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Copper-Mediated Non-Enzymatic Formation of Nitrite from Ammonia and Hydrogen peroxide at Alkaline pH

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Abstract: Ammonia is quantitatively oxidized by hydrogen peroxide to form nitrite, 7.63 ± 0.14 nmoles μmole^{-1} H_2O_2. The reaction is accomplished within a minute occurring both at 4 and 20 ^{o}C ($P>0.1$, $n = 5$). The reaction is mediated by copper (II) as it fails to occur in absence of copper; and is quantitatively reduced in presence of copper sequestering agents. The reaction requires the presence of free ammonia as it fails to occur at acidic and neutral pH or when ammonia is substituted with equivalent molar mass of ammonium chloride. Formation of cuprous oxide is presumably the primary step to facilitate the generation of nitrite since copper reduction has been quantitatively linked to nitrite formation. The study provides an alternative method for nitrite generation from oxidation of ammonia by hydrogen peroxide mediated by copper.

Key words: Non-enzymatic, Nitrite, Hydrogen peroxide, Copper, Ammonia.

Introduction

Nitrite is generated from reduction of nitrate, oxidation of ammonia or from autoxidation of nitric oxide. Enzymatic generation of nitrite results from: (i) reduction of nitrate by nitrate reductase by denitrifying organisms [1], (ii) oxidation of ammonia by nitrifying organisms employing ammonia monooxygenase and hydroxylamine oxidoreductase[2,3], and (iii) oxidation of nitric oxide in biological system by ceruloplasmin [4]. Non-enzymatic production of nitrite results from (i) reduction of nitrate with cadmium [5], vanadium [6], zinc [7, 8], hydrazine[9] or hydrochloric acid [10]; (ii) oxidation of ammonia[11-13]; (iii) autoxidation of nitric oxide [14];
(iv) conversion of nitrogen trioxide or equimolecular concentrations of nitric oxide and nitrogen dioxide in water [15]; (v) action of shock-waves on solutions of hydrogen peroxide and arginine [16]; and (vi) photodegredation of humic substances from natural waters [17].

An alternative non-enzymatic process for generation of nitrite was mooted on the following considerations (i) hydrogen peroxide is a strong reducing agent (*electron donor*) in presence of hydroxide ions [15, 18] (ii) copper (II) and its reduced form copper (I) are soluble in ammonia forming amine complexes [15] (iii) ammonia can be oxidized to nitrous acid by hydrogen peroxide [15, 18] (iv) nitrous acid forms ammonium nitrite with ammonia; and (v) nitrite can be specifically determined by modified Griess reaction. Consequently, the experiments were designed to optimize the conditions favoring non-enzymatic generation of nitrite from oxidation of ammonia by hydrogen peroxide in presence of copper ions.

Materials and Methods

The experiments were carried out at an ambient temperature of 20.0 ± 0.3 ^{0}C. The chemicals used were of analytical grade purity, and obtained from reputed sources of India.

Reagents

Copper (II) solution: Stock copper (II) solution was prepared from copper acetate monohydrate as 0.1 %(w/v) in water to provide approximately 5 µmoles copper mL^{-1}.

Copper (I) solution: Stock copper (I) solution was prepared approximately as 0.005 M by dissolving 30 mg of cuprous oxide in 401 mL of 0.75 M ammonia solution.

Ammonia solution: Stock solution of ammonia was prepared approximately as 1 M by diluting 2 mL of available ammonia stock solution (25 %, w/v) to 270 mL with water. The solution was standardized using standards NaOH (primary standard 0.1 M oxalic acid) and H_2SO_4 with methyl red solution 0.1 %(w/v) in 96 % (v/v) ethanol as indicator.

Hydrogen peroxide solution: Stock solution of 100 µmoles H_2O_2 mL^{-1} water was prepared by mixing 6 mL of 30% available stock solution in water to make 529 mL. The solution was standardized titrimetrically against standard $KMnO_4$, previously standardized with 0.05 M oxalic acid as primary standard.

Nitrite solution: Stock solution of nitrite, 100 µmoles mL^{-1} water, was prepared by dissolving sodium nitrite 1 g in water to make 143 mL

Sulfanilamide solution: 1 % (w/v) in 1 % HCl, prepared once in a fortnight.

Coupling solution: Freshly prepared solution of N-(1-napthyl) ethylenediamine dihydrochloride (NEDA) as 0.1 % (w/v) in 1 % HCl.

Modified Saltzman solution (MSS): The reagent contained sulfanilamide 1.25 mg and NEDA powder 2.5 mg mL^{-1} 1 M citric acid solution.

Rochelle salt solution: 0.1 M stock solution of potassium sodium tartrate in water.

Acetate buffer: Approximately 0.6 M buffer was prepared by taking 2.425 g sodium hydroxide pellets and 3.5 mL glacial acetic acid in water to make 101 mL. The pH of the buffer was adjusted to 6.75 and 7.45 using dilute acetic acid and sodium hydroxide.

Concentrated HCl: The strength was titrimetrically evaluated as approximately 11.7 M.

Stock solutions of sodium hydroxide, ammonium chloride, citric acid monohydrate and tri-sodium citrate were each made as 1 M in water. Appropriate working solutions from the stock solutions were made freshly from the stock solutions by dilution in water.

Standard protocol for generation of nitrite

Each milliliter of copper (II) solution, 0.5 µmole, was added 1 mL of ammonia, 200 µmoles, followed by 0 and 1.25 through 10 µmoles of hydrogen peroxide in 2 mL water. The samples were incubated at room temperature for about 5 minutes. Each sample was added 0.1 mL of sulfanilamide solution followed by 0.5 mL citric acid solution, allowed diazotization for 1 to 2 minutes, and then added 0.2 mL of coupling solution. The color developed was monitored, after about one hour spectrophotometrically at 540nm (UV–vis Spectrophotometer SL-150, Elico (India) Ltd., Hyderabad). The mass of nitrite was estimated in terms of simultaneously run calibration curve using 12 through 24 nmoles nitrite per sample.

Order of addition of primary reagents

The reagents were used at standard concentrations of copper, ammonia and hydrogen peroxide respectively, as 0.5, 200 and 5 µmoles with additions made in three orders:

(i) $NH_3 \rightarrow H_2O_2 \rightarrow Cu$, (ii) $Cu \rightarrow H_2O_2 \rightarrow NH_3$ and (iii) $Cu \rightarrow NH_3 \rightarrow H_2O_2$. The reaction in each case was allowed to proceed at room temperature for about five minutes following the addition of the last reactant, and then analyzed for nitrite as per outlined protocol.

Effect of varying ammonia, and copper mass

At standard concentrations of Cu (II) and H_2O_2, the mass of ammonia varied from 0 through 500 µmoles while at standard concentrations of NH_3 and H_2O_2, the mass of copper varied from 0 through 4000 nmoles.

Effect of varying ambient temperatures

Nitrite generation was monitored at standard concentrations of the reactants while maintaining one set of the samples at room temperature (20 ^oC), and keeping the second set in ice-cold water (4 ^oC) for the period of nitrite generation and up to completion of diazotization. The coupling was allowed to proceed at room temperature.

Optimizing time course of the reaction

One milliliter aliquots were withdrawn from the reaction mixture at 1, 3, 5, 10, 20 and 30 minutes following addition of the hydrogen peroxide. Each aliquot (containing 0.5 µmole Cu (II), 200 µmoles NH_3, and 5 µmoles H_2O_2) was added to a solution containing 0.8 mL MSS and 2.2

mL water, allowed standing for about one hour at room temperature, and monitored for absorbance at 540 nm.

Assessing necessity for copper and free ammonia

The samples at standard concentrations of the primary reactants were monitored for nitrite generation: (i) in presence of various concentrations of Rochelle salt, tri-sodium citrate or citric acid; (ii) while substituting ammonia with equimolar mass of ammonium chloride in absence and presence of different masses of sodium hydroxide; and (iii) in presence of acetate buffer with pH adjusted to 6.75 and 7.45. The samples, containing the reactants in appropriate concentrations, were run separately for pH analysis using pocket pH meter (pH Scan 3, Eutech Instruments, Malaysia) with sensitivity 0.01 pH unit.

Monitoring copper (II) reduction with concomitant diazotization

Each experiment was conducted sequentially in two steps. Firstly, the samples containing the reactants in optimized concentrations of ammonia (20 through 200 µmoles), copper (0.5 and 1.0 µmole) and hydrogen peroxide (2 through 30 µmoles) were monitored at 450 nm to assess the reduction of copper (II) to copper (I) oxide. Secondly, the samples if deficient in ammonia were added the same to make 200 µmoles to match the mass present in standard protocol. The samples were thereafter subjected to diazotization and coupling with appropriate reagents and then monitored at 540 nm for nitrite generation. The absorbance values observed at 450 nm, reflecting copper reduction, were correlated to the absorbance values observed at 540 nm, indicating nitrite generation.

Nitrite generation in presence of exogenous copper (I) oxide

Nitrite generation with the required reactants was monitored in presence of exogenous copper (I) oxide using copper (I) equivalent to 0.5 and 1 µmole, ammonia 100 and 500 µmoles, and hydrogen peroxide 2.5 through 10 µmoles.

Effect of the reactants on nitrite recovery

The effect of the reactants on the recovery of added nitrite was evaluated when added at standard concentrations in different combinations just before addition of citric acid during nitrous acid formation, or 20 minutes following development of the chromophore.

Table I. Cu (II)-mediated nitrite generation from NH_3 and H_2O_2.

Hydrogen peroxide (µmoles)	Absorbance	Nitrite generated[a] (nmoles)
1.25	0.090 ± 0.001	12.9 ± 0.1
2.5	0.194 ± 0.002	24.2 ± 0.3
3.75	0.292 ± 0.004	34.9± 0.4
5.0	0.368 ± 0.002	43.2 ± 0.2
7.5	0.554 ± 0.004	63.3 ± 0.4
10.0	0.706 ± 0.002	79.9 ± 0.3
Statistical features		
r ± s.e.	0.999 ± 0.001	
b ± s.e	0.070 ± 0.001	7.63 ± 0.14

[a] Nitrite estimated in terms of simultaneously run calibration curve.

The values are mean ± s.e. of five observations each.

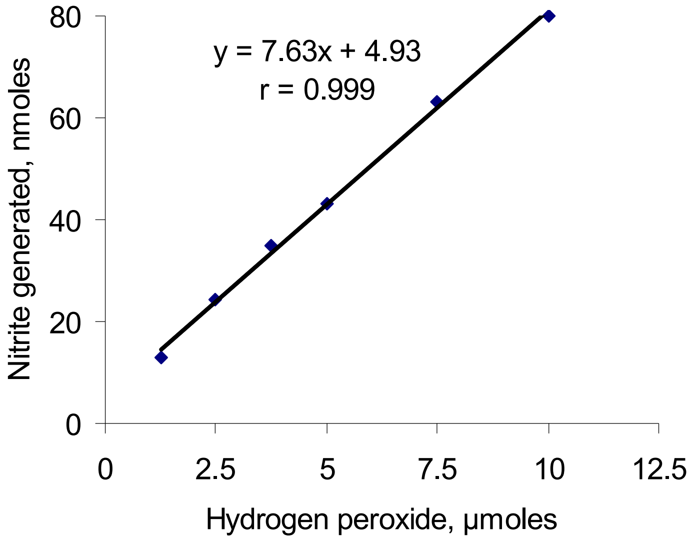

Fig.1. Cu (II) (0.5 µmole) mediated generation of NO_2 from NH_3 200 µmoles and H_2O_2 at room temperature

Results and Discussion

Optimum conditions for nitrite generation

The Griess reaction provides a simple and specific method for nitrite analysis [19]. The method was used to monitor generation of nitrite in the reaction mixtures. As evident **(Table1, Fig.1),** ammonia in presence of copper (II) is quantitatively oxidized to nitrite by varying masses of hydrogen peroxide. At standard concentrations of copper (II) 0.5 µmole, ammonia 200 µmoles and hydrogen peroxide 5 µmoles, the mean absorbance value at 540 nm compiled over the time period of the studies, 0.340 ± 0.002 (n=200), does not differ from the value obtained at corresponding concentration of hydrogen peroxide from the standard assay, 0.349±0.004 (n=5) (P>0.1) (Table1). The estimated mass of nitrite, nmoles $\mu mole^{-1}$ H_2O_2, over the study period has ranged from 6.7 to 10.0 with the mean value, 8.0 ± 0.1 (n=200) comparable to the regression estimate, 7.63 ± 0.14 (n=6) provided by the assay (Table 1) (P>0.1). The reaction occurs at room temperature with mean absorbance value at 4 ^{0}C, 0.334±0.004, comparable to the value, 0.338 ± 0.002, observed at 20 ^{0}C (P>0.1, n = 5 each). The reaction is complete within a minute providing uniform absorbance values throughout 30-min observation period with absorbance at 1-min, 0.345±0.003, similar that observed at 30-min, 0.345±0.003 (P>0.1, n = 5 each). An incubation period of about five minutes was used throughout the studies for practical convenience. The order of addition of the reagents variously affects the extent of nitrite formation (Table 2). For instance, the magnitude of diazotization in presence of copper is little affected with the additions of hydrogen peroxide or of ammonia towards the end (P>0.1). However, nitrite generation is reduced by about 29 % if copper is added towards the end in comparison to the standard practice of adding hydrogen peroxide towards the end (P<0.01). The standard order of addition of the reagents (Cu $\rightarrow NH_3 \rightarrow H_2O_2$) followed during the study ensures better precision, COV 3 %, compared to other modes of addition (Table 2). Copper and ammonia, respectively, as low as 20 nmoles and 20 µmoles with 5 µmoles of hydrogen peroxide generate estimated mean nitrite 3.42 ± 0.08 nmoles $\mu mole^{-1}$ H_2O_2 (n=5). At standard mass of 5 µmoles of hydrogen peroxide, diazotization increases linearly with increase in ammonia from 20 through 200 µmoles at 0.5 µmole copper (r=0.95, b=0.0007), and with increase in copper from 0.02 through 0.5 µmole at 200 µmoles ammonia (r=0.99, b=0.114) (Table 3). Peak diazotization is achieved with 0.5 µmole copper and 200 µmoles ammonia. At higher concentrations, the absorbance values fall linearly with increase in ammonia, 300 to 500 µmoles (r= -1.0) or with increase in copper, 1 to 4 µmoles (r= -0.99). These observations suggest that at higher concentrations the reactants are interfering nitrite formation, diazotization and/or stability of the chromophore.

Table II. Effect of order of reagent addition on diazotization and nitrite generation (5 µmoles H_2O_2, 0.5 µmole Cu and 200 µmole NH_3).

Sequence of addition	Absorbance	Nitrite (nmoles)	COV (%)
$NH_3 \rightarrow H_2O_2 \rightarrow Cu$	0.230 ± 0.010[a]	27.9 ± 1.1	15.3
$Cu \rightarrow H_2O_2 \rightarrow NH_3$	0.278 ± 0.010[b]	33.4 ± 1.2	13.9
$Cu \rightarrow NH_3 \rightarrow H_2O_2$	0.331 ± 0.003[b]	39.1 ± 0.3	3.0

The values are mean ± s.e. of fifteen observations each executed as three experiments on different days (n=5 each). The values bearing identical superscripts are not different (P>0.1) than those with different superscripts (P<0.01).

Table III. Effect of varying ammonia and copper on diazotization

Ammonia[a] (µmole)	Absorbance	Copper[b] (µmole)	Absorbance
0	0	0	0
20	0.204 ± 0.004	0.02	0.255 ± 0.006
50	0.214 ± 0.006	0.05	0.268 ± 0.003
100	0.290 ± 0.002	0.25	0.286 ± 0.005
200	0.325 ± 0.007	0.5	0.314 ± 0.006
300	0.320 ± 0.005	1.0	0.276 ± 0.002
400	0.275 ± 0.003	2.0	0.243 ± 0.003
500	0.230 ± 0.009	4.0	0.208 ± 0.003

[a] Copper 0.5 µmole; [b] Ammonia 200 µmoles; H_2O_2 5 µmoles..

The values are mean ± s.e.of five observations each.

Nitrite generation requires availability of copper and free ammonia

Nitrite is not formed in absence of copper (**Table 3)** implying that the metal is necessarily required to facilitate the oxidation of ammonia by hydrogen peroxide. This is further confirmed from the results obtained in presence of copper chelators. Diazotization falls linearly with increasing mass of Rochelle salt, 25 through 1000 nmoles, causing reduction in absorbance from 30.0 ± 0.6 through 90 ± 1 per cent over the test range with perfect linearity over 25 through 100 nmoles of the chelator (r= 0.99). Similar effects are observed with citric acid and tri-sodium citrate. Absorbance values at 30 and 50 nmoles are, respectively, reduced by 59±1 and 95±1 per cent with citric acid and by 40 ±1 and 76 ± 1 per cent with tri-sodium citrate. Evidently, the tartrate anion is nearly 1000 times more effective than either form of citrate. Comparatively citric acid is 1.4 times more effective than the citrate salt as the former exerts a dual action – reducing the availability of free ammonia, by reduction of pH and formation of ammonium citrate, and of copper by its chelation. Nitrite generation is obviously linked to free ammonia or to alkaline pH of the medium. It does not occur if ammonia, mean pH at nitrite generation as 11.40 ± 0.06 (n = 5), is substituted with equimolar mass of ammonium chloride, mean pH 6.09 ± 0.18 (n = 5). Similarly it fails to occur if pH is dropped to 3.07 ± 0.01 with addition of citric acid, 500 µmoles, before the addition of ammonia, or if pH is adjusted to 6.75 or 7.45 with acetate buffer (n =5 each). Diazotization commences if pH is raised by addition of standard sodium hydroxide. For instance, the absorbance values at 100, 200 and 400 µmoles of sodium hydroxide in presence of ammonium chloride increase, respectively, to 0.228 ± 0.004, 0.250 ± 0.011 and 0.160 ± 0.003 compared to the zero absorbance in presence of ammonium chloride alone, and control value of 0.314 ± 0.005 in presence of ammonia (n=5 each). The mean pH values increase with sodium hydroxide, correspondingly, as 9.98 ± 0.01, 11.09 ± 0.08 and 12.92 ± 0.01. The extent of diazotization is positively correlated to increase in pH over the alkaline range 7.45 through 11.4 (r = 0.98). This is presumably due to an increase in the proportion of free ammonia with increase in pH keeping in view that the pKa of NH_4/NH_3 is 9.02, and pH less than 9.0 causes considerable reduction in proportion of free ammonia [20]. These observations imply the necessity of presence of free ammonia and copper for generation of nitrite from oxidation of ammonia by hydrogen peroxide.

Nitrite formation is linked to reduction of copper (II) to copper (I)

Nitrite formation is closely linked to reduction of copper (II) to copper (I) in presence of hydrogen peroxide. Initially copper (II) is quantitatively reduced by hydrogen peroxide forming a reddish-yellow color with maximum absorbance at 450 nm. The effect is particularly discernible at higher mass of copper, 10 µmoles, and limited mass of ammonia, 20 µmoles, while using hydrogen peroxide 5 through 20 µmoles (r= 0.95, n = 5 each). Higher mass of ammonia, 200 µmoles, as used in the standard protocol tends to obscure the appearance of the color. This is attributed to the solubility of reddish-yellow copper (I) oxide in ammonia forming colorless solution of diamminecopper, [Cu $(NH_3)_2$] OH [15]. These observations necessitated to optimize the protocol for monitoring and linking of copper reduction to generation of nitrite: (i) ammonia mass was added towards the end, and varied from 20 through 200 µmoles; (ii) the samples at reduction step were run individually to completion of the reaction; (iii) the monitoring of the samples at 450 nm was completed within one minute following addition of ammonia; and (iv) the mass of ammonia if deficient was made to 200 µmoles per sample before the commencement of the diazotization. As evident (Table 4) increase in reduction of copper is directly related to increase in diazotization and formation of nitrite both at 0.5 and 1 µmole of copper (II). The regression coefficients for copper reduction, expressed in units of absorbance at 450 nm $\mu mole^{-1}$ H_2O_2, range from 0.0013 through 0.0094 (r = 0.98 to 1.0), and those for estimated nitrite formation, expressed as nmoles nitrite $\mu mole^{-1}$ H_2O_2, range from 1.33 through 4.69 depending upon concentration of ammonia (r=0.97 to 0.99). The regression coefficients linking increase in nitrite formation in nmoles to increase in absorbance at 450 nm show an increase from 138 through 2727 depending upon concentration of hydrogen peroxide (r= 0.96 to 0.99). The magnitude of the coefficients varies linearly with increase in ammonia from 20 through 200 µmoles (r = 0.99, b= 14.2) (**Fig.2).** These observations indicate that nitrite formation is a linear function of concentrations of hydrogen peroxide and ammonia linked to copper reduction. However, an increase in ammonia from 20 through 200 µmoles tends to mask the absorbance at 450 nm leading to fall in regression coefficients from 0.0094 through 0.0013. The masking effect is unrelated to copper reduction or to nitrite formation. For instance, doubling ammonia mass from 20 to 40 µmoles show comparable absorbance values at 450 nm (P>0.1) while nitrite formation increases about 2-folds both at 10 and 20 µmoles of hydrogen peroxide (**Table 4**; P <0.01). The color disappears quickly while using 200 µmoles of ammonia. Therefore, readings at 450 nm were completed within less than a minute.

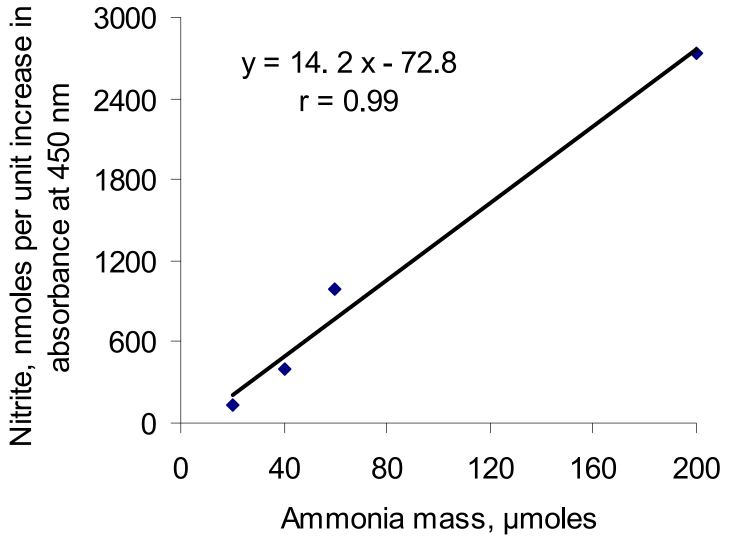

Fig.2. Cu (II)-reduction linked nitrite formation with varying mass of NH_3. The values on y-axis are regression coefficients relating mass of nitrite formed by H_2O_2 per unit increase in absorbance at 450 nm

Table IV. Hydrogen peroxide mediated copper reduction linked to nitrite formation.

Reactants, µmole			Absorbance		Mean nitrite
Cu	NH_3	H_2O_2	450^b nm	540^c nm	(nmoles)
1.0	20	2	0.023 ± 0.002	0.031 ± 0.001	6.5
		5	0.050 ± 0.001	0.067 ± 0.003	10.4
		10	0.093 ± 0.003	0.173 ± 0.007	22.0
		15	0.140 ± 0.001	0.197 ± 0.009	24.6
		20	0.193 ± 0.003	0.243 ± 0.003	29.6
	40	2	0.016 ± 0.002	0.047 ± 0.003	8.3
		5	0.048 ± 0.004	0.133 ± 0.004	17.6
		10	0.094 ± 0.004	0.334 ± 0.009	39.4
		15	0.110 ± 0.004	0.448 ± 0.020	51.8
		20	0.185 ± 0.003	0.632 ± 0.015	71.8
0.5	60	10	0.028 ± 0.004	0.396 ± 0.007	46.2
		20	0.060 ± 0.011	0.941 ± 0.046	105.4
		30	0.118 ± 0.005	1.2860 ± 0.009	142.9
	200	10	0.017 ± 0.003	0.427 ± 0.003	49.6
		20	0.030 ± 0.001	0.820 ± 0.020	92.3
		30	0.043 ± 0.003	1.080 ± 0.070	120.5

[a] The concentration adjusted to 200 µmoles before diazotization; [b] Parameter for copper reduction; [c] Parameter for diazotization. The values are mean ± s.e. of five observations each.

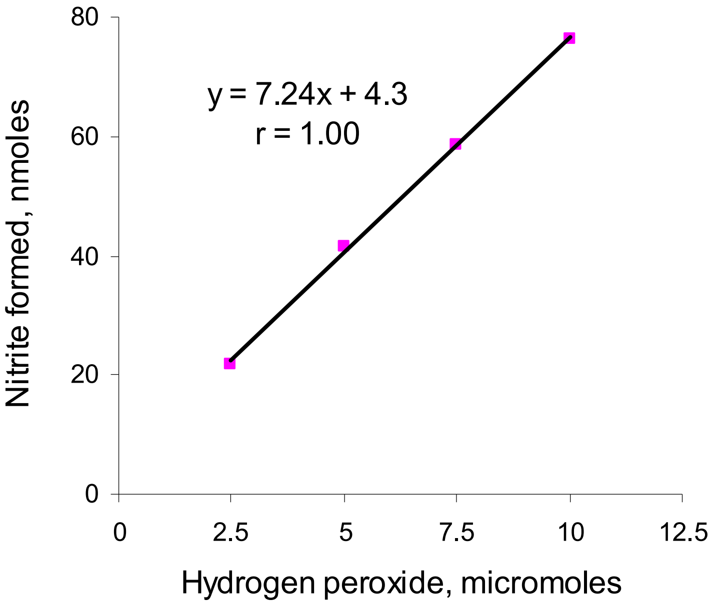

Fig.3. Nitrite formation by H_2O_2 in presence of Cu (I) oxide containing 0.5 µmole equivalent of Cu (I) and 200 µmoles of NH_3.

Table V. Effect of the reactants and Cu (I) oxide on nitrite recovery.

Reactant (s)	n	Nitrite added (nmoles)	Nitrite recovery		
			(nmoles)	(%)	Mean (%)
Copper (II)[a] + ammonia	4	12	8.8 ± 0.3	73.7 ± 2.5	76.2 ± 1.2
	4	18	13.7 ± 0.3	76.1 ± 1.7	
	4	24	18.9 ± 0.3	78.8 ± 1.4	
Hydrogen peroxide	4	12	9.8 ± 0.1	83.1 ± 0.8	61.7 ± 2.2
	14	18	11.2 ± 0.3	62.1 ± 1.4	
	8	24	12.2 ± 0.5	51.0 ± 2.0	
Reaction mixture[b]	8	12	11.3 ± 0.4	94.1 ± 3.2	91.3 ± 1.2
	12	18	16.7 ± 0.2	93.0 ± 1.1	
	12	24	21.1 ± 0.5	87.9 ± 2.0	
Copper (I)[c] + ammonia	5	12	12.8 ±0.4	106.9 ± 3.3	102.4 ± 1.7
	5	24	23.5 ±0.3	97.8 ± 1.2	
	5	36	36.3 ± 0.2	101.0 ± 0.7	

[a, c] The mass of Cu (I) and Cu (II) as 0.5 μmole with 200 μmoles of NH_3;
[b] Reaction mixture, Cu (II) + NH_3 + H_2O_2.

Exogenous copper (I) oxide mediated nitrite formation

Experiments with exogenous copper (I) oxide yielded results similar to those observed with copper (II) that generates copper (I) *in situ* in presence of hydrogen peroxide:

(i) the mass of nitrite formed is linearly related to the concentration of hydrogen peroxide over 2.5 through 10 μmoles in presence of 200 μmoles of ammonia and ca. 0.5 μmole of copper (I) oxide (Fig.3) as is seen with copper (II) (Fig.1);

(ii) the regression coefficient estimate, b ± S.E., in units of nmoles nitrite μmole^{-1} hydrogen peroxide as 7.24 ± 0.16 (r= 0.999 ± 0.001)(Fig.3) is similar to the value, 7.63 ± 0.14 obtained with copper (II) (Fig 1);

(iii) increase in ammonia from 200 to 500 μmoles at 0.5 μmole of copper and 5 μmoles of hydrogen peroxide cause significant reduction in absorbance with both forms of copper from 0.352 ± 0.006 to 0.150 ± 0.003 with copper (I), and from 0.325 ± 0.007 to 0.230 ± 0.009 with copper (II) (Table 3) ($P<0.01$, n=5 each) ; and

(iv) increase in copper from 0.5 to 1.0 μmole at 200 μmoles of ammonia and 5 μmoles of hydrogen peroxide causes significant reduction in absorbance from 0.352 ± 0.006 to 0.288 ± 0.007 with copper (I), and from 0.314 ± 0.006 to 0.276 ± 0.002 with copper (II) (Table 3) ($P<0.01$, n=5 each). In fact, the regression estimate, nmoles nitrite per μmole hydrogen peroxide, drops by about 21 per cent from 7.24 ± 0.16 to 5.69 ± 0.08 (r= 0.996 ± 0.004) when copper (I) content is increased from 0.5 to 1 μmole.

The ability of cuprous oxide to facilitate generation of nitrite suggests that it is an intermediate product to facilitate oxidation of ammonia by hydrogen peroxide.

Effect of primary reactants, and of copper (I) oxide on nitrite recovery

Copper (II)[5] and reducing agents are known to cause interference in determination of nitrite by diazotization[5, 6]. Hydrogen peroxide has high reducing potential especially in presence of hydroxide ions [15, 18]. These considerations provided rationale for finding the effect of the components of the reaction mixture individually, and in combination on the absorbance values of added nitrite. When copper and hydrogen peroxide are separately added during the formation of nitrous acid from standard nitrite, the recovery of nitrite is reduced, respectively, to about 24 and 38 per cent compared to about 10 per cent reduction when the reactants are added together (Table 5). Reduction in nitrite recovery by copper (II) is presumably due to its effect on the azo dye since per cent reduction in absorbance with addition of copper (II) at 20 minutes following addition of the coupling agent, 25.2 ± 3.4 (n=11) is not different from the value, 27.6 ± 2.3 (n= 18), obtained in presence of copper (II) during nitrous acid formation ($P>0.1$). Copper (I) does not interfere with the recovery of added nitrite (**Table 5**). Hydrogen peroxide-induced reduction in the recovery of added nitrite is presumably due to its interference with the generation of nitrous acid since its presence at this stage causes a marked reduction in absorbance, 44.5 ± 2.1 per cent (n = 36). It has apparently no effect on the stability of the chromophore since addition of hydrogen peroxide 20 minutes following addition of coupling agent failed to affect the absorbance values

($P>0.1$, $n=10$). In presence of the reaction mixture containing ammonia, copper (II) and hydrogen peroxide together at standard concentrations, the recovery of added nitrite is considerably improved (Table 5), and the mean absorbance of the samples with added nitrite 12 to 24 nmoles, 0.429 ± 0.023, is not different from the mean of sum of the absorbance values obtained separately with nitrite and the reaction mixture, 0.448 ± 0.025 ($P > 0.1$, n= 8 each). This is apparently due to the consumption of the reactants during the formation of the nitrite, and the observed loss of about 10 per cent in estimated recovery of nitrite is presumably due to the residual unutilized reactants. On these considerations, it is reasonable to presume that the mass of nitrite actually generated by the reaction is about ten per cent more than the estimated value. The foregoing observations reveal that nitrite is formed from oxidation of free ammonia by hydrogen peroxide in presence of either copper (II) or copper (I). The reactions involved in the generation of nitrite may be summarized as follows:

Hydrogen peroxide with lowest recorded redox potential of - 0.68 V compared to that of Cu^{++} / Cu^{+}, + 0.15 V[15] acts as a strong reducing agent particularly in presence of hydroxide ions [13, 18] to donate electrons to copper (II) forming copper (I) oxide,

$$H_2O_2 + 2\,OH^- \rightarrow 2\,H_2O + O_2 + 2\,e^- \quad (1)$$
$$2\,Cu^{++} + 2\,e^- + H_2O_2 \rightarrow Cu_2O + H_2O \quad (2)$$

Reddish-yellow cuprous oxide is rendered colorless in presence of sufficient ammonia to form diamminecopper (I)[15],

$$Cu_2O + 2\,NH_4OH \rightarrow 2\,[Cu\,(NH_3)_2]\,OH + H_2O \quad (3)$$

Diamminecopper (I), generated from reduction of copper (II) or added exogenously facilitates oxidation of ammonia, a reducing agent [14], by hydrogen peroxide,

$$NH_3 + 3\,H_2O_2 \xrightarrow{Cu\,(NH_3)_2]\,OH} HNO_2 + 5\,H_2O \quad (4)$$

Further studies are required to elucidate the actual role of diamminecopper (I) in the reaction; whether it is converted to tetramminecopper (II), or undergoes a reversible changes during the process. The cuprous oxide or its diamminecopper (I) cation form presumably acts as a reducing agent to facilitate oxidation of ammonia by release of oxygen from hydrogen peroxide. Ammonia has tendency to remove oxygen from a number of metal oxides including that from cupric oxide forming water and nitrogen gas [18]. On the contrary, cuprous oxide in presence of hydrogen peroxide seems to favor conversion of ammonia to nitrous acid. The mass of ammonia required for the reaction is quite high as it is needed to maintain: (i) solubility of copper; (ii) optimal alkalinity for expression of reducing potential of hydrogen peroxide; (iii) adequate concentration of free ammonia; and (iv) conversion of nitrous acid to ammonium nitrite.

Conclusions

The present study has revealed non-enzymatic generation of nitrite at room temperature from oxidation of free ammonia by hydrogen peroxide in presence of copper. The reactants involved in the process are commonly occurring in the living systems and in the environment. Ammonia, copper and hydrogen peroxide are extensively used since they have wide range of industrial, agricultural and environmental applications [1, 21]. Ammonia and hydrogen peroxide are increasingly used together to deliginify industrial and agricultural wastes [22-24], and in nutritional research studies in ruminants [25, 26]. The contention of the authors that ammonia and hydrogen peroxide are environmentally benign reagents [22] may not be so in presence of copper in light of the present studies. Further investigations are required to evaluate the extent of contribution from the reaction to overall load of nitrite/nitrate in different biological and environmental settings. Such studies are particularly relevant in view of growing understanding about the physiological and pathological roles of nitric oxide [27] vis-à-vis those of copper [28-30], ammonia[31, 32] and hydrogen peroxide[33-35] in neuronal functioning. Nitrite is both source as well as a stable metabolite of nitric oxide.

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