The detection of glycine from the treatment of glyoxylic acid with iron(II) sulfate and ammonia in water

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A glycine/$(NH_4)_2SO_4$ mixture was isolated by treatment of glyoxylic acid with $FeSO_4$ and $aqNH_3$ in H_2O. The yields of glycine were estimated by ^{1}H NMR. Pyruvic acid was not reduced to alanine under these conditions. This method for forming glycine might have occurred prebiotically alongside the Urey-Miller arc discharge method for making amino acids because glyoxylic acid is formed by arc discharge through a N_2/CO_2 atmosphere and both NH_3 and Fe(II) occurred in the earth's early oceans. The carboxylic acid directs the reduction of 2,4-dinitrobenzoic acid to give 2-amino-4-nitrobenzoic acid.

In 1938, an English translation of Oparin's 1936 Russian book 'Origin of Life' was published.[1] One of Oparin's key proposals was that early earth had a reducing atmosphere of methane, ammonia, hydrogen and water. He suggested methane and ammonia as constituents because they are present on Jupiter and Saturn and both hydrogen and water are ubiquitous. In 1953 a gas mixture of this composition was subjected to an arc discharge now known as the Urey–Miller experiment.[2] This gave some amino acids which were detected by TLC after spraying with ninhydrin. However, later research has suggested that the earth's early atmosphere was more likely to be composed of carbon dioxide and nitrogen with a partial pressure of carbon dioxide as high as 10 bars.[3] This is based on geological evidence since the absence of continents on early earth meant that carbon was not stored as sedimentary carbonate so more may have been in the atmosphere. The occurrence of present day carbonates, chalk, limestone and dolomite, as carbon dioxide was also proposed by Haldane in 1929.[4] A second arc discharge experiment was performed by Miller and Lazcano using an atmosphere of carbon dioxide and nitrogen to investigate a non reducing atmosphere.[5] Provided a reducing agent such as ascorbate or Fe(II) was present amino acids were formed. Other key products were nitrite, nitrate, formaldehyde, glycoaldehyde and glyoxylic acid. These compounds might therefore have been present as a flux into the early earth's oceans. In 1993 Summers and Chang provided a possible prebiotic route to ammonia by studying the reduction of nitrite with Fe(II).[6–7]

$$6 \, Fe(II) + 7H^+ + NO_2^- = 6Fe(III) + 2H_2O + NH_3$$
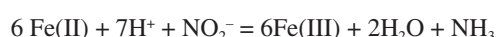
The earth's early oceans are considered to have been rich in Fe(II) salts, a few mg L^{-1}, which is 100 times more concentrated than today.[8] The evidence for this comes from studies of banded iron-formations (BIF) such as that of the Hamersley Basin of Australia which has a circumference of 1000 miles. The deposition of Fe(III), which is less soluble than Fe(II), occurred at such a rate that weathering and transport of Fe(II) by rivers to the ocean is unlikely. A number of BIF's are known.[9] With the presence of Fe(II) and ammonia in the earth's early oceans it seems reasonable to study the reductive chemistry of the arc discharge products from a CO_2/N_2 atmosphere. Both nitrite and glyoxylic acid can be formed by arc discharge

through carbon dioxide and nitrogen.[5] Ammonia can be formed by the reduction of nitrite with Fe(II).[6–7] In persuit of further potential prebiotic routes for making amino acids we examined the reductive amination of glyoxylic acid with $Fe(II)SO_4$ $7H_2O$ and NH_3 in H_2O. α-Keto acids have previously been converted to protonated α-imino acids and reduced with an NADH analogue in low yields but not in water.[10] Glyoxylic acid has been reduced to glycine with Pd/H_2 and $aqNH_3$.[11] α-Keto acids, but not glyoxylic acid, have been reduced to amino acids using precipitated FeS and $Fe(OH)_2$ and amines.[12] $Fe(II)SO_4$ $7H_2O$ was first used to reduce the nitro group with $Ba(OH)_2$ in 1880[13] then with $aqNH_3$ in 1882[14] and later in 1917[15] but has not been used for reductive aminations. [$Fe(II)SO_4$ $7H_2O$ has been called green vitriol, copperas and melanterite. In the early German papers, it is called 'Eisenvitriol'.]

Our studies were specifically to focus on water as solvent as this could mimic a prebiotic solvent of early earth. Possible roles of glyoxylic acid in prebiotic chemistry have been reviewed.[16]

Discussion

Glyoxylic acid hydrate and $Fe(II)SO_4$ $7H_2O$ were treated with ammonia and left in water for 24 h at 75 °C and for 24 h at room temperature (Scheme 1). $Fe(II)SO_4$ $7H_2O$ is not a strong reducing agent but complexation to ammonia makes it electron rich so it readily donates an electron forming an Fe(III) salt. We considered two possible reaction pathways. Firstly, the reaction of glyoxylic acid and ammonia to form an imine. We considered that this reversibly formed imine could complex to Fe(II) followed by reduction. The imine is conjugated to a carbonyl group which should facilitate electron transfer from Fe(II).

Secondly, if $Fe(II)(NH_3)_x(H_2O)_y$ [$x + y = 6$] could complex to the glyoxylic acid carboxyl group and deliver ammonia to the electrophilic aldehyde to give the Fe(II) glyoxylic acid imine complex (shown in Scheme 1), the formation of an unbound, hydrolytically unstable imine need not occur. This could be helpful under dilute prebiotic conditions. Although the stoichiometric reaction requires 2 equiv. of Fe(II) it was found that a cleaner product filtrate was observed using only 1 equiv. The reaction was worked up by carefully decanting firstly the still solution onto silica followed by the iron salts, washing with
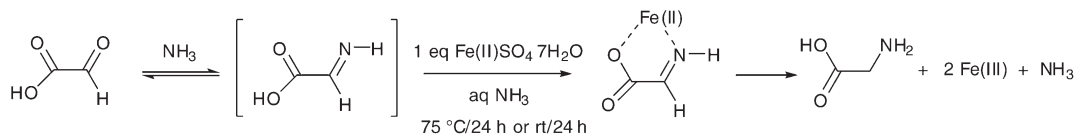
Scheme 1

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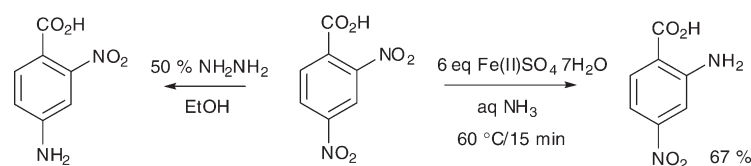
Scheme 2

water and concentration *in vacuo*. The iron salts block the filtration on silica. Both reactions gave similar analyses which are now described. Analysis by TLC on a silica plate (2.5 cm plate width, eluant 10% conc NH_3/MeOH, development with a ninhydrin dip of 1 mg mL^{-1} MeOH) showed a strong glycine spot to be present compared with a glycine standard. After running the silica plate in conc NH_3/MeOH solution it was dried at 100 °C for 1 min to remove traces of ammonia. If the reaction itself is analysed with less than 10% NH_3 in the eluent (which might be owing to evaporation) the glycine streaks but this is caused by the iron salts and was demonstrated by analysing a mixture of glycine with some Fe(II)SO$_4$ 7H$_2$O. Glycine from the filtered solution did not streak on the TLC plate. Analysis of the IR spectrum showed a strong carbonyl stretch at 1600–1700 cm^{-1} the same as that for glycine. Glyoxylic acid showed a much higher carbonyl stretch between 1700 and 1760 cm^{-1} which proved that the carbonyl stretch was not from glyoxylic acid. Three broad resonances at 1380–1410, 1080–1120 and 600–620 cm^{-1} match those of $(NH_4)_2SO_4$ confirming that this is present in the sample. Analysis of the sample by UV confirmed that an amino acid was present. Some of the sample and separately authentic glycine was treated with ninhydrin and K_2CO_3 in hot water for 1 min. Both solutions behaved identically and turned a deep red/brown colour. Both displayed UV spectra with a long wavelength λ_{max} beyond 500 nm similar to that of an authentic sample of murexide. The sample was analysed by NMR in an attempt to quantify a yield for the reaction. An internal standard of sodium benzoate could be seen showing that there were no paramagnetic impurities. A sample that had been purified by trituration with methanol twice showed no signal for glycine at 3.4 ppm. However crude material after filtration through silica and drying did display clear evidence for glycine with a peak at 3.4 ppm. The authenticity of this peak was proved by spiking the NMR tube with glycine. The yield of glycine admixed with ammonium sulfate was estimated to be 18% for the reaction done at 75 °C and 24% for the room temperature reaction (Table 1, entries 1 and 2). Some glycine may have been lost in the work-up since it could bind to the iron salts although treatment of the reaction mixture with excess ammonia prior to work-up did not increase the quantity of glycine formed (16% yield, entry 3). However, a reaction performed for 24 h at room temperature with an excess of ammonia (20 mL) in water (100 mL) had more glycine present. A yield of 28 and 27% was estimated by H^1 NMR (entry 4) from two separate reactions. The excess of ammonia presumably drives the imine formation.

However, the previously reported hydrogenation of glyoxylic acid with aqNH$_3$ over Pd/C took 6 days before hydrogen uptake ceased. The slow uptake of hydrogen by a reactive imine might have been due to its equilibrium with glyoxylic acid and NH$_3$. We therefore performed another experiment in which glyoxylic acid was left to stand with Fe(II)SO$_4$ 7H$_2$O and dilute aqNH$_3$ at room temperature for one week. The presence of glyoxylic acid in the reaction was monitored by staining TLC plates with Brady's reagent (eluted with 10% conc NH$_3$/MeOH). TLC analysis after 24 h of the reactions heated at 75 °C showed the absence of any glyoxylic acid which suggested it may have decarboxylated by heating. After one week at room temperature the yield of glycine in the product salts was estimated as 20% (entry 5).
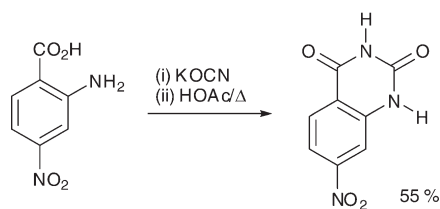
Treatment of pyruvic acid with Fe(II)SO$_4$ 7H$_2$O and NH$_3$ gave no alanine (analysed by TLC and ninhydrin dip) suggesting that the carbonyl group is not electrophilic enough to react with ammonia in water at this level of dilution. Pyruvic acid is a liquid and not a hemi-ketal whereas glyoxylic acid is a solid with a hydrated aldehyde group.

To investigate the directing effects of a carboxylic acid on an Fe(II) reduction 2,4-dinitrobenzoic acid was treated with Fe(II)SO$_4$ 7H$_2$O and aqNH$_3$ at 60 °C for 15 min (Scheme 2). Fe(II)SO$_4$ 7H$_2$O and ammonia can reduce nitro groups to amines,[14,15] originally discovered by Gabriel, but this experiment would show if the carboxylic acid group coordinates to Fe(II) and has a directing effect. Six equivalents of Fe(II)SO$_4$ 7H$_2$O were used which is the stoichiometric amount required to reduce one nitro group to an amine. The product of this reaction was exclusively 2-amino-4-nitrobenzoic acid[17] identified by its spectroscopic properties, a comparison of them to authentic commercial material and by its conversion to a cyclic quinazoline-2,4-dione.

Furthermore a TLC sample of the isomer 4-amino-2-nitrobenzoic acid was prepared which has a different R$_f$ value.[18] The exclusive synthesis of 2-amino-4-nitrobenzoic acid suggests that the carboxylic acid coordinated to the Fe(II) group and catalysed the reduction of the nitro group. This is also the more hindered nitro group so coordination to the Fe(II) would be required for faster reduction. Treatment with NH$_2$NH$_2$ in EtOH gives the isomer 4-amino-2-nitrobenzoic acid reducing the less hindered nitro group.[18] A second batch of 2-amino-4-nitrobenzoic acid was obtained by treating the insoluble Fe(III) salts with aqueous ammonia. This showed that the product was complexing to the insoluble Fe(III) salts. 2-Amino-4-nitrobenzoic acid is soluble in methanol (see Experimental) allowing separation from the iron and ammonium salts. Glycine from the previous section is harder to purify because it is poorly soluble in methanol. This experiment provides precedent that the carboxyl group might facilitate the reduction of glyoxylic acid imine by complexing to Fe(II). 2-Amino-4-nitrobenzoic acid was converted to 7-nitroquinazoline-2,4-dione by condensation with KOCN following a preparation for making quinazoline-2,4-dione (Scheme 3).[19]

6-Nitroquinazoline-2,4-dione has been used in nucleotide studies and the 7-nitro isomer was previously unknown.[20]

In summary a potential prebiotic synthesis of the amino acid glycine is proposed via the reductive amination of glyoxylic acid with soluble Fe(II)SO$_4$ and ammonia. Arc discharge

Table 1   The maximum theoretical yield is 50% for all entries using 1 equiv. of Fe(II)SO$_4$ 7H$_2$O

Entry	Conditions (mL of conc NH$_3$ made up to 100 mL H$_2$O with 1eqFe(II)SO$_4$ 7H$_2$O, temp, time)	Yield /%
1	1.5 mL conc NH$_3$ 75 °C 24 h	18
2	1.5 mL conc NH$_3$ room temperature 24 h	24
3	1.5 mL conc NH$_3$ room temperature 24 h work up (20 mL conc NH$_3$)	18
4	20 mL conc NH$_3$ room temperature 24 h	28
5	1.5 mL conc NH$_3$ room temperature 7 days	20

Scheme 3

experiments through a CO_2/N_2 atmosphere[5] might mimic a prebiotic flux of amino acids, nitrite and glyoxylic acid. Fe(II) needed to reduce nitrite to ammonia and for this reduction may have been present in prebiotic oceans.[8] The yield of amino acids is estimated at only 0.01% in the arc-discharge experiments.[5] Although the yields of glycine reported here are higher the rates will slow down upon dilution. It is thought that to originate and sustain life a mechanism of pre-concentration of reagents from a dilute ocean might have occurred.[21]

Experimental

IR spectra were recorded on an ATI Mattson FTIR spectrometer using potassium bromide or sodium chloride discs. UV spectra were recorded using a Perkin-Elmer Lambda 25 UV-VIS spectrometer with EtOH as the solvent. ^{1}H and ^{13}C NMR spectra were recorded at 250 MHz and 62.9 MHz respectively using a Bruker AC 250 spectrometer. In some cases, ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 100.5 MHz respectively using a Varian 400 spectrometer. Chemical shifts, δ are given in ppm relative to the residual solvent and coupling constants, J are given in Hz. Low resolution and high resolution mass spectra were obtained at the University of Wales, Swansea using electron impact ionisation, chemical ionisation and electrospray ionisation methods. Melting points were determined on a Kofler hot-stage microscope.

Glassware was cleaned with conc HCl to remove insoluble Fe(III) salt residues.

Reductive aminations
Glycine (Method 1): Glyoxylic acid hydrate (600 mg, 6.52 mmol) and Fe(II)SO$_4$ 7H$_2$O (1.8 g, 6.48 mmol) were dissolved in water (100 mL) and treated with concentrated ammonia (1.5 mL). The mixture was heated at 75 °C with occasional swirling for 24 h. After cooling and standing still the mixture was filtered through a 2 cm pad of silica to give an almost clear solution. The filtration was done by carefully decanting the solution from the iron salts onto the silica pad because the iron salts block the filter. The iron salts were then poured onto the silica pad and washed with a small amount of water. The filtrate gives a pale yellow solution on concentration *in vacuo* on a rotary evaporator which was evaporated to dryness at 60–65 °C to give an off-white solid (1.15 g) consisting of glycine and ammonium sulfate. The lack of green colouration suggests that no Fe(II) cation is present: $λ_{max}$ (water)/nm 540–560 (developed by heating some sample with ninhydrin and K$_2$CO$_3$ in water in a test tube); $ν_{max}$ (KBr)/cm^{-1} 2800–3600, 1600–1700, 1080–1120 and 600–620 cm^{-1}; $δ_H$ (400 MHz; D$_2$O) An expected peak for glycine was found at 3.4 ppm. A sample was spiked with glycine to prove the authenticity of this peak. The internal standard of sodium benzoate was observed at 7.28(3H) and 7.68(2H); Glycine analysis by ^{1}H NMR (18%). TLC on a silica plate showed a strong glycine spot compared to authentic glycine (2.5 cm plate width, eluent 10% conc NH$_3$/MeOH, development with a ninhydrin dip of 1 mg mL^{-1} MeOH). The plate is dried at 100 °C for 1 min after elution with NH$_3$/MeOH solution to remove traces of ammonia before using the ninhydrin dip. The ammonia slowly evaporates over a few days which reduces the R$_f$ value. Initially it is about 0.6–0.8 with the sides of the spot angled upwards. With less ammonia the R$_f$ value is less and the sides of the broader spot angle downwards. For some sensitive reactions using an NH$_3$/MeOH eluent and ninhydrin dip, the wider silica plate of 2.5 cm is preferred and gives more resolved spots. Note that traces of salts (such as brine) on TLC spots can decrease the R$_f$ value of amino acids on silica plates. The glycine in the reaction mixture sometimes streaks because of the iron salts (depending upon how much NH$_3$ is left in the eluent) but after filtration through silica the spot elutes normally.

Glycine (Method 2): Glyoxylic acid hydrate (500 mg, 5.43 mmol) and Fe(II)SO$_4$ 7H$_2$O (1.5 g, 5.39 mmol) were dissolved in water (100 mL) and treated with concentrated ammonia (1.5 mL). The mixture was left at room temperature with occasional swirling for 24 h and then worked up as described previously to give an off white solid (1.17 g). Glycine analysis by ^{1}H NMR (24%).

Glycine (Method 3): Glyoxylic acid hydrate (600 mg, 6.52 mmol) and Fe(II)SO$_4$ 7H$_2$O (1.8 g, 6.48 mmol) were dissolved in water (100 mL) and treated with concentrated ammonia (1.5 mL). The mixture was heated at 75 °C with occasional swirling for 24 h The mixture was treated with conc NH$_3$ (20 mL) then worked up as described previously (1.1 g). Glycine analysis by ^{1}H NMR (18%).

Glycine (Method 4): Glyoxylic acid hydrate (500 mg, 5.43 mmol) and Fe(II)SO$_4$ 7H$_2$O (1.5 g, 5.39 mmol) were dissolved in water (80 mL) and treated with concentrated ammonia (20 mL). The mixture was left to stand at room temperature for 24 h and worked up as described previously to give an off white solid (1.0 g). Glycine analysis by ^{1}H NMR (28 and 27%).

Glycine (Method 5): Glyoxylic acid hydrate (500 mg, 5.43 mmol) and Fe(II)SO$_4$ 7H$_2$O (1.5 g, 5.39 mmol) were dissolved in water (100 mL) and treated with concentrated ammonia (1.5 mL). The mixture was stoppered then left to stand at room temperature for 7 days and worked up as described previously to give an off white solid (1.1 g). Glycine analysis by ^{1}H NMR (20%).

Syntheses
2-Amino-4-nitrobenzoic acid: 2,4-Dinitrobenzoic acid (2.0 g, 9.81 mmol) was added to water (50.0 mL), in a beaker, followed by concentrated NH$_3$ (1.0 mL) to aid dissolution. The mixture was heated to 50 °C to dissolve the solids. Six equivalents of Fe(II)SO$_4$·7H$_2$O (15.7 g, 56.6 mmol) were added to water (50.0 mL), in a separate beaker, and heated at 50 °C until dissolved. The two solutions were combined and concentrated NH$_3$ (20.0 mL) was added slowly, with stirring. The reaction was left to proceed at 60 °C for 15 min. The mixture was filtered through a Buchner funnel whilst hot and washed with water. The red aqueous filtrate was transferred into a round-bottomed flask, and evaporated *in vacuo* at 50 °C, leaving the product as well as the remaining salts. The product was taken up in MeOH and decanted from the salts into a separate round-bottomed flask, a procedure, which was repeated until there was no visible sign of the product in the salts (MeOH stayed clear, and not yellow, when added to the solid). The brown iron salts, left in the Buchner funnel, were added to H$_2$O (100 mL) in a beaker and heated to 55 °C. Concentrated NH$_3$ (20.0 mL) was added to the mixture, with vigorous stirring. The mixture was left for 10 mins, after which it was filtered and washed with H$_2$O. The above treatment with concentrated NH$_3$ was repeated once more on the iron salts. The two resulting aqueous filtrates were combined and the water was removed *in vacuo*. The product was taken up in MeOH and filtered from the remaining iron salts. The methanol was combined with the first methanol fraction, concentrated *in vacuo*, then purified by chromatography on silica gel eluting with MeOH: DCM 1:9, to give the title compound as a brown solid (1.15 g, 67%) m.p. 244–245 °C (lit. 264 °C[22] and 269 °C[17]). A separate analysis of each batch gave firstly 0.75g (44%) and secondly 0.39 g (23%); $λ_{max}$ (ethanol)/nm 402 (log ε 2.95); $ν_{max}$ (KBr)/cm^{-1} 3510, 3395, 2750, 1680 and 1300; $δ_H$ (250 MHz; CD$_3$OD) 7.29 (1H, d, J = 8.9 Hz), 7.59 (1H, s) and 7.99 (1H, d, 8.9 Hz); $δ_C$ (62.9 MHz; CD$_3$OD) 109.6, 111.9, 116.0, 134.4, 152.9, 153.6 and 170.3; *m/z* (EI) C$_7$H$_6$N$_2$O$_4$ 182 (M$^+$ 100%); (ASAP) Calcd 183.0400. Found 183.0395 (M$^+$ + H).

7-Nitro-1H-quinazoline-2,4-dione 2-Amino-4-nitrobenzoic acid (0.25 g, 1.37 mmol) was dissolved in H$_2$O (30.0 mL) and treated with aqueous NaOH (5.0 mL, 5M). Powdered KOCN (1.0 g, 12.3 mmol) then glacial acetic acid (2.0 mL) was added to the mixture. The reaction mixture was stirred for 2 h then aqueous HCl (5 mL, 1.0M) was added. A green precipitate formed which was filtered, dried and weighed (0.21 g). The filtered solid was dissolved in glacial acetic acid (75.0 mL), heated to 55 °C and stirred for 3 h. The HOAc was evaporated on the rotary evaporator and the resulting solid was dissolved in MeOH. The product was decanted into a separate round-bottomed flask separating it from impurities. The MeOH was evaporated *in vacuo* and the mixture purified by chromatography on silica gel (eluent MeOH 1:9 DCM then MeOH) to give the *title compound* (0.16 g, 55%) as a light green/yellow solid, m.p. 178–179 °C; $λ_{max}$ (ethanol)/nm 328 (log ε 3.2), 353 (3.3) and 383 (3.1); $ν_{max}$ (KBr)/cm^{-1} 3445, 3330, 1685, 1530 and 1305; $δ_H$ (250 MHz; DMSO-d$_6$) 6.74

(1H, s), 7.70 (1H, d, J = 8.5Hz), 8.13 (1H, d, J = 8.5Hz), 9.30 (1H, s) and 11.04 (1H, s); δ_C (62.9 MHz; DMSO-d$_6$) 113.2, 113.8, 122.8, 132.3, 143.4, 149.5, 155.7 and 168.3; m/z (APCI) 208 (M$^+$ 100%); (APCI) Calcd 208.0353 (M$^+$+ H). Found 208.0347.

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