^{-2}\ s^{-1}$ respectively) the nitrosation of penicillamine (Pen) and N-acetylpenicillamine (N-Ac pen) was studied, as these compounds are structurally related to cysteine and N-acetylcysteine in an attempt to establish the generality of N-acetyl substitution upon the reactivity of S-nitrosation.

$HS\ CH_2\ CH\ (NH_2)\ CO_2H$ — cysteine

$HS\ C\ (CH_3)_2\ CH\ (NH_2)\ CO_2H$ — penicillamine

The product of the nitrosation of penicillamine is assumed to be the S-nitroso derivative. The product of N-nitrosation, however, has been isolated,[48] when the reaction was carried out with an excess of sodium nitrite, presumably by a mechanism analogous to that already described for cysteine.

Bonnett et al carried out ^{15}N N.M.R. studies[8] on the green solution resulting from the reaction of N-acetylpenicillamine with acidified nitrite solution and determined the product to be the thionitrite derivative. This thionitrite has been isolated and characterized by X-ray crystallography.[7] Indeed it is one of the most stable thionitrites so far isolated.

Aldred, Williams and Garley[19] undertook a kinetic investigation of the nitrosation of N-acetylpenicillamine at 31°C and showed that the rate equation

$$\text{rate} = k_3 [HNO_2][H^+][\text{N-Ac pen}]$$

applied here. They also showed catalysis of this reaction by chloride, bromide, thiocyanate and iodide ions.

The nitrosation of penicillamine and N-acetylpenicillamine was studied by investigating the dependence of the rate constant on the substrate concentration. The results of these studies are given in Tables 2.34 and 2.35.

Table 2.34: Nitrosation of penicillamine

$[HClO_4]$ = 0.2 M

$[NaNO_2]$ = 1 x 10^{-4} M

$10^3[Pen]/M$	k_0/s^{-1}
3	0.0533 ± 0.001
4.5	0.0796 ± 0.002
6	0.105 ± 0.004

Table 2.35: Nitrosation of N-acetylpenicillamine

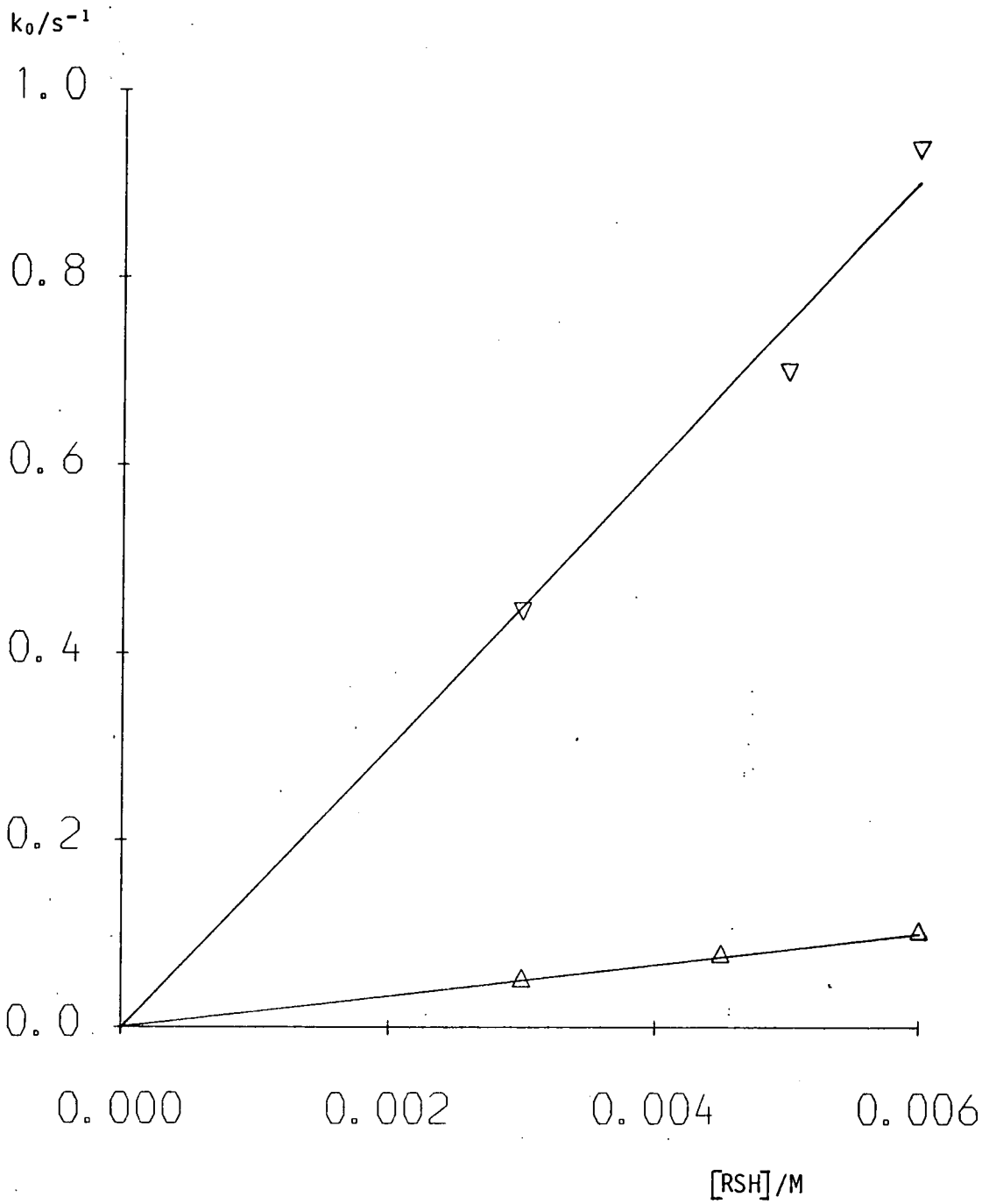
$[HClO_4]$ = 0.2 M

$[NaNO_2]$ = 1 x 10^{-4} M

$10^3[N\text{-Ac pen}]/M$	k_0/s^{-1}
3	0.446 ± 0.016
4.5	0.699 ± 0.022
6	0.94 ± 0.016

Figure 2.10 shows that the expected first order dependence on the thiol concentration is observed. Using these data k_3 was determined as 90 l^2 mol^{-2} s^{-1} and 786 l^2 mol^{-2} s^{-1} for the nitrosation of penicillamine and N-acetylpenicillamine respectively. Comparison of the rate constants of the N-acetyl derivative to the thiol for cysteine and penicillamine, the ratios are for cysteine 4 : 1 and penicillamine 9 : 1. Therefore the same effect is noticed for penicillamine and N-acetyl-penicillamine as observed for cysteine and N-acetylcysteine. A possible explanation for this is given in section 2.8.

Figure 2.10 : Dependence of k_0 on [penicillamine] and [N-acetylpenicillamine]

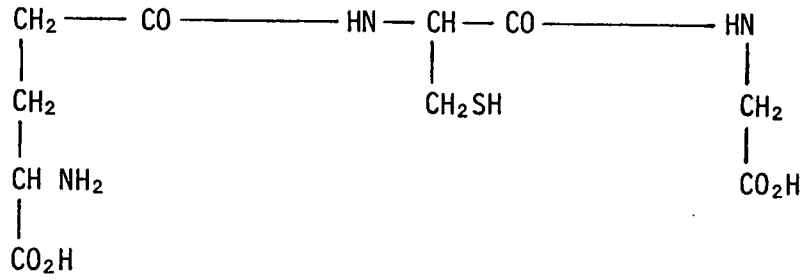


▽ RSH = N-acetylpenicillamine

△ RSH = penicillamine

2.6 Nitrosation of Glutathione

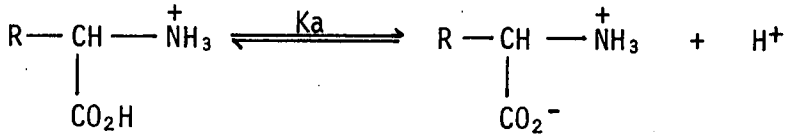
Glutathione (GSH) is a naturally occurring tripeptide of glutamic acid, cysteine and glycine.



The nitrosation of glutathione was studied by investigating both the acid and nucleophile catalyzed reactions.

2.6.1 Acid Catalyzed Nitrosation

The dependence of the rate constant on the acid and glutathione concentrations was investigated. The glutamic acid residue of glutathione can exist in both the zwitterionic (x) and N-protonated (xi) forms



(xi) (x)

pKa = 3.59[53]

As the acid and glutathione concentrations are comparable, the acid concentration has to be corrected for loss of acidity due to protonation of the zwitterion to form the N-protonated species. Therefore the actual acid concentration was calculated for each concentration of glutathione and acid used by solving the quadratic equation

$$[xi]^2 + [xi]\,(Ka + [GSH]_T + [H^+]_T) - [GSH]_T[H^+]_T = 0$$

where $[GSH]_T$ and $[H^+]_T$ are the added concentrations of glutathione and acid respectively.

The corrected acid concentration, $[H^+]_C$, can then be calculated from

$$[H^+]_C = [H^+]_T - [xi]$$

Table 2.36: Dependence of k_0 on [GSH]

$$[HClO_4]_T = 2 \times 10^{-2}\ M$$
$$[NaNO_2] = 1 \times 10^{-4}\ M$$

$10^3[GSH]/M$	$10^2[H^+]_C/M$	k_0/s^{-1}
2.5	1.76	0.0432 ± 0.001
5	1.51	0.08 ± 0.0004
10	1.02	0.133 ± 0.001

Due to the fact that the acid concentration is different for each of the glutathione concentrations used, a graph of k_0 against glutathione concentration cannot be plotted. Therefore the individual values of k_3 were calculated from the equation

$$k_0 = k_3\,[GSH]\,[H^+]_C$$

The results of this calculation are shown in Table 2.37 and 2.38

Table 2.37: Individual k_3 values

$10^2[GSH]/M$	$k_3/l^2\ mol^{-2}\ s^{-1}$
2.5	984
5	1.06×10^3
10	1.1×10^3

Table 2.38: Dependence of k_0 on acid concentration

$[GSH] = 1 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2 [H^+]_c$/M	k_0/s^{-1}
1.03	0.141 ± 0.004
3.05	0.327 ± 0.003
4.69	0.508 ± 0.02
7.08	0.789 ± 0.02

Figure 2.11 shows that the nitrosation of glutathione is first order with respect to the acid. Calculation of k_3 from the gradient of the line of k_0 against acid concentration leads to a value of k_3 of 1.08×10^3 l^2 mol^{-2} s^{-1}.

Figure 2.11 shows a slight positive intercept, reflecting the fact that glutathione is reacting in both the zwitterionic and N-protonated forms.

2.6.2 Nucleophile Catalysis

The effect of the addition of chloride, bromide and thiocyanate ions on the nitrosation of glutathione was studied by investigating the dependence of the rate constant on the nucleophile concentration.

Figure 2.11 : Dependence of k_0 on acid concentration

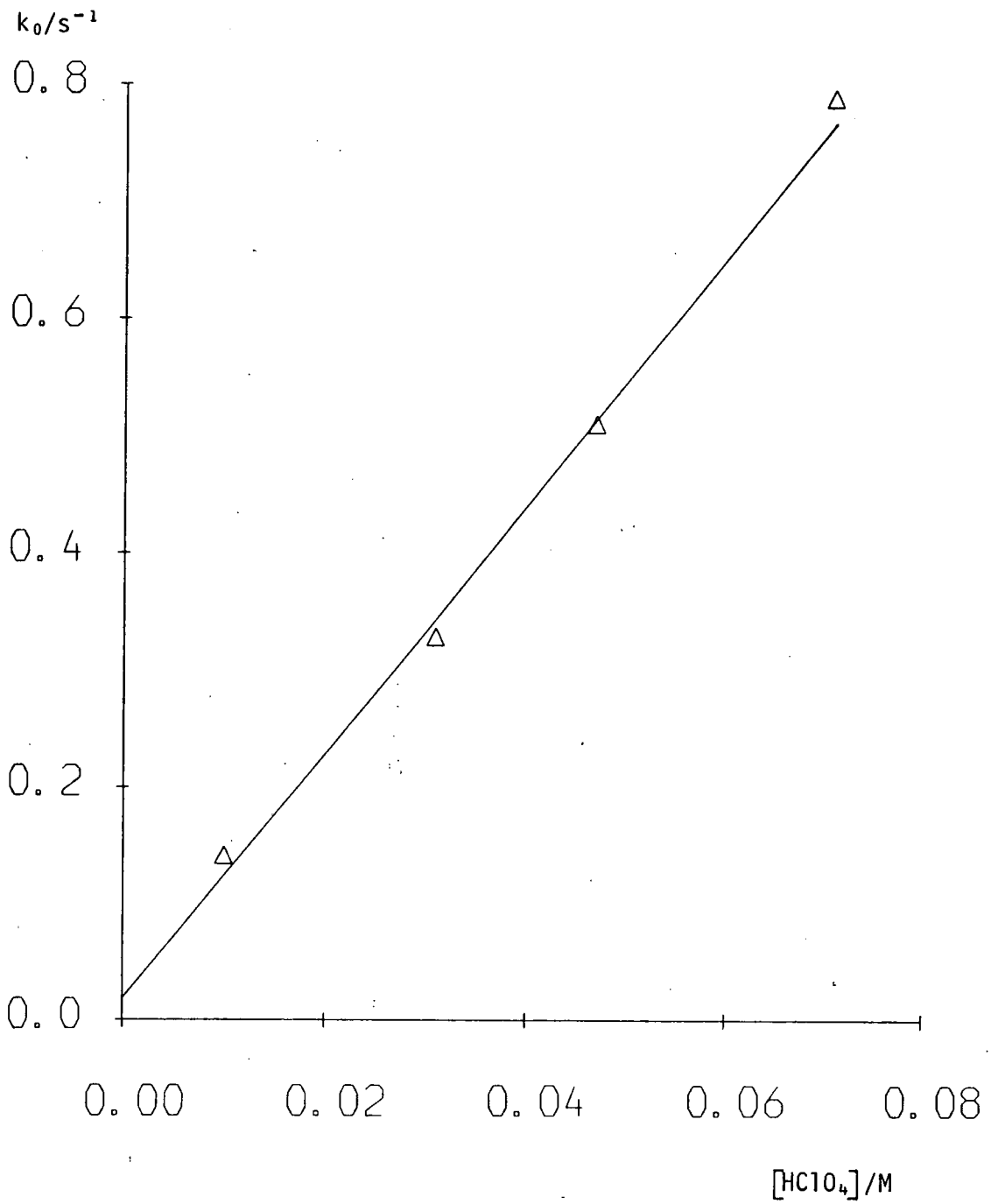


Table 2.39: Chloride ion catalysis

$[GSH] = 1 \times 10^{-2}$ M

$[HClO_4]_C = 1.04 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Cl^-]$/M	k_0/s^{-1}
0.1	0.273 ± 0.007
0.2	0.391 ± 0.013
0.25	0.454 ± 0.01
0.5	0.801 ± 0.011

Table 2.40: Bromide ion catalysis

$[GSH] = 1 \times 10^{-2}$ M

$[HClO_4]_C = 1.04 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Br^-]$/M	k_0/s^{-1}
0.05	0.279 ± 0.009
0.25	0.858 ± 0.021
0.5	1.61 ± 0.03

Table 2.41: Thiocyanate ion catalysis

$[GSH] = 1 \times 10^{-2}$ M

$[HClO_4]_C = 1.04 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[SCN^-]$/M	k_0/s^{-1}
0.5	0.201 ± 0.009
1	0.265 ± 0.009
2	0.386 ± 0.008

These data are presented graphically in Figure 2.12 which shows that a first order dependence on the nucleophile is observed. This is expected from the nucleophile catalyzed nitrosation rate equation

$$\text{rate} = k_2 K_{NOX} [HNO_2][H^+][GSH][X^-] + k_3[HNO_2][H^+][GSH]$$

The intercept refers to the non nucleophile catalyzed nitrosation. Using the slope of these lines the k_2 values were determined and are shown in Table 2.42.

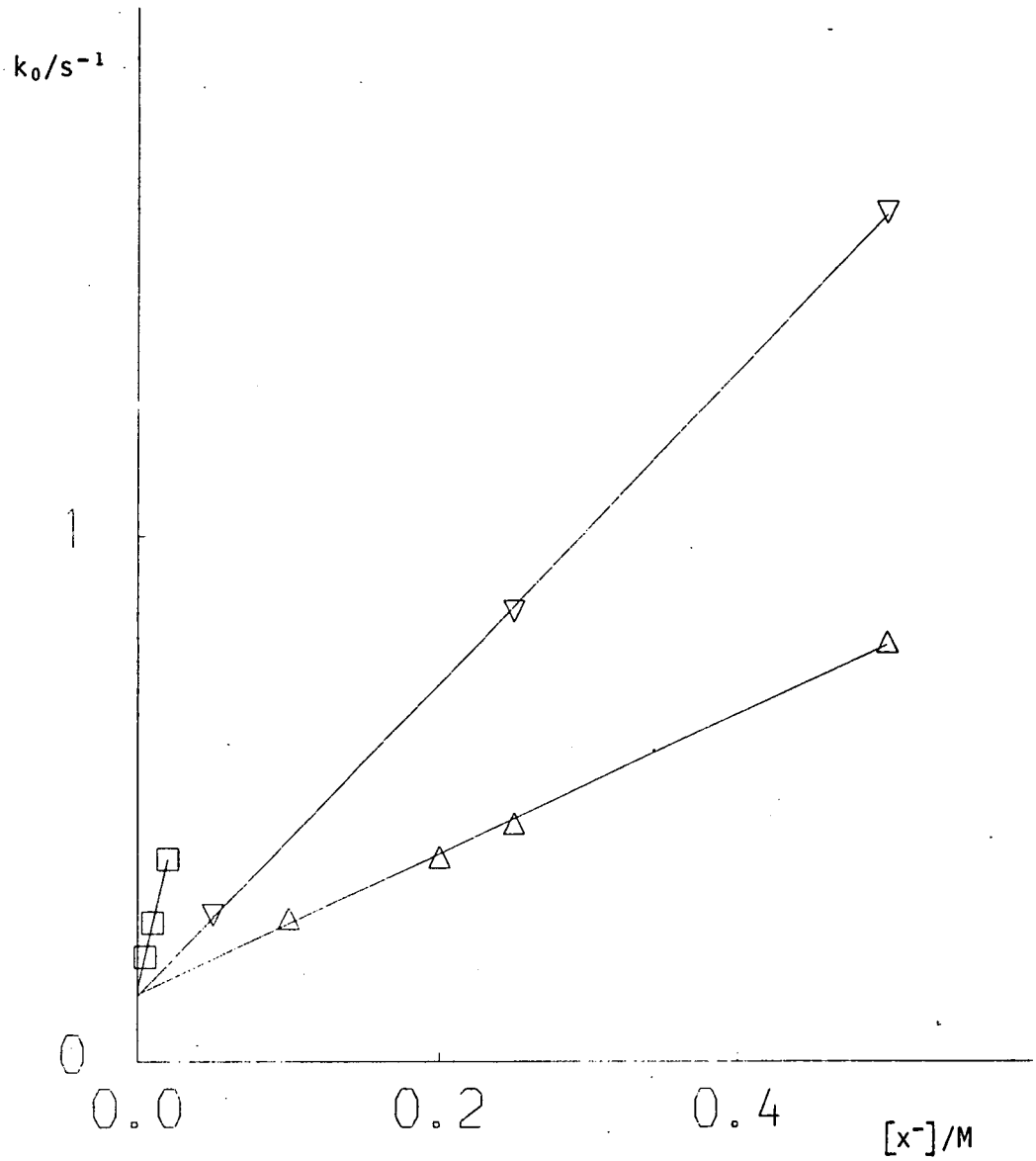
Table 2.42: k_2 values

Nucleophile	k_2/l mol^{-1} s^{-1}
Cl^-	1.1×10^6
Br^-	5.5×10^5
SCN^-	3.7×10^3

These k_2 values show the order of catalysis NOCl > NOBr > NOSCN.

The variation of glutathione concentration in the presence of chloride, bromide and thiocyanate ions was also studied. For these experiments a higher acid concentration was used so that the loss of acid through protonation of the zwitterion was negligible. The results of these experiments are presented in Tables 2.43 to 2.45.

Figure 2.12 : Dependence of k_0 on nucleophile concentration



□ x^- = thiocyanate

▽ x^- = bromide

△ x^- = chloride

Table 2.43: [GSH] variation in the presence of the chloride ion

$[Cl^-]$ = 0.5 M

$[HClO_4]$ = 0.21 M

$[NaNO_2]$ = 1 x 10^{-4} M

$10^2[GSH]/M$	k_0/s^{-1}
0.5	7.13 ± 0.056
1	13.5 ± 0.32
2	24.8 ± 1.1

Table 2.44: [GSH] variation in the presence of the bromide ion

$[Br^-]$ = 0.5 M

$[HClO_4]$ = 0.21 M

$[NaNO_2]$ = 1 x 10^{-4} M

$10^2[GSH]/M$	k_0/s^{-1}
0.5	14.7 ± 0.11
1	28.8 ± 0.62
2	54.1 ± 3.9

Table 2.45: [GSH] variation in the presence of the thiocyanate ion

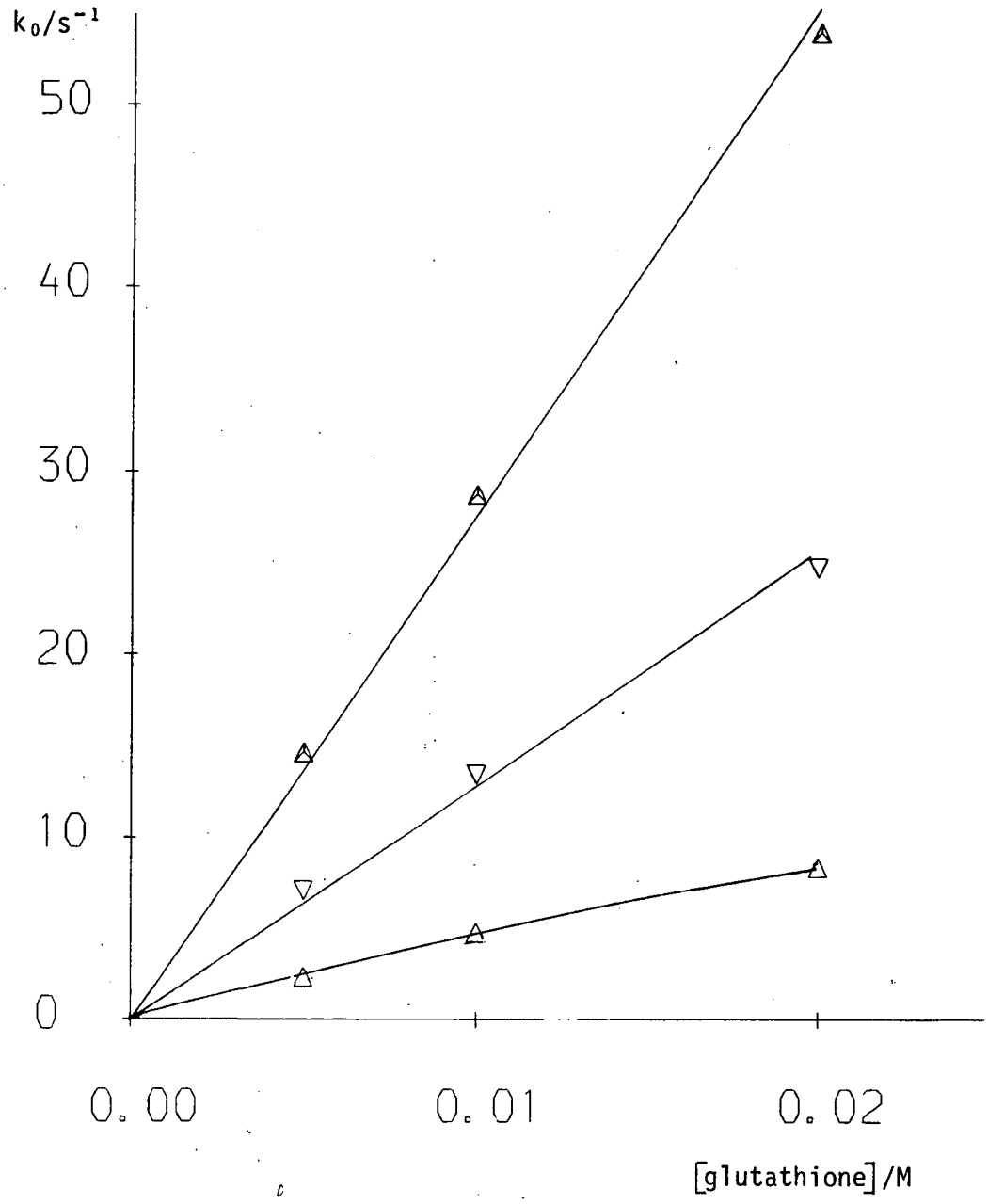
$[SCN^-]$ = 2 x 10^{-2} M

$[HClO_4]$ = 0.194 M

$[NaNO_2]$ = 1 x 10^{-4} M

$10^2[GSH]/M$	k_0/s^{-1}
0.5	2.35 ± 0.043
1	4.75 ± 0.77
2	8.35 ± 0.21

Figure 2.13 : Dependence of k_0 on [glutathione] in the presence of nucleophiles



△ in the presence of bromide
▽ in the presence of chloride
△ in the presence of thiocyanate

Figure 2.13 shows that the substrate dependence remains first order throughout the range of concentrations used in the presence of chloride and bromide ions. In the presence of the thiocyanate ion, however, a slight curvature is observed which can again be interpreted in terms of the rate of formation of NOSCN starting to become significant.

k_2 can be calculated from these results since in the presence of chloride and bromide ions the gradient of the lines in Figure 2.13 is given by

$$\text{slope} = k_3 [H^+] + k_2 K_{NOX} [H^+][X^-]$$

- see section 2.4.2. Using the value of k_3 as 1.08×10^3 l^2 mol^{-2} s^{-1} (determined from the dependence of the rate constant on acid concentration) these k_2 values were determined and are shown in Table 2.46.

Table 2.46: k_2 values

Nucleophile	k_2/l mol^{-1} s^{-1}
Cl^-	1×10^7
Br^-	4.8×10^5

These values are in reasonable agreement with those determined from the dependence of the rate constant on nucleophile concentration (Table 2.42). The corresponding value of k_2 for the thiocyanate ion was not calculated since the graph of k_0 against glutathione concentration was non linear.

2.7 Nitrosation of Thioglycolic Acid

Finally the S-nitrosation of a relatively simple thiol, thioglycolic acid (TGA) was studied by investigating both the

acid catalyzed and nucleophile catalyzed nitrosation of this thiol.

$HO_2C\ CH_2SH$

Bonnett et al[8] carried out ^{15}N N.M.R. studies on the red solution resulting from the reaction of thioglycolic acid with acidified nitrite solution and observed spectra consistent with the formation of the thionitrite

$$HO_2C\ CH_2SH + HNO_2 \xrightarrow{H^+} HO_2C\ CH_2SNO + H_2O$$

2.7.1 Acid Catalyzed Nitrosation

The acid catalyzed nitrosation of thioglycolic acid was investigated by studying the dependence of the rate constant on both the acid and substrate concentrations. The results of these experiments are shown in Tables 2.47 and 2.48, and graphically in Figure 2.14.

Table 2.47: Dependence of k_0 on thiol concentration

$[HClO_4] = 2.03 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[TGA]/M$	k_0/s^{-1}
0.5	0.269 ± 0.006
1	0.562 ± 0.007
2	1.07 ± 0.013

Table 2.48: Dependence of k_0 on acid concentration

$[TGA] = 1 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[H^+]$/M	k_0/s^{-1}
4.05	1.06 ± 0.013
5.8	1.53 ± 0.031
8.1	2.13 ± 0.017

Figure 2.14 shows that the nitrosation of thioglycolic acid is first order with respect to the thiol and acid as expected from the rate equation for acid catalyzed nitrosation.

$$\text{rate} = k_3 [HNO_2][H^+][TGA]$$

k_0 is again the observed first order rate constant defined by

$$\text{rate} = k_0 [HNO_2]$$

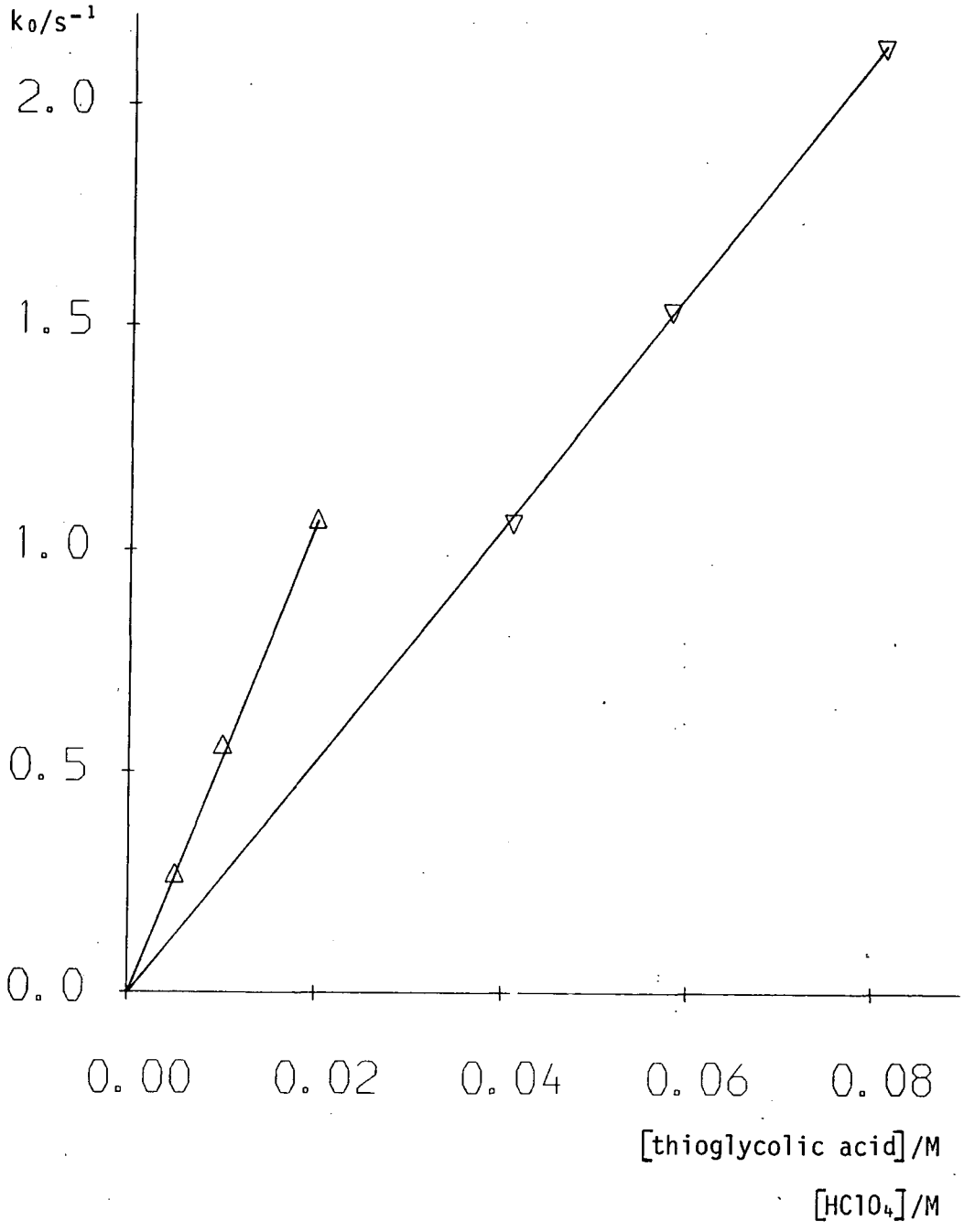
$$\text{where } k_0 = k_3 [H^+][TGA] \qquad (7)$$

Using equation (7) and the results obtained, k_3 was calculated as 2.67×10^3 l^2 mol^{-2} s^{-1} from the dependence of k_0 on the thioglycolic acid concentration and 2.59×10^3 l^2 mol^{-2} s^{-1} from the dependence of k_0 on the acid concentration.

2.7.2 Nucleophile Catalysis

To complete the studies on the S-nitrosation of thioglycolic acid, the effect of chloride, bromide and thiocyanate ions was investigated by studying the nitrosation of thioglycolic acid at different concentrations of nucleophile. The results are laid down in Tables 2.49 to 2.50 and represented graphically in Figure 2.15.

Figure 2.14 : Dependence of k_0 on [thioglycolic acid] and [acid]



▽ $[HClO_4]$ dependence

△ [thioglycolic acid] dependence

Table 2.49: Chloride ion catalysis

$[TGA] = 1 \times 10^{-2}$ M

$[HClO_4] = 2.03 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Cl^-]$/M	k_0/s^{-1}
0.1	0.96 ± 0.012
0.25	1.49 ± 0.047
0.5	2.22 ± 0.033

Table 2.50: Bromide ion catalysis

$[TGA] = 1 \times 10^{-2}$ M

$[HClO_4] = 2.03 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Br^-]$/M	k_0/s^{-1}
0.1	1.57 ± 0.033
0.25	3.06 ± 0.067
0.5	5.42 ± 0.16

Table 2.51: Thiocyanate ion catalysis

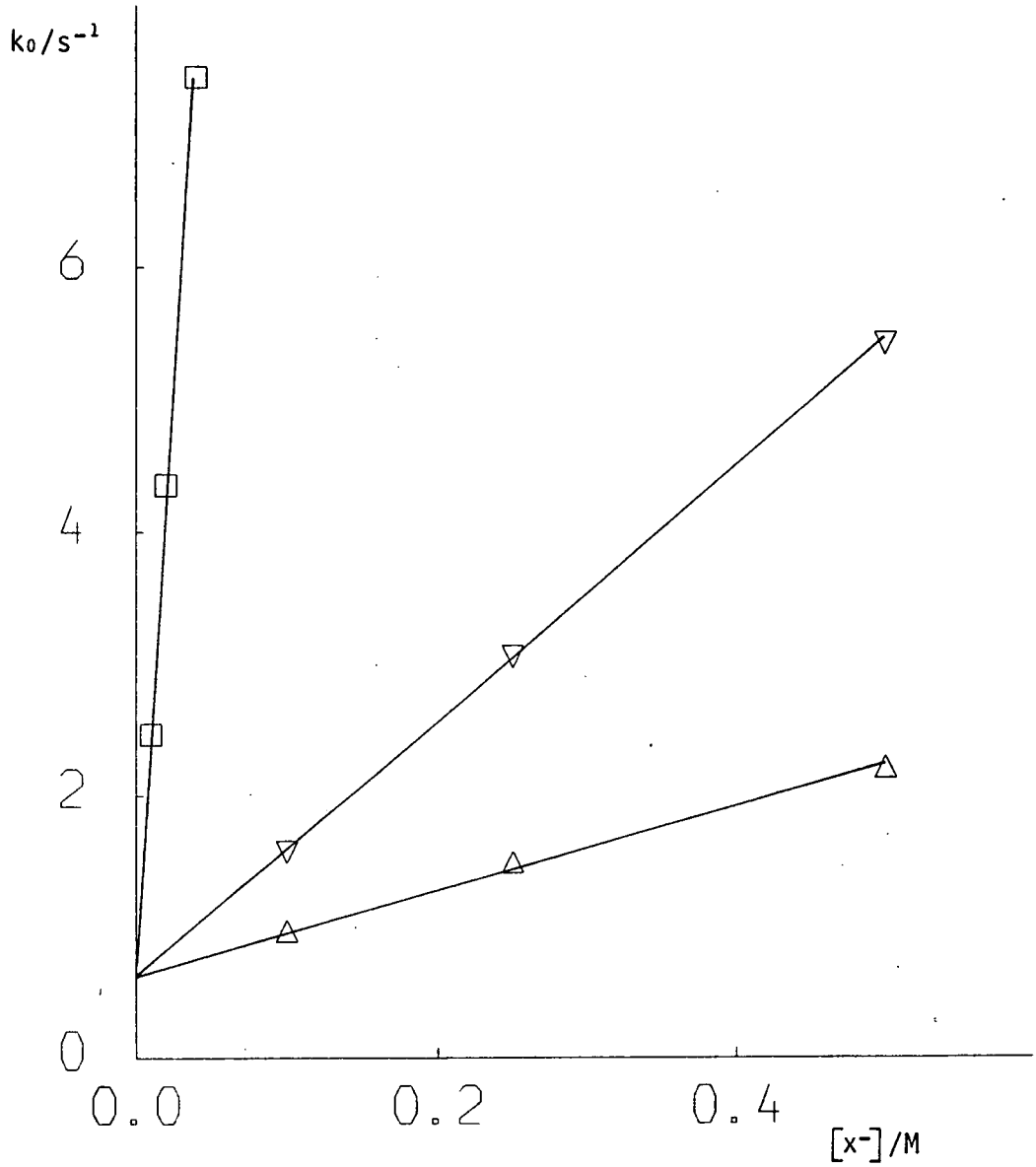
$[TGA] = 1.1 \times 10^{-2}$ M

$[HClO_4] = 2.03 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[SCN^-]$/M	k_0/s^{-1}
1	2.47 ± 0.096
2	4.35 ± 0.049
4	7.45 ± 0.099

Figure 2.15 : Dependence of k_0 on nucleophile concentration



□ x^- = thiocyanate

▽ x^- = bromide

△ x^- = chloride

These results show the first order dependence on the nucleophile expected from the rate equation for nucleophile catalyzed nitrosation.

$$\text{rate} = k_2 \, K_{NOX} \, [HNO_2][H^+][TGA][X^-]$$

Figure 2.15 shows that the catalytic activity of the ions is $Cl^- < Br^- < SCN^-$, whereas the order of catalysis is NOCl > NOBr > NOSCN which can be seen by comparison of the k_2 values in Table 2.52.

Table 2.52: k_2 values

Nucleophile	k_2/l mol^{-1} s^{-1}
Cl^-	1.39×10^7
Br^-	1.13×10^6
SCN^-	2.49×10^4

Again the thioglycolic acid concentration was varied in the presence of these nucleophiles and the results are shown in Tables 2.53 to 2.55.

Table 2.53: [TGA] variation in the presence of the chloride ion

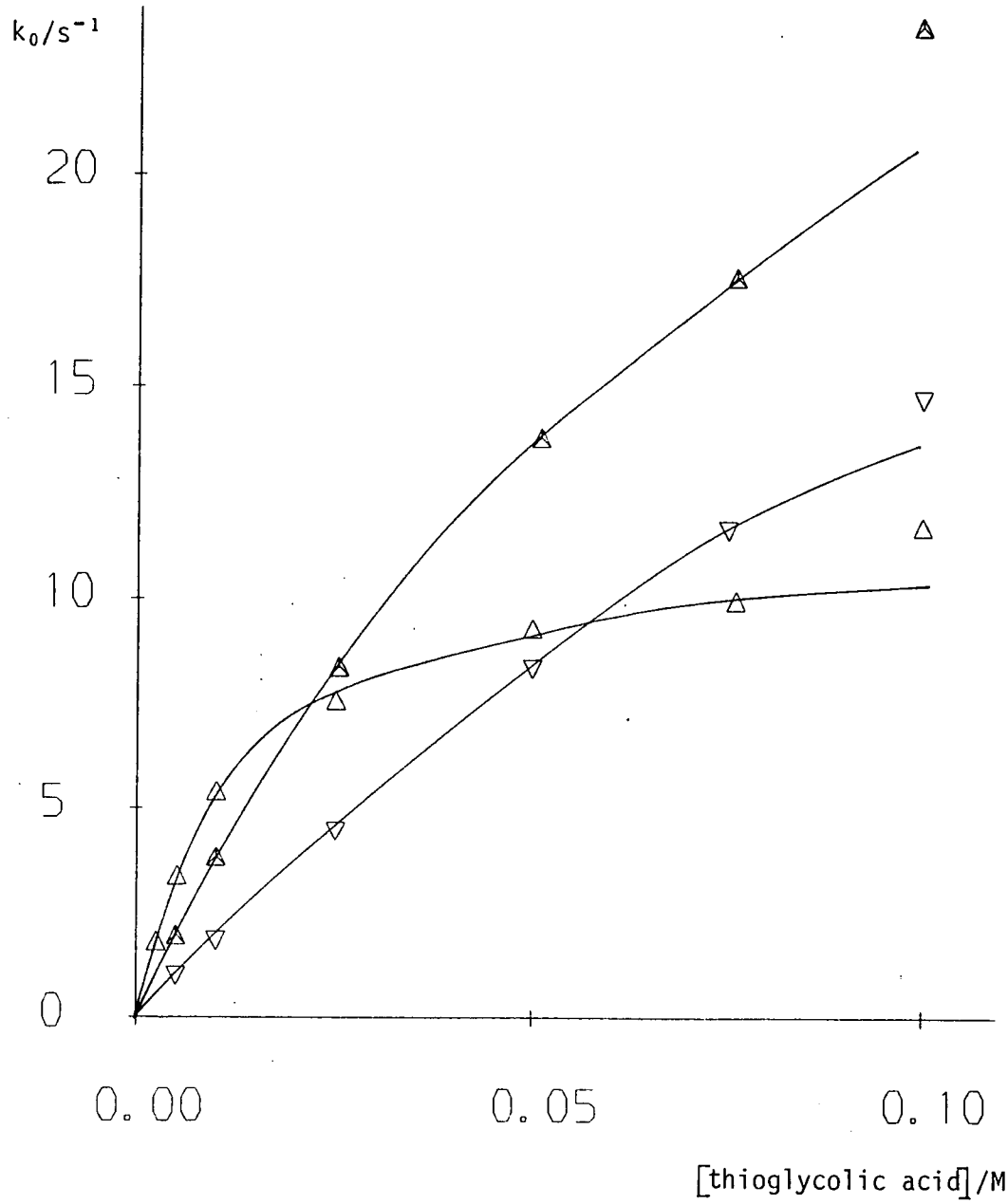
$[Cl^-] = 0.5$ M

$[HClO_4] = 1.87 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[TGA]$/M	k_0/s^{-1}
0.5	0.979 ± 0.021
1	1.82 ± 0.03
2.5	4.44 ± 0.047
5.01	8.35 ± 0.091
7.5	11.6 ± 0.31
10	14.7 ± 0.23

Figure 2.16 : Dependence of k_0 on [thioglycolic acid] in the presence of nucleophiles



△ in the presence of bromide
▽ in the presence of chloride
△ in the presence of thiocyanate

Table 2.54: [TGA] variation in the presence of the bromide ion

$[Br^-] = 0.5$ M

$[HClO_4] = 1.87 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[TGA]/M$	k_0/s^{-1}
0.51	1.96 ± 0.028
1.02	3.82 ± 0.136
2.54	8.41 ± 0.299
5.08	13.8 ± 0.17
7.62	17.6 ± 0.15
10.2	23.6 ± 0.43

Table 2.55: [TGA] variation in the presence of the thiocyanate ion

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

$[HClO_4] = 1.9 \times 10^{-2}$ M

$[NaNO_2] = 1 \times 10^{-4}$ M

$10^2[TGA]/M$	k_0/s^{-1}
0.25	1.81 ± 0.041
0.51	3.37 ± 0.016
1.02	5.41 ± 0.081
2.54	7.59 ± 0.187
5.08	9.31 ± 0.181
7.63	9.97 ± 0.184
10.2	11.7 ± 0.43

As can be seen from Figure 2.16, the first order dependence on thioglycolic acid is not observed throughout the range of concentrations used. All the results lead to curved plots of k_0 against thioglycolic acid concentration, the effect being particularly pronounced in the presence of bromide and thiocyanate ions. As for N-acetylcysteine, this effect can be explained in terms of the fact that the formation of NOX can nolonger be considered as a rapid pre-equilibrium. From the kinetics laid out in section 2.4.2 the equation

$$\frac{1}{k_0 - k_3\,[H^+][RSH]} = \frac{k_{-1}}{k_1\,k_2\,[H^+][X^-][RSH]} + \frac{1}{k_1\,[H^+][X^-]}$$

was shown to apply. Therefore the results were plotted as $1/k_0 - k_3[H^+][RSH]$ against $1/[TGA]$. Calculations of these values are shown in Tables 2.56 to 2.58 and in Figure 2.17. From this analysis values of k_1, k_{-1} and k_2 can be determined. The results of these calculations are presented in Table 2.59.

By Comparison of the k_2 values with those determined from the dependence of k_0 on nucleophile concentration (Table 2.52) it can be seen that reasonable agreement between both determinations is observed. Agreement between these k_1 and k_{-1} values and those determined in the study of the nitrosation of N-acetylcysteine (Table 2.33) is not so good. This probably arises from the large error associated with double reciprocal plots. However, the k_{-1} values, which is the rate constant for the hydrolysis of the NOX species or in other words the O-nitrosation of water, show the expected trend NOCl > NOBr > NOSCN.

Table 2.56 : Calculation of $\frac{1}{k_0 - k_3 [H^+][TGA]}$ and $\frac{1}{[TGA]}$ Reaction in the presence of the chloride ion.

$k_3 = 2.6 \times 10^3\ l^2\ mol^{-1}\ s^{-1}$ (from the acid dependence results)

$[TGA]/M$	k_0/s^{-1}	$k_3[H^+][TGA]/s^{-1}$	$k_0 - k_3[H^+][TGA]/s^{-1}$	$\frac{1}{k_0 - k_3[H^+][TGA]}/s$	$\frac{1}{[TGA]}/M^{-1}$
5.01×10^{-3}	0.979	0.244	0.735	1.36	199.6
1×10^{-2}	1.82	0.487	1.33	0.75	99.8
2.5×10^{-2}	4.44	1.22	3.22	0.31	39.94
5.01×10^{-2}	8.35	2.44	5.91	0.169	19.96
7.51×10^{-2}	11.6	3.65	7.95	0.126	13.32
0.1	14.7	4.87	9.83	0.102	9.98

Figure 2.57 : Calculation of $\frac{1}{k_0 - k_3 [H^+][TGA]}$ and $\frac{1}{[TGA]}$ Reaction in the presence of the bromide ion.

$k_3 = 2.6 \times 10^3\ l^2\ mol^{-2}\ s^{-1}$ (from the acid dependence results)

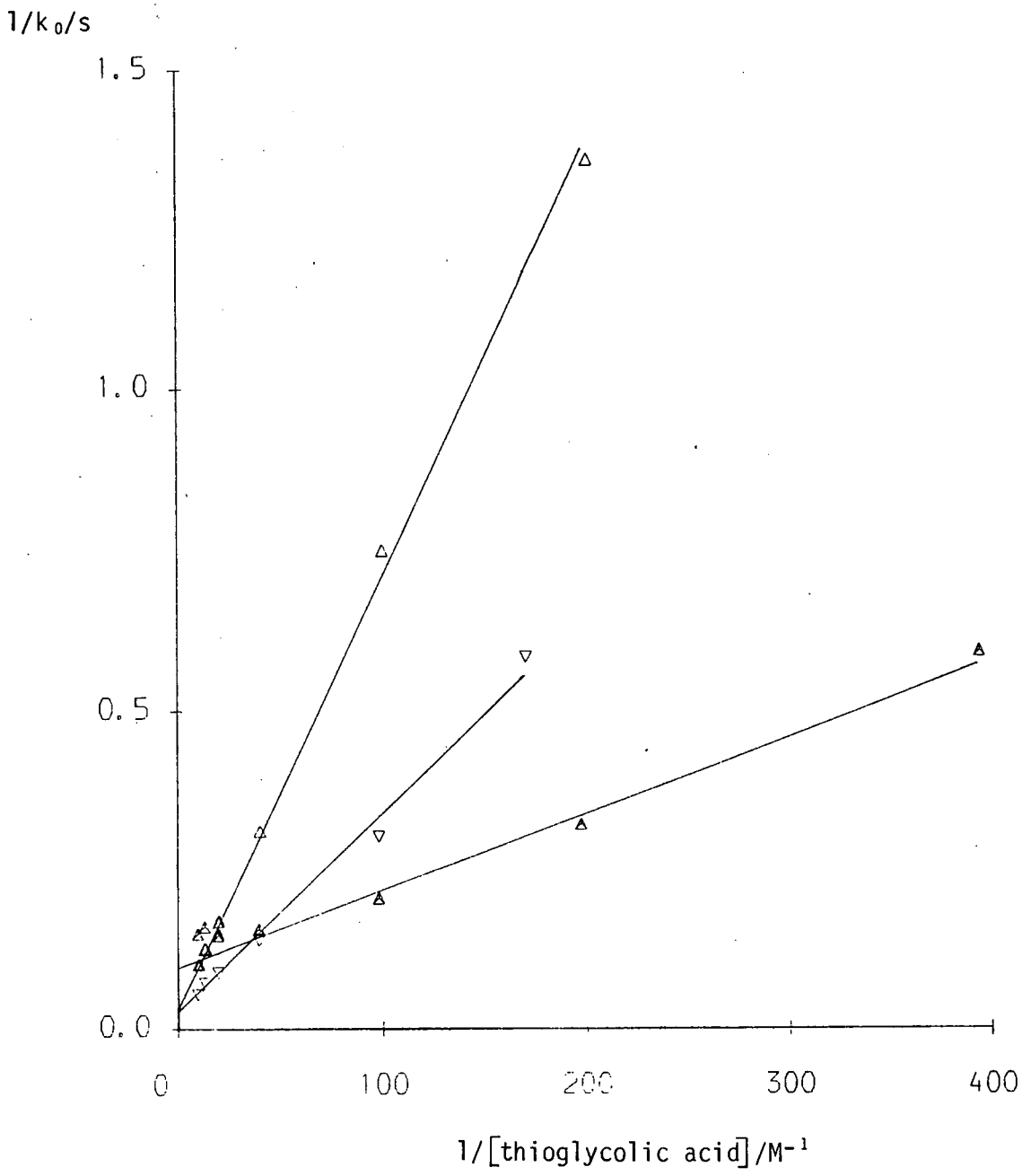
$[TGA]/M$	k_0/s^{-1}	$k_3[H^+][TGA]/s^{-1}$	$k_0 - k_3[H^+][TGA]/s^{-1}$	$\frac{1}{k_0 - k_3[H^+][TGA]}/s$	$\frac{1}{[TGA]}/M^{-1}$
5.08×10^{-3}	1.96	0.247	1.71	0.584	196.9
1.02×10^{-2}	3.82	0.496	3.32	0.301	98
2.54×10^{-2}	8.41	1.24	7.18	0.139	39.4
5.08×10^{-2}	13.8	2.47	11.3	0.0883	19.7
7.62×10^{-2}	17.6	3.71	13.9	0.072	13.1
0.102	23.6	4.96	18.6	0.0536	9.8

Figure 2.58 : Calculation of $\frac{1}{k_0 - k_3 [H^+][TGA]}$ and $\frac{1}{[TGA]}$. Reaction in the presence of the thiocyanate ion.

$k_3 = 2.6 \times 10^3\ l^2\ mol^{-2}\ s^{-1}$ (from the acid dependence results)

$[TGA]/M$	k_0/s^{-1}	$k_3[H^+][TGA]/s^{-1}$	$k_0 - k_3[H^+][TGA]/s^{-1}$	$\frac{1}{k_0 - k_3[H^+][TGA]}$ /s	$\frac{1}{[TGA]}$ /M^{-1}
2.54×10^{-3}	1.81	0.126	1.68	0.594	393.7
5.08×10^{-3}	3.37	0.251	3.12	0.321	197
1.02×10^{-2}	5.41	0.505	4.91	0.204	98
2.54×10^{-2}	7.59	1.26	6.33	0.156	39.4
5.08×10^{-2}	9.31	2.52	6.8	0.147	19.7
7.63×10^{-2}	9.97	3.78	6.19	0.161	13.1
0.102	11.7	5.05	6.65	0.15	9.8

Figure 2.17 : Double reciprocal plots



△ in the presence of thiocyanate
▽ in the presence of bromide
△ in the presence of chloride

Table 2.59: Determined rate constants

	Cl^-	Br^-	SCN^-
$k_1/l^2mol^{-2}s^{-1}$	2.67×10^3	3.29×10^3	1.35×10^4
k_{-1}/s^{-1}	2.34×10^6	6.45×10^4	421
$k_2/l\ mol^{-1}s^{-1}$	1.42×10^7	7.45×10^5	3.32×10^4

2.8 Discussion

All the thiols studied exhibited the same kinetics. In the absence of added nucleophiles, such as chloride, bromide and thiocyanate ions, the rate equation

$$\text{rate} = k_3\ [HNO_2]\,[H^+]\,[RSH]$$

was shown to apply. This rate equation has been interpreted in terms of rate determining electrophilic attack on the sulphur atom by the nitrous acidium ion or nitrosonium ion.

$$HNO_2 + H^+ \underset{}{\overset{\text{fast}}{\rightleftharpoons}} H_2NO_2^+$$

$$H_2NO_2^+ \quad RSH \xrightarrow{\text{slow}} R{-}\overset{+}{S}(H){-}NO + H_2O$$

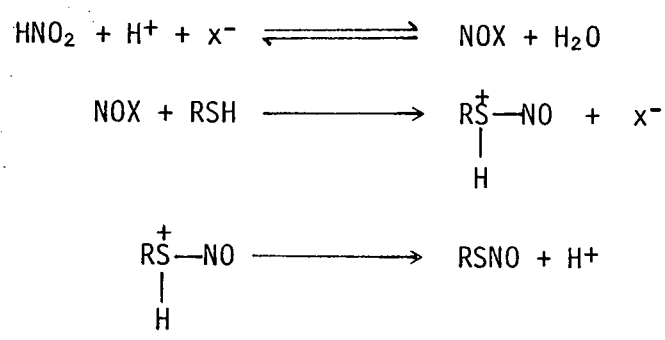
$$R{-}\overset{+}{S}(H){-}NO \xrightarrow{\text{fast}} RSNO + H^+$$

scheme 2.12

In the presence of nucleophiles the addition of the extra kinetic term

$$\text{rate} = k_2 K_{NOX}\ [HNO_2]\,[H^+]\,[X^-]\,[RSH]$$

accounted for the results. This is the familiar rate equation for nitrosation of the substrate by the NOX species.



scheme 2.13

Normally the formation of NOX occurs in a rapid pre-equilibrium. However for the nitrosation of N-acetylcysteine and thioglycolic acid at high thiol concentrations, this is nolonger the case.

A summary of the rate constants determined in this Chapter is given in Table 2.60. The acid catalyzed nitrosation rate constants refer to those determined from the dependence of k_0 on the acid concentration, except for penicillamine and N-acetylpenicillamine where only the substrate dependence was investigated. The nucleophile catalyzed rate constants refer to those determined from the dependence of k_0 on the nucleophile concentration.

Table 2.60: Summary of rate constants determined.

	k_3 $l^2mol^{-2}s^{-1}$	k_2(NOCl) $l\ mol^{-1}s^{-1}$	k_2(NOBr) $l\ mol^{-1}s^{-1}$	k_2(NOSCN) $l\ mol^{-1}s^{-1}$
cysteine	340	1.22×10^6	5.82×10^4	690
cysteine methyl ester	213	1×10^6	4.9×10^4	712
N-acetyl cysteine	1.5×10^3	1×10^7	4.5×10^5	1.6×10^3
penicillamine	90			
N-acetyl penicillamine	786			
glutathione	1.1×10^3	1.1×10^7	5.5×10^5	3.7×10^3
thioglycolic acid	2.6×10^3	1.4×10^7	1.1×10^6	2.5×10^4

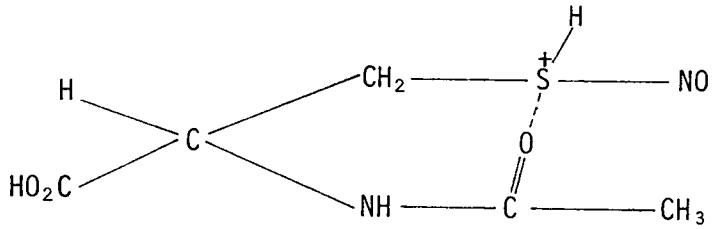
The results for the nucleophile catalyzed nitrosation of the thiols all show the now well established order of catalysis NOCl > NOBr > NOSCN, as expected by consideration of the electronegativities of the x species. The catalytic activity of the ions, however, for all the thiols studied is Cl^- < Br^- < SCN^-. This difference is due to the dominating effect of the K_{NOX} values which have been determined as 1.14×10^{-3} l^2mol^{-2} (NOCl)[22], 5.1×10^{-2} l^2mol^{-2} (NOBr)[23] and 32 l^2mol^{-2} (NOSCN).[24]

The majority of the differences in the rate constants observed for these thiols can be explained in terms of electronic effects.

Firstly, consider the substituent effects observed for the cysteine derivatives. The rate constants for the nitrosation of cysteine and the methyl ester of cysteine are comparable. This is to be expected since the electronegativities of the

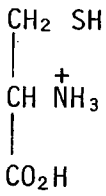
carboxyl group and methyl ester groups are about the same. Therefore changing from $-CO_2H$ to $-CO_2Me$ should have little effect on the sulphur atom and this is shown by the similarity of the rate constants and confirms that under the conditions used, cysteine is mostly reacting in the N-protonated form, rather than in the more reactive zwitterionic form.[46]

Next consider the effect of N-acetyl substitution in the amino group. For both cysteine and penicillamine this has a significant effect on the rate constant of nitrosation. For cysteine, the introduction of a N-acetyl group has an activating effect of ca 4, and for penicillamine the activating effect is rather larger at ca 9. It is difficult to explain this by any through-bond electronic effect, and may be due to internal stabilization in which the N-acetyl group interacts with the positively charged sulphur atom in the transition state as shown for N-acetyl cysteine.

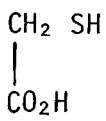


This will then cause the nitrosation of the N-acetyl derivatives to proceed faster than the parent compound.

The rate of nitrosation of thioglycolic acid is considerably faster than cysteine. This can be explained by considering the groups present on each molecule.

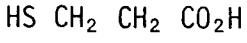


cysteine



thioglycolic acid

The predominant form of cysteine under the given experimental conditions has been drawn here. The main groups that will affect the electron distribution in cysteine are the carboxyl group and the amino group, and in thioglycolic acid the carboxyl group. In going from cysteine to thioglycolic acid, the carboxyl group has moved closer to the sulphur atom (being two carbons away in cysteine and adjacent in thioglycolic acid). This would be expected to cause a decrease in the electron density at the sulphur atom in thioglycolic acid, thereby causing a decrease in the nucleophilicity of the sulphur atom, or in other words decrease the susceptibility of the thiol to electrophilic attack by the nitrosating agent. This should cause a corresponding decrease in the rate of nitrosation. However, in thioglycolic acid the strongly electron withdrawing $-NH_3^+$ group is absent. This group would appear to have the dominating effect on the rate constant for the nitrosation of cysteine. This means that even though the carboxyl group is closer in thioglycolic acid than in cysteine, the absence of the protonated amino group means that it reacts more rapidly. This effect can also be seen by comparing the reactions of cysteine and thioglycolic acid with that of 3-mercaptopropanoic acid.



3-mercaptopropanoic acid

The third order rate constant for acid catalyzed nitrosation (k_3) has been determined[18] as 4,764 l^2 mol^{-2} s^{-1}, considerably larger than that for cysteine, and thioglycolic acid where the carboxyl group is one carbon closer to the sulphur atom.

In glutathione the protonated amino group is also absent, instead being replaced by a peptide linkage.

The acid catalyzed nitrosation rate constants determined for these thiols are compared to the rate constants for the nitrosation of other substrates in Table 2.61. This shows the high reactivity of the thiols towards the nitrosating agent. This means that thiols will be nitrosated rapidly under the acidic conditions present in the stomach. The diffusion controlled limit for the nitrosation of a neutral substrate by the nitrous acidium ion or nitrosonium ion appears to be around 7,000 l^2 mol^{-2} s^{-1}. [32]

Table 2.61: k_3 values at 25°C

Substrate	$k_3/l^2mol^{-2}s^{-1}$	Reference
Urea	0.89	54
Penicillamine	90	this work
HN_3	160	54
Cysteine methyl ester	213	this work
Cysteine	340	this work
Methanol	700	19
N-Acetyl Penicillamine	786	this work
Glutathione	1,100	this work
Mercaptosuccinic acid	1,334	18
N-Acetylcysteine	1,500	this work
Thioglycolic acid	2,600	this work
Thiourea	6,960	31
Sulphanilic acid	7,300	54

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CHAPTER THREE

DECOMPOSITION OF THIONITRITES

3.1 Introduction

Thionitrites in aqueous solution can undergo either hydrolysis, to form the thiol and nitrous acid,

$$RSNO + H_2O \rightleftharpoons RSH + HNO_2 \qquad (1)$$

or decompose to form the disulphide and nitric oxide

$$2RSNO \longrightarrow RSSR + 2NO \qquad (2)$$

Hydrolysis is a relatively slow process compared to nitrosation and, as mentioned in Chapter One, the equilibrium in equation (1) lies well over to the left.[1] It is possible, however, to study the hydrolysis of thionitrites by carrying out the reaction at high acid concentrations and in the presence of a nitrite trap to remove the nitrous acid as it is formed thus driving the reaction to the right. This prevents renitrosation of the thiol. The hydrolysis of S-nitroso - N-acetyl penicillamine, a particularly stable thionitrite[2], has been studied[3] under conditions of high acidity (~3M sulphuric acid). The rate constants determined showed the rate of hydrolysis of thionitrites to be considerably slower than that of alkyl nitrites[4] as expected since the reaction is thought to proceed via protonation of the thionitrite at the sulphur atom or alkyl nitrite at the oxygen atom. The higher basicity of oxygen compared to sulphur makes the alkyl nitrite more susceptible to protonation and hence hydrolysis.

$$RSNO + H^+ \rightleftharpoons R\overset{+}{S}HNO$$

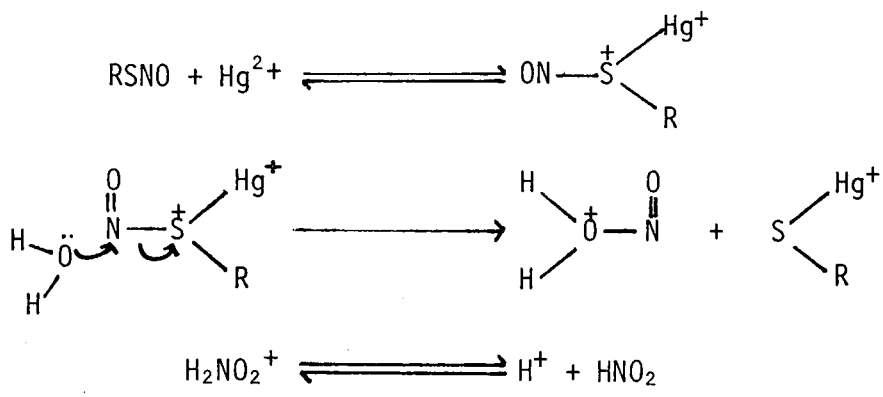
$$R\overset{+}{S}HNO + H_2O \rightleftharpoons RSH + HNO_2 + H^+$$

$$HNO_2 + \text{nitrite trap} \longrightarrow \text{products}$$

scheme 3.1

Catalysis of this reaction by added nucleophiles such as the bromide ion was also shown, and again interpreted in terms of attack of the nucleophilic species on the protonated form of the thionitrite.

The hydrolysis of thionitrites has been shown to be strongly catalyzed by mercuric ions.[5] This is probably due to complex formation with the thionitrite and subsequent attack of the complex by water, as shown in scheme 3.2



scheme 3.2

Hence S-nitrosocysteine, which is relatively stable in acid solution ($t_{\frac{1}{2}}$ 50 hours in 5 x 10^{-2} to 0.5M sulphuric acid) rapidly hydrolyses in the presence of mercuric salts and a nitrite trap to remove the nitrous acid as it is formed. This is the basis of an analytical method for the determination of thiols.

Thionitrites are also thermally and photolytically unstable. In fact synthetic usage has been made of this property of thionitrites for the formation of unsymmetrical disulphides.[6] For example:

$$PhSNO + {}^{t}BuSH \longrightarrow PhSS^{t}Bu$$

The advantage of this procedure is that the reaction can be carried out in one reaction vessel and gives good yields of the disulphide - for example, the above reaction yields 98% disulphide.

The stability of thionitrites seems to depend on the size of the R group; bulkier substituents increase the stability and so certain thionitrites are relatively stable. These include ^{t}butylthionitrite[7] and triphenylmethylthionitrite[8] which are stable as solids for a few days at room temperature. More recently S-nitroso-N-acetylpenicillamine has been prepared[2] which is indefinitely stable as a solid, and which decomposes slowly in solution.

Tasker and Jones[9] found that the product of the reaction of phenylmercaptan with nitrosyl chloride was the disulphide and nitric oxide, which they postulated were formed via the thionitrite.

$$C_6H_5SH + NOCl \longrightarrow HCl + C_6H_5SNO$$

$$2C_6H_5SNO \longrightarrow (C_6H_5S)_2 + 2NO$$

scheme 3.3

Later Rheinboldt looked at the thermal decomposition of triphenylmethylthionitrite[10] and trimethylthionitrite[11] and again found that the products were the disulphide and nitric oxide.

The decomposition process is thought to have a radical component to it, the thionitrite undergoing homolytic fission to generate the thiyl radical and nitric oxide, and the thiyl radical then dimerizes, or possibly reacts with another thionitrite molecule to form the disulphide and nitric oxide, as shown in scheme 3.4.

$$RSNO \rightleftharpoons RS^{\cdot} + NO$$

$$2RS^{\cdot} \longrightarrow RSSR$$

$$RS^{\cdot} + RSNO \longrightarrow RSSR + NO$$

scheme 3.4

Van Zwet and Kooyman[12] studied the decomposition of triphenylmethylthionitrite in more depth and found that the rate of decomposition was not first order with respect to the thionitrite, and was increased by the addition of a radical scavenger. They explained this in terms of the first step, that is formation of the thiyl radical and nitric oxide, being reversible. Further evidence for the involvement of radicals in the decomposition of thionitrites comes from the study by Josephy et al[13], who carried out E.S.R. spin trapping experiments on thionitrites formed from a variety of thiols and isoamylnitrite. They obtained distinctive hyperfine splitting constants which they assigned to the thiyl radical. They also prepared triphenylmethylthionitrite, which gave similar hyperfine parameters.

Recently some kinetic work on the thermal decomposition of S-nitrosothioureas has been carried out[14] and the rate law

$$\text{rate} = k_1[R_2\overset{+}{C}SNO][R_2CS] + k_2[R_2\overset{+}{C}SNO]^2 \qquad (4)$$

has been established. The authors explain the first term in terms of a reaction between the nitrosylthiourea and thiourea, to form a radical cation with loss of nitric oxide.

$$R_2\overset{+}{C}SNO + R_2CS \longrightarrow (R_2CSSR_2)^{\cdot +} + NO \qquad (5)$$

The photolytic decomposition of thionitrites has also been studied,[15] again with the formation of the disulphide and

nitric oxide by a similar radical mechanism, as shown in scheme 3.5

$$RSNO \xrightarrow{h\upsilon} RSNO^* \longrightarrow RS^{\cdot} + NO$$

$$RS^{\cdot} + RSNO \longrightarrow RSSR + NO$$

scheme 3.5

The evidence for the involvement of thiyl radicals and the above mechanism is that monomers of acrylonitrile and methylmethacrylate reduced the quantum yield for the disappearance of the thionitrite.

Similar to this is the photoaddition of thionitrites to carbon carbon double bonds,[16] which also involves the presence of thiyl radicals.

$$R^1SNO \xrightarrow{h\upsilon} R^1S^{\cdot} + NO$$

$$R^1S^{\cdot} + R^2R^3C{=}CH_2 \longrightarrow R^2R^3C^{\cdot}{-}CH_2SR^1$$

$$R^2R^3C^{\cdot}{-}CH_2SR^1 + NO \longrightarrow R^2R^3C(NO){-}CH_2SR^1$$

$$R^2R^3C(NO){-}CH_2SR^1 \longrightarrow (R^2R^3CCH_2SR^1)_2\ NO$$

scheme 3.6

3.2 Decomposition of S-Nitrosocysteine

As mentioned in the introduction thionitrites can either undergo hydrolysis or decomposition

$$RSH + HNO_2 \rightleftharpoons RSNO \downarrow \tfrac{1}{2}\ RSSR + NO$$

scheme 3.7

The equilibrium constant for the nitrosation of cysteine has been determined at 1.84×10^6.[17] As the rate constant

for the acid catalyzed nitrosation has been determined as 340 l^2 $mol^{-2}s^{-1}$, this means that the rate constant for the acid catalyzed hydrolysis of S-nitrosocysteine has a rate constant of the order of 10^{-4} l $mol^{-1}s^{-1}$, that is the hydrolysis of S-nitrosocysteine is a very slow process.

Previous studies on the decomposition of S-nitrosocysteine have shown that it is least stable at pH values near neutrality. In solutions of 5 x 10^{-2}m to 0.5M sulphuric acid S-nitrosocysteine is relatively stable ($t_{\frac{1}{2}}$ 50 hours).[5] Mirna and Hofmann[18] studied the stability of S-nitrosocysteine in the pH range 1.1 to 5.5 and noted that its stability decreased with increasing pH, and proposed that hydrolysis was occurring. This, however, is not consistent with the increased stability of S-nitrosocysteine at high acidity since hydrolysis at acid pH is an acid catalyzed process,[3] nor with the slowness of the hydrolysis. Byler et al[17] studied the equilibrium reaction between cysteine and nitrous acid and found that above about pH 4.3 the rate of decomposition of S-nitrosocysteine became so significant that accurate determinations could not be made. Cantoni et al[19] studied the stability of S-nitrosocysteine in alkaline solution and found its stability to increase with increasing pH. The nitrite ion was isolated as one of the products, presumably from alkaline hydrolysis by a mechanism analogous to that observed for alkyl nitrites.[20]

More recently the decomposition of S-nitrosocysteine hydrochloride has been studied at pH 2, 5.5 and 9.75,[21] and similar results were obtained. Thus at pH 2 no significant decomposition of S-nitrosocysteine had occurred after eight hours, whereas at pH 5.5 and 9.75 the rate of decomposition was faster. The

existence of a radical component to the decomposition was proposed. Dennis et al were able to analyze the decomposition profile at pH 5.5 kinetically, whereas the profile at pH 9.75 proved too complex for kinetic analysis and no mechanism was proposed to account for the observed rate behaviour.

The study of the decomposition of S-nitrosocysteine was undertaken here in view of its being a potential nitrosating agent (see Chapter Four) and purely to gain an indication of how stable it is in aqueous solution and the effect, if any, of other compounds on its stability.

3.2.1 Decomposition of S-Nitrosocysteine at pH 5.5, 7 and 9.8

As can be seen from figures 3.1, 3.2 and 3.3 the effect of pH on the stability of S-nitrosocysteine is as previously found,[21] that is it is least stable at pH7 compared to pH 5.5 and pH 9.8. Due to the complexity of these decomposition profiles, kinetic analyses could not be undertaken.

Under the normal conditions used to study the nitrosation of thiols the reaction goes to completion. However under the conditions used here to generate S-nitrosocysteine (typical concentrations 4×10^{-3} M $NaNO_2$, $HClO_4$ and cysteine) the reaction probably does not go to completion (see Chapter Four), therefore some unreacted thiol will be present in the reaction vessel which may affect the decomposition of S-nitrosocysteine. In view of this and previous studies on the decomposition of thionitrites the possible reactions which may be occurring are shown in scheme 3.8

Figure 3.1 : Decomposition of S-nitrosocysteine at pH 5.5

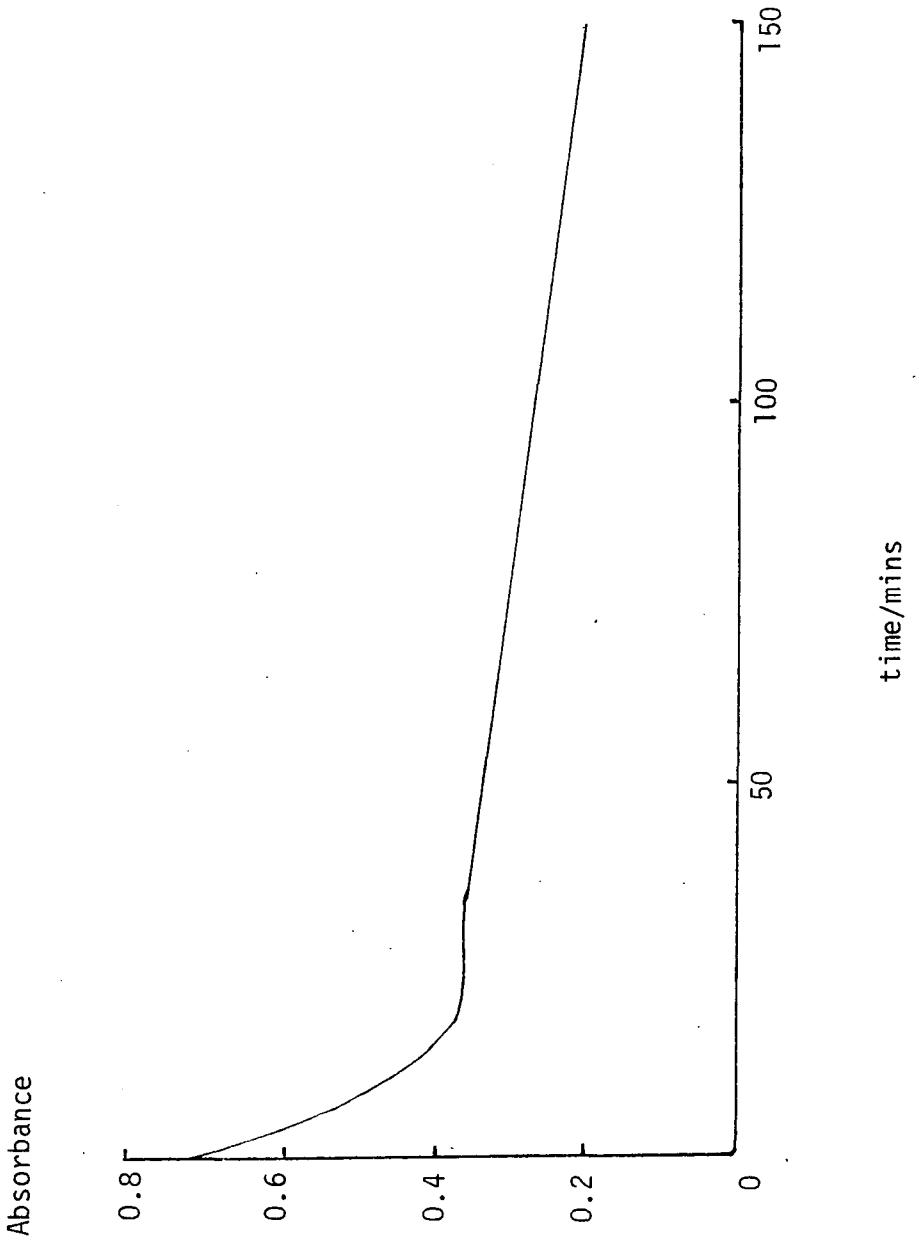


Figure 3.2 : Decomposition of S-nitrosocysteine at pH 7

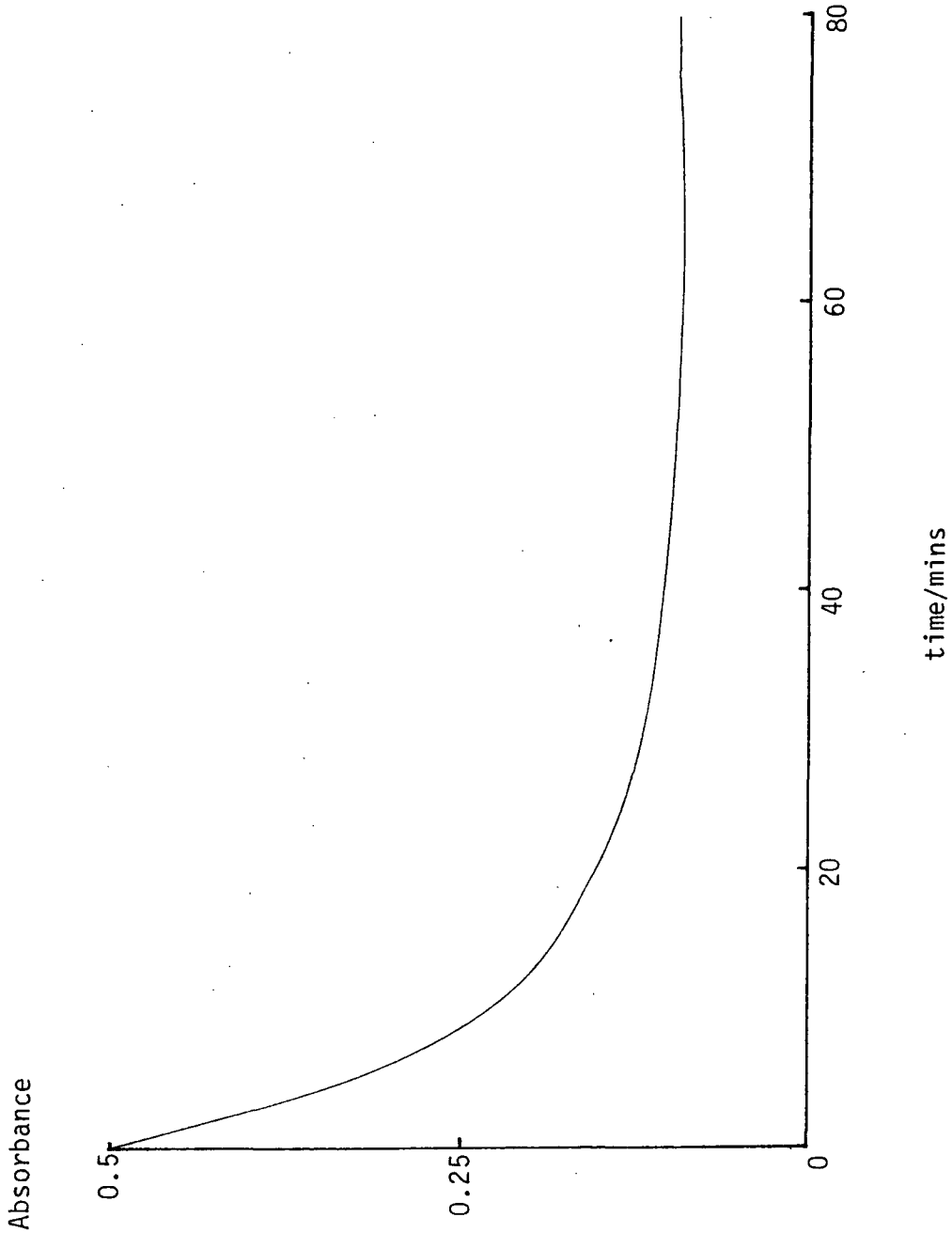
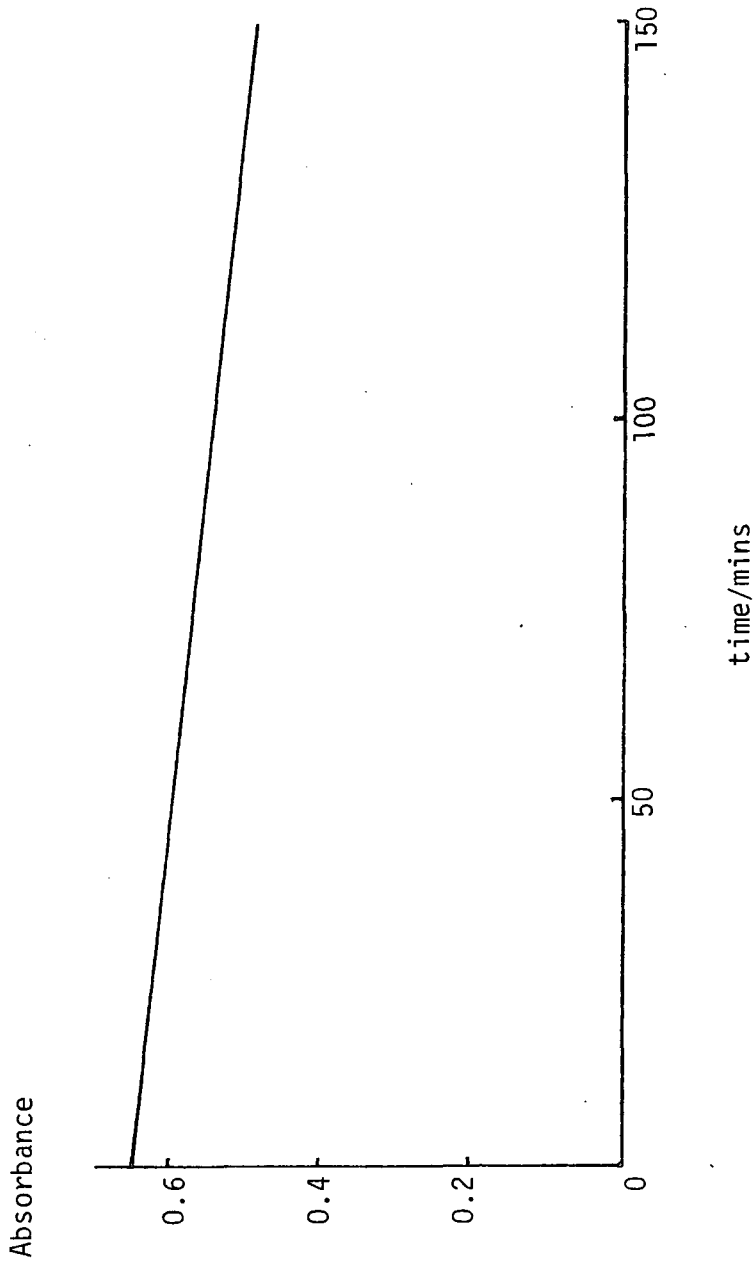


Figure 3.3 : Decomposition of S-nitrosocysteine at pH 9.8



$$RSNO \rightleftharpoons RS^{\cdot} + NO$$

$$RS^{\cdot} + RSNO \longrightarrow RSSR + NO$$

$$RSNO + RSH \longrightarrow RSSR + HNO$$

$$2RS^{\cdot} \longrightarrow RSSR$$

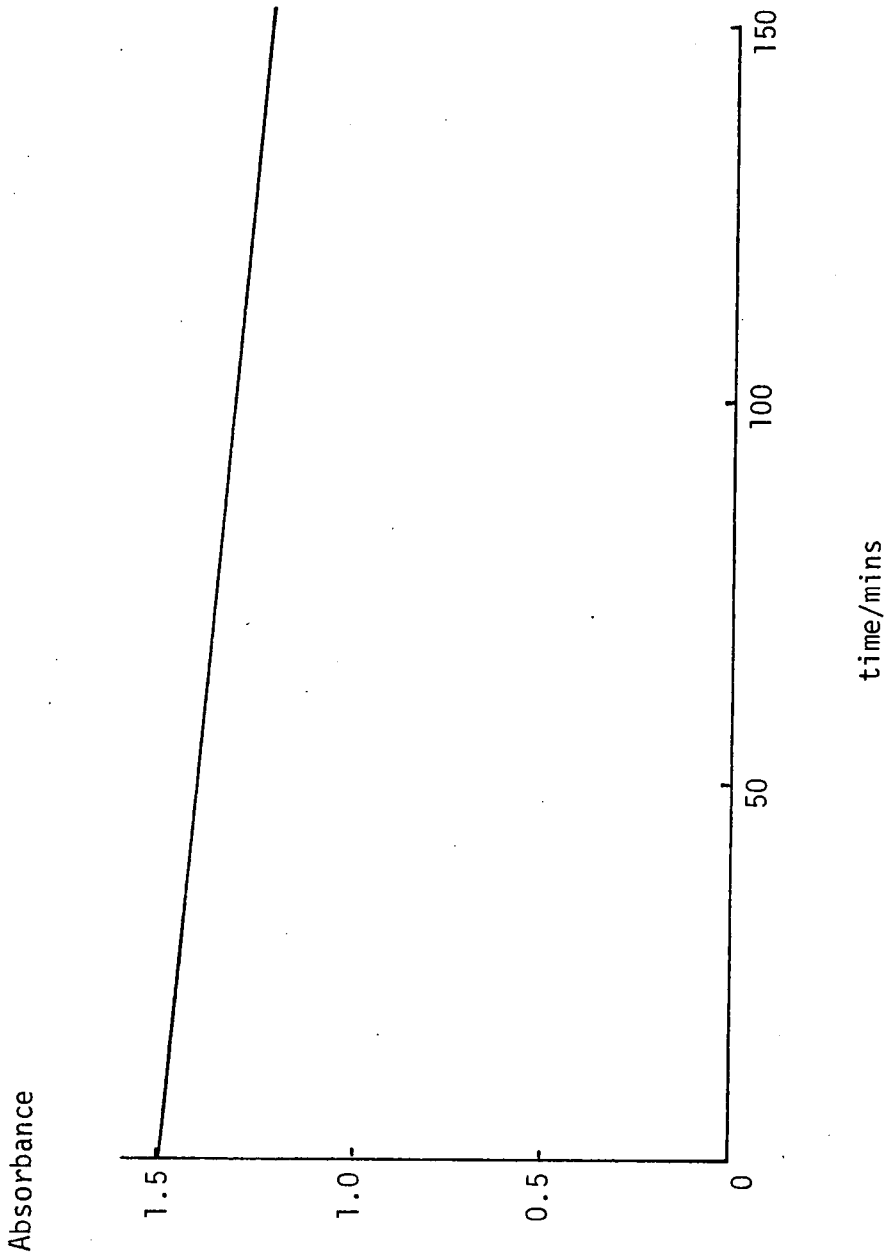
scheme 3.8

The effect of sodium chloride, sodium bromide and sodium thiocyanate on the stability of S-nitrosocysteine at pH 5.5, 7 and 9.8 was also investigated, and also the effect of alanine and sodium bicarbonate at pH 7. Again kinetic interpretation of these profiles was not possible, although comparison of these profiles with the decomposition profile of S-nitrosocysteine at the appropriate pH seems to indicate that none of these compounds had a significant effect, except for sodium thiocyanate at pH 9.8 where a slight catalysis of the decomposition reaction was noted. At pH 5.5 and pH 9.8 sodium thiocyanate had the effect of changing the shape of the decomposition profile.

3.3 Decomposition of S-Nitrosoglutathione

The decomposition of S-nitrosoglutathione, and the effect of certain compounds on the rate of decomposition, at pH 7 was studied and a typical decomposition profile is shown in Figure 3.4. Again this study was carried out to investigate the stability of S-nitrosoglutathione in aqueous solution in view of its potential as a nitrosating agent. Therefore an extensive study into the mechanism was not undertaken, although in the light of the studies on the decomposition of other thionitrites, it would seem likely that a similar mechanism is involved here. It is also possible that there is

Figure 3.4 : Decomposition of S-nitrosoglutathione at pH 7



some unreacted thiol in the reaction vessel due to the conditions used (see Chapter Four) which may effect the decomposition of S-nitrosoglutathione by an analogous mechanism to that proposed in the previous section. The results obtained, however, indicate that S-nitrosoglutathione is more stable in aqueous solution than is S-nitrosocysteine. Again, by comparison of the decomposition profiles of S-nitrosoglutathione in the presence of alanine, sodium chloride and sodium bicarbonate with the decomposition profile of S-nitrosoglutathione at pH 7 suggests that these additives have no significant effect on the rate of decomposition of the thionitrite.

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CHAPTER FOUR

TRANSNITROSATION REACTIONS

4.1 Introduction

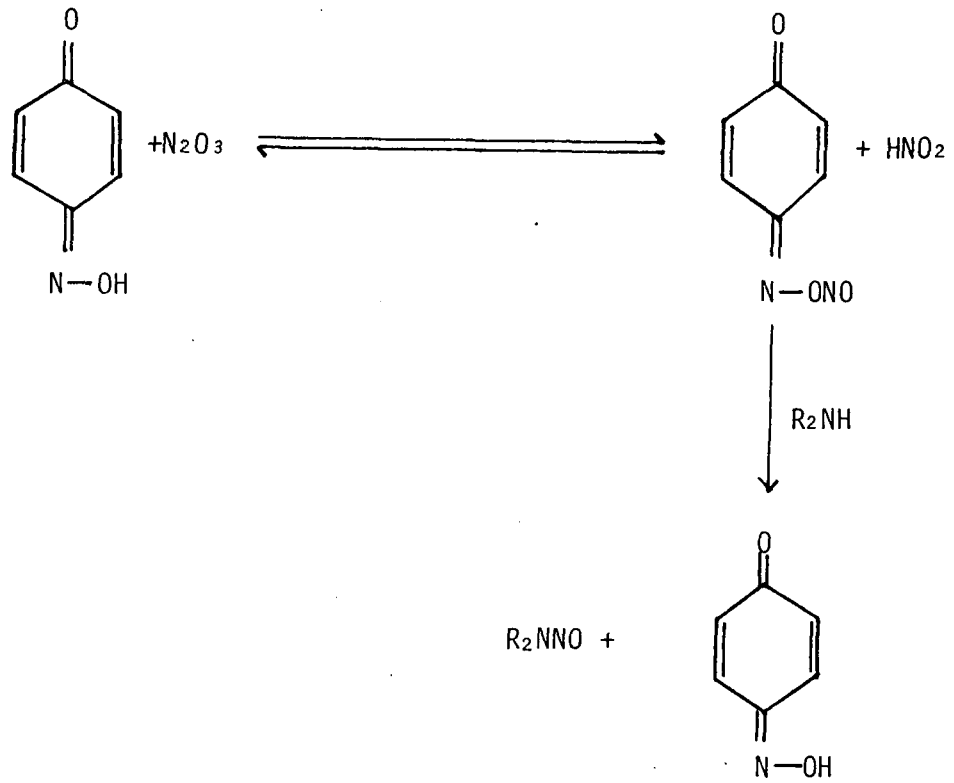
Transnitrosation is the transfer of the nitroso group from one compound to another. Interest in this area then arises due to the possibility that nitroso compounds which are non carcinogenic or weakly carcinogenic may bring about the nitrosation of other substrates and hence form nitroso compounds of higher carcinogenicity.[1]

Alkyl nitrites, as mentioned in Chapter One, have been used for some time to effect the nitrosation of other species.[2] t-Butylnitrite has recently been shown to cause rapid nitrosation of alcohols in non aqueous solvents.[3,4]

Due to the reversibility of O-nitrosation, under acid conditions nitrosation may be occurring via prior hydrolysis of the alkyl nitrite and the liberation of nitrous acid or by a direct transnitrosation. Shenton and Johnson[5] studied the nitrosation of sulphanilamide by cyclohexyl nitrite and nitrous acid and concluded that the nitrosating agent was the same in both cases. Aldred and Williams[6] studied the nitrosation of aniline derivatives in propan-1-ol (to avoid hydrolysis) and showed that propyl nitrite only causes nitrosation very slowly unless nucleophiles such as bromide and thiocyanate ions are present. The inefficiency of alkyl nitrites as nitrosating agents themselves in water or alcohol solvents has also been shown by the presence of alcohols in the nitrosation of N-methylaniline[7] and morpholine[8]. Casado et al studied the nitrosation of morpholine under conditions where nitrous anhydride was the effective nitrosating agent. With the addition of alcohols no first order dependence on the

nitrite concentration was observed which would be expected if the alkyl nitrites became a significant nitrosating agent. Therefore it seems likely that the ability of alkyl nitrites to effect nitrosation in acid solution in the absence of nucleophiles is due to hydrolysis and the liberation of nitrous acid, rather than by direct transnitrosation. However acid catalyzed alcoholysis has been proposed to go via direct transnitrosation,[9] the reaction being inhibited by small amounts of water.

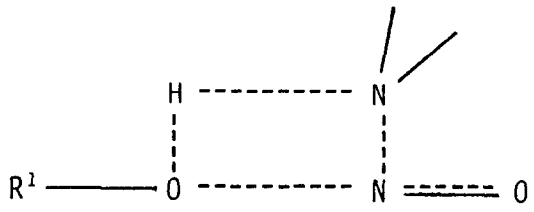
Catalysis of nitrosation reactions by nitrosophenols has been reported.[10] The reaction mechanism proposed involves a direct transnitrosation from the O-nitroso derivative of the oxime, as shown in scheme 4.1



scheme 4.1

A concerted mechanism has also been proposed for the reaction of alkyl nitrites under neutral and alkaline conditions with alcohols[11] and amines.[12,13] Kinetic studies[13] have been

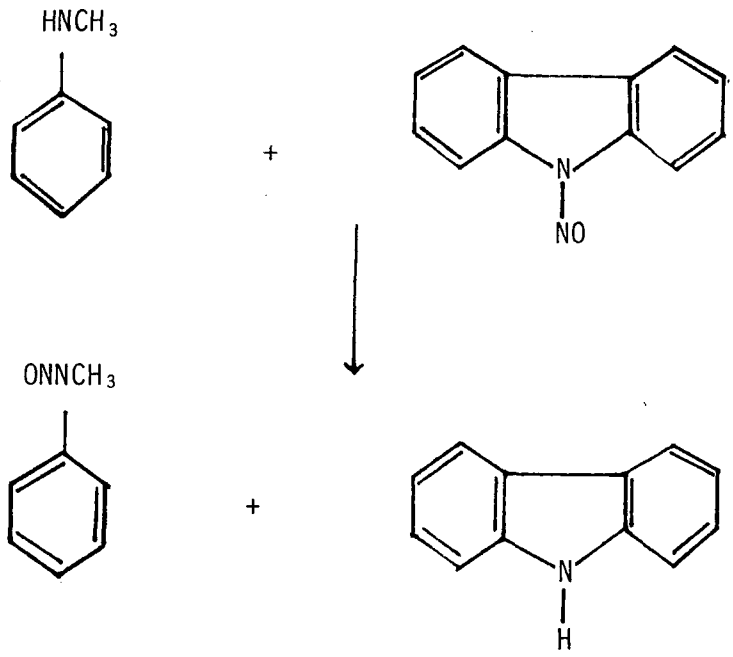
carried out on the reaction between alkyl nitrites and amines and a transition state as shown below proposed,



the same as when this reaction is carried out in 61% dioxan-water.[14]

There are several reports of reactions of nitrosamines with amines, some of which involve hydrolysis of the nitrosamine and then reaction by the liberated nitrous acid, and some of which are thought to go via direct reaction between the nitrosamine and the amine.

Baumgardner et al[15] have shown that N-nitroso-3-nitrocarbazole is an effective nitrosating agent in organic solvents, for example nitrosation of N-methylaniline in boiling benzene.



scheme 4.2

Singer et al have studied the transnitrosation potential of several alicyclic nitrosamines, for example, piperazine, morpholine and piperidine derivatives.[16,17] These reactions were carried out under acid conditions and were catalyzed by the addition of nucleophiles such as chloride, bromide and thiocyanate ions. The order of catalytic activity of the nucleophiles determined was as found for nitrosation reactions, i.e. $SCN^- > Br^- > Cl^-$. They postulated that the reaction was occurring via hydrolysis of the nitrosamine to form an NOX species which is then the effective nitrosating agent.

$$R_2NNO + H^+ \rightleftharpoons R_2\overset{+}{N}HNO$$

$$R_2\overset{+}{N}HNO + X^- \rightleftharpoons R_2NH + NOX$$

$$NOX + R_2{}^1NH \rightleftharpoons R_2{}^1NNO + H^+ + X^-$$

$X^- = Cl^-$, Br^- or SCN^-

scheme 4.3

They did not find any evidence for a direct, uncatalyzed reaction path, i.e. a direct transnitrosation.[17]

Sieper[18] studied the reactions of nitrosodiphenylamine and showed that it could act as a nitrosating agent. Challis and Osbourne[1,19] studied this reaction in more detail and found that the protonated form of the nitrosamine was the reactive entity, and that it reacted by a direct, uncatalyzed mechanism with N-methylaniline. However with sodium azide the reaction was via formation of nitrous acid, as shown in scheme 4.4.

$$Ph_2NNO + H^+ \rightleftharpoons Ph_2\overset{+}{N}HNO$$

$$Ph_2\overset{+}{N}HNO + PhNHMe \rightleftharpoons Ph\overset{+}{N}HMeNO + Ph_2NH$$

$$Ph\overset{+}{N}HMeNO \rightleftharpoons PhNMeNO + H^+$$

$$Ph_2NNO + H^+ \rightleftharpoons Ph_2\overset{+}{N}HNO$$

$$Ph_2\overset{+}{N}HNO + X^- \rightleftharpoons Ph_2NH + NOX$$

$$NOX + N_3^- \rightleftharpoons X^- + NON_3$$

$$NON_3 \rightleftharpoons N_2O + N_2$$

scheme 4.4

The reaction with aniline was found to involve both reaction pathways. Thompson and Williams[20] studied the nitrosation by N-nitrosodiphenylamine of aniline and two primary aliphatic amines in the presence of a nitrite trap to prevent the reaction by free nitrous acid. Of the three amines only aniline was reactive under the conditions used. They determined from the acidity dependence of the rate constant that nitrosation was occurring via the protonated form of the nitrosamine and the anilinium ion, and proposed that the mechanism was similar to that observed for nitrosation of some aromatic amines with pKa >3 whereby the -NO group first interacts with the π electrons on the benzene ring.[21]

Perhaps the best known example of transnitrosation involving S-nitroso species is the catalysis of nitrosation reactions by the thiocyanate ion and thioureas, which was discussed in Chapter One.

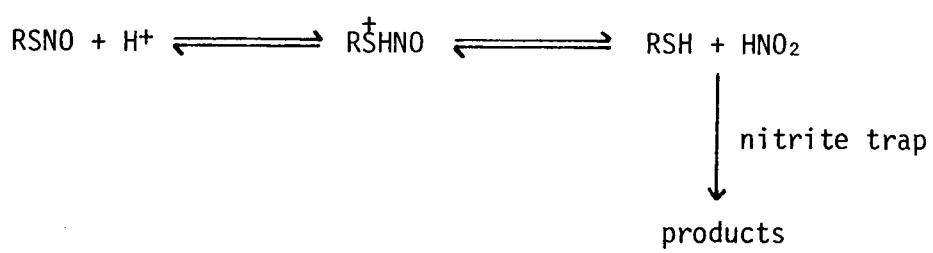
Mirna and Hofmann[22] suggested that thionitrites may act as transnitrosating agents to myoglobin, to form nitrosomyoglobin and the disulphide.

$$2RSNO + 2Mb \longrightarrow RSSR + 2NOMb$$

Dennis et al[23] studied the transnitrosation reaction between S-nitrosocysteine and morpholine, N-methylaniline and pyrrolidine at acid and alkaline pH, and compared this reaction with the direct nitrosation of the amines by nitrous acid. At acid pH (pH 2.65) the transnitrosation reaction was found to be slower than the direct nitrosation, and led to a smaller yield of nitrosamine. At pH 5.5 the rates and yields of the two reactions were comparable, and they found that S-nitrosocysteine could cause nitrosation in alkaline solution (pH 9.8). That the transnitrosation reaction is slower under acid conditions and yields less nitrosamine than nitrosation by nitrous acid may explain why no catalysis by cysteine of the nitrosation of morpholine was observed,[24] and why the addition of thiols to the nitrosation of amines inhibited the reaction.[7] Dennis et al[25] also studied the reaction between S-nitrosoglutathione and a protein-bound nitrite model and N-methylaniline at pH 5.5 and found that transnitrosation by S-nitrosoglutathione was slower than by S-nitrosocysteine. Kunisaki and Hayashi[26] studied the effects of cysteine, glutathione and ergothioneine on dimethylamine nitrosation at pH 2 to 7. Ergothioneine increased the yield of nitrosodimethylamine from pH 3 to 7, whereas cysteine and glutathione increased the yield of nitrosodimethylamine from pH 5 to pH 7.

Other examples of thionitrites acting as nitrosating agents include those reported by Cae et al[27] and Field et al.[28]

These reactions could be taking place by a variety of mechanisms. Hydrolysis of thionitrites only occurs under conditions of high acidity and in the presence of a nitrite trap to remove the nitrous acid.



scheme 4.5

Al-Kaabi et al[29] studied the nitrosation of N-methyl-4-nitroaniline by S-nitroso-N-acetylpenicillamine under these conditions and observed quantitative conversion of the amine to the nitrosamine. However no nitrosamine was formed when the reaction was carried out in the presence of a nitrite trap and concluded that the reaction was occurring via hydrolysis of the thionitrite and then reaction with the liberated nitrous acid.

Under conditions where hydrolysis is less significant, it is possible that either a direct reaction between the thionitrite and the amine is occurring, or that the reaction is occurring due to the decomposition of the thionitrite, which yields nitric oxide.

$$2RSNO \longrightarrow RSSR + 2NO$$

The nitric oxide can then be oxidized to form dinitrogen trioxide and dinitrogen tetroxide, both of which have been shown to be effective nitrosating agents.[30]

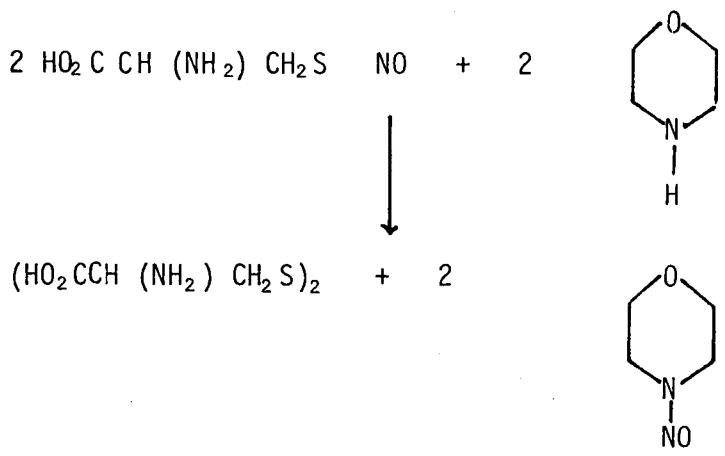
Recently S-nitrosothiosulphate[31] has been shown to be a direct acting nitrosating agent under acid conditions.

The aim of the following studies was to investigate the potential of thionitrites as nitrosating agents at neutral to alkaline pH (the typical small intestine pH is between 6.6 and

7.6)[32] In addition to this the effect of certain species which may be encountered in the small intestine on these reactions was studied.

4.2 Transnitrosation Between S-Nitroso-L-Cysteine and Morpholine

The transnitrosating potential of S-nitrosocysteine (L-cysteine, as mentioned earlier, is a naturally occurring amino acid) towards morpholine was studied.



In this scheme the disulphide is written as the product, which is based on the results of previous work on trans-nitrosation reactions. No attempt was made to isolate this product or identify it.

The effect of acetate, alanine (a naturally occurring amino acid), sodium chloride, sodium bicarbonate (both of which are secreted by the pancreas) and glucose on this reaction was investigated.

4.2.1 Reaction at pH 7 and pH 8

The transnitrosation reaction between S-nitrosocysteine and morpholine was investigated at pH 7 and the effect of the morpholine concentration on the yield of N-nitrosomorpholine was established. The reaction at pH 8 was also studied.

H.P.L.C. was used for analysis of the reaction solutions, the separated peaks being observed at 242 nm.[23] The N-nitrosomorpholine (NMOR) concentration was determined using calibration curves based on peak height measurements. The results of these studies are shown in Tables 4.1, 4.2 and 4.3. The acid concentration quoted in all the Tables refer to the concentration used for the formation of S-nitrosocysteine. These reactions were carried out in duplicate as indicated by presenting the results in terms of "experiments" 1 and 2. This terminology is used throughout this chapter.

Table 4.1: Reaction at pH 7 with equal concentrations of S-nitrosocysteine and morpholine.

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2.1 \times 10^{-3}$ M

$[Morpholine] = 2.3 \times 10^{-3}$ M

Experiment	$10^5[NMOR]/M$
1	6 ± 0.2
2	3.8 ± 0.04

Table 4.2: Reaction at pH 7 with an excess of morpholine

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.8 \times 10^{-2}$ M

Experiment	$10^5[NMOR]/M$
1	9.5 ± 0.1
2	13.3 ± 0.03

Table 4.3: Reaction at pH 8

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.3 \times 10^{-2}$ M

Experiment	$10^5[NMOR]/M$
1	3.65 ± 0.07
2	5.7 ± 0.05

The results given in Tables 4.1 and 4.2 show that the expected dependence of the yield of N-nitrosomorpholine on the morpholine concentration is observed, that is a higher morpholine concentration results in a higher yield of N-nitrosomorpholine.

Under the conditions used the reaction forming the thionitrite was calculated to give 40% S-nitrosocysteine, in agreement with the findings of Byler, Gosser and Susi.[33] Therefore at pH 7 with an excess of morpholine, the reaction goes to give approximately 18% N-nitrosomorpholine based on the actual concentration of S-nitrosocysteine.

The reaction between S-nitrosocysteine and morpholine was also studied using U.V. spectrophotometry measuring the absorbance at 260 nm.[34] A yield of 1.55×10^{-4} M N-nitrosomorpholine was determined, in good agreement with the values obtained using H.P.L.C. (Table 4.2).

Because the reaction to form S-nitrosocysteine only goes to give 40% thionitrite, there is present in the reaction solution a significant amount of unreacted sodium nitrite. Nitrosation by aqueous sodium nitrite solutions has been shown to be

negligible above approximately pH 5.5.[35] However, the direct nitrosation of morpholine was carried out at pH 7. As can be seen from Table 4.4, the yield of N-nitrosomorpholine is much lower than in Table 4.2. Therefore, as expected, direct nitrosation of morpholine by the unreacted sodium nitrite is only playing a small role in the overall reaction.

Table 4.4: Direct nitrosation of morpholine at pH 7.

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

[Morpholine] $= 4.9 \times 10^{-2}$ M

Experiment	10^6 [NMOR]/M
1	3.5
2	2

There are two possible ways that this reaction may be occurring. Either S-nitrosocysteine is acting as a direct nitrosating agent

$$2\ RSNO + 2\ R_2NH \longrightarrow RSSR + 2\ R_2NNO$$

or is decomposing, liberating nitric oxide which, in the presence of oxygen, has been shown to be an effective nitrosating agent in this pH range[30] due to oxidation to nitrogen dioxide and then reaction by dinitrogen trioxide or dinitrogen tetroxide.

$$2\ RSNO \longrightarrow RSSR + 2NO$$

$$NO \xrightarrow{O_2} NO_2$$

$$NO + NO_2 \rightleftharpoons N_2O_3$$

$$N_2O_3 + R_2NH \longrightarrow R_2NNO + HNO_2$$

$$HNO_2 \longrightarrow NO_2^- + H^+$$

$$2NO_2 \rightleftharpoons N_2O_4$$

$$N_2O_4 + R_2NH \longrightarrow R_2NNO + HNO_3$$

$$HNO_3 \longrightarrow NO_3^- + H^+$$

scheme 4.7

The second of these reaction mechanisms is dependent on the presence of oxygen. Nitric oxide itself, in the absence of oxygen or other catalysts has been shown to be an ineffectual nitrosating agent.[30] Therefore with a view to trying to establish which reaction mechanism is occurring in these reactions, the reaction at pH 7 was repeated and nitrogen was bubbled through the solution (see Chapter Five).

Table 4.5: The effect of oxygen

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2.1 \times 10^{-3}$ M

$[Morpholine] = 4.1 \times 10^{-2}$ M

Experiment	10^5 [NMOR]/M
1	7.15
2	8.6

If these results are compared to the results in Table 4.2, it can be seen that this only had a slight effect on the yield of N-nitrosomorpholine. These results suggest that the pathway does not involve nitric oxide and the reactions

outlined in scheme 4.7.

4.2.2 Effect of Some Added Species on The Transnitrosation Reaction

The effect of alanine, sodium acetate, sodium chloride, sodium bicarbonate and glucose on the transnitrosation reaction between S-nitrosocysteine and morpholine was investigated at pH 7. The results are presented in Tables 4.6 to 4.10. The two N-nitrosomorpholine concentrations given for each concentration of the additive represent the results from repeat experiments.

Table 4.6: Effect of alanine

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.8 \times 10^{-2}$ M

10^2 [alanine]/M	10^4 [NMOR]/M
2	1.09 ± 0.03
	1.06 ± 0.01
1	1 ± 0.03
	0.95 ± 0.001
0.2	1.14 ± 0.01
	1.12 ± 0.005

Table 4.7: Effect of Sodium Acetate

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.6 \times 10^{-2}$ M

10^2 [sodium acetate]/M	10^4 [NMOR]/M
2	1.27 ± 0.02
	1.36
1	1.98
	2.01 ± 0.06
0.2	1.62 ± 0.02
	0.87 ± 0.003

Table 4.8: Effect of Sodium Chloride

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.6 \times 10^{-2}$ M

10^2 [NaCl]/M	10^4 [NMOR]/M
2	1.24 ± 0.005
	1.65 ± 0.07
1	1.27 ± 0.01
	1.82 ± 0.005
0.2	1.34 ± 0.008
	1.85 ± 0.005

Table 4.9: Effect of Sodium Bicarbonate

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.6 \times 10^{-2}$ M

$10^2 [NaHCO_3]$/M	$10^4 [NMOR]$/M
2.2	1.45 ± 0.003
	1.87 ± 0.003
1.1	1.29 ± 0.03
	1.88 ± 0.01
0.22	1.1
	1.5 ± 0.03

Table 4.10: Effect of Glucose

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.7 \times 10^{-2}$ M

$10^2 [glucose]$/M	$10^4 [NMOR]$/M
2	1.48 ± 0.02
	2.17 ± 0.03
1	1.37 ± 0.02
	2.01 ± 0.003
0.2	1.4 ± 0.01
	1.19 ± 0.008

Comparison of these results with those in Table 4.2 shows that none of these compounds has a significant effect on the

yield of N-nitrosomorpholine. It might be expected that alanine and glucose would cause a decrease in the yield of N-nitrosomorpholine by competing with morpholine for the effective nitrosating agent. However, no such effect is observed. This lack of effect will be discussed in more detail in section 4.5.

The reaction between S-nitrosocysteine and morpholine in the absence and presence of alanine was monitored as a function of time and the results are shown in Tables 4.11 and 4.12, and graphically in Figure 4.1. The time between points is a function of the analysis time. All times are relative to the time of the first analysis.

Table 4.11: [NMOR] as a function of time

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.8 \times 10^{-2}$ M

Time/mins	10^4[NMOR]/M
0	0.68
19	1.01
38	1.02
56	1.04
76	1

Table 4.12: [NMOR] as a function of time in the presence of alanine.

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

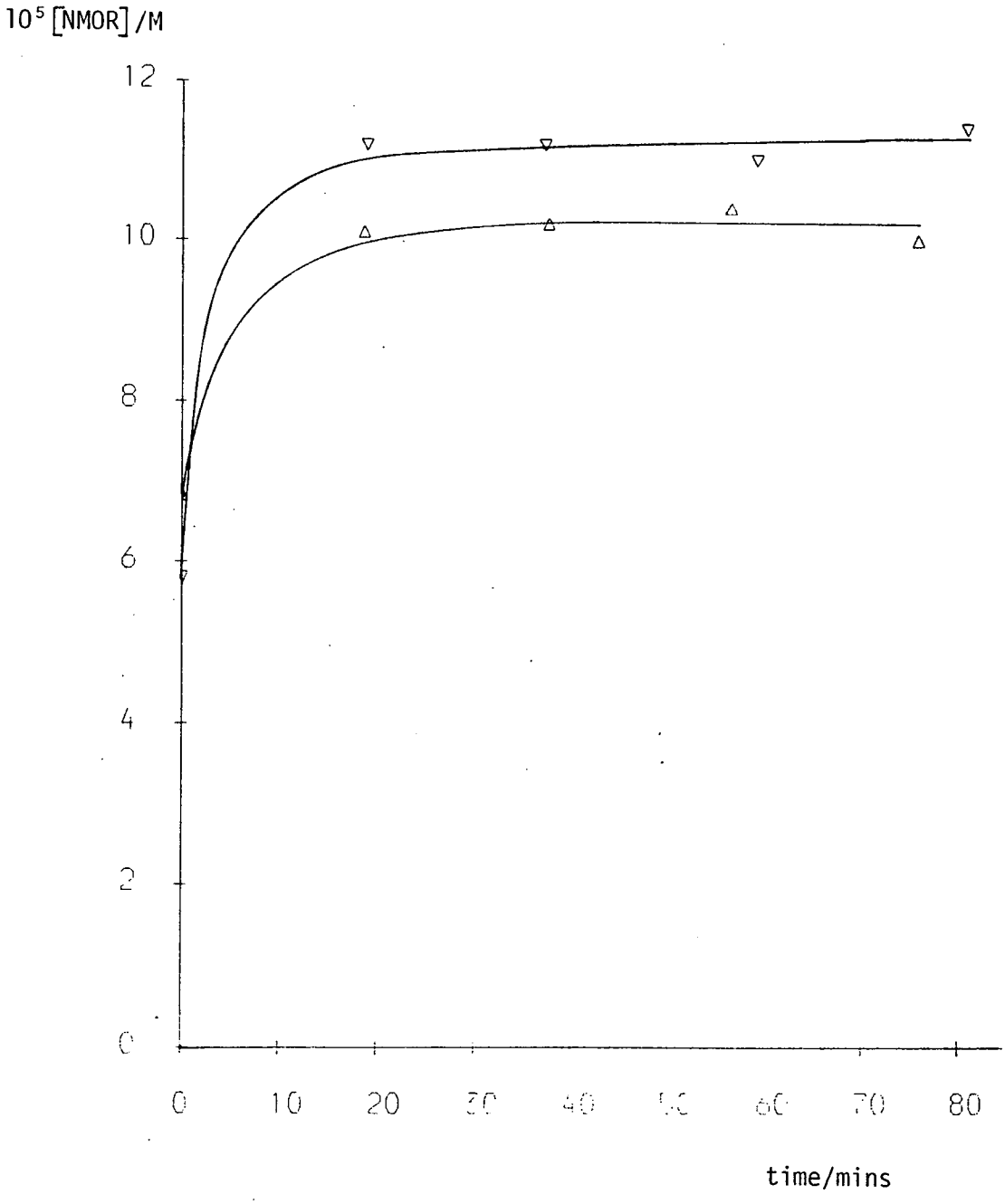
$[Morpholine] = 4.8 \times 10^{-2}$ M

$[Alanine] = 2 \times 10^{-2}$ M

Time/mins	10^4[NMOR]/M
0	0.58
19	1.12
37	1.12
59	1.1
81	1.14

These results show the rapidity of the reaction, a significant amount of N-nitrosomorpholine being formed by the first analysis, and being complete by the second analysis. The reaction also levels off completely, there being no significant increase in the N-nitrosomorpholine concentration from the second analyses to analyses carried out after approximately 24 hours. This, and the lack of effect when the reaction was carried out in the presence of nitrogen, lends weight to the possibility that the reaction may be a direct one. Comparison of the rate of nitrosation of morpholine with the decomposition of S-nitrosocysteine (Chapter Three) shows that after twenty minutes not all the S-nitrosocysteine has decomposed, and if the reaction was occurring via oxidation of the evolved nitric oxide, and then nitrosation by dinitrogen trioxide and/or dinitrogen tetroxide, completion of the reaction at an early stage would not be expected.

Figure 4.1 : Transnitrosation between S-nitrosocysteine and morpholine in the presence and absence of alanine as a function of time.



▽ in the presence of alanine

△ in the absence of alanine

Due to the speed of the reaction, a significant amount of N-nitrosomorpholine is formed during adjustment of the pH of the solution (see Chapter Five). An indication of this is given by the fact that the first analysis of the solution was carried out immediately after the pH of the solution had been adjusted. Therefore the reaction at pH 7 in the presence of glucose was repeated with adjustment of the pH of the solutions of S-nitrosocysteine and morpholine to pH 7 being carried out separately. The results of this experiment are shown in Table 4.13.

Table 4.13: pH adjustment of S-nitrosocysteine and morpholine prior to mixing.

$[Cysteine] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.8 \times 10^{-2}$ M

$10^2[glucose]/M$	$10^4[NMOR]/M$
2	1.12 ± 0.008
	0.73 ± 0.01
1	1.04 ± 0.008
	0.74 ± 0.02
0.2	1.57 ± 0.003
	1.1 ± 0.008

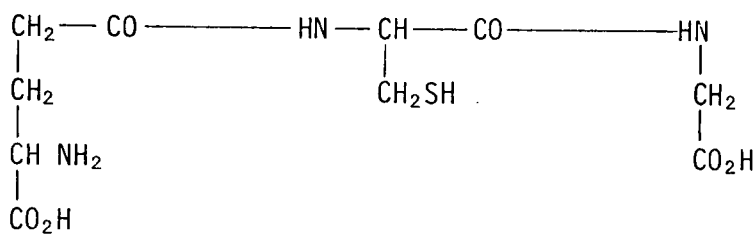
This method did not have a significant effect on the yield of N-nitrosomorpholine as can be seen by comparison of these data with the results in Table 4.10.

Identification of the N-nitrosomorpholine peak in the analyses of the reaction mixtures was done by comparison of the

retention time with the retention time for pure N-nitrosomorpholine. However, retention time is not conclusive proof that the peak observed in the reaction mixture is, in fact, N-nitrosomorpholine. To obtain further evidence for the validity of this assignment, one of the reaction mixtures and a solution of pure N-nitrosomorpholine were analyzed at two wavelengths. If the two peaks are due to the same compound, the ratio of the peak areas at the two wavelengths should be the same. In both cases the ratio was 0.89.

4.3 Transnitrosation Between S-Nitrosoglutathione and Morpholine

Similar studies to those with S-nitrosocysteine were carried out using S-nitrosoglutathione as the thionitrite. Glutathione, as mentioned earlier, is a naturally occurring tripeptide of glutamic acid, cysteine and glycine.



As for the reactions involving S-nitrosocysteine, the acid concentrations quoted in the following Tables refers to the concentration used in the formation of S-nitrosoglutathione.

4.3.1 Reaction at pH 7 and pH 8

The transnitrosation reaction between S-nitrosoglutathione and morpholine was investigated at pH 7 and pH 8 and the results are shown in Tables 4.14 and 4.15.

Table 4.14: pH 7

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.8 \times 10^{-2}$ M

Experiment	$10^4[NMOR]/M$
1	3.37 ± 0.008
2	3.31 ± 0.02

Table 4.15: pH 8

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.6 \times 10^{-2}$ M

Experiment	$10^4[NMOR]/M$
1	2.02 ± 0.005
2	1.86 ± 0.008

The same trend is observed here as with S-nitrosocysteine and morpholine, that is less N-nitrosomorpholine is formed at pH 8 compared to pH 7. On the face of it, it looks as if S-nitrosoglutathione reacts to form more N-nitrosomorpholine. However, if the percentage yield based on the actual concentration of the thionitrite is calculated (the reaction in this case goes to form 80% thionitrite), it shows that this reaction goes to yield 21% N-nitrosomorpholine.

4.3.2 Effect of Some Added Species on The Transnitrosation Reaction

The effect of alanine, sodium acetate, sodium chloride, sodium bicarbonate and glucose on the transnitrosation reaction

between S-nitrosoglutathione and morpholine was investigated at pH 7. The results are presented in Tables 4.16 to 4.20.

Table 4.16: Effect of alanine.

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

[Morpholine] = 4.8×10^{-3} M

10^2 [alanine]/M	10^4 [NMOR]/M
2	3.79 ± 0.03
	2.97 ± 0.03
1	2.83
	3.41 ± 0.09
0.2	2.63 ± 0.02
	2.68 ± 0.01

Table 4.17: Effect of sodium acetate.

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

[Morpholine] = 4.6×10^{-2} M

10^2 [sodium acetate]/M	10^4 [NMOR]/M
2	1.74 ± 0.02
	2.41 ± 0.003
1	1.99 ± 0.005
	1.58 ± 0.003
0.2	1.81 ± 0.05
	1.83 ± 0.02

Table 4.18: Effect of sodium chloride.

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.8 \times 10^{-2}$ M

$10^2[NaCl]/M$	$10^4[NMOR]/M$
2	2.33 ± 0.09
	1.82 ± 0.01
1	1.06
	3.15 ± 0.01
0.2	2.23
	2.65 ± 0.05

Table 4.19: Effect of sodium bicarbonate

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.8 \times 10^{-2}$ M

$10^2[NaHCO_3]/M$	$10^4[NMOR]/M$
2	2.42 ± 0.003
	2.71 ± 0.1
1	2.7 ± 0.2
	2.36 ± 0.02
0.2	1.98 ± 0.03
	2.33 ± 0.03

Table 4.20: Effect of glucose.

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[Morpholine] = 4.7 \times 10^{-2}$ M

10^2 [glucose]/M	10^4 [NMOR]/M
2	2.33 ± 0.03
	3.12 ± 0.03
1	3.73 ± 0.04
	2.27
0.2	2.03
	2.78 ± 0.02

If the data in these Tables are compared to the results in Table 4.14, it can be seen that, as for the experiments involving S-nitrosocysteine, none of these compounds has a significant effect on the yield of N-nitrosomorpholine.

The reaction between S-nitrosoglutathione and morpholine at pH 7 in the absence and presence of alanine was monitored as a function of time. The results are presented in Tables 4.21 and 4.22 and graphically in Figure 4.2. Again the times given are relative to the first analysis.

Table 4.21: [NMOR] as a function of time.

$[GSH] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2.01 \times 10^{-3}$ M

[Morpholine] = 4.72×10^{-3} M

Time/mins	10^4[NMOR]/M
0	0
47	0.42
96	0.97
168	1.87
227	2
292	2.09

Table 4.22: [NMOR] as a function of time in the presence of alanine.

$[GSH] = 2.01 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

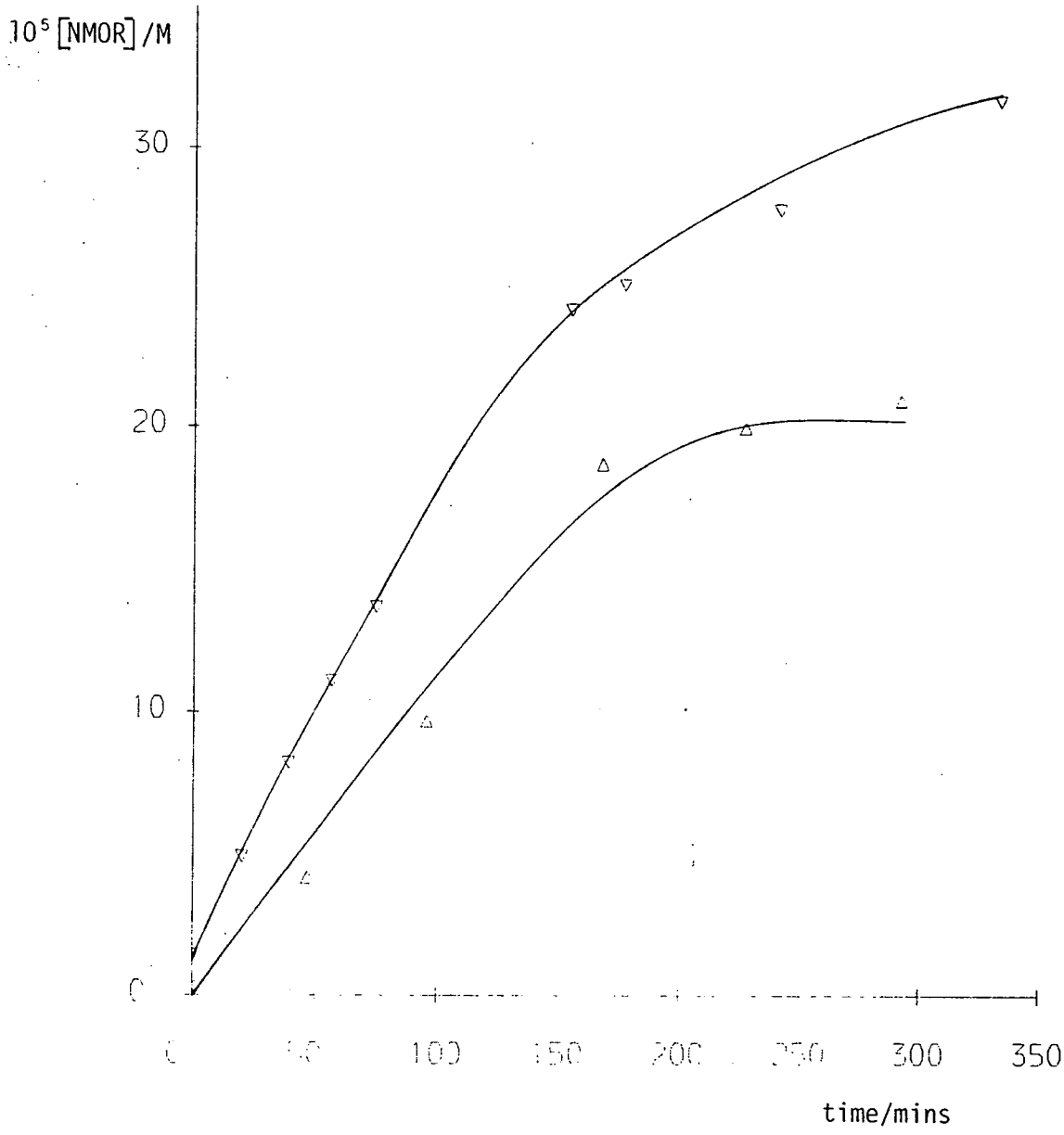
$[NaNO_2] = 2.02 \times 10^{-3}$ M

[Morpholine] = 4.83×10^{-2} M

[Alanine] = 2×10^{-2} M

Time/mins	10^4[NMOR]/M
0	0.14
20	0.49
39	0.82
57	1.11
75	1.37
155	2.42
177	2.51
241	2.71
333	3.17

Figure 4.2 : Transnitrosation between S-nitrosoglutathione and morpholine in the presence and absence of alanine as a function of time.



▽ in the presence of alanine

△ in the absence of alanine

As can be seen from these results the reaction in the presence of alanine is somewhat faster than the reaction in the absence of alanine. It is not clear why this is so.

Again the reaction is complete before all the thionitrite has decomposed (compare with the results in Chapter Three). By the same reasoning used earlier, this increases the likelihood of the transnitrosation being a direct reaction.

4.4 Transnitrosation Between S-Nitroso-N-Acetylpenicillamine and Morpholine

Finally the transnitrosation reaction between S-nitroso-N-acetylpenicillamine and morpholine was studied at pH 7 and pH 8. At pH 7 S-nitroso-N-acetylpenicillamine is less stable than S-nitrosoglutathione, being more comparable with S-nitrosocysteine. The results of these experiments are presented in Tables 4.23 and 4.24.

Table 4.23: pH 7

$[\text{N-Acpen}] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[\text{Morpholine}] = 4.6 \times 10^{-2}$ M

Experiment	$10^4[\text{NMOR}]/\text{M}$
1	2.31 ± 0.04
2	2.15 ± 0.02

Table 4.24: pH 8

$[N\text{-Acpen}] = 2 \times 10^{-3}$ M

$[HClO_4] = 2.1 \times 10^{-3}$ M

$[NaNO_2] = 2 \times 10^{-3}$ M

$[\text{Morpholine}] = 4.3 \times 10^{-2}$ M

Experiment	$10^4[NMOR]/M$
1	2.52 ± 0.02
2	2.42 ± 0.008

The reaction to form the thionitrite of N-acetyl-penicillamine goes to yield 80% S-nitroso-N-acetyl-penicillamine. Calculation of the percentage yield of N-nitrosomorpholine based on the actual thionitrite concentration gives the result that this transnitrosation reaction goes to yield 14% nitrosamine.

The reaction at pH 7 was repeated using U.V. spectrophotometry. The concentration of S-nitroso-N-acetylpenicillamine was approximately 1.5×10^{-3} M and the yield of N-nitrosomorpholine was 3.73×10^{-4} M, or the reaction gave a 25% yield of nitrosamine. This is not in such good agreement as the experiments using S-nitrosocysteine, but in this case the experimental details were different. (see Chapter Five).

4.5 Discussion

As mentioned in section 4.2.1, there are two possible mechanisms by which this reaction may be occurring. The first of these is a direct reaction between the thionitrite and the amine.

$$2\ RSNO + 2\ R_2NH \longrightarrow 2\ R_2NNO + RSSR$$

The second is that the thionitrite decomposes, liberating nitric oxide, which can then be oxidized by oxygen in the headspace of the vessel to form nitrogen dioxide, which then leads to reaction by dinitrogen trioxide and dinitrogen tetroxide, both of which have been shown to be effective nitrosating agents under the conditions used.[30]

$$2\ RSNO \longrightarrow RSSR + NO$$

$$NO \longrightarrow NO_2$$

$$NO_2 + NO \rightleftharpoons N_2O_3$$

$$N_2O_3 + R_2NH \longrightarrow R_2NNO + HNO_2$$

$$OH^- + HNO_2 \longrightarrow H_2O + NO_2^-$$

$$2NO_2 \rightleftharpoons N_2O_4$$

$$N_2O_4 + R_2NH \longrightarrow R_2NNO + HNO_3$$

$$OH^- + HNO_3 \longrightarrow H_2O + NO_3^-$$

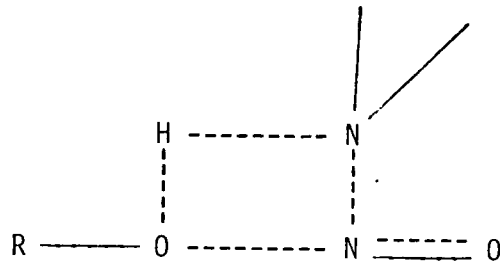
scheme 4.8

Although the first reaction mechanism has not been conclusively proved, there are a number of reasons why this mechanism is preferred to account for the observations described in this Chapter.

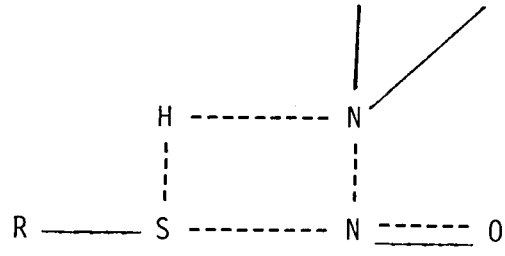
The second mechanism is dependent on the presence of oxygen. Nitric oxide itself, in the absence of oxygen or catalysts, has been shown to be an ineffective nitrosating agent under the conditions used.[30] When the transnitrosation reaction between S-nitrosocysteine and morpholine was carried out under

nitrogen, a significant decrease in the yield of N-nitrosomorpholine, which would be expected if the reaction was occurring via the second mechanism, was not observed. Studies on the reaction as a function of time show that the transnitrosation reaction is complete before all the thionitrite has decomposed. If the nitrosation of morpholine involves prior decomposition of the thionitrite to generate nitric oxide and then reaction by dinitrogen trioxide or dinitrogen tetroxide, then completion of the reaction at this early stage would not be expected.

Studies using alkyl nitrites to nitrosate amines have been carried out under alkaline conditions[13] and in 61% dioxan-water,[14] the reaction being a direct one which involves a transition state as shown below,



the alkyl nitrite reacting with the unprotonated amine. It is possible, therefore, to conceive of a similar transition state being involved in the reaction between thionitrites and amines of the type.



The product of this type of intermediate would be the thiol. In the previous reaction schemes the product has been written as the disulphide. However, no attempt to isolate this end

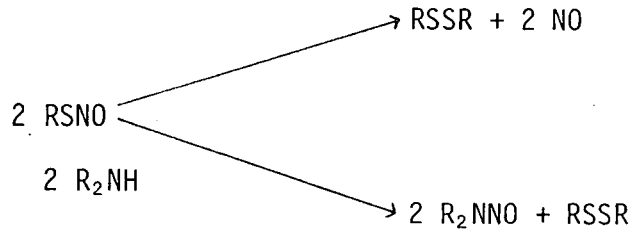
product was attempted, and so either the thiol or disulphide could be the product.

Comparison of the yields of N-nitrosomorpholine formed by the three thionitrites shows that the percentage yields are comparable irrespective of the thionitrite used.

Table 4.25: Percentage yields of NMOR at pH 7

Thionitrite	Percentage yield
S-nitrosocysteine	18%
S-nitrosoglutathione	21%
S-nitroso-N-acetylpenicillamine	14%

The stabilities of these thionitrites are varied. S-nitrosoglutathione is more stable than S-nitrosocysteine or S-nitroso-N-acetylpenicillamine. If the reaction is a direct one, then it would be expected that the more stable the thionitrite, the higher the yield of N-nitrosomorpholine since the thionitrite can either undergo reaction with the amine or decomposition.



Therefore the similarity in percentage yields is a surprising result, and it is not clear why this is so.

Mechanistic studies on the nitrosation of amines by alkyl nitrites[13] have shown that the alkyl nitrite reacts with the unprotonated amine. Casado et al[13] studied the nitrosation of dimethylamine by propyl nitrite in alkaline solution and

found that the nitrosation of dimethylamine was first order with respect to unprotonated amine, the rate of nitrosation increasing with increasing pH until a maximum was reached. Comparison of the yields of N-nitrosomorpholine from the nitrosation of morpholine by the three thionitrites shows that with S-nitrosocysteine and S-nitrosoglutathione the yield of N-nitrosomorpholine is higher at pH 7 compared to pH 8, the difference in the yields being less significant for S-nitrosoglutathione. S-nitroso-N-acetylpenicillamine yields approximately the same N-nitrosomorpholine concentration at both pH 7 and pH 8. This is, again, a surprising result. If the reaction between the thionitrites and morpholine was happening by a similar mechanism, the rate of nitrosation of morpholine should be faster at pH 8 than pH 7 due to the increased concentration of unprotonated amine. Therefore at pH 8 more of the thionitrite would be expected to undergo reaction with the amine rather than decompose, since these pathways are in competition. Studies have also shown the stability of S-nitrosocysteine to increase with increasing pH under neutral to alkaline conditions.[36] Therefore, for a number of reasons an increase in the yield of N-nitrosomorpholine would be expected at pH 8.

Comparison of the rate of nitrosation of morpholine by S-nitrosocysteine and S-nitrosoglutathione at pH 7 shows that the reaction is faster with S-nitrosocysteine, consistent with the findings of Dennis et al.[23,25]

The addition of alanine, sodium acetate, sodium chloride, sodium bicarbonate and glucose to the reaction between S-nitrosocysteine or S-nitrosoglutathione and morpholine had

no significant effect on the yield of N-nitrosomorpholine. It might be expected that glucose and alanine would have an effect since these are both nitrosatable substrates. In considering the effect of glucose on the yield of N-nitroso-morpholine, it has to be borne in mind that the nitrosation of glucose is a reversible reaction,[37] and so a big effect would not be expected. The pKa values for alanine and morpholine are 9.87[38] and 8.5[34] respectively. It has been shown in the reaction of alkyl nitrites with amines[12] that the higher the pKa value of the amine, the more reactive it is towards the alkyl nitrite. However, if the reaction is occurring between the thionitrite and the unprotonated form of the amine, the higher pKa of alanine will mean that it is more extensively protonated than morpholine and hence reaction with alanine is less likely to occur. Also Oae et al[14] studied the nitrosation of several amines by phenethyl nitrite in 61% dioxan-water and found that primary amines were less reactive than secondary amines of similar pKa values by factors of 10 - 470. This may therefore account for the lack of effect of the addition of alanine.

The rate constants for S-nitrosation, as shown by the results in Chapter Two, mean that under gastric conditions the nitrosation of naturally occurring thiols, for example cysteine and glutathione, will occur readily. The transnitrosation results show that under alkaline conditions, such as the small intestine, they can quite rapidly effect the nitrosation of amines and that certain species liable to be encountered in vivo have little effect on the extent of this reaction.

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CHAPTER FIVE

EXPERIMENTAL DETAILS

5.1 Techniques Used

5.1.1 Stopped-Flow Spectrophotometry

The rate constants for the nitrosation of the thiols studied were determined using stopped-flow spectrophotometry, a technique which enables first order rate constants of between approximately 0.01 s^{-1} to 200 s^{-1} to be measured.

The two solutions (one normally being sodium nitrite, and the other containing all the other components of the reaction) are stored in the reservoirs and from there enter two identical syringes. A single piston drives the two syringes so that equal volumes of each solution are mixed (that is, the concentration of each solution is halved on mixing). When the plunger of the third syringe hits the stop, the flow stops, and at the same time presses the trigger which starts the monitoring of the reaction. (Figure 5.1)

The reaction is monitored using a beam of monochromatic light which passes through the cell. This signal is then amplified by a photomultiplier, which has a voltage of about -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 on top of a large voltage, so an equal but opposite voltage is added to the signal, that is, about +6 volts, or the signal is "biased off". Therefore with just 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 on a storage oscilloscope, so that voltage readings could be taken and the rate constants determined.

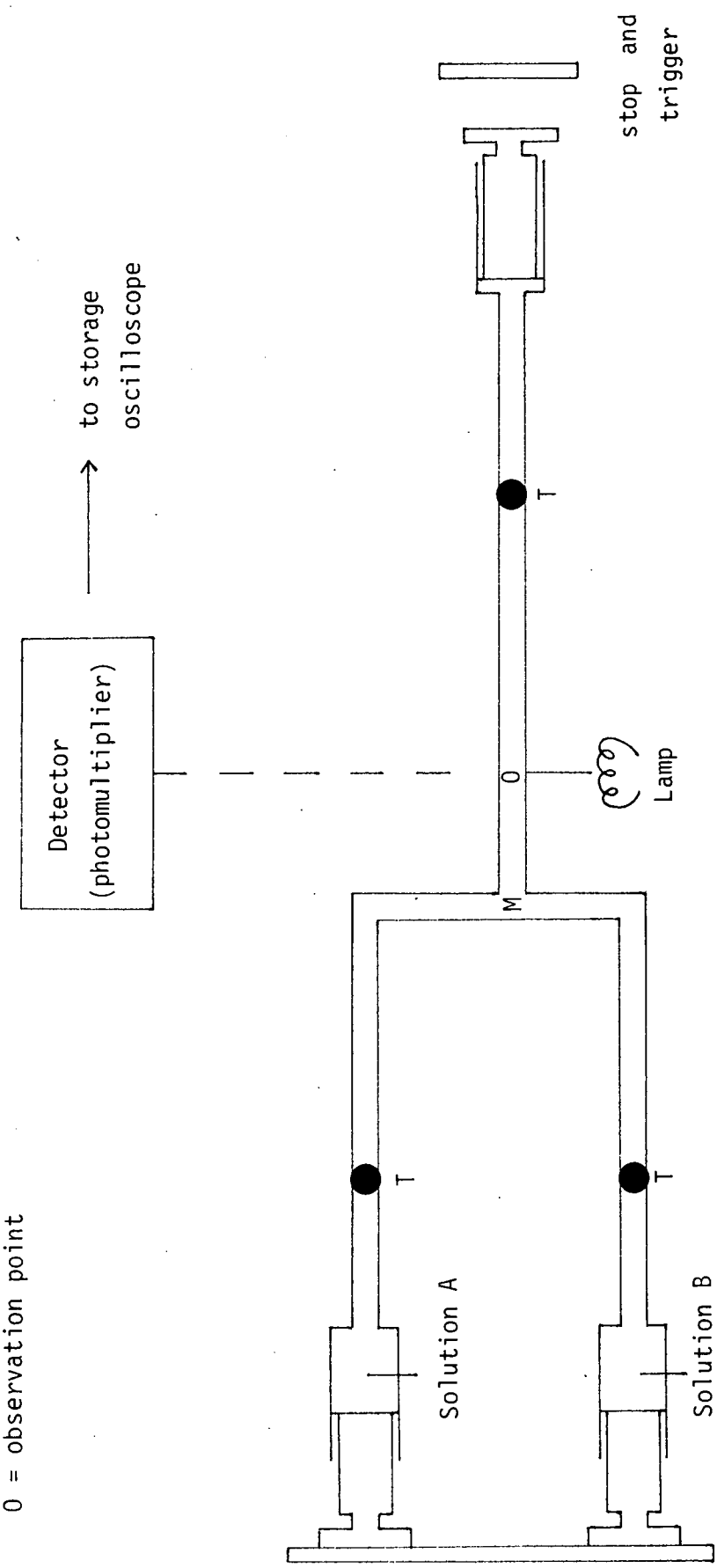


Figure 5.1 : Schematic Diagram of a Stopped-Flow Spectrophotometer

T = three way taps

M = mixing point

O = observation point

5.1.2 U.V./Visible Spectrophotometry

The decomposition of the thionitrites and some of the transnitrosation reactions between S-nitrosocysteine or S-nitroso-N-acetylpenicillamine and morpholine were followed using a thermostated Beckman model 25 recording spectrophotometer. A 1 cm silica cell was filled with part of the solution to be analyzed, and placed in the sample cell holder. An identical cell was filled with solvent (in most cases water, except for the solutions involving S-nitroso-N-acetylpenicillamine, where 5% dioxan in water was used) for the reference. The difference in absorbance between the sample and reference cells was then monitored as a function of time.

5.1.3 High Performance Liquid Chromatography (H.P.L.C.)

The transnitrosation product analysis results were obtained using reversed-phase H.P.L.C., reversed-phase being where separations are carried out on a non-polar column using polar solvents. A Gilson system was used, comprising two model 303 pumps, pressure gauge, injector, column and detector. The solvents used were methanol and water, the methanol being Analar grade, and the water was distilled demineralized water. The solvents were filtered using nylon 66 membrane filters with 0.45 um pore size and ultrasonically degassed under vacuum every day.

The sample was introduced onto the column using a rheodyne model 7125 syringe loading sample injector fitted with a 20 µl loop. This type of injector has six ports; two are connected to the sample loop; two ports are for the solvent flow through the injector; and two are vents. When the injector is in the "load" position, solvent flow bypasses the

sample loop, one end of the loop being connected to the needle port, and the other to one of the vents. 100 µl of the sample of interest was then injected onto the loop using a microlitre syringe. This excess of volume ensures that the loop is flushed from solvent and contains sample only, the excess solution running to waste via one of the vents. To introduce the sample onto the column, the valve rotor is turned to the "inject" position. The solvent flow is then diverted through the sample loop and so onto the column. Separation was then effected using a 25 cm column, i.d. 4.5 mm, packed with 5 um spherisorb O.D.S.2 (i.e. fully capped). The separated components were then observed using a holochrome H/MD detector. The system was operated via an Apple II e microcomputer using Gilson software.

5.2 Determination of the Observed Rate Constants

The rate constants for the nitrosation of the thiols were determined under first order conditions.

For the reaction $R \longrightarrow P$ then

$$-\frac{dR}{dt} = \frac{dP}{dt} = k[R]$$

Integrating

$$\ln \frac{[R]_0}{[R]_t} = -kt \qquad (1)$$

Under the conditions used on the stopped-flow spectrophotometer, that is the voltage change during the reaction is less than 10% of the signal voltage (-6V), the output signal voltage is proportional to the absorbance, which in turn is related to the concentration of the absorbing species by the Beer Lambert law

$$A = \varepsilon c l$$

Since the voltage is proportional to the absorbance

$$V = gA$$

where g is the constant of proportionality. Therefore

$$V = g\varepsilon c l$$

The voltage at time t is then given by

$$V_t = g\varepsilon_R [R]_t \, l + g\varepsilon p \, [P]_t \, l$$

where $[R]_t$ and $[P]_t$ are the concentrations of R and P respectively, and ε_R and εp are their extinction coefficients.

$$\text{But } [P]_t = [R]_0 - [R]_t$$

$$\therefore V_t = g\varepsilon_R [R]_t \, l + g\varepsilon p \, ([R]_0 - [R]_t) \, l$$

$$V_t = [R]_t \, (g\varepsilon_R \, l - g\varepsilon p \, l) + [R]_0 \, g \, \varepsilon p \, l$$

$$\text{but } [R]_0 = [P]_\infty$$

$$\text{so } [R]_0 \, g \, \varepsilon p \, l = [P]_\infty \, g \, \varepsilon p \, l = V_\infty$$

$$\therefore [R]_t = \frac{V_t - V_\infty}{g\varepsilon_R l - g \, \varepsilon p \, l}$$

$$V_0 = g \, \varepsilon_R \, [R]_0 \, l$$

$$\text{and } V_\infty = g \, \varepsilon p \, [P]_\infty \, l = g \, \varepsilon p \, [R]_0 \, l$$

$$\text{and so } V_0 - V_\infty = [R]_0 \, (g \, \varepsilon_R \, l - g \, \varepsilon p \, l)$$

$$\therefore [R]_0 = \frac{V_0 - V_\infty}{g \, \varepsilon_R \, l - g \, \varepsilon p \, l}$$

$$\therefore \frac{[R]_t}{[R]_0} = \frac{V_t - V_\infty}{V_0 - V_\infty}$$

substituting into equation (1) gives

$$\ln\left(\frac{V_t - V_\infty}{V_0 - V_\infty}\right) = -kt$$

or $\ln (V_t - V_\infty) = -kt + \ln (V_0 - V_\infty)$

Therefore $\ln (V_t - V_\infty)$ was plotted against time, which gave straight line graphs, the gradient of which corresponds to -k.

Errors in measurement of fast reactions of this type are generally greater than they are for conventional spectrophotometry. Therefore the mean k_0 values quoted are the mean of at least five separate measurements, and the error quoted is the standard deviation between the individual k_0 values.

5.3 Chemical Reagents

All the thiols used were purchased commercially and, except for cysteine and thioglycolic acid, stored desiccated in a refrigerator. Thioglycolic acid was stored in a refrigerator.

S-nitroso-N-acetyl-D,L, penicillamine was stored refrigerated. Alanine was purchased commercially and stored in a refrigerator.

Sodium chloride, sodium bromide, sodium thiocyanate, sodium bicarbonate, sodium acetate, glucose and sodium nitrite were all available commercially and used as supplied.

Morpholine was purchased commercially, distilled under reduced pressure prior to use and was stored refrigerated.

Perchloric acid solutions were prepared by the appropriate dilution of 60-62% perchloric acid and were standardized against sodium hydroxide using phenol phthalein as the indicator.

Sodium nitrite solutions were prepared regularly.

5.4 Kinetic and Product Study Measurements

5.4.1 Nitrosation of Thiols

The rate measurements were carried out using a stopped-flow spectrophotometer. The reaction was started, as explained earlier, by mixing together equal amounts of the two solutions, one containing sodium nitrite, the other containing the thiol and acid and a nucleophile where appropriate. The reaction was followed by monitoring the increase in absorbance at 330 nm[1] due to the appearance of the thionitrite. The conditions used were such that the concentrations of all the other species were in excess of the sodium nitrite concentration, and good first order plots of ln $(V_\infty - V_t)$ against time were obtained. Typical kinetic runs for each of the thiols studied are shown in Tables 5.1 and 5.3 to 5.8 along with the instantaneous values of k_0 which are included merely to show the error within a run. Table 5.2 shows a typical set of individual k_0 values from which the mean k_0 value was calculated.

Table 5.1: A typical kinetic run for the acid catalyzed nitrosation of cysteine.

$[NaNO_2] = 1 \times 10^{-4}$ M

[Cysteine] $= 1 \times 10^{-2}$ M

$[HClO_4] = 0.194$ M

t/s	V_t/mV	k_0/s^{-1}
0	225	
0.5	262.5	0.697
1	290	0.713
1.5	307.5	0.694
2	320	0.683
2.5	330	0.694
∞	352.5	

mean $k_0 = 0.696 \pm 0.01\ s^{-1}$

Table 5.2: A typical set of k_0 values

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Cysteine] = 1 \times 10^{-2}$ M

$[HClO_4] = 0.194$ M

Run	k /s^{-1}
1	0.682
2	0.697
3	0.689
4	0.701
5	0.689
6	0.685

mean $k_0 = 0.69 \pm 0.007$ s^{-1}

Table 5.3: A typical kinetic run for the acid catalyzed nitrosation of cysteine methyl ester hydrocholoride

$[NaNO_2] = 1 \times 10^{-4}$ M

[MeCys] = 1×10^{-2} M

$[HClO_4] = 0.198$ M

t/s^{-1}	V_t/mV	k_0/s^{-1}
0	217.5	
0.5	238.8	0.506
1	252.5	0.46
1.5	265	0.462
2	275	0.465
2.5	282.5	0.461
∞	312.5	

mean $k_0 = 0.471 \pm 0.018\ s^{-1}$

Table 5.4: A typical kinetic run for the acid catalyzed nitrosation of N-acetylcysteine

$[NaNO_2] = 1 \times 10^{-4}$ M

$[N\text{-}AcCys] = 5 \times 10^{-3}$ M

$[HClO_4] = 0.195$ M

t/s	V_t/mV	k /s^{-1}
0	150	
0.2	195	1.55
0.4	230	1.61
0.6	255	1.62
0.8	272.5	1.62
1	285	1.61
1.2	295	1.63
∞	318.8	

mean $k_0 = 1.61 \pm 0.026$ s^{-1}

Table 5.5: A typical kinetic run for the acid catalyzed nitrosation of penicillamine.

$[NaNO_2] = 1 \times 10^{-4}$ M

$[Pen] = 6.01 \times 10^{-3}$ M

$[HClO_4] = 0.197$ M

t/s	V_t/mV	k /s^{-1}
0	160	
2	195	0.105
4	225	0.108
6	245	0.103
8	260	0.097
10	277.5	0.101
12	290	0.101
14	300	0.101
∞	345	

mean $k_0 = 0.102 \pm 0.003$

Table 5.6: A typical kinetic run for the acid catalyzed nitrosation of N-acetylpenicillamine

$[NaNO_2] = 1 \times 10^{-4}$ M

[N-Acpen] $= 3 \times 10^{-3}$ M

$[HClO_4] = 0.197$ M

t/s	V_t/mV	k_0/s^{-1}
0	140	
0.5	170	0.455
1	192.5	0.44
1.5	212.5	0.451
2	228.8	0.46
2.5	240	0.453
3	250	0.456
∞	287.5	

mean $k_0 = 0.453 \pm 0.006\ s^{-1}$

Table 5.7: A typical kinetic run for the acid catalyzed nitrosation of glutathione.

$[NaNO_2] = 1 \times 10^{-4}$ M

$[GSH] = 1 \times 10^{-2}$ M

$[HClO_4] = 1.02 \times 10^{-2}$ M

t/s	V_t/mV	k_0/s^{-1}
0	170	
2	205	0.133
4	232.5	0.135
6	252.5	0.133
8	270	0.137
10	280	0.132
12	290	0.134
∞	330	

mean $k_0 = 0.134 \pm 0.002\ s^{-1}$

Table 5.8: A typical kinetic run for the acid catalyzed nitrosation of thioglycolic acid

$[NaNO_2] = 1 \times 10^{-4}$ M

$[TGA] = 2 \times 10^{-2}$ M

$[HClO_4] = 2.03 \times 10^{-3}$ M

t/s	V_t/mV	k_0/s^{-1}
0	100	
0.2	140	0.97
0.4	177.5	1.04
0.6	205	1.03
0.8	227.5	1.03
1	247.5	1.05
1.2	262.5	1.04
∞	327.5	

mean $k_0 = 1.03 \pm 0.03\ s^{-1}$

5.4.2 Decomposition of Thionitrites

All experimental measurements concerning thionitrite decomposition were made using a Beckman model 25 recording spectrophotometer thermostated at 25°C. The S-nitroso derivative was formed in situ from equimolar amounts of sodium nitrite, perchloric acid and thiol, followed by the

addition of any other reactant as appropriate (total volume 20 ml). The pH of the solution was then adjusted, using a PTI-6 universal digital pH meter, by the addition of perchloric acid and sodium hydroxide until the desired pH of the solution was obtained. A small part of the solution was then transferred into a 1 cm silica cuvette and measurements were made as described in section 5.1.2 at 330 nm.

5.4.3 Transnitrosation Reactions

The majority of the results described in Chapter Four were obtained using H.P.L.C. The S-nitroso derivative was generated in situ as for the decomposition studies, then the additive was added as required, and finally the amine (total volume 25 ml). The pH of this solution was then adjusted using a PTI-6 universal digital pH meter, and then placed in a water bath thermostated at 37^0C. The pH of the solutions was measured prior to analysis; 80% staying within 0.3 pH of the desired pH for those involving S-nitrosocysteine, and 80% remaining within 0.5 pH of the desired pH for solutions containing S-nitrosoglutathione. Solutions were then analyzed as described in section 5.1.3, using a solvent composition of 35% methanol, 65% water and the separated peaks were observed at 242 nm^2. Analysis of the resulting chromatograms was done by peak height measurements. A calibration curve was constructed by the analysis of known concentrations of N-nitrosomorpholine (NMOR) by the above method and a graph of peak height against NMOR concentration was plotted. As can be seen from Figures 5.2 and 5.3 these calibration points gave good linear plots going through the origin. The NMOR

Figure 5.2 : Standard curve of peak height against [NMOR]

.5 range

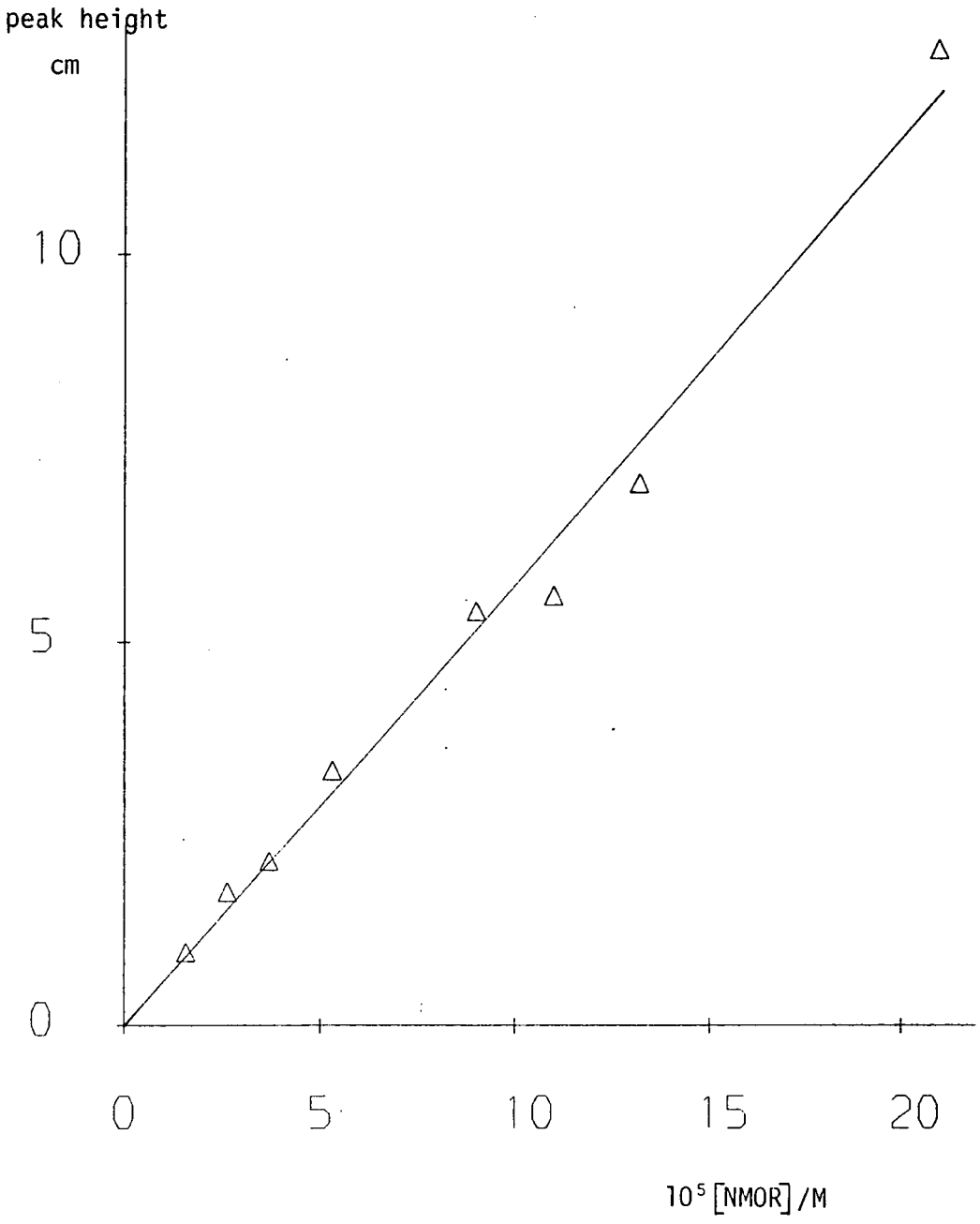
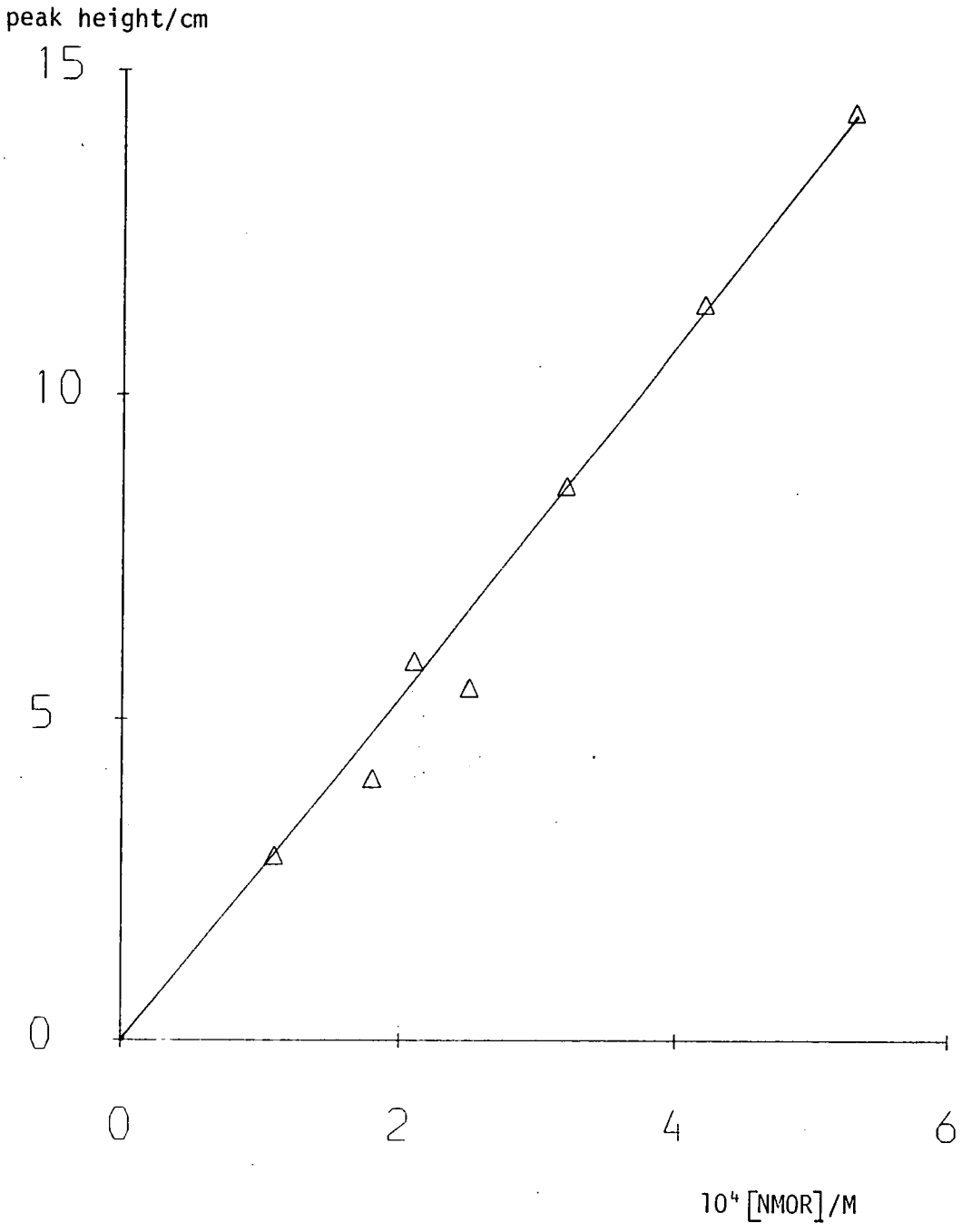


Figure 5.3 : Standard curve of peak height against [NMOR]

1 range



concentrations in the reaction solutions could then be read off these graphs.

As the solvent composition used was of insufficient strength to elute the morpholine, after every five analyses the solvent composition was increased to 100% methanol to clean the column.

The experiment to study the effect of oxygen on the reaction was done by adjusting the pH of the morpholine solution to pH 7 and then nitrogen was bubbled through the solution for about five minutes. Nitrogen was bubbled through the S-nitrosocysteine solution for five minutes prior to pH adjustment as this solution is acidic and S-nitrosocysteine is relatively stable at low pH. The passing of nitrogen through this solution was continued whilst the pH of the solution was adjusted to pH 7, the morpholine added, and the final adjustment of the pH of the solution was carried out. The stoppers to the flask were well greased.

For those experiments carried out using the Beckman model 25 recording spectrophotometer, thermostated at 37°C, the solutions involving S-nitrosocysteine were prepared as before and then analyzed by monitoring the increase in absorbance due to the formation of NMOR at 260 nm[3]. As S-nitrosocysteine also absorbs at this wavelength, a solution containing only S-nitrosocysteine at the same concentration was also prepared, and the difference in the infinity absorbances between these two solutions gave the absorbance due to NMOR. This was converted into NMOR concentration using the extinction coefficient of 3540 mol $l^{-1}cm^{-1}$ at 260 nm[3].

The results using S-nitroso-N-acetylpenicillamine as the thionitrite and u.v. spectrophotometry were obtained using the solid S-nitroso derivative. Due to the instability of the thionitrite in solution approximately 0.0065g was weighed out (giving approximately 1.5×10^{-3} M thionitrite in 20 ml) and then dissolved immediately before use, 1 ml of dioxan being added to facilitate dissolution. The solution was then analyzed as above. Again the thionitrite absorbs at 260 nm and so a solution of only the thionitrite was prepared of the same concentration. The difference in infinity absorbances of these two solutions gave the absorbance due to NMOR.

REFERENCES

1. L.R. Dix and D.L.H. Williams; J. Chem. Soc. Perkin Trans. 2; 1984, 109

2. M.J. Dennis, R. Davies and D.J. McWeeny; J. Sci. Food Agric.; 1979, 30, 639.

3. T.-Y. Fan and S.R. Tannenbaum; J. Agric. Food Chem.; 1973, 21, 237.

APPENDIX

Lectures and Seminars Organized by the Department of Chemistry and Durham University Chemical Society during the Period 1983 - 1986.

(* denotes lectures attended)

* 5.10.83 Prof. J. P. Maier (Basel, Switzerland).
"Recent Approaches to Spectroscopic characterization of Cations."

12.10.83 Dr. C. W. McLeland (Port Elizabeth, Australia).
"Cyclization of Aryl Alcohols Through the Intermediacy of Alkoxy Radicals and Aryl Radical Cations."

19.10.83 Dr. N. W. Alcock (Warwick).
"Aryl Tellurium (iv) Compounds, Patterns of Primary and Secondary Bonding."

20.10.83 Prof. R. B. Cundall (Salford).
"Explosives".

26.10.83 Dr. R. H. Friend (Cavendish, Cambridge).
"Electronic Properties of Conjugated Polymers."

3.11.83 Dr. G. Richards (Oxford).
"Quantum Pharmacology."

* 10.11.83 Dr. J. H. Ridd (U.C.L.).
"Ipso-Attack in Electrophilic Aromatic Substitution."

17.11.83 Dr. J. Harrison (Sterling Organic).
"Applied Chemistry and the Pharmaceutical Industry."

24.11.83 Prof. D. A. King. (Liverpool)
"Chemistry in 2-Dimensions."

30.11.83 Prof. I.M.G. Cowie (Stirling).

"Molecular Interpretation of Non-Relaxation Processes In Polymer Glasses."

1.12.83 Dr. J. D. Coyle (The Open University).

"The Problem with Sunshine."

2.12.83 Dr. G. M. Brooke (Durham).

"The Fate of the Ortho-Fluorine in 3,3-Sigmatropic Reactions Involving Polyfluoro-Aryl and -Heteroaryl Systems."

14.12.83 Prof. R. J. Donovan (Edinburgh).

"Chemical and Physical Processes Involving the Ion-Pair States of the Halogen Molecules."

10.1.84 Prof. R. Hester (York).

"Nanosecond Laser Spectroscopy of Reaction Intermediates."

18.1.84 Prof. R. K. Harris (U.E.A.).

"Multi-Nuclear Solid State Magnetic Resonance."

26.1.84 Prof. T. L. Blundell (Birkbeck College, London).

"Biological Recognition : Interactions of Macromolecular Surfaces."

2.2.84 Prof. N.B.H. Jonathan (Southampton).

"Photoelectron Spectroscopy - a Radical Approach."

8.2.84 Dr. B. T. Keaton (Kent).

"Multi-Nuclear N.M.R. Studies."

15.2.84 Dr. R. M. Paton (Edinburgh).

"Heterocyclic Syntheses Using Nitrile Sulphides."

16.2.84 Prof. D. Phillips (The Royal Institute).

"Luminescence and Photochemistry - a Light Entertainment."

23.2.84 Prof. F.G.A. Stone, F.R.S. (Bristol).

"The Use of Carbene and Carbyne Groups to Synthesize Metal Clusters."

1.3.84 Prof. A. J. Leadbetter (Rutherford Appleton Labs.)

"Liquid Crystals."

7.3.84 Dr. R. T. Walker (Birmingham)

"Synthesis and Biological Properties of some 5-Substituted Uracic Derivatives; Yet Another Example of Serendipity in Anti-Viral Chemotherapy."

8.3.84 Prof. D. Chapman (Royal Free Hospital School of Medicine, London).

"Phospholipids and Biomembranes, Basic Science and Future Techniques."

21.3.84 Dr. P. Sherwood (Newcastle).

"X-Ray Photoelectron Spectroscopic Studies of Electrode and Other Surfaces."

21.3.84 Dr. G. Beamson (Durham/Kratos).

"EXAFS : General Principles and Applications."

23.3.84 Dr. A. Ceulmans (Leuven).

"The Development of Field-Type Models of the Bonding in Molecular Clusters."

28.3.84 Prof. H. Schmidbaur (Munich, F.R.G.)

"Ylides in Coordination Sphere of Metal : Synthetic, Structural and Theoretical Aspects."

2.4.84 Prof. K. O'Driscoll (Waterloo).

"Chain Ending Reactions in Free Radical Polymerization."

3.4.84 Prof. C. H. Rochester (Dundee).

"Infra-red Studies of Adsorption at the Solid-Liquid Interface."

25.4.84 Dr. R. M. Acheson (Biochemistry, Oxford).

"Some Heterocyclic Detective Stories."

27.4.84 Dr. T. Albright (Houston, U.S.A.)

"Sigmatropic Rearrangements in Organometallic Chemistry."

14.5.84 Prof. W. R. Dolbier (Florida, U.S.A.)

"Cycloaddition Reactions of Fluorinated Allenes."

16.5.84 Dr. P. J. Garrett (U.C.L.)

"Syntheses with Dilithiated Vicinal Diesters and Carboximides."

22.5.84 Prof. F. C. de Schryver. (Leuven)

"The Use of Luminescence in the Study of Micellar Aggregates" and
"Configurational and Conformational Control in Excited State Complex Formation."

23.5.84 Prof. M. Tada (Waseda, Japan).

"Photochemistry of Dicyanopyrazine Derivatives."

31.5.84 Dr. A. Haaland (Oslo).

"Electron Diffraction Studies of Some Organo-Metallic Compounds."

11.6.84 Dr. J. B. Street (I.B.M., California).

"Conducting Polymers Derived From Pyrroles."

19.9.84 Dr. C. Brown (I.B.M., California)

"New Superbase Reactions with Organic Compounds."

21.9.84 Dr. H. W. Gibson (Signal UOP, Illinois).

"Isomerization of Polyacetylene."

18.10.84 Dr. N. Logan (Nottingham).

"N_2O_4 and Rocket Fuel."

19.10.84 Dr. A. Germain (Languedoc, Montpellier).

"Anodic Oxidation of Perfluoro Organic Compounds in Perfluoroalkane Sulphonic Acids."

24.10.84 Prof. R. K. Harris (Durham).

"N.M.R. Studies."

25.10.84 Dr. W. J. Feast (Durham).

"Synthesis of Conjugated Polymers : How and Why?"

28.10.84 Dr. R. Snaith (Strathclyde).

"Exploring Lithium Chemistry : Novel Structures, Bonding and Reagents."

7.11.84 Prof. W. W. Porterfield (Hampden - Sydney College, U.S.A.)

"There is no Borane Chemistry (only Geometry)"

7.11.84 Dr. H. S. Munroe (Durham).

"New Information From E.S.C.A. Data."

8.11.84 Prof. B. J. Aylett (Queen Mary College, London)

"Silicon - Dead Common or Refined?"

15.11.84 Prof. B. T. Golding (Newcastle-upon-Tyne).

"The Vitamin B_{12} Mystery."

21.11.84 Mr. N. Everall (Durham).

"Picosecond Pulsed Laser Raman Spectroscopy."

*22.11.84 Prof. D. T. Clark (I.C.I. New Science Group).

"Structure, Bonding, Reactivity and Synthesis as Revealed by E.S.C.A."

27.11.84 Dr. W. J. Feast (Durham).

"A Plain Man's Guide to Polymeric Organic Metals."

*28.11.84 Dr. T. A. Stephenson (Edinburgh).

"Some Recent Studies in Platinum Metal Chemistry."

*29.11.84 Prof. C.J.M. Stirling (University College of North Wales)

"Molecules Taking the Strain."

* 6.12.84 Prof. R. D. Chambers (Durham).

"The Unusual World of Fluorine."

12.12.84 Dr. K. B. Dillon (Durham).

"^{31}P NMR Studies of Some Anionic Phosphorous Complexes."

*11.1.85 Emeritus Prof. H. Suschitzkey (Salford).

"Fruitful Fissons of Benzafuroxanes and Isobenzimidazoles (Umpolung of o-Phenyl-Enediamines."

*24.1.85 Dr. A. K. Covington (Newcastle-upon-Tyne).

"Chemistry with Chips."

*31.1.85 Dr. M.L.H. Green (Oxford).

"Naked Atoms and Negligee Ligands."

* 7.2.85 Prof. A. Ledwith (Pilkington Bros.)

"Glass as a High Technology Material."

13.2.85 Dr. G.W.J. Fleet (Oxford).

"Synthesis of Some Alkaloids From Carbohydrates."

*14.2.85 Dr. J. A. Salthouse (Manchester).

"Son et Lumière"

*19.2.85 Dr. D. J. Mincher (Durham).

"Stereoselective Synthesis of Some Novel Anthracyclinones Related to the Anti-Cancer Drug Adriamycin and to the Steffimycin Antibiotics."

21.2.85 Prof. P. M. Maitlis, F.R.S. (Sheffield).

"What Use is Rhodium?"

27.2.85 Dr. R. E. Mulvey (Durham).

"Some Unusual Lithium Complexes"

6.3.85 Dr. P. J. Kocienski (Leeds).

"Some Synthetic Applications of Silicon - Mediated Annulation Reactions."

7.3.85 Dr. P. J. Rodgers (I.C.I. Plc. Agricultural Division, Billingham.)

"Industrial Polymers From Bacteria,"

* 7.3.85 Dr. P. W. Atkins (Oxford).

"Magnetic Reactions."

*12.3.85 Prof. K. J. Packer (B.P. Ltd./East Anglia).

"NMR Investigations of the Structure of Solid Polymers."

*14.3.85 Prof. A. R. Katritzky, F.R.S. (Florida).

"Some Adventures in Heterocyclic Chemistry."

20.3.85 Dr. M. Poliakoff (Nottingham).

"New Methods for Detecting Organometallic Intermediates in Solution."

28.3.85 Prof. H. Ringsdorf (Mainz).

"Polymeric Lipsomes as Models for Biomembranes and Cells?"

24.4.85 Dr. M. C. Grossel (Bedford College, London).

"Hydroxypyridone Dyes - Bleachable One - Dimensional Metals?"

*25.4.85 Major S. A. Shackleford (U.S. Air Force).

"In Situ Mechanistic Studies on Condensed Phase Thermochemical Reaction Processes : Deutenium Isotope Effects in HMX Decomposition, Explosives and Combustion."

1.5.85 Dr. D. Parker (I.C.I. Plc. Petrochemical and Plastics Division, Wilton).

"Applications of Radioisotopes in Industrial Research."

* 7.5.85 Prof. G. E. Coates (Formerly of University of Wyoming U.S.A.)

"Chemical Education in England and America : Successes and Deficiencies."

* 8.5.85 Prof. D. Tuck (Windsor, Ontario).

"Lower Oxidation State Chemistry of Indium."

8.5.85 Prof. G. Williams (U.C.W. Aberystwyth).

"Liquid Crystalline Polymers."

* 9.5.85 Prof. R. K. Harris (Durham).

"Chemistry in a Spin : Nuclear Magnetic Resonance."

14.5.85 Prof. J. Passmore (New Brunswick, U.S.A.)

"The Synthesis and Characterization of Some Novel Selenium-Iodine Cations, Aided by ^{77}Se NMR Spectroscopy."

*15.5.85 Dr. J. E. Packer (Auckland, New Zealand).

"Studies of Free Radical Reactions in Aqueous Solution Using Ionizing Radiation."

17.5.85 Prof. I. D. Brown (McMaster University, Canada).

"Bond Valence as a Model for Inorganic Chemistry."

21.5.85 Dr. D.L.H. Williams (Durham).

"Chemistry in Colour.

22.5.85 Dr. M. Hudlicky (Blacksburg, U.S.A.)

"Preferential Elimination of Hydrogen Fluoride From Vicinal Bromofluorocompounds."

*22.5.85 Dr. R. Grimmett (Otago, New Zealand).

"Some Aspects of Nucleophilic Substitution in Imidazoles."

*4.6.85 Dr. P. S. Belton (Food Research Institute, Norwich).

"Metal-Sulphur-Nitrogen Complexes."

*14.6.85 Prof. Z. Rappoport (Hebrew University, Jerusalem).

"The Rich Mechanistic World of Nucleophilic Vinylic Substitution."

19.6.85 Dr. T. N. Mitchell (Dorkmund).

"Some Synthetic and NMR-Spectroscopic Studies of Organotin Compounds."

26.6.85 Prof. G. Shaw (Bradford).

"Synthetic Studies of Imidazole Nucleosides and the Antibiotic Caformycin".

12.7.85 Dr. K. Laali (Hydrocarbon Research Institute, University of Southern California.)

"Recent Developments in Superacid Chemistry and Mechanistic Considerations in Electrophilic Aromatic Substitutions, a Progress Report."

13.9.85 Dr. V. S. Parmar (Delhi).

"Enzyme Assisted E.R.C. Synthesis."

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