

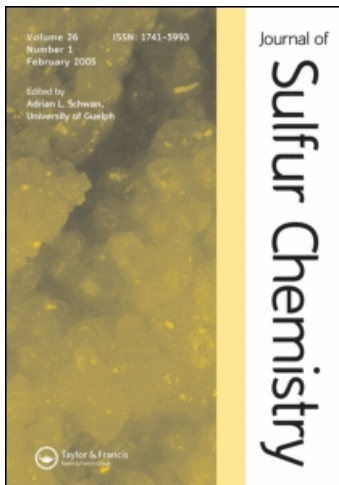
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Selective and an efficient oxidative conversion of glutathione to glutathione disulfide with *N*-bromosuccinimide in aqueous acidic solution: kinetic and mechanistic chemistry

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Oxidative conversion of thiols to disulfides is an important chemical transformation in organic synthesis. A tripeptide, glutathione (GSH), composed of glutamate, cysteine and glycine, has been found to be the most abundant low molecular weight thiol in most biological systems. Its importance in mammal systems is believed to be related to its functions in oxidative metabolism and detoxification. It is noted that despite the importance of this substrate, less information is available in the literature on the oxidation of this substrate viewed from its kinetic and mechanistic studies. *N*-Bromosuccinimide (NBS) is a mild and selective oxidant for many organic compounds, and hence, it has been used as an oxidant for the present redox system. Consequently, the kinetics of oxidation of GSH with NBS in aqueous HClO₄ medium has been investigated at 283 K. The reaction rate exhibits first-order dependence on [NBS]₀ and fractional-order dependence each on [GSH]₀ and [H⁺]. The effect of added succinimide, ionic strength and dielectric constant of the medium on the rate of the reaction has been studied. The solvent isotope effect was studied using D₂O. The reaction was studied at different temperatures and thermodynamic parameters have been computed. Glutathione disulfide is characterized as the oxidation product of GSH. The protonated species RN⁺HBr (here R = (CH₂O)₂–) of the NBS is assumed to be the reactive oxidizing species. The reaction constants involved in the mechanism were evaluated. The observed results have been explained by a plausible mechanism, and the related rate law has been deduced.

Keywords: glutathione; *N*-bromosuccinimide; oxidation; kinetics; acid medium

1. Introduction

The thiol group is generally oxidized to the corresponding disulfide, although instances of oxidation behind disulfide stage are reported depending on the reaction conditions and nature of the reactants (1). The disulfides are important intermediates for the preparation of sulfinyl and sulfenyl compounds and also have industrial applications (2). The mechanistic chemistry of the

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oxidative conversion of thiols to disulfides has received considerable attention in understanding the reaction pathways of a particular redox system (3). This kind of chemical transformation is an important reaction because the disulfide bridges are important for the three-dimensional structure of proteins. Glutathione (GSH) (γ -glutamyl-cysteinyl-glycine) is a tripeptide and its importance in mammal systems is believed to be related to its functions in oxidative metabolism and detoxification (4). Controlled oxidative conversion of GSH to glutathione disulfide (GSSH) forms a very important synthetic route in biochemical reactions. In the literature, many reports are available on the oxidation of GSH with various reagents (4–7). However, most of these methods are complex in nature and not economical. And also, information on the kinetic and mechanistic aspects of these redox reactions are lacking. By keeping this in view, in the present research, we have developed a systematic route for the formation of GSSH from GSH which has significant advantages such as: simple with short reaction time, cost-effectiveness, the use of relatively non-toxic reagents, ease of isolation of products and the ability to be scaled up for industrial operations. The study has been extended to explore the mechanistic aspects of this oxidation through kinetic investigations.

N-halosuccinimides are important group of *N*-halo reagents and relatively stable compounds when compared with other *N*-halo reagents (8). Depending on the reaction conditions, these reagents behave as a source of both halonium ion as well as a nitrogen anion (9). As a result, these reagents react with a wide range of functional groups leading to an array of molecular transformation (8, 9). The important member of this class is *N*-bromosuccinimide (NBS). NBS is a potent oxidant and it has been used in the quantitative estimation as well as oxidation-kinetic study of diverse group of substrates (9–12). As a consequence, in the present kinetic study, NBS has been used as an oxidant in order to establish optimum conditions for the formation of GSSH and also to examine the mechanistic behavior of this reagent toward GSH oxidation.

In the light of the above facts and in continuation of our research work (2) on the oxidation of some thiols, the present paper reports a systematic investigation on the kinetics and mechanism of oxidation of GSH with NBS in perchloric acid medium. The very objective of the present work is to develop optimum conditions for the smooth oxidative conversion of GSH to GSSH through kinetic and mechanistic investigations.

2. Results and discussion

Preliminary kinetic experiments revealed that the reactions between NBS and GSH were too rapid to be measured in a neutral or alkaline medium at an ambient temperature. To investigate the kinetic and mechanistic aspects of this redox system, detailed kinetic experiments were carried out at 283 K in perchloric acid medium and optimum conditions for the quite oxidation of GSH with NBS were established. The kinetics of oxidation of GSH with NBS was investigated at several different initial concentrations of the reactants in HClO_4 medium.

2.1. Effect of varying reactant concentrations on the rate of the reaction

With the substrate in excess, at constant $[\text{GSH}]_0$, $[\text{HClO}_4]$ and temperature, plots of $\log [\text{NBS}]$ versus time were linear ($R^2 > 0.9839$), indicating a first-order dependence of rate on $[\text{NBS}]_0$. Further, the values of k' were unaltered with the variation in $[\text{NBS}]_0$ (Table 1), confirming the first-order dependence on $[\text{NBS}]_0$. Under the same experimental conditions, the reaction rate increased in $[\text{GSH}]_0$ (Table 1) and a plot of $\log k'$ versus $\log [\text{GSH}]_0$ was linear ($R^2 = 0.9921$) with a slope of 0.71, indicating a fractional-order dependence of rate on $[\text{GSH}]_0$.

Table 1. Effect of varying concentrations of NBS, GSH and HClO₄ on the rate of reaction at 283 K.

10^4 [NBS] ₀ (mol dm ⁻³)	10^3 [GSH] ₀ (mol dm ⁻³)	10^3 [HClO ₄] (mol dm ⁻³)	$10^4 k'$ (s ⁻¹)
6.0	8.0	5.0	2.14
7.0	8.0	5.0	2.08
8.0	8.0	5.0	2.15
9.0	8.0	5.0	2.20
10.0	8.0	5.0	2.18
8.0	1.0	5.0	0.51
8.0	3.0	5.0	1.10
8.0	5.0	5.0	1.62
8.0	8.0	5.0	2.15
8.0	12.0	5.0	3.34
8.0	8.0	1.0	0.87
8.0	8.0	2.0	1.36
8.0	8.0	5.0	2.15
8.0	8.0	10.0	3.08
8.0	8.0	18.0	4.28

2.2. Effect of varying perchloric acid concentration on the rate of the reaction

Values of k' increased with the increase in [HClO₄] (Table 1) and a plot of $\log k'$ versus \log [HClO₄] was linear ($R^2 = 0.9991$) with a slope of 0.55, indicating a fractional-order dependence on [HClO₄].

2.3. Effect of added succinimide concentration on the rate of the reaction

Addition of the reduction product of NBS, succinimide (4.0×10^{-4} mol dm⁻³), had no effect on the rate signifying that it is not involved in the pre-equilibrium with the oxidant.

2.4. Effect of ionic strength of the medium on the rate of the reaction

The effect of ionic strength on the reaction rate was studied in the presence of 0.20 mol dm⁻³ KClO₄ solution. It was observed that the ionic strength had no significant effect on the rate of the reaction. This suggests the involvement of non-ionic species in the rate-determining step (rds). Consequently, no attempts were made to fix the ionic strength of the medium constant for kinetic runs.

2.5. Effect of varying dielectric constant of the medium on the rate of the reaction

In order to find the nature of reactive species, the dielectric constant (D) of the medium was varied by adding MeOH (0–20% v/v) to the reaction mixture by keeping all other experimental conditions constant. The reaction rates ($10^4 k' \text{ s}^{-1}$) at 76.73, 74.50, 72.37, 67.48 and 62.71 of D were found to be 2.15, 2.42, 2.89, 3.40 and 4.15, respectively. A plot of $\log k'$ versus $1/D$ was linear ($R^2 = 0.9959$) with a positive slope indicating an ion–dipole type of interaction. Blank experiments showed that there was no oxidation of methanol by NBS during the experimental period. Values of D were taken from the literature (13).

Table 2. Effect of varying temperature on the rate of reaction and thermodynamic parameters for the oxidation of GSH by NBS in acid medium.

Temperature (K)	$10^4 k' (\text{s}^{-1})$
278	1.42
283	2.15
288	3.28
293	4.02
E_a (kJ mol ⁻¹)	46.3
ΔH^\ddagger (kJ mol ⁻¹)	43.9 ± 0.01
ΔG^\ddagger (kJ mol ⁻¹)	89.5 ± 0.20
ΔS^\ddagger (JK ⁻¹ mol ⁻¹)	-160 ± 0.13
log A	4.85 ± 0.15

Note: $[\text{NBS}]_0 = 8.0 \times 10^{-4} \text{ mol dm}^{-3}$; $[\text{GSH}]_0 = 8.0 \times 10^{-3} \text{ mol dm}^{-3}$; $[\text{HClO}_4] = 5.0 \times 10^{-3} \text{ mol dm}^{-3}$.

2.6. Effect of solvent isotope on the rate of the reaction

The rate studies in D₂O medium showed that the rate of the reaction is faster in heavy water. The value of k' in D₂O was $3.32 \times 10^{-4} \text{ s}^{-1}$ while the corresponding value in H₂O was $2.15 \times 10^{-4} \text{ s}^{-1}$ leading to a solvent isotope effect $k'(\text{H}_2\text{O})/k'(\text{D}_2\text{O}) = 0.65$.

2.7. Effect of varying temperature on the rate of the reaction

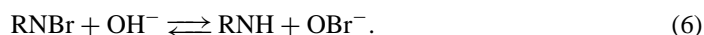
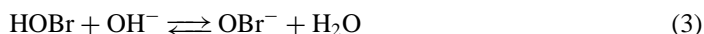
The reaction was studied at different temperatures (278–293 K) and from the linear Arrhenius plot of $\log k'$ versus $1/T$ ($R^2 = 0.9908$), values of activation energy and other thermodynamic parameters (ΔH^\ddagger , ΔS^\ddagger , ΔG^\ddagger and $\log A$) were deduced. All these data were tabulated in Table 2.

2.8. Test for free radicals

The addition of reaction mixture to aqueous acrylamide solution did not initiate polymerization showing the absence of free radical species. The proper control experiments were also performed under similar experimental conditions without the oxidant.

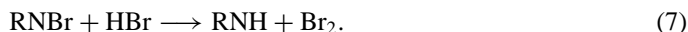
2.9. Reactive species of NBS

NBS (RNBr here R = (CH₂O)₂–) acts as a mild oxidant in both acidic and alkaline media (8, 9). In general, NBS undergoes a two electron change in its reactions. As a consequence, this reagent reacts with a wide range of functional groups affecting an array of molecular transformations (8, 9). Depending on the pH of the medium, NBS furnishes different types of equilibria in acid and alkaline solutions (14, 15) as shown below:



Therefore, the possible oxidizing species in the acid medium are NBS itself (RNBr), protonated NBS (RN^+HBr) and Br^+ , and in alkaline NBS solution, they are NBS, HOBr and OBr^- .

In present investigations, all the kinetic runs were carried out in the presence of mercuric acetate ($5.0 \times 10^{-3} \text{ mol dm}^{-3}$) in order to avoid any possible bromine oxidation. It was observed that addition of mercuric acetate had no significant effect on the rate of the reaction. This indicates that it is not involved in NBS oxidation and functions only as a bromide ion trapping agent (16). It also suppresses completely the oxidation by Br_2 , which would have been formed by the interaction of HBr and NBS as



Mercuric acetate exists as HgBr_4^{2-} or unionized HgBr_2 and thus ensures that the oxidation takes place through NBS. Further, the acceleration of the rate by an increase in the concentration of H^+ ions and insignificant effect of added succinimide (RNH) on the reaction rate assumes that RN^+HBr is the most probable oxidizing species in the present case.

2.10. Reaction scheme

In view of the above kinetic observations, Scheme 1 is proposed to explain the mechanism of oxidation of GSH with NBS in perchloric acid medium. An initial equilibrium involves the protonation of NBS forming the active oxidizing species RN^+HBr as shown in Step (i) of Scheme 1. In the next fast pre-equilibrium step (Step (ii)), the lone pair of electrons of sulfur atom of GSH attacks the positive bromine of the RN^+HBr species forming an intermediate Complex-I. This Complex-I, in a slow and rds (Step (iii)), disproportionates to form another intermediate Complex-II with the elimination of succinimide. In the next step (Step (iv)), the intermediate Complex-II interacts with another mole of substrate, with the elimination of bromide, to yield the final product, GSSH.

2.11. Rate law

Based on Scheme 1, the rate law for the above redox system can be derived as follows:

The total effective concentration of NBS is $[\text{NBS}]_t$, then

$$[\text{NBS}]_t = [\text{RN-Br}] + [\text{RN}^+\text{HBr}] + [\text{Complex-I}]. \quad (8)$$

From Step (i) of Scheme 1,

$$K_1 = \frac{[\text{RN}^+\text{HBr}]}{[\text{RNBr}][\text{H}^+]} \text{ or } [\text{RNBr}] = \frac{[\text{RN}^+\text{HBr}]}{K_1[\text{H}^+]}. \quad (9)$$

From Step (ii) of Scheme 1,

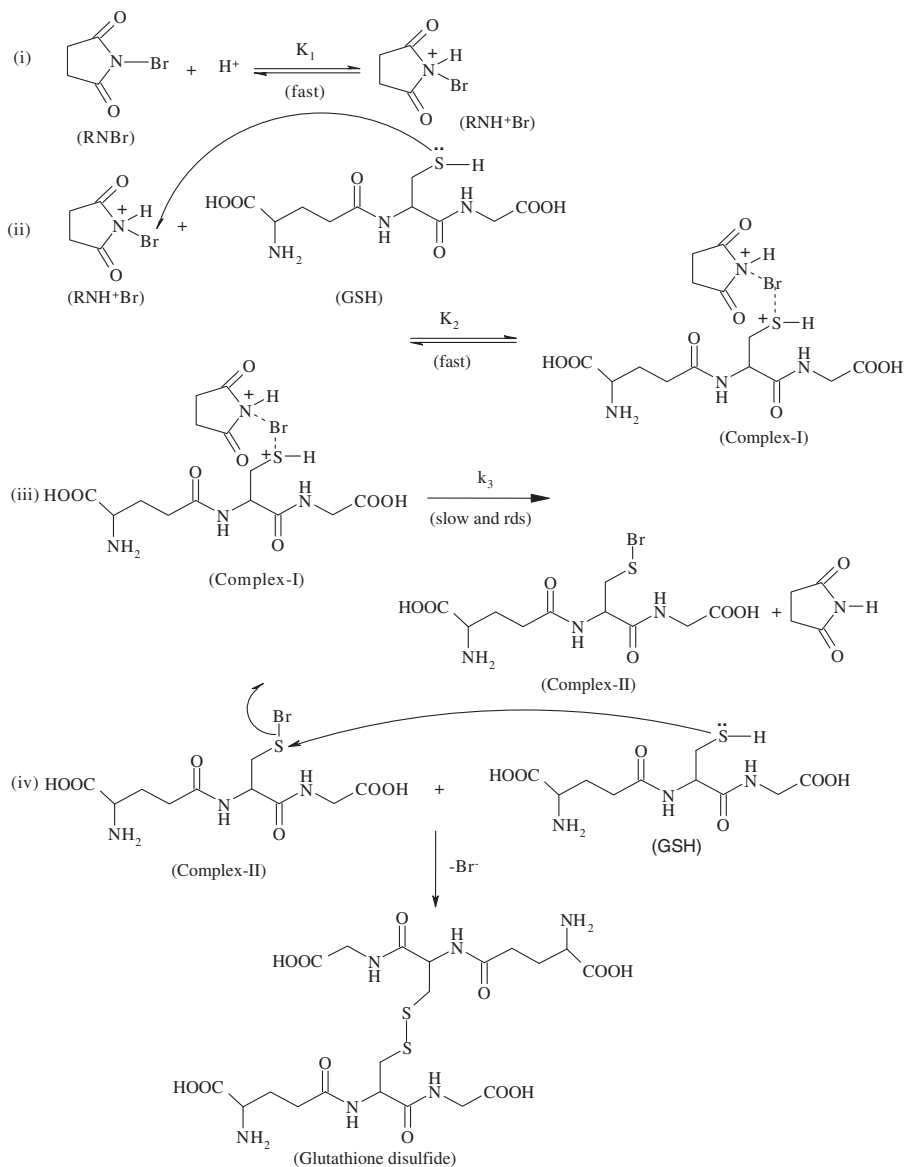
$$K_2 = \frac{[\text{Complex-I}]}{[\text{RN}^+\text{HBr}][\text{GSH}]} \text{ or } [\text{RN}^+\text{HBr}] = \frac{[\text{Complex-I}]}{K_2[\text{GSH}]}. \quad (10)$$

By substituting for $[\text{RN}^+\text{HBr}]$ from Equation (10) into Equation (9) one obtains,

$$[\text{RNBr}] = \frac{[\text{Complex-I}]}{K_1 K_2 [\text{GSH}][\text{H}^+]}. \quad (11)$$

By substituting for $[\text{RN}^+\text{HBr}]$ and $[\text{RNBr}]$ from Equations (10) and (11), respectively, into Equation (8) and solving for $[\text{Complex-I}]$, we get

$$[\text{Complex-I}] = \frac{K_1 K_2 [\text{NBS}]_t [\text{GSH}][\text{H}^+]}{1 + K_1 [\text{H}^+] K_1 K_2 [\text{GSH}][\text{H}^+]}. \quad (12)$$



Scheme 1. A general mechanistic scheme for the oxidation of GSH by NBS in acid medium.

From slow/rds (Step (iii)) of Scheme 1,

$$\text{Rate} = \frac{-d[\text{NBS}]_t}{dt} = k_3[\text{Complex-I}]. \quad (13)$$

By substituting for [Complex-I] from Equation (12) into Equation (13), the following rate law (Equation (14)) is obtained:

$$\text{Rate} = \frac{K_1 K_2 K_3 [\text{NBS}]_t [\text{GSH}] [\text{H}^+]}{1 + K_1 [\text{H}^+] + K_1 K_2 [\text{GSH}] [\text{H}^+]}. \quad (14)$$

Since rate = $k' [\text{NBS}]_t$, Equation (14) can be transformed into Equation (15)

$$k' = \frac{K_1 K_2 K_3 [\text{GSH}] [\text{H}^+]}{1 + K_1 [\text{H}^+] K_1 K_2 [\text{GSH}] [\text{H}^+]}. \quad (15)$$

Equation (15) may be rearranged into Equations (16) and (17), and they are suitable for verification.

$$\frac{1}{k'} = \frac{1}{K_1 K_2 K_3 [\text{GSH}] [\text{H}^+]} + \frac{1}{k_3 K_2 [\text{GSH}]} + \frac{1}{k_2} \quad (16)$$

$$\frac{1}{k'} = \frac{1}{[\text{GSH}]} \left\{ \frac{1}{K_1 K_2 k_3 [\text{H}^+]} + \frac{1}{k_3 K_2} \right\} + \frac{1}{k_2}. \quad (17)$$

Double reciprocal plots of $1/k'$ versus $1/[\text{GSH}]$ and $1/k'$ versus $1/[\text{H}^+]$ were found to be linear (Figure 1; $R^2 > 0.9912$). From the intercepts and slopes of such plots, values of protonation constant K_1 , equilibrium constant K_2 and decomposition constant k_3 could be derived as $10.50 \times 10^4 \text{ dm}^3 \text{ mol}^{-1}$, $0.45 \times 10^3 \text{ dm}^3 \text{ mol}^{-1}$ and $3.44 \times 10^{-4} \text{ s}^{-1}$, respectively. Further, the proposed mechanism and derived rate law are supported by the following experimental facts.

2.12. Effect of solvent isotope

As expected for an H^+ catalyzed reaction, the reaction rate is increased in the D_2O medium. It is well known that D_3O^+ is a stronger acid than H_3O^+ by a factor of 2–3, a solvent isotope of this magnitude is to be expected (17, 18). The observed solvent isotope effect of $k'(\text{H}_2\text{O})/k'(\text{D}_2\text{O}) < 1$ conform to the concept of solvent isotope effect. However, the magnitude of increase in rate in D_2O is small which can be attributed to the fractional-order dependence of rate on $[\text{H}^+]$. The increase in rate in D_2O medium also implies a fast equilibrium hydrogen ion transfer (Step (i) of Scheme 1).

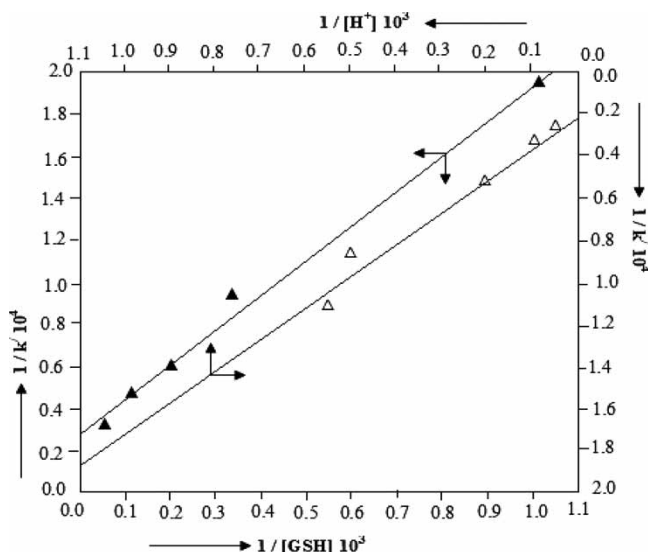


Figure 1. Double reciprocal plots of $1/k'$ versus $1/[\text{GSH}]$ and $1/k'$ versus $1/[\text{H}^+]$. Experimental conditions are as in Table 1.

2.13. Effect of dielectric constant

The influence of solvent on the rate and frequency factors of reactions in solutions can be explained on the basis of the theory of absolute reaction rate (19). The effect of varying solvent composition on the reaction rate has been described in several publications (19–22). For the limiting case of zero angle of approach between two dipoles or an ion–dipole system, Amis (19) has shown that a plot of $\log k'$ versus $1/D$ yields a straight line, with a negative slope for a negative ion–dipole or dipole–dipole interaction, and positive slope results for a positive ion–dipole interaction. The positive dielectric effect observed in the present study supports the interaction between a positive ion and a dipole in the reaction sequence (Scheme 1).

2.14. Thermodynamic parameters

The proposed mechanism is also supported by the moderate value of energy of activation. The positive values of free energy of activation and enthalpy of activation signify that the transition state is highly solvated. The high negative entropy of activation reflects that the transition state is more rigid than initial state with lesser degrees of freedom. Further, the change in ionic strength of the medium did not alter the rate, indicating that the non-involvement of ionic species in the rds. The reduction product of NBS, succinimide, does not influence the rate, showing that it does not participate in pre-equilibrium. All these experimental observations provide additional evidence for the proposed mechanism and the derived rate law.

3. Conclusion

The kinetics of oxidation of GSH by NBS in the acid medium obeys the rate law $-d[\text{NBS}]/dt = k[\text{NBS}]_0[\text{GSH}]_0^{0.71}[\text{HClO}_4]^{0.55}$. Optimum conditions for the smooth oxidative conversion of GSH to GSSH were established. Thermodynamic parameters and reaction constants have been deduced. Protonated NBS has been postulated as the reactive oxidizing species. A general mechanism consistent with the observed kinetics has been proposed and discussed.

4. Experimental

4.1. Materials

An aqueous solution of NBS (Merck) was prepared afresh each time and its strength was ascertained by the iodometric method and preserved in brown bottles to prevent its photochemical deterioration. Fresh aqueous solution of GSH (Aldrich, 98%) was prepared whenever required. Mercuric acetate (E. Merck) was used. Solvent isotope studies were made with D_2O (99.4%) supplied by the Bhabha Atomic Research Centre, Mumbai, India. All other reagents used were of AnalaR grade. Double-distilled water was used throughout the course of the reaction. Regression analysis of experimental data was carried out using Microsoft Excel, from which the regression coefficient (R^2) was calculated.

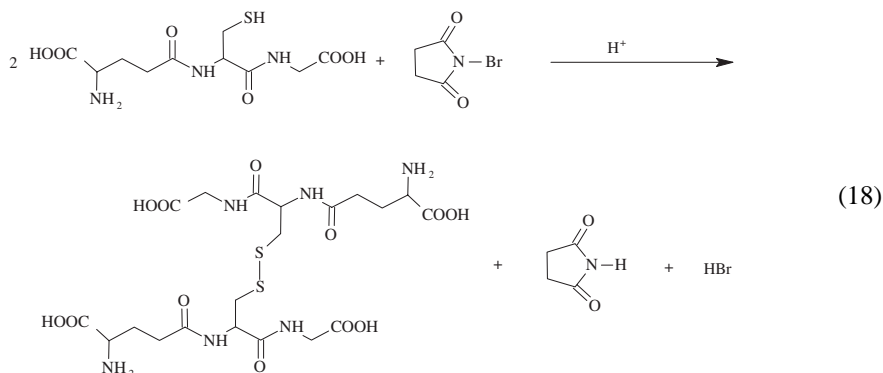
4.2. Kinetic procedure

All the kinetic runs were performed under pseudo-first-order conditions by maintaining the excess of $[\text{GSH}]_0$ over $[\text{NBS}]_0$, and the procedure followed is similar to that reported earlier (2). The

course of the reaction was studied for at least two half-lives. The pseudo-first-order rate constants, k' , calculated from linear plots of $\log [\text{NBS}]$ versus time, were reproducible within 3–5%.

4.3. Stoichiometry

The stoichiometry of the reaction was determined by equilibrating varying ratios of [GSH] and [NBS] in presence of $5.0 \times 10^{-3} \text{ mol dm}^{-3} \text{ HClO}_4$ at 283 K for 48 h under kinetic conditions. Iodometric determination of unreacted NBS showed that the oxidation of GSH involves a single electron change with NBS, which is stoichiometrically represented as



4.4. Characterization of products

The products in the GSH–NBS reaction mixture were extracted several times with diethyl ether. The combined ether extract was evaporated and subjected to column chromatography on silica gel (60–120 mesh) using gradient elutions (chloroform). After initial separation, the products were further purified by recrystallization. The oxidation product of GSH is GSSH. The presence of disulfide in the reaction products was detected by paper chromatography with PhOH saturated with H_2O as a solvent and ninhydrin as a spray reagent ($R_f = 0.088$). GSSH was further confirmed by gas chromatography–mass spectroscopy (GC–MS). The mass spectrum showing an M^+ parent ion peak at 613 amu clearly confirms GSSH. Other peaks observed in the spectrum can be interpreted in accordance with the observed structure. The reduction product of NBS, succinimide, was identified by spot test (23). It was also observed that there was no further oxidation of these products under the present reaction conditions.

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