Contribution from the Chemistry Department, The University, Sheffield, S3 7HF, United Kingdom.

The "Ferric-Thiol" Chromophores of Penicillamine and Cysteamine-N-acetic Acid

C. M. Bell, E. D. McKenzie,* and J. Orton

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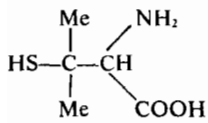
The « ferric-thiol » chromophores of penicillamine, and cysteamine-N-acetic acid are markedly more robust in solution than those of cysteine, and their rate of decomposition is such that they can be studied by conventional spectrophotometric techniques at ambient temperatures. In agreement with previous work on cysteine, recently reinterpreted, both red and violet chromophores of these other thiolamino-acids (with maximum absorption in the visible region at 490 and 570 nm, respectively) are characterised. The slow decomposition reaction of the penicillamine species in aqueous solution is paralleled by a slow formation reaction.

Introduction

Previous work[1-10] on the ferric compounds of cysteine, and related thiol species, has concentrated mainly on solution studies of the rate of reaction of the very labile, highly-coloured species which are formed. More recently, Tomita *et al.*[11] have observed that the compounds can be stabilised in ethanol or aqueous ethanol at temperatures near −70°C.

The present paper gives results of a preliminary study in solution of the ferric compounds of two other amino-acids – penicillamine(I) and cysteamine-N-acetic acid(II)–both of which have the same possible donor



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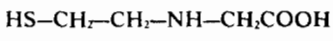

II

atoms as cysteine but different steric requirements. There is a marked stabilisation of their «ferric-thiol » chromophores in solution at room temperature, which allows observation, by conventional techniques, of some novel features of such « ferric-thiol » chromophores, and points the way to further study of such systems.

Experimental Section

Penicillamine in Water. Solutions used were ferric ammonium sulphate in water (10^{-2} *M*) and penicillamine (D or D, L) in aqueous ' tris ' buffer (5×10^{-2} *M* ligand and 5×10^{-2} *M* tris at pH 9; tris = tris-(hydroxomethyl)-methylamine). Usually 0.1-0.5 ml of the iron solution was mixed with the required volume of the ligand solution, and the mixture diluted to 5.00 ml with aqueous buffer solution (pH 9).

In the following, ' aerobic ' conditions refer to such solutions mixed in air, but then transferred immediately to a stoppered spectrophotometer cell, in which they were kept throughout the subsequent measurements. For anaerobic conditions, a small glass vessel was used, to which the spectrophotometer cell was attached by a ground-glass joint. Two separate arms of the apparatus contained separately the iron solution and the ligand solution (each diluted to 2.5 ml). Opposite each arm of the apparatus was a glass neck, sealed with a rubber serum cap. The solutions were de-aerated with a stream of purified nitrogen through hypodermic needles inserted through the serum caps. After de-aeration, the solutions were mixed, and the spectrophotomer cell was fllled simply by up-ending the apparatus. Measurement of the spectra could begin almost immediately.

Spectra were recorded on a Perkin-Elmer 137 UV instrument with repeat-scan atachment.

Penicillamine in DMF and DMSO. All experiments were of the aerobic type described above. Solutions used were 10^{-2} *M* $FeCl_3$ in « lab. reagent » solvents, and 5×10^{-2} *M* ligand in solvent with added $[N(n\text{-}Bu)_4]OH$. The amount of base used was not measured, just sufficient to dissolve the ligand was used. Experiment showed that there was no detectable change of concentration of the chromophore over a wide range of hydroxide ion concentration.

Cysteamine-N-acetic Acid. DMF solutions of $FeCl_3$ and the acid were 10^{-2} *M* and 5×10^{-2} *M*, respectively. All measurements were made under anaerobic conditions as above. The violet and red chromopho-

(*) Author to whom correspondence should be addressed.

(1) L. J. Harris, *Biochem. J.*, *16*, 739 (1922).
(2) L. Michaelis, *J. Biol. Chem.*, *84*, 777 (1929).
(3) B. K. Cannon, and G. W. Richardson, *Biochem. J.*, *23*, 1242 (1929).
(4) M. Schubert, *J. Amer. Chem. Soc.*, *54*, 4077 (1932).
(5) F. M. Page, *Trans. Faraday Soc.*, *51*, 919 (1955).
(6) N. Tanaka, I. M. Kolthoff, and W. Stricks, *J. Amer. Chem. Soc.*, *77*, 1996 (1955).
(7) D. L. Leussing, and L Newman, *J. Amer. Chem. Soc.*, *78*, 552 (1956).
(8) H. Lamfrom, and S. O. Nielsen, *J. Amer. Chem. Soc.*, *79*, 1966 (1957).
(9) D. L. Leussing, J. P. Mislan, and R. J. Coll., *J. Phys. Chem.*, *64*, 1070 (1960).
(10) J. E. Taylor, J. F. Yan, and J. L. Wang, *J. Amer. Chem. Soc.*, *88*, 1663 (1966)
(11) A. Tomita, H. Hirai, and S. Makishima, *Inorg. Chem.*, *6*, 1764, (1967) *ibid.*, *7*, 760 (1968).

determined volume of $[N(n\text{–}Bu)_4]OH$ solution to the ligand solution.

Results

Penicillamine. Even in aqueous alkaline solutions in air at room temperature of a 1:1 solution of iron(III) and penicillamine, the red-violet chromophore persists for some hours.

Aqueous buffer solution. A typical curve of optical density at the absorption maximum versus time is given in Figure 1a. Easily measurable formation of the chromophore is followed by a slower decomposition. Figure 2 is a typical set of absorption spectra showing the novel slow formation. The decomposition (bleaching) reaction is probably the redox reaction

$$2Fe^{3+} + 2RS^- \longrightarrow 2Fe^{2+} + RS\text{–}SR$$

which is irreversible under these conditions. Figure 3 shows the visible absorption spectra at maximum formation for different ligand to iron ratios, under both aerobic and anaerobic conditions. There appear to be at least two different chromophores in these solutions, with absorption maxima at ~490 and 570 nm, respectively, that is, the red and violet species detected for cysteine and mercaptoacetic acid by other workers (see also below). In agreement with the results of Tomita *et al.*,[11] the violet species is the predominant one at high ligand to iron ratios.

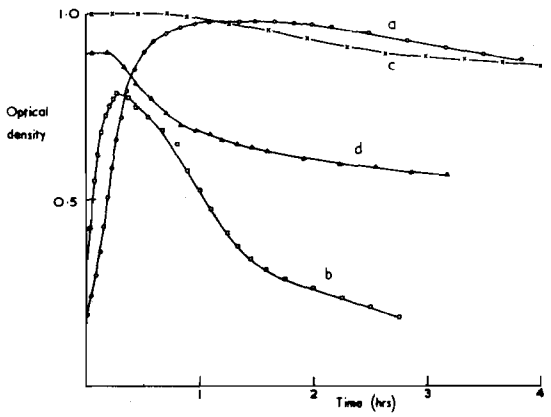

Figure 1. The formation and decomposition of the iron(III)-penicillamine species followed spectrophotometrically at 565 nm. All solutions were of a 6:1 ligand to iron ratio, and concentrations quoted are of the latter. (a) 2.2×10^{-4} *M* solution in 5×10^{-2} *M* aqueous tris buffer at pH 9. (b) 3×10^{-4} *M* solution in aqueous buffer, but the solution was mixed in the presence of oxygen. (c) 3×10^{-4} *M* solution in DMSO. (d) 2.5×10^{-4} *M* solution in DMF.

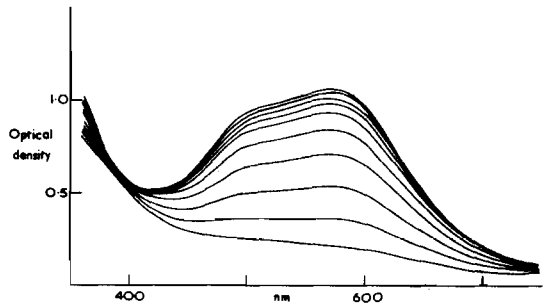

Figure 2. A typical series of spectra run at 5 minute intervals showing the slow formation of the iron(III)-penicillamine chromophore in aqueous tris buffer (pH 9).

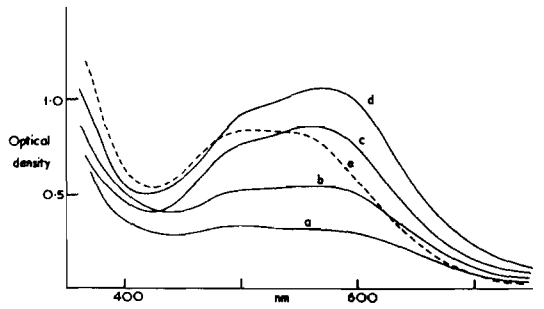

Figure 3. The spectra at maximum formation of the red-violet iron(III)-penicillamine chromophore(s) in aqueous tris buffer for different ligand to iron ratios (a) 1:1, (b) 2:1, (c) 3:1, (d) 6:1 under anaerobic conditions, and (e) 6:1 in the presence (initially) of oxygen.

Under aerobic conditions, both the formation and decomposition reactions are faster. For example, in a 6:1 solution, maximum chromophore formation occurred in 20 min and the calculated ε_{max} was 2500; whereas under anaerobic conditions, maximum formation was delayed to one hour and an optical density corresponding to an ε_{max} of 5000 was recorded.

The stoichiometry of the species cannot be assigned from the spectra. Even at a 6:1 ligand to iron ratio, the formation of the chromophore(s) is incomplete (*cf.* the DMSO and DMF solutions below).

DMSO and DMF solutions. Even greater stabilisation of the ferric-penicillamine chromophores is observed in these dipolar aprotic solvents. Figure 1c gives the plot of optical density versus time for a DMSO solution in which the ligand to iron ratio is 6:1. This curve is comparable with curve 1b for aqueous solution. Formation in such solvents is too fast to be measured by our method, but significant bleaching of the violet chromophore is detectable only after ~50 minutes. Thus there is a distinct initiation period before decomposition is measurable.

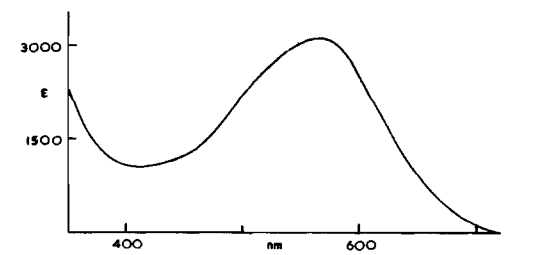

Figure 4. The spectrum of the violet iron-penicillamine chromophore in DMSO solution for a 6:1 ligand to iron ratio.

In such solutions, in contrast to the aqueous solut-

ions, we find only the violet species with ε_{max} at 565 nm (Figure 4). The formation of the violet chromophore is still increasing up to ligand to iron ratios of 15:1 ($\varepsilon_{max} \approx 5000$).

The decomposition with time curve for the violet chromophore in a 6:1 solution in DMF is given in Figure 1d. The bleaching is faster than in DMSO, with estimated half-life of ~7 hrs, and maximum formation of the chromophore occurs near a 6:1 ligand to iron ratio ($\varepsilon_{max} \approx 3500$).

By contrast, for cysteine, at a 15:1 ligand to iron ratio in DMF, the bleaching reaction is markedly decreasing the optical density before we can measure the spectrum.

At high hydroxide ion concentrations, the solutions are colourless, and, the lack of any precipitation of basic ferric species indicates the formation of a different, colourless complex. Dilution of these solutions leads to the formation of the violet species.

Cysteamine-N-acetic Acid. The iron(III) compounds of this ligand were examined in DMF at two different hydroxide ion concentrations each arbitrarily defined by the different chromophores produced. All results refer to anaerobic conditions.

In approximately neutral solution, a red chromophore is produced with a band maximum in the visible region at 485 nm; whilst in more alkaline solutions a violet chromophore is produced with maximum absorption in the visible region at 400 and 555 nm (Figure 5).

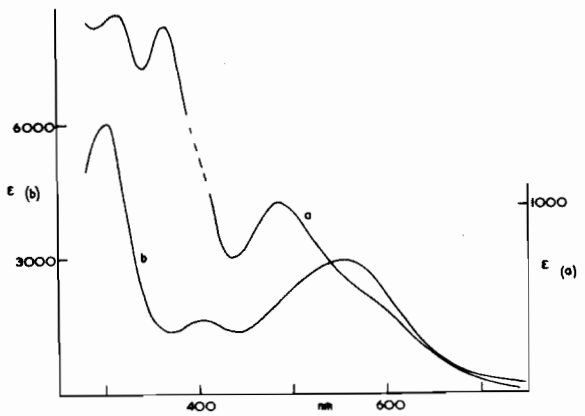

Figure 5. The spectra of the red (a) and violet (b) iron-cysteamine-N-acetate chromophores in DMF. The values of ε_{max} refer to calculations based on the iron concentration. No attempt was made to estimate ε_{max} for the red species in the ultra-violet region.

The red species is much less robust than the violet one. The half-life of the former in a 5:1 solution is less than 35 minutes, whilst the violet species is of a similar order of robustness to the penicillamine chromophore in aqueous alkaline solution. The stoichiometry of both chromophores appears from our spectrophotometric study to be 2:1. At least, for different ligand to metal ratios, maximum formation of the chromophores occurs at this ratio.

Discussion

There is a distinct kinetic stabilisation of the « ferric-thiol » chromophores of these two amino-acids, as compared with the previously studied cysteine species. This parallels, at least in the case of penicillamine, the observed lack of catabolism of the amino-acids by the body, a property which is important in allowing its use in the removal of excess copper in the treatment of Wilson's disease.[18] Undoubtedly much of the stabilisation of these iron chromophores results from the different steric effects of these ligands compared to cysteine. However, in the absence of any knowledge of their structure, we do not speculate about how this arises in detail.

Number of Species. There are at least two coloured iron(III) compounds of these thiol-amino-acids—red and violet ones. Until the work of Tomita *et al.*,[11] the two cysteine species had been confused in the literature. They are now also well-characterised for cysteamine-N-acetic acid, and although the red species is not yet characterised separately for penicillamine, there is evidence that it occurs in admixture with the violet one in aqueous solution.

Stoicheiometry. The present data suggest that both the red and violet chromophores, at least of cysteamine-N-acetate, may be 2:1 species. They do not prove this only that maximum formation of the chromophore is detected from the spectra at such concentrations. By contrast, Page[5] found maximum formation of the red iron-cysteine chromophore at a 3:1 ratio. The discrepancy between the two figures could be a result of partial decomposition of the chromophore in his apparatus.

Structure. It is not possible to infer detailed structure of the chromophore from such ligand-metal charge-transfer spectra, except perhaps the presence of metal-sulphur bonding. The deductions about structure made by Tomita *et al.*[11] unfortunately are wrong in principle, besides being wrong in detail. Most of the cobalt(III) species, to which they wrongly draw parallels, are not firmly characterised; but at least it has been convincingly shown by Gorin[12] that the red isomer of triscysteinatocobalt(III) is a thiol-bridged polymer or oligomer, and not a $[CoS_3O_3]$ chromophore, as earlier proposed by Neville.[13] There is, of course, no evidence that any of the iron species discussed above is mononuclear.

The impossibility of using the visible spectra to infer structure in the iron-sulphur compounds is well-illustrated by two recent X-ray structural determinations. The red compound $[C_5H_7S_2]_2[FeCl_4]$ has a structure[14] containing dithiolium units and tetrahedral $[FeCl_4]^{2-}$ anions, with a closest iron-sulphur approach of 3.5 Å. Yet its diffuse reflectance spectrum[15] in the visible region is very similar to the absorption spectra of the red species characterised above, and by Page.[5] Maximum absorption occurs at ~500 nm,

(12) B. J. McCormick, and G. Gorin, *Inorg. Chem.*, *2*, 928 (1963).
(13) R. G. Neville, and G. Gorin, *J. Amer. Chem. Soc.*, *78*, 4893 (1956).
(14) R. Mason, E. D. McKenzie, G. B. Robertson, and G. A. Rusholme, *Chem. Comms.*, 1673 (1968).
(15) E. D. McKenzie, and G. A. Rusholme, unpublished results.

with a poorly defined shoulder at ~570 nm. The other pertinent structure is that of the iron-protein rubredoxin.[16]. This has an iron atom, apparently in oxidation state +3, which is surrounded tetrahedrally by four cysteine sulphur atoms. Again the absorption spectrum (of the oxidised protein)[17] is very close that of the red iron cysteamine-N acetate chromophore (Figure 5), which appears by contrast to be an $[Fe(S)_2L_x]$ species (where L and x are unknown).

The e.s.r. spectra of $FeCl_3$ and cysteamine-N-acetic acid in DMF (1:5), and the same mixture with hydroxide added to produce the violet chromophore, both give resonances at $g \approx 4.28$, as does oxidised rubredoxin.[17]

More work is required with other different physical techniques before any sort of informed guess can be made about the structure of these ferric-thiol chromophores. However, the observation of a distinct stabilisation of the chromophores of penicillamine and cysteamine-N-acetic acid shows the possibility of using more tractable systems than those studied to date.

(16) J. R. Herriott, L. C. Sieker, L. H. Jensen, *Acta Cryst., A25*, S186 (1969) and J. R. Herriott, L. C. Sieker, L. H. Jensen, and W. Lovenberg, *J. Mol. Biol., 50*, 391 (1970)

(17) W. Lovenberg, and B. E. Sobel, *Acta Cryst. 54*, 193 (1965); R. D. Gillard, E. D. McKenzie, R. Mason, S. G. Mayhew, J. L. Peel, and J. E. Stangroom, *Nature, 208*, 769 (1965).

(18) H. V. Aposhian in « *Metal-Binding in Medicine* » Ed. by M. J. Seven, Lippincott (Philadelphia and Montreal) 1960, p. 293.