COBALT^{III} MACROCYCLES AS POSSIBLE CYANIDE ANTIDOTES

by

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Several cobalt-containing macrocyclic compounds have been examined for their ability to bind cyanide rapidly with a large association constant. These macrocycles were synthesized and studied, resulting in one in particular (Cobalt^{III} *meso*-tetra(4-*N*-methylpyridyl)porphine (Co^{III}TMPyP)) having the physical characteristics necessary for potential use as a cyanide antidote. The binding of cyanide to the oxidized form, forming $Co^{III}TMPyP(CN)_2$, at pH 7.4, 25°C, has been shown to be completely cooperative ($\alpha_H = 2$) with an association constant of 2.1 ± 0.2 x 10^{11}. The kinetics were investigated by stopped-flow spectrophotometry and revealed a complicated net reaction exhibiting four phases at pH 7.4 under conditions where cyanide was in excess. The data suggest molecular HCN (rather than CN^-) to be the attacking nucleophile around neutrality. Additionally, the administration of Co^{III}TMPyP one minute after a lethal dose of cyanide to mice resulted in a marked increase in survival (67%) compared to controls (33%). The time required for the Co^{III}TMPyP-treated mice to right themselves from a supine position was also significantly decreased (9 ± 2 min.) compared to the controls (33 ± 2 min.).

Since blood contains ascorbate, the rate of reduction of Co^{III}TMPyP by ascorbate is of interest. Indeed, the rate of reduction of Co^{III}TMPyP by ascorbate is fast, with second order rate constants of 8.3 x 10^4 $M^{-1}s^{-1}$ at 25°C and 1.4 x 10^5 $M^{-1}s^{-1}$ at 37°C. Addition of cyanide to Co^{II}TMPyP results in the binding of cyanide as evidenced by electric paramagnetic resonance spectroscopy but cyclic voltammetry and kinetic investigations indicate that cyanide induces

oxidation to the Co^{III} dicyano species. The equilibrium binding constant derived from the addition of cyanide to the reduced form was found to be $2.1 \pm 0.1 \times 10^{10}$ (K_β), at pH 7.4, 25°C. Electron paramagnetic resonance spectra of mouse blood taken after the addition of the Co^{III}TMPyP show that the cobalt is reduced and that subsequent addition of cyanide to the blood results in the disappearance of this signal indicating that cyanide scavenging has taken place. The kinetics of cyanide binding to Co^{II}TMPyP are complicated; four phases were found, which were shown to be dependent on the cyanide concentration. A mechanism for the binding of cyanide to the reduced form is proposed.

With regard to public health, HCN is a known toxic component of modern fires and smoke inhalation victims are probably the most frequent patients exhibiting symptoms of acute cyanide toxicity presenting at emergency rooms in Europe and the U.S. Furthermore, there is a paucity of effective cyanide antidotes at present. As such, the development of efficacious cyanide antidotes is desirable.

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1.0 INTRODUCTION

1.1 CYANIDE EXPOSURE & TOXICITY

Humans are primarily exposed to cyanide in two ways.[1] First, through inhalation of contaminated air, including tobacco smoke; and, second, by ingestion of foods derived from cyanogenic plants. Exposure through air is usually a continuous, low-dose (*i.e.* chronic) process, but with the important exception of more acute exposure through modern fire smoke.[2] In the third world, chronic toxicity associated with diets high in cassava and/or sorghum products is a major concern.[3] Consumption of cyanide-laced foods or beverages can, however, result in symptoms of both acute and chronic cyanide poisoning.[1] Failed suicide attempts by ingestion have traditionally represented the most common cases of potentially treatable cyanide intoxications presenting in the emergency room with acute symptoms.[4] Consumption of cyanogenic plant materials by livestock[5] is the most common form of acute cyanide poisoning encountered in the U.S. today. At least in this country, accidental exposures during industrial/mining operations are of relatively low concern.[6] Recent research efforts in the field of antidotes/countermeasures to acute toxicity have largely been driven by the fear that the deliberate release of HCN in confined spaces (*e.g.*, modern buildings, mass-transit vehicles, tunnels *etc.*) might prove an effective means for terrorists to inflict mass casualties on a targeted population.[7]

Aside from the ubiquitous low-dose exposures and the subsiding fear of chemical terrorism mentioned above, the route of exposure, at present, by which acute hydrogen cyanide exposure typically occurs is via the inhalation of fire smoke. Such exposure is made possible by the increasing prevalence of anthropogenic polymeric material (*e.g.*, plastics) that contains nitrogen. Namely, products such as nylon, polyurethane, and polyacrylonitrile release hydrogen cyanide when in the presence of a hot, enclosed fire. Moreover, the numerous components of fire smoke (*e.g.*, carbon monoxide) pose the possibility of a synergistically toxic impact upon fire victims and first responders.[2]

The molecular mechanism(s) by which chronic cyanide toxicity impacts human health is(are) unknown. The levels at which suspected health problems linked to chronic cyanide exposure have been epidemiologically identified[8] are not currently known to be associated with significant inhibition or down regulation of any enzyme systems, or other critical biomolecules. Acute cyanide toxicity, on the other hand, is clearly associated with inhibition of cytochrome *c* oxidase, also known as complex IV of the mitochondrial electron-transport chain.[1,9,10] Dozens of other enzyme systems may be affected, but only at orders-of-magnitude higher levels of the toxin. It has been pointed out[11] that inhibition of these other enzymes occurs in patients who are treated while undergoing slow gastric cyanide absorption having ingested many times the LD_{50} dose. It is quite inappropriate to suggest that inhibition of anything other than complex IV is lethal in the acute scenario. Whatever else is alleviated, any successful therapy must restore electron-transport chain (ETC) activity by overcoming cyanide inhibition of complex IV. The most sensitive tissue to mitochondrial inhibition seems to be the central nervous system (CNS) and the autonomic nervous system serving respiration particularly so. Consequently, death by acute cyanide poisoning is due to respiratory failure[1,9,10] and follows within 1-2 minutes of an

injected or inhaled lethal toxic dose. Sub-lethal toxicity in relation to fire smoke is also an emerging concern because a symptom of sub-lethal cyanide intoxication is disorientation and narcosis. It is thought that this symptom of sub-lethal intoxication in fire victims can lead to poor escape decisions being made resulting in deaths that might otherwise have been avoided.[2,12] Certainly, the large number of facilities using cyanide salts and their broad distribution throughout the U.S.[13] is extremely worrying with regard to the possible theft of said cyanides for other criminal purposes. These concerns, regarding the possibility of imminent cyanide chemical terrorism, have repeatedly been re-stated in the recent emergency preparedness literature.[14-18]

1.2 EXTANT CYANIDE ANTIDOTES

1.2.1 Sodium Nitrite

For more than half a century, the combined administration of intravenous sodium nitrite solution followed by sodium thiosulfate solution was the standard therapy for cyanide poisoning in the U.S. This type of cyanide antidote kit is still carried by medical emergency response teams. The sodium nitrite component of the kit was believed to function by oxidizing deoxyhemoglobin to methemoglobin in the bloodstream.[19] Methemoglobin has a reasonable binding affinity for cyanide and should therefore lower the level of circulating free cyanide ($HCN + CN^-$) thereby ameliorating toxicity. There are, however, several lines of evidence showing this previously accepted mechanism to be wrong:[20] (i) if methemoglobin formation is suppressed, sodium nitrite is still fully protective;[21] (ii) the formation of methemoglobin is known to lag behind the beneficial effect of nitrites;[22] (iii) nitrite is antidotal to cyanide in

cultured cells not containing any hemoglobin, or significant levels of other oxygen transport hemoproteins;[23-25] (iv) toxicity is ameliorated when the level of circulating cyanide is an order of magnitude greater than the nitrite level.[20] It is to be stressed that sodium nitrite is certainly an effective and rapidly acting cyanide antidote, but its mode of action is almost certainly through its NO-donor capability[20] as NO is able to reverse cyanide inhibition of cytochrome *c* oxidase.[25,26] Another important matter is that sodium nitrite alone is an effective cyanide antidote, giving the other component of the old standard combination therapy, sodium thiosulfate, is only beneficial if slow absorption of more than 4 x LD_{50} of the toxin is the case.[27]

1.2.2 Sodium Thiosulfate

Thiosulfate is a substrate for the enzyme rhodanese that converts cyanide to the much less toxic thiocyanate anion, which is excreted through the kidneys.[19] Intriguingly, rhodanese activity is concentrated in the liver and kidneys rather than the CNS where it is principally required for maximal antidotal effect toward cyanide. This issue aside, in sub-lethal scenarios of cyanide intoxication, thiosulfate is not beneficial and actually makes matters worse;[28] seemingly, by a reaction with blood leading to production of sulfide, which is comparably toxic to cyanide.[20] There are better and considerably less toxic sulfur-donor type cyanide antidotes currently under development.[29,30]

1.2.3 Hydroxocobalamin

"Cobalamin" (*i.e.*, hydroxocobalamin, vitamin B_{12}) is a $cobalt^{III}$-containing corrinoid structure (Figure 1) that is able to bind cyanide anion in the bloodstream and, thus, works as an

antidote by lowering the toxic level of circulating free cyanide. It has been available for emergency medical use in Europe for a number of years and has more recently been approved for use in the U.S. Interestingly, intravenous cobalamin is used to treat "acute" cyanide poisoning in victims of smoke inhalation, often an hour or more after they have been removed from the cyanide source.[31-33] In truly acute scenarios death is within minutes if lethal doses are involved and at sub-lethal doses the effects of cyanide requiring intervention do not seem to persist for more than 30-60 minutes.[20] Consequently, fire smoke inhalation, where the toxic dose can be spread over many minutes, appears to include both acute and more chronic patterns of cyanide intoxication. If smoke inhalation patients benefit from cyanide-decorporation procedures hours after the exposure to the source has ceased, there must be a presently undelineated pathobiochemistry where cyanide becomes anabolically "fixed" during the exposure and then slowly released as HCN later in the clinic. Nevertheless, despite the mechanistic uncertainty, much evidence abounds[31-33] that there is some value in this type of treatment and it follows that development of improved cyanide-scavenging agents is desirable.

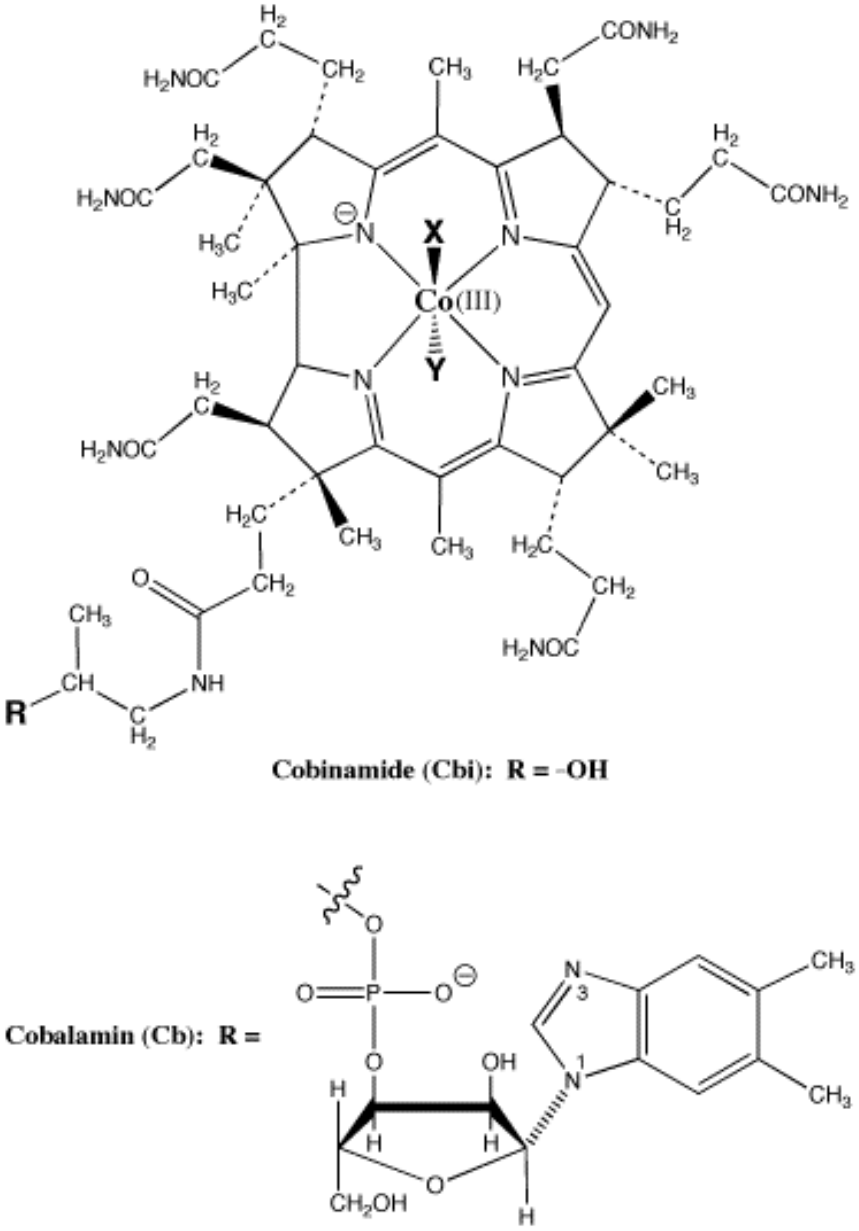


Figure 1: Chemical structure of cobalamin and cobinamide.

1.2.4 Cobinamide

Cobalamin is a less than ideal cyanide scavenger and its biological precursor, cobinamide (Figure 1), has been shown to be 3-10 times more effective in animal studies.[34,35] Both of these

rather complicated biomolecules are, however, expensive to produce commercially, cobinamide the more so. Cobinamide clearly has two axial ligand positions available to bind cyanide, compared with the single available axial position in cobalamin.

1.2.5 Other Potential Antidotes

The conjugate bases of certain keto acids, like pyruvate and α-ketoglutarate, are able to trap cyanide as the essentially non-toxic cyanohydrins, thereby affording a mechanism *in vivo* of lowering the level of any free cyanide present. It is not surprising then that α-ketoglutarate, in particular, has been periodically reported to offer at least prophylactic protection against cyanide intoxication.[36-39] Furthermore, an auxiliary role for molecular oxygen in the antidotal effect has been suggested[37] even though oxygen cannot displace cyanide from cytochrome *c* oxidase.[24,26] Oxygen is, however, also a substrate for nitric oxide synthase and the antidotal effect of supplemental oxygen could be due to production of NO, which clearly is protective.[20]

1.3 ATTEMPTED CYANIDE ANTIDOTES

Given the proclivity of hydroxocobalamin and cobinamide to stably bind cyanide, it is useful to probe into the nature of that ability. A cursory purview of the compounds suggests that the cobalt^{III} state of oxidation is an important factor. Indeed, cobalt^{III}-containing macrocycles provide a stable electronic environment as they contain 18 valence electrons given the octahedral complex that is formed.[40] Such a configuration for transition elements is akin to the application of the "octet rule" to main group elements as said configuration results in all the molecular

orbitals of a transition element possessing a pair of electrons. As such, the pursuit of cobalt^{III}-containing macrocycles underscored the following attempts to produce alternate antidotes to counter acute cyanide toxicity.

1.3.1 Cobalt^{III} Tetramido Macrocycles

The genesis for this project involved the pursuit of a novel class of cobalt^{III}-containing macrocycles that would serve as cyanide scavenging agents. Macrocyclic tetramides, in particular, have been shown to stably bind cobalt^{III}.[41] That compound is synthetically complex rendering it too expensive for the aims of this project. Nonetheless, it provides a useful proxy about which to contemplate similar, tetramido, compounds. In that vein, altogether insoluble tetramido macrocycles have long been known.[42] These macrocycles alternate amido-linkages with pyridinyl moieties and provides another proxy about which to design a novel class of tetramido-compounds. Ultimately, macrocyclic tetramides in which amido-linkages alternated with phenyl moieties were pursued. The selection of phenyl moieties is useful in that the phenylenediamine starting materials necessary to produce a tetramido macrocycle exist with modifiable pendant functional groups so as to provide a synthetic route to aqueous solubility. Lastly, a study of a similar compound that served as an anion receptor[43] suggested that an anionic template would serve as an aid to synthetic yield and purity.

At the outset, the tetramido macrocycles were deemed to possess a number of especially desirable properties for development as pharmaceuticals. The targeted compounds (*e.g.*, Figure 2) can be synthesized in a small number of steps from inexpensive starting materials. Precisely because they are macrocyclic they should be relatively stable; that is, not require refrigeration – an advantage for stockpiling. Also, this stability should translate into minimal problems with

toxicity – given the similarities, these compounds should have comparably modest toxicities and be excreted in the same fashion as porphyrins and phthalocyanines.[44,45] The required ability to bind two cyanide anions with suitable affinity and at reasonable reaction rate could be practically guaranteed given they would finally contain Co^{III} in a roughly square-planar arrangement of nitrogen donors with two axial positions available for ligand substitutions (Figure 2). Therefore, one should be able to set about fine-tuning their other characteristics by suitable derivatization (introducing additional functionalities on either or both of the starting materials) without eliminating the critical required cyanide-binding property.

Unfortunately, purification of these compounds was found to be extremely difficult and cobalt insertion was never confirmed – either directly, or indirectly by cyanide binding to preparations.

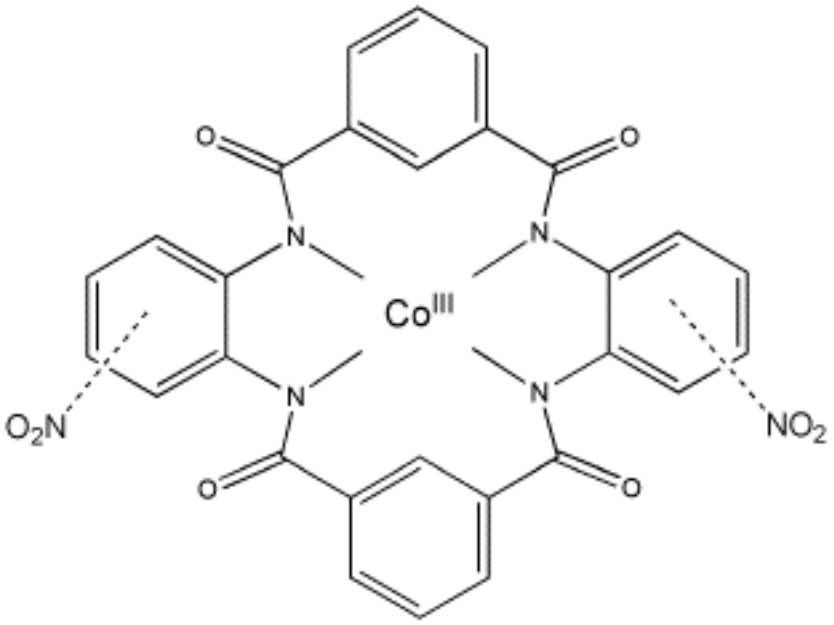


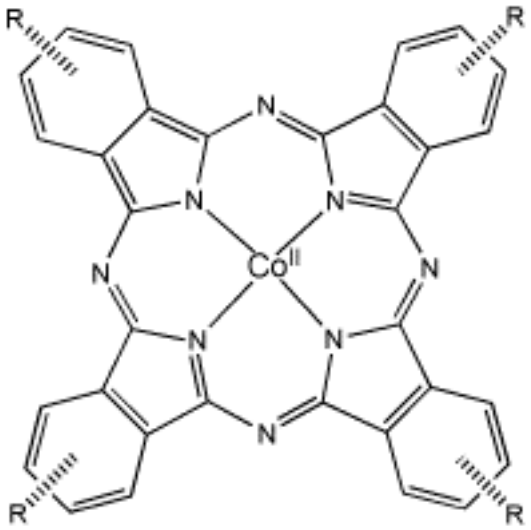
Figure 2: Chemical structure of the proposed Co^{III}-containing tetramido macrocycle.

1.3.2 Cobalt^{III} Phthalocyanines

Phthalocyanines are structurally similar to the corrinoid structure of hydroxocobalamin. Likewise, they readily complex cobalt (albeit as cobalt^{II} requiring a subsequent oxidation to cobalt^{III} once fashioned) in a manner that leaves two axial ligand positions available to bind cyanide anions.[46] There is a plethora of existing derivatization chemistry already documented for phthalocyanines[46] and several known peripheral substituents render phthalocyanines soluble in water. Therefore, there were obvious and attractive reasons to suppose that Co^{III} phthalocyanines might be useful compounds pursuant to the goals of this project.

1.3.2.1 Cobalt^{II} 4,4',4",4'"-tetrasulfophthalocyanine

Cobalt^{II} 4,4',4",4'"-tetrasulfophthalocyanine (Co^{II}-TSPc) can be synthesized in one step from commercially available starting materials and is soluble in aqueous media.[47] Co^{II}-TSPc undergoes a single electron oxidation to Co^{III}-TSPc in the presence of cyanide.[48] It was found, however, that under physiologically relevant concentrations of cyanide, the oxidation and binding of cyanide proceeds too slowly (a time scale of days) to further pursue Co^{II}-TSPc as a potential antidote to acute cyanide toxicity.



Cobalt(II) Phthalocyanines

Figure 3: Chemical structure of Cobalt^{II} 4,4',4",4'"-tetrasulfophthalocyanine (R = SO_3^-) and Cobalt^{II} 4,4',4",4'"-tetraaminophthalocyanine (R= NH_4^+).

1.3.2.2 Cobalt^{II} 4,4',4",4'"-tetraminophthalocyanine

At physiologic pH, Cobalt^{II} 4,4',4",4'"-tetraminophthalocyanine (Co^{II}TAPc) was found to be insoluble and further study was abandoned.

1.3.3 Cobalt^{III} meso tetra(4-*N*-methylpyridyl)porphine

Cobalt^{III} *meso*-tetra(4-*N*-methylpyridyl) (Co^{III}TMPyP) has been widely studied and can be synthesized in three steps from commercially available starting materials in reasonable yield.[49-53] Co^{III}TMPyP is monomeric over a wide range of pH[49] and thus, has the required two axial ligand positions available to bind cyanide anions suggesting an ability to be as efficacious

as cobinamide. In noting these qualities, Co^{III}TMPyP was focused upon for all further study that comprises the dissertation.

1.4 SCOPE OF THE DISSERTATION AND STATEMENT OF HYPOTHESIS

Based on the premise that having two axial ligand positions available to bind cyanide anions must be the key structural advantage of cobinamide over cobalamin, the hypothesis that other, less expensive to produce, $cobalt^{III}$-containing macrocyclic structures with their two axial ligand-binding positions available should have useful cyanide scavenging capabilities is proposed. The likelihood of this proposal not being correct must surely be remote in the extreme – much of what is thought to be known about cyanide toxicity and how to ameliorate it would have to be significantly incorrect – and, indeed, the present studies with Co^{III}TMPyP have ultimately supported the hypothesis (Chapter 3). Perhaps the more intriguing previously reported[54] observation is that CoTMPyP does not work prophylactically as a cyanide antidote. Reasoning that reduction of the compound to Co^{II}TMPyP by ascorbate would occur *in vivo*, additional reasoning suggested that this would lead to deactivation of cyanide binding capability and absence of prophylaxis and set out to test this second hypothesis (Chapter 4).

2.0 MATERIALS & METHODS

2.1 MATERIALS

All reagents were ACS grade, or better, used without further purification and, unless otherwise noted, purchased from Aldrich or Sigma. Argon gas (99.998%) was acquired from Matheson. Concentrations of bovine serum albumin (BSA) solutions were determined spectrophotometrically using the average extinction coefficient.[55] Cobinamide was a kind gift from Prof. Gerry Boss, U. of California, San Diego.

2.2 INSTRUMENTATION

A Vacuum Atmospheres Company Omni-Lab glove box, equipped with an electrolytic fuel cell to measure oxygen levels to below 1 part per million, was used to provide an anaerobic environment when necessary. Shimadzu UV-1650PC and UV-2501PC spectrophotometers were employed for the measurement of electronic absorption spectra and photometric titrations. Stopped-flow kinetics were measured with an Applied Photophysics stopped-flow/laser-flash

spectrometer (LKS.60-SX.18MV-R system) (figure 4) and the resultant data was fit with the PC Pro-K software (!SX.18MV) provided by the manufacturer. Cyclic voltammetry was performed using a Princeton Applied Research Potentiostat/galvanometer 283. X-band (9 GHz) electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford ESR 910 cryostat for ultra-low-temperature measurements. The microwave frequency was calibrated by a frequency counter and the magnetic field was calibrated with a gauss meter. This instrument was obtained with the assistance of a shared-instrumentation grant (NSF) and is maintained at Carnegie Mellon University. The software (SpinCount) used to analyze the EPR spectra was graciously provided by Professor Mike Hendrich, Carnegie Mellon University. A Finnegan LCQ quadrupole field ion trap mass spectrometer equipped with an electrospray ionization source (ESI-MS) was provided by Professor Mark Bier, Carnegie Mellon University.

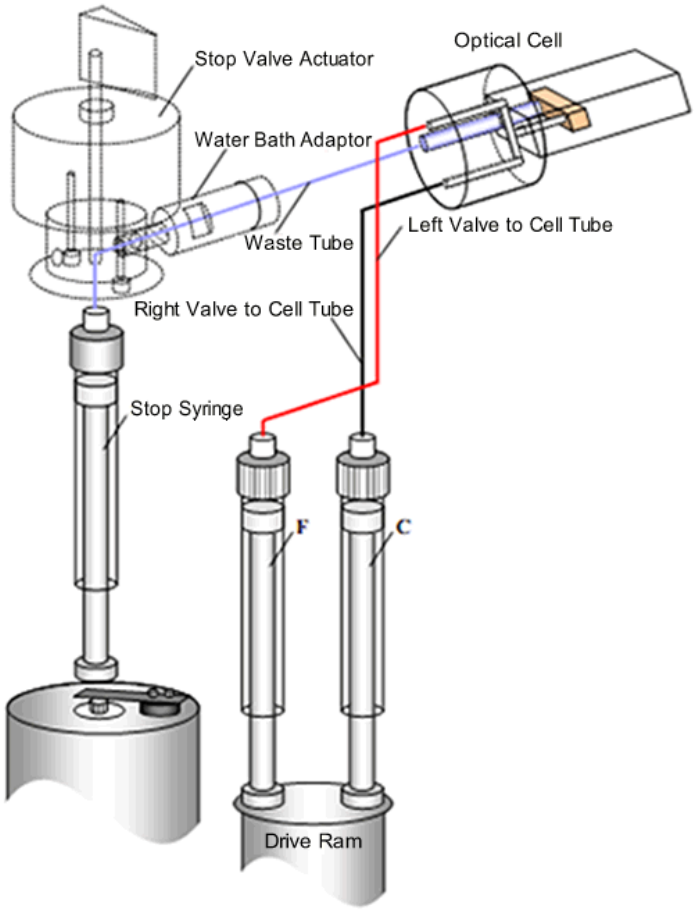


Figure 4: A schematic of a typical stopped-flow design.

The stopping mechanism in this iteration is the stop syringe. Two drive syringes (C and F) contain reactants. The drive ram engages the syringes' pistons so that reactants are pushed through their respective tubing into a mixer and, finally, the observation cell. The prior contents of the cell are consequentially pushed through the waste tubing, impacting the stop syringe until it triggers a switch that stops the flow while also initiating the collection of data. The reactants, at this point in time, have been mixed in the observation cell for roughly one millisecond. Determination of the exact time between mixing and data collection (the dead time) is dependent upon the design of both the stopped-flow instrument and the observation cell. This figure was adopted from Applied Photophysics Ltd.[56]

2.3 SYNTHESES

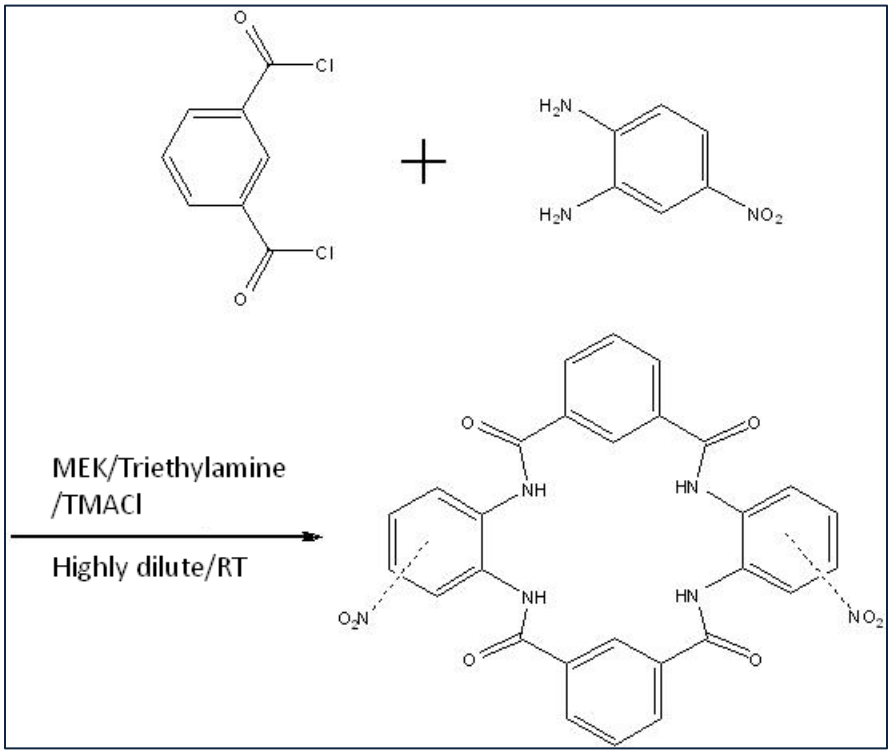


Figure 5: The reaction scheme by which the final product of Chapter 2.3.1 is acquired.

2.3.1 Co^{III} tetramido macrocycles

Equimolar quantities of 4-nitro-*o*-phenylenediamine and isophthaloyl dichloride were condensed at room temperature in methyl ethyl ketone (MEK) in the presence of catalytic quantities of triethylamine and tetramethylammonium chloride. The reaction was performed via the concurrent addition of both starting materials (as dissolved in MEK) via dropping funnels to a round bottom flask containing catalysts dissolved in MEK, under argon, over a period of two

hours. The resultant macrocycle (Figure 5) precipitated from solution along with triethylamine•HCl byproduct. The product was washed with 1M aqueous hydrochloric acid effectively removing the contaminating byproduct as confirmed by ESI-MS (Figure 6). The three peaks of the anionic mass spectrum correlate to the product with a loss of a proton (*m/z* 565), the product binding a chloride anion (*m/z* 601), and either an octomeric macrocycle binding a chloride anion or a sandwich complex consisting of two molecules of the product binding a single chloride anion (*m/z* 1167). The lack of hexameric or decameric macrocycles in the anionic mass spectrum suggests that the final peak is, indeed, a sandwich complex. A yield of ~50% was reliably recovered.

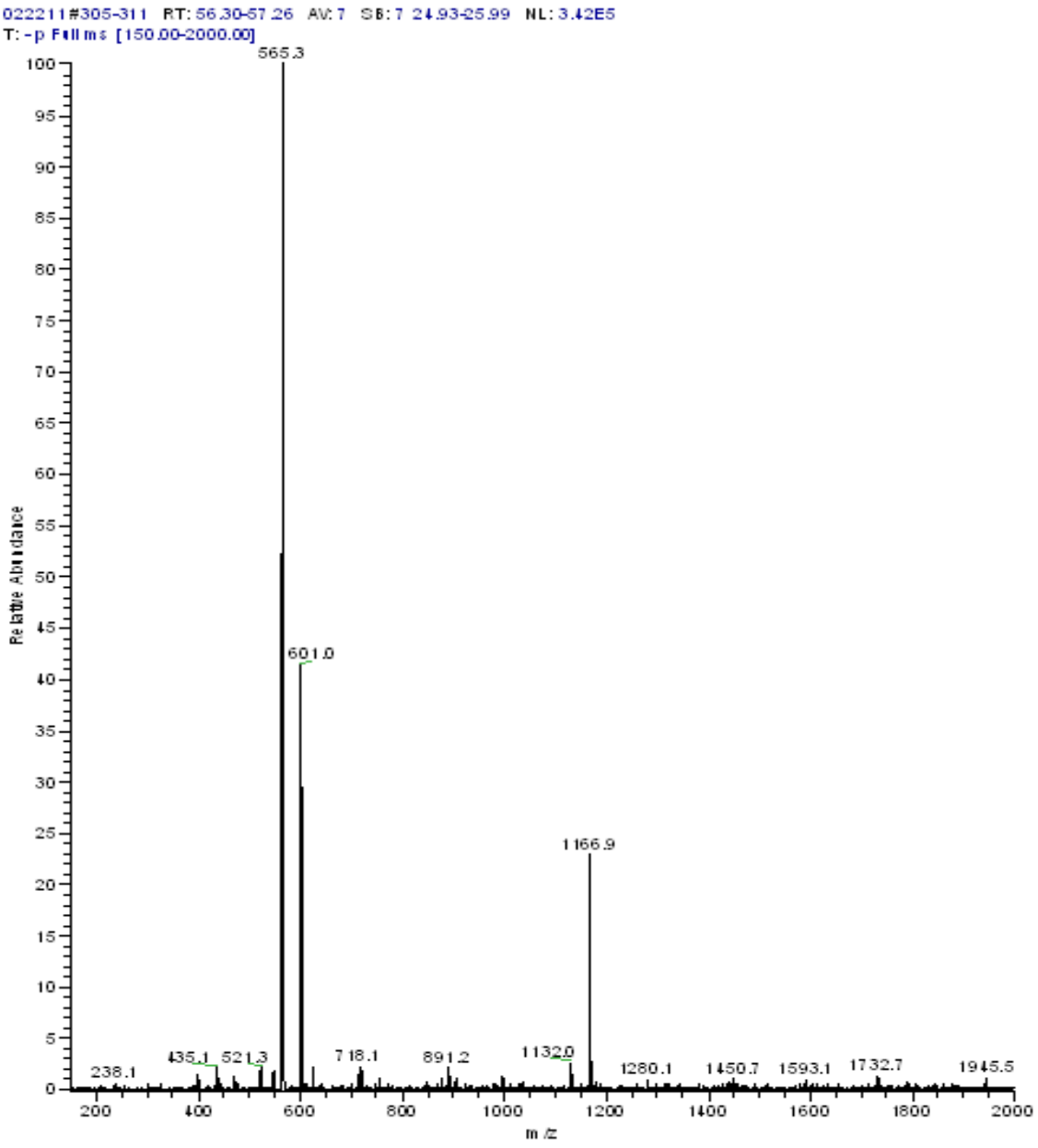


Figure 6: ESI-MS anionic mass spectrum providing evidence of the relatively pure product of Chapter 2.3.1.

The three peaks of the anionic mass spectrum correlate to the product with a loss of a proton (*m/z* 565), the product binding a chloride anion (*m/z* 601), and a sandwich complex consisting of two molecules of the product binding a single chloride anion (*m/z* 1167).

2.3.2 Co^{II} phthalocyanines

2.3.2.1 Co^{II} 4,4',4",4'"-tetrasulfophthalocyanine

The procedure below was conducted via the methods of Weber and Busch.[47] The trisodium salt of 4-sulfophthalic acid, Cobalt chloride, ammonium chloride, ammonium molybdate, and urea were placed in a mortar and ground into a homogenous mixture with a pestle. Meanwhile, nitrobenzene was added to a round-bottomed flask and heated to 180°C in an oil bath. To the flask, the solid mixture was added in a piecemeal fashion over the course of an hour. The resultant, heterogeneous mixture was stirred for 6 hours while the temperature was maintained at 180°C. Next, the product was washed with methanol, repeatedly, until the aroma of nitrobenzene was no longer present. The refined product was then subjected to 1 M (aq) hydrochloric acid that was saturated with sodium chloride and briefly refluxed. Upon cooling, undissolved product was filtered and dissolved in 100 mM (aq) sodium hydroxide. Said solution was heated to 80°C after which impurities precipitated and were filtered. To cooled solution, sodium chloride was added. Upon stirring and heating to 80°C ammonia evolved and product precipitated. Once such evolution ceased, the product was collected via filtration. The noted acid and base washes were twice repeated.

2.3.2.2 Co^{II}4,4',4",4'"-tetraaminophthalocyanine

The procedure below was conducted via the methods of Achar *et al*.[57] 4-nitrophthalic acid, cobalt chloride, ammonium chloride, ammonium molybdate and urea were placed in a mortar and ground into a homogenous mixture with a pestle. Meanwhile, nitrobenzene was

added to a round-bottomed flask and heated to 180°C in an oil bath. To the flask, the solid mixture was added in a piecemeal fashion over the course of an hour. The resultant, heterogeneous mixture was stirred for 6 hours while the temperature was maintained at 180°C. Next, the product was washed with methanol, repeatedly, until the aroma of nitrobenzene was no longer present. The refined product was then subjected to 1 M (aq) hydrochloric acid that was saturated with sodium chloride and briefly refluxed. Upon cooling, undissolved product was filtered and dissolved in 100 mM (aq) sodium hydroxide. Said solution was heated to 80°C, after which impurities precipitated and were filtered. To cooled solution, sodium chloride was added. Upon stirring and heating to 80°C ammonia evolved and product precipitated. Once such evolution ceased, the final product, Co^{II} 4,4',4",4'"-tetranitrophthalocyanine (CoTNPc), was collected via filtration. The noted acid and base washes were twice repeated. To form the tetraamine, CoTNPc was slurried in water to which sodium sulfide nonahydrate was added and stirred at 50°C for 5 hours. The resultant solid was filtered and slurried with 1 M (aq) hydrochloric acid. Product was again filtered and slurried with 1 M (aq) sodium hydroxide. Lastly, the product was washed with copious amounts of water prior to being dried in an oven at 100°C for 1 hour.

2.3.3 Co^{III} meso tetra(4-*N*-methylpyridyl)porphine

H_2TMPyP was synthesized by the method of Hambright *et al*.[49] Cobalt was inserted into the macrocycle using the method of Pasternack *et al*.[50] Briefly, $CoCl_2$ was refluxed under argon with aqueous H_2TMPyP overnight. Upon cooling, the resultant Co^{III}TMPyP was precipitated with excess potassium iodide. The flat brown flakes of precipitated

metalloporphyrin iodide were then dissolved in a 5% acetone aqueous solution and re-precipitated with excess potassium iodide. The final solid product was filtered from the mixture, washed with dilute aqueous potassium iodide, and dried in an oven at 100°C for 1 hour.

2.4 METHODS

2.4.1 Anaerobiosis

When a critical anaerobic environment was required, work was conducted in a glove box equipped with an argon atmosphere. Less critically, a vacuum manifold was repurposed with argon and employed to reconfigure the environment of septum sealed cuvettes.

2.4.2 Titration experiments

For determinations of cyanide binding equilibria all solutions were maintained in "capped" (septum sealed, with head volumes minimized) vessels and transfers made with gas-tight syringes. Small aliquots of relatively concentrated cyanide solutions (buffered with 50 mM sodium borate; pH 11) were titrated into larger volumes of relatively dilute solutions of Co^{III}TMPyP (buffered with 50 mM sodium phosphate and 1 mM EDTA; pH 7.4) to maintain neutrality at both 25°C and 37°C. As multiple fast and slow cyanide binding forms are feasible, solutions were allowed to equilibrate for ~10 minutes after the addition of cyanide prior to recording the resultant absorption changes, by which time constant readings were obtained.

Binding constants were determined by the construction of Hill plots. The saturation of the Co^{III}TMPyP with cyanide was determined from the absorbance changes, where Y represents the proportion of sites occupied by cyanide (or fractional saturation) was plotted against the concentration of the free cyanide concentrations. Fits of the data yielded cooperativity values and binding constants for the association of cyanide to Co^{III}TMPyP.

2.4.3 Kinetics experiments

All reactions (cyanide, azide, or ascorbate) were run under pseudo first order conditions and the temperature controlled by a thermostatted reaction chamber. The individual rates observed are the average of at least three runs. The average deviation of these runs is less than 5%. Rate constants were then obtained from linear fits to the observed rates versus the substrate: total cyanide (CN- + HCN), sodium azide, or sodium ascorbate. Rapid reaction kinetics were performed with a stopped-flow spectrophotometer.

2.4.4 Animals, exposures, and righting recovery determinations

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Number 1008725). Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss Webster (CFW) mice weighing 35-45 g were purchased from Charles River Laboratories, Wilmington, MA. All animals were 16-20 weeks old and were housed four per cage. The mice were allowed access to food and water *ad lib*. and experiments commenced after the animals were allowed to adapt to their new environment for one week. All solutions were administered

through ~0.1 mL intraperitoneal (i.p.) injections. At the end of exposures and tests, mice were euthanized with 150 mg/kg (i.p.) sodium pentobarbital followed by cervical dislocation. Righting recovery times were assessed based on the method of Crankshaw *et al*.[29] with minor changes as described by Cambal *et al*.[20] Following injections, mice were placed in clear plastic tubes in a supine position. The time from the cyanide injection until the mouse flipped from a supine to a prone position in the plastic tube was taken as the endpoint. After righting, the tube was rolled to make sure the mouse could maintain the prone position, thereby avoiding false positive results. Trials were conducted with three different experiments: saline, 5 mg/kg sodium cyanide, and 20.8 mg/kg Co^{III}TMPyP.

2.4.5 Cyclic voltammetry

Cyclic voltammograms were acquired from relatively dilute solutions of Co^{III}TMPyP in excess cyanide (1 mM), 0.1 M $NaNO_3$, 50 mM phosphate buffer at pH 7.4 at 25°C under argon. A three-electrode configuration was employed. Polished platinum wire was used as both the working and counter electrode. A Ag|AgCl|KCl(sat.) electrode was used as a reference electrode. All potentials were reported with respect to this reference electrode.

2.4.6 Electron paramagnetic resonance

The following methods employed have previously been well established in this laboratory.[58-60] Quantification of EPR signals was performed by simulating the spectra using known (or determined) parameters for each sample in question. Simulations employed a least-squares fitting method to match the line shape and signal intensity of a selected spectrum.

Simulated spectra were expressed in terms of an absolute intensity scale, which could then be related to sample concentration through comparison with a Cu^{II}(EDTA) spin standard of known concentration.

3.0 THE METALLOPORPHYRIN Co^{III}TMPyP AMERLIORATES ACUTE SUB LETHAL CYANIDE TOXICITY IN MICE

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[Adapted from a publication[98] in *Chemical Research in Toxicology*]

3.1 ABSTRACT

The administration of Co^{III}TMPyP one minute after a lethal dose of cyanide to mice resulted in a marked increase in survival (67%) compared to controls (33%). The time required for the Co^{III}TMPyP-treated surviving mice to right themselves from a supine position was also significantly decreased (9(±2) min.) compared to the controls (33(±2) min.). The formation of

$Co^{III}TMPyP(CN)_2$ at pH 7.4 has been shown to be completely cooperative (α_H =2) with an association constant of 2.1 (±0.2) x 10^{11}. The kinetics were investigated by stopped-flow spectrophotometry and revealed a complicated net reaction exhibiting four phases at pH 7.4 under conditions where cyanide was in excess. The data suggest molecular HCN (rather than CN^-) to be the attacking nucleophile around neutrality. The two slower phases do not seem to be present when cyanide is not in excess and the other two phases have rates comparable to that observed for cobalamin, a known effective cyanide scavenger. Addition of bovine serum albumin (BSA) did not affect the cooperativity of cyanide binding to $Co^{III}TMPyP$, only lowered the equilibrium constant slightly to 1.2 (±0.2) x 10^{11} and had an insignificant effect on the observed rate. All observations were consistent with the demonstrated antidotal activity of $Co^{III}TMPyP$ operating through a cyanide binding (*i.e.*, scavenging) mechanism.

3.2 INTRODUCTION

Cases of cyanide poisoning in which successful clinical intervention was possible have frequently involved very high doses of cyanogenic material (multiples of the LD_{50}) being slowly absorbed and distributed systemically. The antidotal use of cyanide-scavenging agents is an effective part of the therapy in such cases.[31,61,62] While only recently approved for use in the U.S., cobalamin (*i.e.*, hydroxocobalamin, a vitamin B_{12} derivative) has been accepted to be a safe and effective cyanide antidote for some years in Europe, with the central $cobalt^{III}$ ion directly binding cyanide anion. Cobalamin is, however, a less than ideal cyanide antidote requiring intravenous administration in gram quantities.[31] Its immediate biological precursor, cobinamide,

presently under development, is three to ten times more efficacious *in vivo*.[35,63] Unfortunately, from the pharmaceutical perspective, both of these cyanide scavengers are complicated molecules, costly to produce, cobinamide significantly more so than cobalamin. The alternative sodium nitrite-thiosulfate combination therapy is more cost effective, but there are toxicity issues beginning to emerge in relation to this treatment.[20,29] It follows that there remains a need to find improved cyanide antidotes that can be produced at reasonable cost and, ideally, stored at ambient temperatures.

Both cobalamin and cobinamide contain cobalt^{III} chelated within the same macrocyclic corrin-ring structure, but cobinamide lacks the 5,6-dimethylbenzimidazole ribonucleotide tail normally occupying the fifth ligand position in cobalamin. Clearly, it is advantageous that each cobinamide molecule has two axial coordination sites at the cobalt^{III} ion available to bind two cyanide anions compared with only one by cobalamin. Based upon this observation, it is reasonable to assert that cobalt^{III} complexes of other more easily synthesized macrocycles, like certain porphyrins and phthalocyanines, should exhibit cyanide-binding properties suitable for their application to antidotal cyanide scavenging. Consequently, the results of an earlier study[54] showing cobalt^{III} porphyrins to be ineffective as cyanide antidotes when given to mice prophylactically are surprising.

Hypothesizing that there may be one or more pathways through which the putative antidote could become slowly deactivated *in vivo*, an investigation was undertaken into the possible therapeutic use of a water-soluble cobalt^{III}-containing porphyrin as cyanide-scavenging antidote when given after the toxin. Cobalt^{III} *meso*-tetra(4-*N*-methylpyridyl) (Co^{III}TMPyP, Figure 7A, insert) can be synthesized in three steps from commercially available starting materials in reasonable yield. Co^{III}TMPyP is monomeric over a wide range of pH[49] and thus, has

the required two axial ligand positions available to bind cyanide anions. The results of this dissertation show that the association and rate constants for cyanide binding by Co^{III}TMPyP are such that this macrocyclic compound appears to be a suitable candidate for use as a cyanide scavenger, together with proof-of-concept data supporting its efficaciousness in mice.

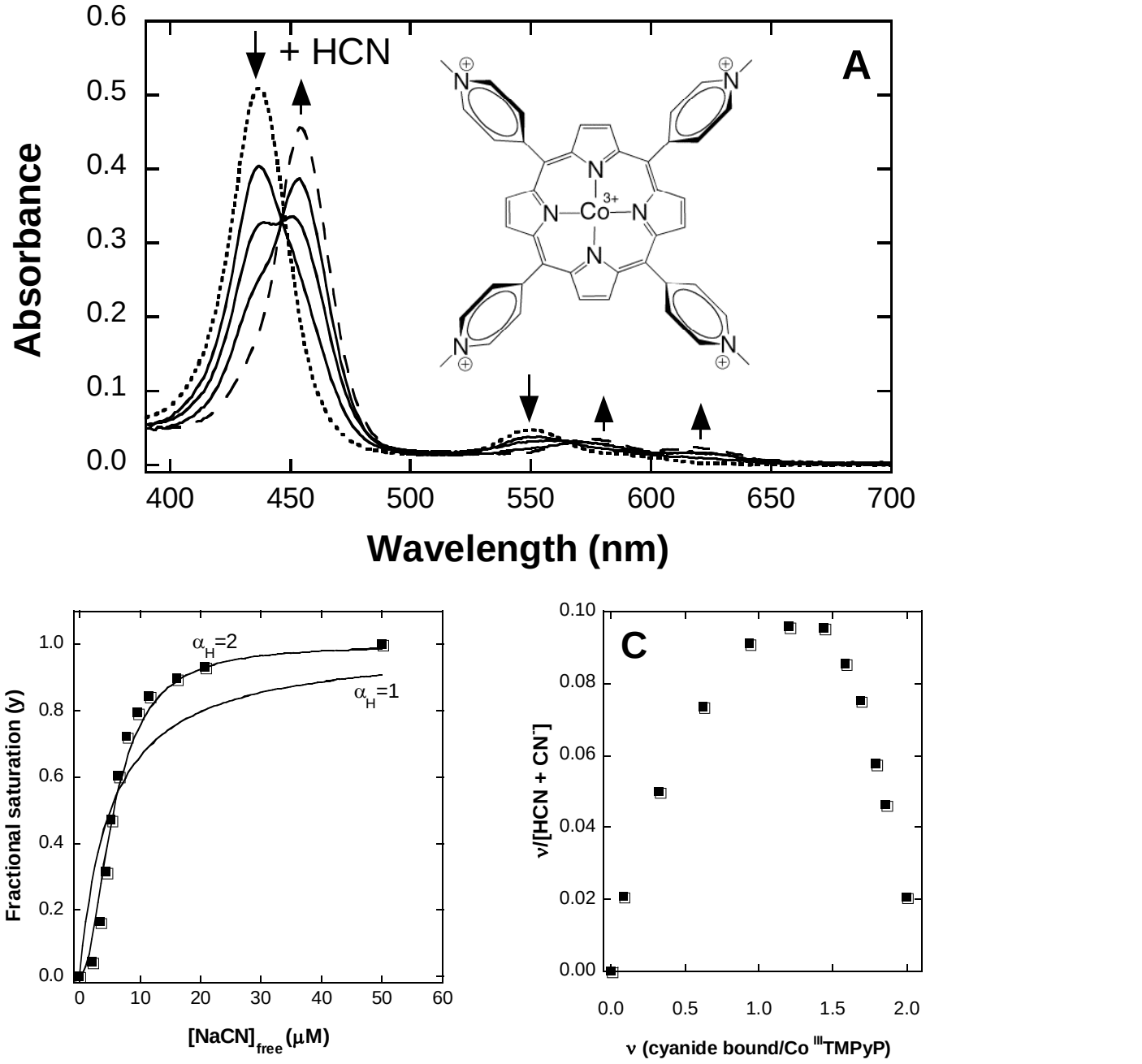

Figure 7: Titrations of $Co^{III}TMPyP(OH)(H_2O)$ with cyanide at pH 7.4 and 25°C.

Small aliquots of sodium cyanide solution in 5 mM sodium tetraborate buffer (pH 11) were titrated into a solution of $Co^{III}TMPyP(OH)(H_2O)$ **(A)** Electronic absorption spectra of $Co^{III}TMPyP(OH)(H_2O)$ species titrated with NaCN, 1.00 cm pathlength. **Main panel:** Initial $Co^{III}TMPyP(OH)(H_2O)$ (dotted trace); subsequent absorbance changes observed during the addition of cyanide to concentrations of 5, 10, and 15 μM (solid traces); final spectrum of $Co^{III}TMPyP(CN)_2$ (dashed trace) after addition of cyanide to 100 μM. **Inset:** Structure of $Co^{III}TMPyP$. **(B)** Titration of 3.48 μM $Co^{III}TMPyP(OH)(H_2O)$ with cyanide following the absorbance changes at 454 nm. The solid line represents a nonlinear least squares fit to the data using the Hill equation. **(C)** Scatchard plot (number of cyanide molecules bound per $Co^{III}TMPyP(OH)(H_2O)$ molecule (ν) versus ν/cyanide concentration) of the data from B.

3.3 RESULTS

Binding of cyanide to Co^{III}TMPyP at physiological pH: In aqueous media at pH 7.4, Co^{III}TMPyP axially binds two water molecules, one of which is deprotonated to form Co^{III}TMPyP(OH)(H_2O) with a pK_{a1} of 6.[51] The second deprotonation occurs at a much higher pH (pK_{a2}= 10.0) and, therefore, at the physiological pH of 7.4, the aquo/hydroxo form of Co^{III}TMPyP predominates (95%). Previous work also established that one molecule of Co^{III}TMPyP binds two molecules of cyanide.[51] The formation of the bis(cyano) adduct occurs in a stepwise fashion, characterized by the following equations:

(1) Co-TMPyP(OH)(H_2O) + HCN $\leftrightarrows$ Co-TMPyP(H_2O)(CN) + H_2O K_{f1}

(2) Co-TMPyP(H_2O)(CN) + HCN $\leftrightarrows$ Co-TMPyP$(CN)_2$ + H_3O^+ K_{f2}

Hambright and Langley[52] report an equilibrium constant for the second process (K_{f2}, monocyano to dicyano) to be about 5.6 (± 0.3) x 10^7. The equilibrium process in this and many other Co^{III} macrocycles is thought to be completely cooperative, that is the second cyanide binds as soon as the first is attached to the cobalt atom. In order to obtain the overall equilibrium constant for the binding of two cyanides at physiological pH, a titration of Co^{III}TMPyP(OH)(H_2O) with cyanide in 50 mM phosphate buffer, 1 mM EDTA, pH 7.4, was carried out using a spectrophotometric method. Co^{III}TMPyP(OH)(H_2O) in a sealed cuvette with little to no headspace was monitored spectrophotometically as known amounts of cyanide (kept in borate buffer to prevent HCN loss) were added using a gas tight syringe and a time interval of 10 minutes was allowed between additions for the solution to equilibrate before recording spectra (see Chapter 2.4.2). The electronic absorption spectra obtained during the titration of Co^{III}TMPyP(OH)(H_2O) with cyanide at 25°C display well-maintained isosbestic

points (Figure 7A) indicating the presence of only two species: the aquo/hydroxo and the dicyano species. As the wish was to report a physiologically relevant reaction, an effective formation constant (K'_{β}) can be defined as $K'_{\beta} = [Co^{III}TMPyP(CN)_2] / ([Co^{III}TMPyP(H_2O)_2] [HCN]^2)$ at pH 7.4 where the hydrogen ion concentration is ignored. A further complication arises from the fact that cyanide has a pK_a of 9.2 at 25°C, so that 94% of the cyanide free in solution is protonated, but the form bound to $Co^{III}TMPyP$ is the anion. From the spectra (at 454 nm) the fraction of the $Co^{III}TMPyP(CN)_2$ per total porphyrin (fractional saturation, Y) was determined, and thus the free cyanide concentration (protonated and unprotonated) could be calculated. In Figure 7B the free cyanide is plotted versus the fractional saturation (Y) and the data was fit using a nonlinear regression and the Hill equation:

$$Y= [(CN)\alpha_H/K'_{\beta}]/[1+(L)\alpha_H/K'_{\beta}]$$

Using $\alpha_H = 2$ gave the best fits of the data ($\alpha_H = 1$ is shown for comparison) confirming the complete cooperativity of the binding and K'_{β} was found to be $2.1(\pm0.2) \times 10^{11}$. A Scatchard plot (Figure 7C) of the data also shows the cooperative nature of the interaction by the convex-curve shape obtained. If independent, non-interacting sites of cyanide binding existed, the Scatchard plot obtained would be linear. Thus the data in Figure 1 confirms the all or none reaction of $Co^{III}TMPyP(H_2O)(OH)$ with cyanide to form the dicyano complex as has been noted for many other water soluble porphyrin and corrin systems. The same calculations were applied to the data obtained at 37°C resulting in a $K'_{\beta 37°C}$ of $2.4(\pm0.2) \times 10^{11}$.

Kinetics of cyanide binding to $Co^{III}TMPyP$: The substantial magnitude of the equilibrium binding constant of $Co^{III}TMPyP$ to cyanide hints at its potential as a cyanide-chelating antidote. To be efficacious, it is also necessary that the reaction of $Co^{III}TMPyP$ with cyanide is reasonably fast. In order to verify the suitably fast binding of cyanide to

$Co^{III}TMPyP(OH)(H_2O)$, stopped-flow spectrophotometric experiments were conducted under pseudo-first order cyanide conditions (at least a 20-fold excess of total cyanide, CN^- + HCN), monitoring the appearance of the $Co^{III}TMPyP(CN)_2$ Soret maximum (454 nm) at two temperatures. At pH 7.4 and 25°C, the resulting data clearly did not represent a single process and was best fit by at least three exponentials (Figure 8A). It was apparent from the sum of the amplitudes of these three phases that the overall process was incomplete and, consequently, there must be at least one additional slower phase. The additional phase was inconveniently slow for measurement in the stopped-flow spectrometer, so these measurements were performed independently in a conventional spectrophotometer. At both 25°C and 37°C four rate constants were obtained that all showed a linear dependence on cyanide concentration with zero or near-zero intercepts (Figures 8B, 8C, 8D and 8E). As expected all four rate constants increased upon raising the temperature from 25°C to 37°C (Table 1). The amplitude of the absorbance changes was, however, increased for k_1 and decreased for k_2, while k_3 and k_4 remained roughly constant (Table 1).

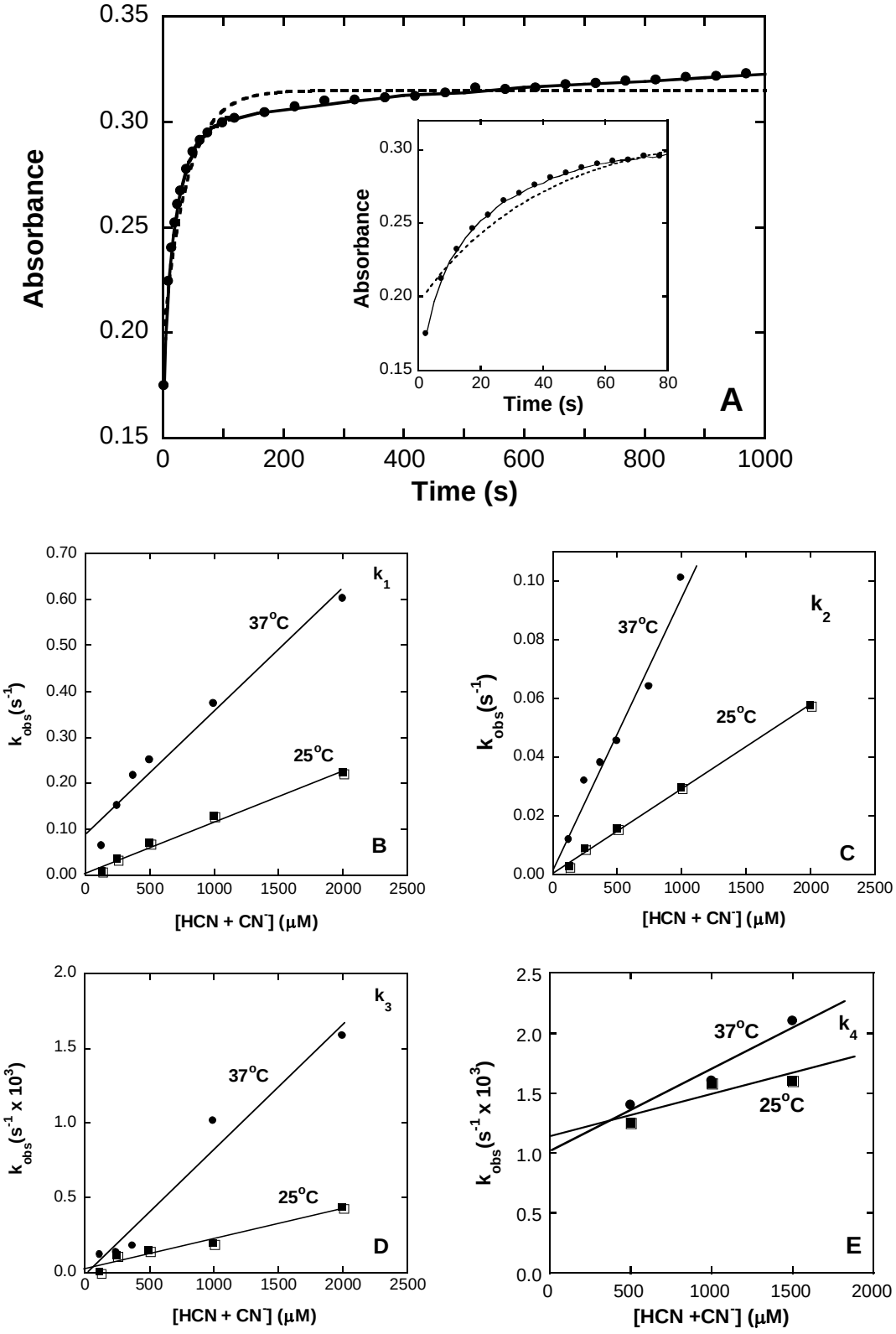

Figure 8: Kinetics of the reaction of cyanide with $Co^{III}TMPyP(OH)(H_2O)$ under pseudo first order conditions.

The reaction of sodium azide with $Co^{III}TMPyP(OH)(H_2O)$ was also examined (pH 7.4, 25°C) and only one exponential was observed (Figure 9, main panel) apparently in keeping with the results of an earlier study involving thiocyanate anion and this same metalloporphyrin.[51] From the slope of the linear fit of the observed rates with the azide concentrations (Figure 9, insert) a rate constant of 16.2 (±0.8) $M^{-1}s^{-1}$ (based on total cyanide) was calculated. This is less than the previously reported[53] value of ~10^2 $M^{-1}s^{-1}$ for the rate constant for the reaction of thiocyanate with $Co^{III}TMPyP(OH)(H_2O)$ at pH 8 (presumably at room temperature) and intermediate between the k_2 (29 M^{-1} s^{-1}) and k_3 (9 M^{-1} s^{-1}) found here for the reaction of cyanide with $Co^{III}TMPyP(OH)(H_2O)$ at pH 7.4 and 25°C (Table 1). The observed monophasic azide-binding (Figure 9) and similar thiocyanate-binding[53] kinetics strongly suggest that the multiphasic nature of the cyanide-binding reaction (Figure 8A) is specific to the HCN/CN^- system rather than the metalloporphyrin. The previous observations of multiphasic kinetic behavior for the analogous cobinamide reaction by Baldwin *et al.*[64] support this assertion. It has previously been noted[65,66] that the available data regarding anion binding to metalloporphyrins suggests a dissociative mechanism; specifically in the present context, it is the dissociation of water molecules that is expected to be rate limiting and the identity of the incoming nucleophile should be relatively unimportant in determining the substitution rate. Therefore, the similarity of the rate constants found for the reaction of $Co^{III}TMPyP$ with N_3^-, cyanide, and that previously reported for SCN^- is[53] i) unremarkable and ii) does not provide any unambiguous clues as to whether HCN or CN^- might be the attacking species in any of the phases documented in Table 1.

Table 1: Second order rate constants and absorbance amplitudes for the formation of $Co^{III}TMPyP(CN)_2$ at 25-37°C, pH 7.4-8.4 in 0.1 M sodium phosphate buffers.

	Phase 1		Phase 2		Phase 3		Phase 4	
Conditions	k_1 ($M^{-1}s^{-1}$)	ΔA%	k_2 ($M^{-1}s^{-1}$)	A%	k_3 ($M^{-1}s^{-1}$)	A%	k_4 ($M^{-1}s^{-1}$)	A%
pH 7.4, 25 °C	111 (±7)	6	29 (±1)	40	9 (±1)	8	0.35 (±.05)	46
pH 7.4, 37 °C	270 (±24)	14	93 (±9)	25	20 (±3)	7	0.7 (±.05)	49
pH 8.4, 25 °C	-----	0	30 (±5)	9	5 (±1)	18	0.4 (±.05)	43

The observed kinetics could be resolved into four exponential phases. Rate constants are calculated from linear fits of observed rates to the cyanide concentration as shown in Figure 8 (cyanide was in at least 20-fold excess over porphyrin). The percent absorbance change (amplitude) for each phase is also given. Uncertainties shown in parentheses are standard deviations.

Seeking clarification regarding the possible involvement of HCN and/or CN^-, the kinetics of cyanide binding at pH 8.4, where the porphyrin is still expected to be predominately (> 95%) in the aquo/hydroxo form (pK_as = 6.0 and 10.0)[53] were examined. At pH 8.4, in 50 mM phosphate buffer, only two exponential phases were revealed in the stopped-flow data (not shown) plus the slowest phase observed by conventional spectrophotometry remained. The fastest rate, k_1, could no longer be found and the slower rate constants were essentially unchanged (Table 1). As the fast rate, corresponding to k_1 (Figure 8B) may have increased and been lost in the dead-time, the kinetics were carefully examined at lower cyanide concentrations, on a millisecond time scale, and even at a temperature of 10°C in an attempt to observe the "missing" phase. It was found that the total absorbance changes were very similar to those observed at pH 7.4 (except slower-corresponding to the second rate constant) and thus our conclusion is that the "fast" rate, with rate constant k_1, is not present at pH 8.4.

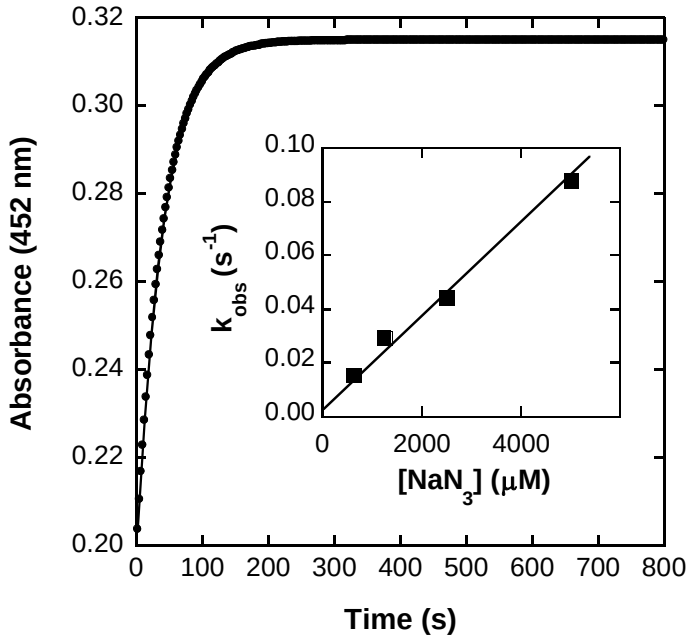

Figure 9: Kinetics of the reaction of azide with Co^{III}TMPyP(OH)(H_2O) under pseudo first order conditions.

The reaction was followed at 454 nm in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 25°C under conditions of excess sodium azide (500 to 5000 μM). **Main panel:** Representative stopped flow kinetic transient for the reaction of azide with Co^{III}TMPyP(OH)(H_2O) under pseudo first order conditions (solid circle) with a single exponential fit (solid line). **Inset:** Linear fit of the observed rates with increasing azide concentration, slope = 16.2(±0.8) $M^{-1}s^{-1}$.

Cyanide binding to Co^{III}TMPyP in the presence of bovine serum albumin (BSA): *In vivo* Co^{III}TMPyP will encounter many biomolecules potentially able to interfere with its cyanide-scavenging capability. Serum albumin is present at relatively high concentration in the bloodstream and thus a likely participant in such interference. Therefore, examination of the reaction of Co^{III}TMPyP with cyanide in the presence of a physiologically relevant amount of bovine serum albumin (10 μM BSA) should be instructive. In the presence of BSA the electronic absorption spectra of both Co^{III}TMPyP(OH)(H_2O) and Co^{III}TMPyP($CN)_2$ (Figures

10A and B) exhibited very minor differences (red shifts and decreases in intensity) compared with the spectra of the same species in BSA-free buffer. BSA is a cysteine-rich protein and it is reasonable to expect more dramatic spectral changes than those evident (Figures 10A and B) if substantial displacement of the water-derived axial ligands in $Co^{III}TMPyP(OH)(H_2O)$ by cysteine thiols occurred. The small shifts and intensity differences observed most probably indicate an interaction between BSA and the metalloporphyrin primarily involving the macrocyclic moiety rather than the $cobalt^{III}$ ion. A titration experiment was then conducted to assess any effect of the presence of BSA on cooperativity and binding equilibrium in the reaction of cyanide with $Co^{III}TMPyP$. The titration curve (fractional saturation versus free cyanide, Figure 10C) indicated that the cooperativity of the cyanide binding was essentially maintained and the equilibrium was only slightly lowered; $K'_{\beta BSA} = 1.2\ (\pm 0.2) \times 10^{11}$ at 25°C. Additionally, when the rate of cyanide binding in the presence and absence of BSA was conducted, it was clear that the initial rates were virtually identical (Figure 10D). So, contrary to expectation, these observations collectively suggest that the presence of proteinaceous species (such as amines or thiols) as potential axial ligands does not, in fact, interfere significantly with the cyanide-scavenging ability of $Co^{III}TMPyP$.

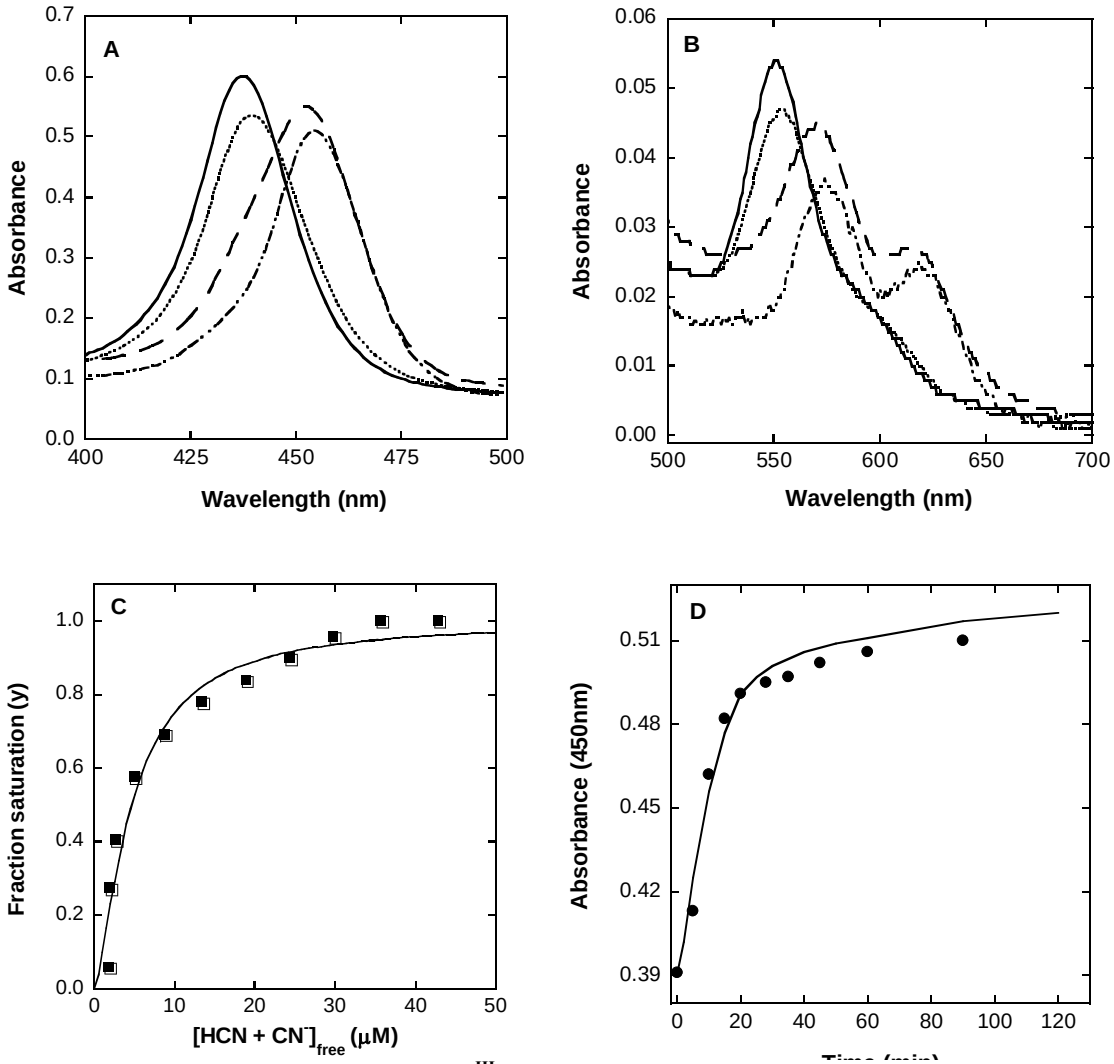

Figure 10: Titrations and kinetics of $Co^{III}TMPyP(OH)(H_2O)$ with cyanide in the presence of BSA at pH 7.4 and 25°C.

(A) Electronic absorption spectra from 400 nm to 500 nm of 3.48 μM $Co^{III}TMPyP(OH)(H_2O)$ (solid trace), $Co^{III}TMPyP(OH)(H_2O)$ + 10 μM BSA (dotted trace). **(B)** Electronic absorption spectra from 500 nm to 700 nm of 3.48 μM $Co^{III}TMPyP(CN)_2$ (solid trace) and $Co^{III}TMPyP(CN)_2$ + 10 μM BSA in the presence of excess cyanide (dash-dot trace). **(C)** Small aliquots of sodium cyanide solution in 5 mM sodium tetraborate buffer (pH 11) were titrated into a solution of $Co^{III}TMPyP(OH)(H_2O)$ (3.48 μM in 50 mM sodium phosphate buffer, pH 7.4, 1 mM in EDTA) in the presence of 10 μM BSA using gas-tight syringes and a 1.00 cm pathlength septum-sealed cuvette at 25°C (see Chapter 2.4.2 for further details). The solid line represents a nonlinear least squares fit to the data using the Hill equation. **(D)** The rate of cyanide binding in the presence (solid dots) and absence (solid trace) of BSA. Conditions: 3.48 μM $Co^{III}TMPyP(OH)(H_2O)$ in 50 mM sodium phosphate buffer, pH 7.4, 1 mM in EDTA and 0.1 mM in NaCN.

Animal Experiments: A previous study with mice in which Co^{III}TMPyP was given as a prophylactic, 15 and 60 minutes prior to the administration of lethal doses of cyanide, found no beneficial antidotal effect.[54] To the contrary, in the present work with Swiss Webster mice (males, 16-20 weeks of age) the effectiveness of Co^{III}TMPyP as a cyanide antidote when administered after the toxin (Figure 11) was demonstrated. These data require some comments for clarification. The LD_{50} for NaCN given *i.p.* to the same kind of mice as employed in this work has been reported to be 5.7 mg/kg by Baskin *et al.*[67] Clearly, since 12 out of 18 control mice died (Figure 11A) the 5.0 mg/kg NaCN used in the present experiments was greater than the LD_{50}. The reason for the discrepancy probably resides in the ease with which volatile HCN can be lost from working cyanide solutions. Despite attempting to reduce these losses by working in sealed vessels with minimized headspaces and making all transfers with gas-tight syringes, in our laboratory the effective LD_{50} for *i.p.* NaCN has appeared to vary between the reported 5.7 mg/kg and about 4.5 mg/kg – dependent upon the particular combinations of glassware and personnel employed. Given the likely systematic error involved (HCN loss) during manipulations, it is our opinion that the true value for the LD_{50} for *i.p.* NaCN must lie closer to 4.5 mg/kg than 5.7 mg/kg for these mice. For descriptive purposes, the current 5.0 mg/kg NaCN dose is labeled as "just lethal." When Co^{III}TMPyP (21 mg/kg, *i.p.*) was administered within 1 minute of this cyanide dose, survival increased from 33% to 67% (Figure 11A). Recently, 85% survival for mice given 12 mg/kg sodium nitrite as an antidote 2 min. after the same just-lethal NaCN dose[20] was observed. The ability of surviving intoxicated individuals placed upon their backs to right themselves (see Chapter 2.4.4) is a convenient behavioral assessment of sub-lethal intoxication and recovery. The ability of surviving mice to right

themselves was found to be significantly improved by administration of $Co^{III}TMPyP$ compared to controls given just saline, with recovery times the same as those previously found for sodium nitrite[20] being observed (Figure 11B). In summary, when given shortly after the toxin, $Co^{III}TMPyP$ is clearly antidotal to cyanide intoxication in mice.

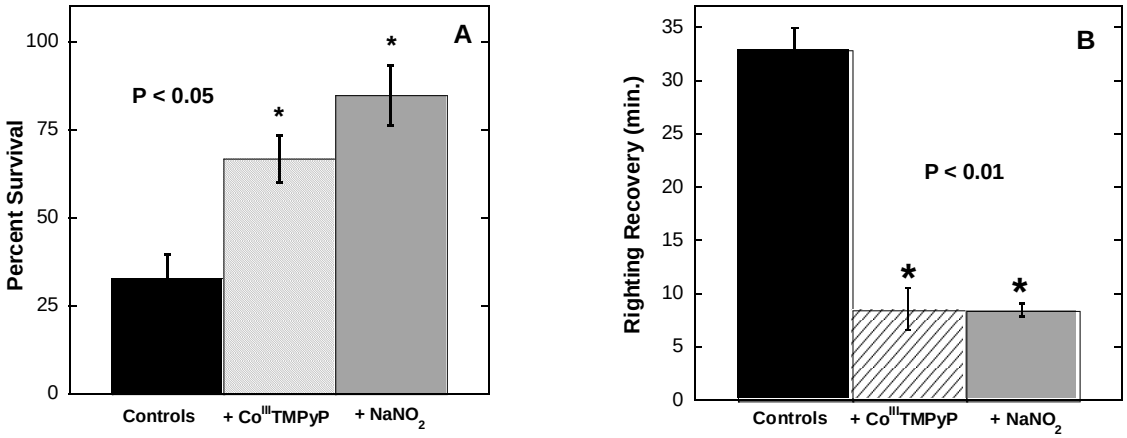

Figure 11: The antidotal effect of $Co^{III}TMPyP$ on cyanide-intoxicated mice.

(A) Survival rates showing the efficacy of $Co^{III}TMPyP$ (20.8 mg/kg, 20% LD_{50}, *i.p.*) and sodium nitrite (12 mg/kg, *i.p.*) in Swiss-Webster mice (males, 16-20 weeks of age) given a just-lethal dose of NaCN (5 mg/kg *i.p.*). Following the administration of toxin (t = 0) the antidote was quickly delivered ($Co^{III}TMPyP$ at t = 1 min, or sodium nitrite at 2 min, both *i.p.*). **(B)** The righting recovery time of the surviving cyanide-intoxicated mice was decreased in those administered $Co^{III}TMPyP$ (20.8 mg/kg) or sodium nitrite (12 mg/kg, *i.p.*). Following the administration of toxin (t = 0) and antidote, the surviving mice were placed on their backs and the times at which the animals righted were recorded (see Chapter 2.4.4 for details).

3.4 DISCUSSION

Antidotal effectiveness of $Co^{III}TMPyP$: Equilibrium constants for the binding of cyanide to various cobaltic macrocycles have been reported, but the numerical values depend on assumptions made about the binding model –in particular, i) whether [CN^-] or [HCN] is written on the left hand side of equations 1 & 2 and ii) should the hydrogen-ion concentration be

included.[54,68-72] For example, the formation constant for the (mono)cyano adduct of the FDA approved cyanide-scavenging agent cobalamin (or vitamin B_{12}) was originally (1960) evaluated in terms of a model assuming CN^- to be the incoming nucleophile:[72]

(3) $B_{12}(H_2O) + CN^- \leftrightarrows B_{12}(CN^-) + H_2O$; $K_{f\{1960\}} = [B_{12}(CN^-)] / ([B_{12}(H_2O)][CN^-]) \approx 10^8$

In a seminal paper almost 20 years later (1979) Reenstra and Jencks[69] showed, however, that HCN is the relevant nucleophile around neutrality:

(4) $B_{12}(H_2O) + HCN \leftrightarrows B_{12}(CN^-) + H_3O^+$; $K_{f\{1979\}} = [B_{12}(CN^-)][H_3O^+] / ([B_{12}(H_2O)][HCN])$

Here, in the case of Co^{III}TMPyP, it is agreed upon that two cyanide molecules displace the water-derived axial ligands from the aquo/hydroxo form of Co^{III}TMPyP, but make no other assertion as to the species of cyanide (HCN or CN^-) involved, or whether a proton is consumed/released. That is, to facilitate comparison with ligand-binding constants as normally reported for metalloproteins, an essentially biochemical convention is adopted and the result as K'_β with a value of $2.1(\pm0.2) \times 10^{11}$ is presented, where the equilibrium constant has been evaluated in terms of total cyanide (= $[HCN + CN^-] \approx [HCN]$ at pH 7.4) and the product $[H_3O^+]$ has simply been left out. Consequently, to meaningfully compare the present result with the earlier formation constant for cyanocobalamin of George *et al.*,[72] the earlier result should be multipied by $[CN^-]/[HCN]$ at neutrality: $10^8 \times (\sim10^{-2}) \approx 10^6$. Therefore, inspection of the relevant equilibrium constants suggests that Co^{III}TMPyP could be a significantly better cyanide scavenger than cobalamin.

Hambright *et al.* have reported[52] a formation constant of 5.6×10^7 for the binding of a second cyanide anion to the monocyanoCo^{III}TMPyP complex. Re-calculating this result to reflect total cyanide rather than the anion concentration (as *per* the earlier authors) gives K'_{f2} =

9.0×10^5, again a significantly smaller numerical value, confirming that some care needs to be exercised in comparing results from different studies. It follows that $K_{f1} = K'_{\beta} - K'_{f2} = 2.1 \times 10^{11} - 9.0 \times 10^5 \approx 10^5$. These estimates for the two formation constants (*i.e.*, K_{f1} and $K'_{f2} \approx 10^5$ and 10^6 respectively) are in keeping with the binding of cyanide to Co^{III}TMPyP being cooperative and, indeed, a Hill constant of 2.0 fits the equilibrium data much better than a constant of 1.0 (Figure 7B) or any intermediate number. Titrations performed in the presence of one equivalent BSA (Figure 10C), a possible source of interference in serum, show no alteration in the cooperativity of cyanide binding to Co^{III}TMPyP and only modest changes in the equilibrium constant (1.2×10^{11} in the presence of BSA *vs.* 2.1×10^{11} without). Moreover, a kinetic comparison (Figure 10D) shows that there is virtually no difference in the rate of cyanide binding to Co^{III}TMPyP in the presence and absence of BSA. These findings appear to confirm the promising candidacy of Co^{III}TMPyP as a potentially useful cyanide antidote.

The second order rate constant for the binding of cyanide to cobalamin at 25ºC and approximately neutral pH has been reported to be 80 $M^{-1}s^{-1}$.[69] This is comparable to the faster phases of the reaction observed for cyanide with Co^{III}TMPyP (Table 1). Therefore, there does not seem to be any overriding kinetic reason why Co^{III}TMPyP should not, by virtue of its cyanide-scavenging ability, have reasonable antidotal activity toward cyanide intoxication. Consequently, the reported[54] lack of any such antidotal activity in mice is troublesome, as based upon these findings the accepted cyanide-scavenging mechanism by which cobalamin surely works could logically be questioned.

In the previous study[54] the Co^{III}TMPyP (and other metalloporphyrins) were given prophylactically, 15 and 60 min. before the (lethal) cyanide dose, with no detectable beneficial effect. In the present proof-of-concept study the Co^{III}TMPyP was delivered soon (1 min.) after

the cyanide and found readily measurable protection both in terms of fewer deaths (Figure 11A) and quicker recovery of survivors (Figure 11B) for mice given the antidote compared to controls. The failure of Co^{III}TMPyP as a prophylactic cyanide antidote must be due to its inactivation within 1-15 min. *in vivo*. The possible mechanism(s) responsible for this inactivation are currently under investigation and will be presented in due course.

Complexity of the reaction between Co^{III}TMPyP and cyanide: The kinetics and mechanisms of the substitution reactions of various anionic ligands with water-soluble porphyrins have been studied by several groups, but in particular, Pasternack & Cobb[53] found that the addition of thiocyanate (SCN^-) and a solvent proton to $Co^{III}TMPyP(OH)(H_2O)$ resulted in an intermediate, $Co^{III}TMPyP(H_2O)(SCN)$, which then quite rapidly acquired a second thiocyanate ion to form $Co^{III}TMPyP(SCN)_2$. The presence of the thiocyanate group was found to exert a *trans* influence in the $Co^{III}TMPyP(H_2O)(SCN)$ intermediate resulting in the fast addition of the second thiocyanate, so that the addition of the first thiocyanate is the rate-determining step. The limited data set obtained for the reaction of Co^{III}TMPyP with azide (N_3^-) (Figure 9) appears to be in keeping with an analogous mechanism. Based upon such observations, it is reasonable to expect that cyanide should behave similarly and, indeed, in the case of another water-soluble metalloporphyrin, $Cobalt^{III}$-tetrakis(4-sulfonatophenyl)porphyrin (Co^{III}TSPP), a two-step reaction in which the first step is rate limiting was found.[52] In their report primarily on the Co^{III}TSPP-cyanide reaction, Hambright and Langley[52] briefly noted that the first cyanide addition to $Co^{III}TMPyP(OH)(H_2O)$ had a rate constant of 1.1×10^3 $M^{-1}s^{-1}$ at pH 7.4 and 25°C – but then made no further mention of Co^{III}TMPyP, did not show any of the relevant data and their opinion regarding the attacking species, cyanide anion or HCN, was unclear.

As is now shown, the kinetics of cyanide binding to Co^{III}TMPyP are remarkably complicated with three to four rate constants (depending on pH, see Table 1) all of which depend on the cyanide concentration (Figure 8). It is highly unlikely that unidentified impurities in the Co^{III}TMPyP preparations are responsible for any of these phases as the otherwise analogous azide reaction exhibits only a single phase (Figure 9). Similar, Baldwin *et al.*[64] observed three exponential phases in their study of the rate of reaction of cyanide with cobinamide, a Co^{III}-containing corrin, where the fastest phase was lost on raising the pH from 7 to 8. Therefore, the observed kinetics of the association reaction between cyanide and Co^{III}TMPyP (Figure 8, Table 1) has more features in common with the analogous cobinamide reaction[64] than with the Co^{III}TSPP reaction.[52] The pK_a of HCN is 9.24 at 25ºC[73] and it follows that in the kinetic experiments at pH 8.4 the concentration of cyanide anion was an order of magnitude greater than at pH 7.4. Therefore, if CN^- were the incoming nucleophile in any of the kinetic phases, there should be a significant increase in the observed rate for that phase at pH 8.4 compared to pH 7.4. No such effect was observed and, consequently, the data indicate molecular HCN to be the more important attacking nucleophile. This is in keeping with the previously reported findings for cobalamin where HCN was also shown to be the attacking nucleophilic species around neutral pH rather than the anion.[69] To the contrary, the reaction of cyanide with Co^{III}TSPP does appear to involve both HCN and CN^- under neutral conditions, as the observed reaction rate increases by 30% between pH ~7 and ~8.[52] At pH 7.4, a small portion of Co^{III}TMPyP (~4%, pK_a = 6) exists as the diaquo complex ($Co^{III}TMPyP(OH_2)_2$) whereas at pH 8.4, a small portion (~ 3%, pK_a = 10) is present as the dihydroxo complex ($Co^{III}TMPyP(OH)_2$). In the case of both $cobalt^{III}$ corrins[64] and porphyrins,[52] displacement of the axial aquo ligands in the bis(aquo) complexes by cyanide is very slow and, consequently, the presence of the rapid phase 1 at pH 7.4 (and its

absence at pH 8.4) (Table 1) cannot be explained on the basis of this phase involving reaction of the bis(aquo) Co^{III}TMPyP.

It is apparent in the kinetic traces (*e.g.*, Figure 8A) that the reaction was still continuing more than 15 min. after it was initiated. This was not the case in the titration experiments where absorbance changes had ceased within 10 min. of cyanide additions being made and suggests that at least the phase associated with k_4 (and perhaps also that associated with k_3) is(are) only present when cyanide is in large (> 20-fold) excess over Co^{III}TMPyP. There are plausible explanations for such behavior. For example, cyanide may self-associate to form complex species (such as NC—H---NCH, $[NC—H---NC]^-$, *etc.*) that could form metastable complexes with Co^{III}TMPyP, inhibiting its final conversion to $Co^{III}TMPyP(CN)_2$. Supporting this argument, the amplitude of phase 4 was observed to diminish at high ionic strength (0.3 M KCl, not shown). Since the focus of the present study was, however, the potential use of Co^{III}TMPyP as an antidotal cyanide scavenger, the details of reactions in which cyanide is in large excess are of marginal interest and investigation of the slow phases was abandoned.

Resultantly, the two quicker phases must be considered in relation to cyanide scavenging *in vivo*, where the cyanide cannot be in large excess over the antidote if the intervention is to be successful. The kinetics of the reaction between pyridine and Co^{III}TMPyP have previously been shown to be biphasic at pH 8 and explained on the basis of rapid substitution by the first pyridine followed by slower substitution of the second to yield the bis(pyridyl) product.[50] The cyanide results, monitored at the absorption maximum for $Co^{III}TMPyP(CN)_2$ (454 nm), are inconsistent with a similar biphasic mechanism at pH 8.4. If phase 2 were to represent formation of the mono(cyano) adduct followed by phase 3 leading to the final bis(cyano) product, then the amplitude of phase 3 would have to be the same or greater than the phase 2 amplitude, whereas

the reverse situation was actually observed (Table 1). The amplitudes of phase 2 and phase 4 (Table 1) are consistent with a two-step mechanism similar to the pyridine reaction, but if this were the case, phase 4 would have to dominate the later stages of any titration procedures rather than be absent as already stated. Based upon these observations, it is concluded that the presence of more than one independent phase in the cyanide-reaction kinetics necessarily means there are multiple mechanistic pathways to product formation and suggest the following scheme (Figure 12) to account for the two faster processes (Phases 1 and 2):

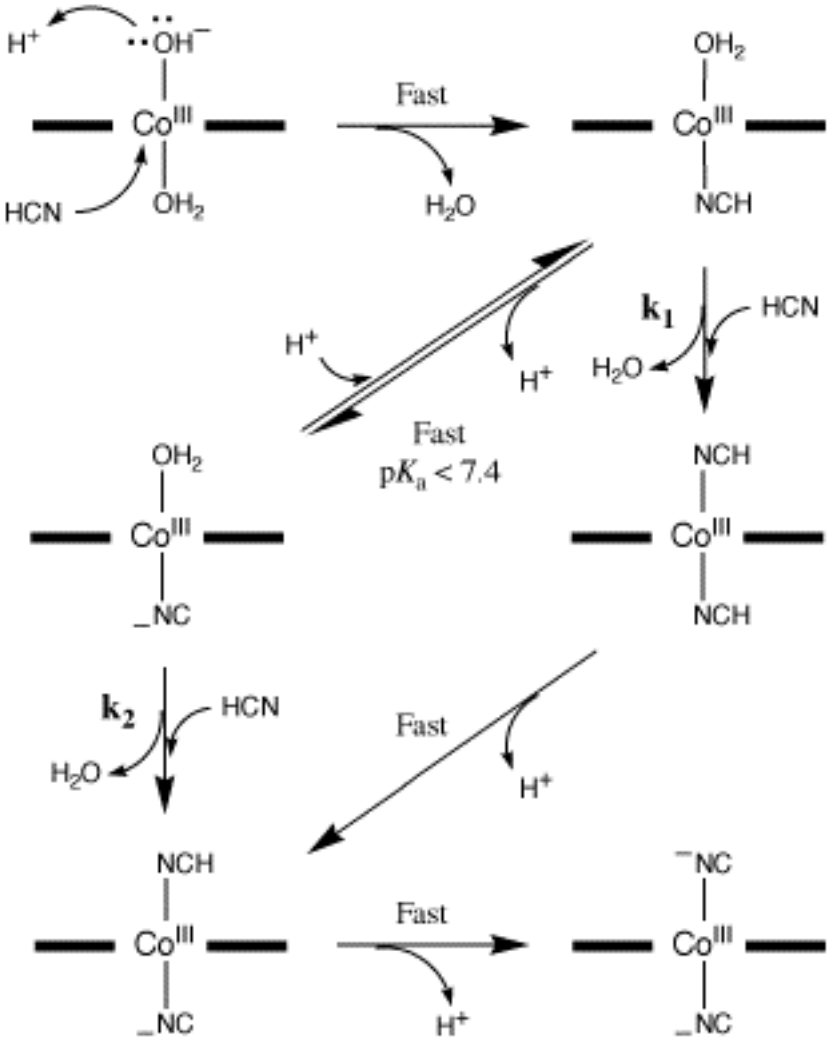

Figure 12: A plausible scheme for the two fastest phases of the reaction between Co^{III}TMPyP and HCN at pH 7.4-8.4.

The key features of this scheme are i) interconversion of liganded HCN and CN^- in the intermediate, with an associated pK_a somewhat less than 7.4, accounts for the absence of phase 1 under mildly alkaline conditions (Table 1) and ii) the differing *trans* effects of HCN and CN^- account for the two distinct rate constants (k_1 and k_2). Of course, our assertion that HCN is the most important attacking nucleophile only applies to the experimentally detected rate-

determining processes. For reasons discussed above, these findings cannot be reconciled with the substitution of the first cyanide being rate limiting in the reaction with Co^{III}TMPyP. Consequently, in the initial fast step leading to formation of the mono(cyano) intermediate, the nucleophile could be CN^- rather than HCN (as drawn) or a combination of both.

Concluding Remarks: However perplexing the mechanistic details may be, the rate of the cyanide reaction with Co^{III}TMPyP and the magnitude of the association constant are large enough to render this metalloporphyrin an effective antidote to cyanide intoxication in experimental animals (Figure 11). The multiple positive charges on the molecule (Figure 7A, inset) lead us to suspect that it may partition into mitochondria and have an undesirable toxicity not readily apparent in the present proof-of-concept study. Nevertheless, the results are encouraging in so far as they suggest that many cobaltic macrocycles should be antidotal to cyanide intoxication, broadening the range of potential candidate structures to include simpler, less expensive molecules than the corrinoids currently either available, or under development.

4.0 THE EFFECT OF ASCORBATE ON THE CYANIDE SCAVENGING CAPABILITY OF $Co^{III}TMPyP$

4.1 ABSTRACT

The rate of reduction of $Co^{III}TMPyP$ by ascorbate is quite rapid, with second order rate constants of 8.3 x 10^4 $M^{-1}s^{-1}$ at 25°C and 1.4 x 10^5 $M^{-1}s^{-1}$ at 37°C. Addition of cyanide to $Co^{II}TMPyP$ results in the binding of cyanide as evidenced by EPR spectroscopy but CV and kinetic investigations indicate that cyanide induces oxidation to the Co^{III} dicyano species. The equilibrium binding constant derived from the addition of cyanide to the reduced form (K_β = 2.1 (±0.1) x 10^{10}) is quite similar to the previously determined binding constant for cyanide binding to the oxidized form ($Co^{III}TMPyP$, K_β = 2 x 10^{11}). EPR spectra of mouse blood taken after the addition of the $Co^{III}TMPYP$ show that the cobalt is reduced and that subsequent addition of cyanide to the blood results in the disappearance of this signal indicating that cyanide scavenging has taken place. The kinetics of cyanide binding to $Co^{II}TMPyP$ are complicated; four phases were found, which were shown to be dependent on the cyanide concentration. A mechanism for the binding of cyanide to the reduced form is proposed.

4.2 INTRODUCTION

In a recent proof-of-concept study the efficacy of Cobalt^{III} *meso*-tetra(4-*N*-methylpyridyl)porphine (Co^{III}TMPyP) as an antidote for cyanide toxicity (Chapter 3) was demonstrated. Therefore, the results of an earlier investigation[54] showing cobalt^{III} porphyrins to be ineffective as cyanide antidotes when given to mice prophylactically remain intriguing. While some effort was expended on Co^{III}TMPyP and other water-soluble cobalt porphyrins in the past,[49, 52-54] there seems to be no recent work addressing the possible development of these compounds as cyanide scavengers. This is surprising given the renewed interest in the availability of such compounds to treat victims of smoke inhalation.[74-76] As such, further effort was expended in pursuit of finding cyanide-binding agents that are cheaper and similarly effective to the known compounds hydroxocobalamin and cobinamide. Hypothesizing that the reported lack of prophylaxis exhibited by Co^{III}TMPyP in cyanide-intoxicated mice[54] might be a consequence of the reduction of the complex to the Co^{II} form by reductants circulating in the blood, the following has been undertaken in this chapter: (i) an investigation into the kinetics of reduction of Co^{III}TMPyP by ascorbate; (ii) a determination of the equilibrium and kinetic cyanide-binding characteristics of the reduced product, Co^{II}TMPyP; (iii) verification that the same reactions occur in the mouse bloodstream.

4.3 RESULTS

Ascorbate reduction of Co^{III}TMPyP: The reduction of Co^{III}TMPyP to Co^{II}TMPyP has been shown to have a quasi-reversible reduction potential with an $E^{0'}$ of 0.42 V in 0.1 M H_2SO_4 vs. NHE using either cyclic voltammetry (CV) or by spectroelectrochemistry employing an optically-transparent thin-layer electrode (OTTLE) in the absence of oxygen.[77,78] Pasternack and Cobb,[51] verified by Chan *et al.*,[77] determined Co^{III}TMPyP to have two pK_as of 6.0 (±0.1) and 10.0 (±0.1) for the following acid-base equilibria:

(5) $Co^{III}TMPyP(H_2O)_2 \leftrightarrows Co^{III}TMPyP(H_2O)(OH) + H^+$ K_{a1}

(6) $Co^{III}TMPyP(H_2O)(OH) \leftrightarrows Co^{III}TMPyP(OH)_2 + H^+$ K_{a2}

Thus at neutral pH, the aquo/hydroxo form predominates and the pertinent equation for reduction at pH > 6, as determined by Chan *et al.*, is as follows:

$$Co^{III}TMPyP(H_2O)(OH) + e^- + H^+ \leftrightarrows Co^{II}TMPyP$$

The absence of axial ligands in the product should be taken to mean that the presence of either one, two or no water molecules is somewhat controversial, although Stich *et al.* have concluded that there are no axial ligands present in reduced corrinoids.[79] Chan *et al*. also determined the reduction potential at pH 7.4 to be 0.33 V vs. NHE. The product of the reaction was previously determined by exhaustive electroreduction of Co^{II}TMPyP in an OTTLE cell.[77] Ascorbate, found in circulating plasma at a concentration of ~60 μM,[80] has a reduction potential of -0.06[81] and therefore should easily be able to reduce $Co^{III}TMPyP(OH)(H_2O)$. The rate of reduction under pseudo-first order conditions was observed by stopped-flow spectrophotometry by following the disappearance of the 440 nm band of $Co^{III}TMPyP(OH)(H_2O)$. Linear fits of the observed rates to sodium ascorbate concentrations were used to determine a rate constant of 8.3×10^4 $M^{-1}s^{-1}$ at

25°C and 1.4 x 10^5 $M^{-1}s^{-1}$ at 37°C (Figure 13). So, given that the level of ascorbate in mouse blood is ~60 μM and the effective antidotal dose of Co^{III}TMPyP should lead to approximate blood concentrations of ~200 μM soon after injection, the anticipated initial rate of Co^{III}TMPyP(OH)(H_2O) reduction is given by 1.4 x 10^5 $M^{-1}s^{-1}$ x (60 x 10^{-6}) x (200 x 10^{-6}) = 1.7 x 10^{-3} M/s. That is, the antidote will become reduced to its Co^{II} form essentially as it is absorbed into the bloodstream, any residual Co^{III}TMPyP(OH)(H_2O) is only likely to persist for a matter of seconds.

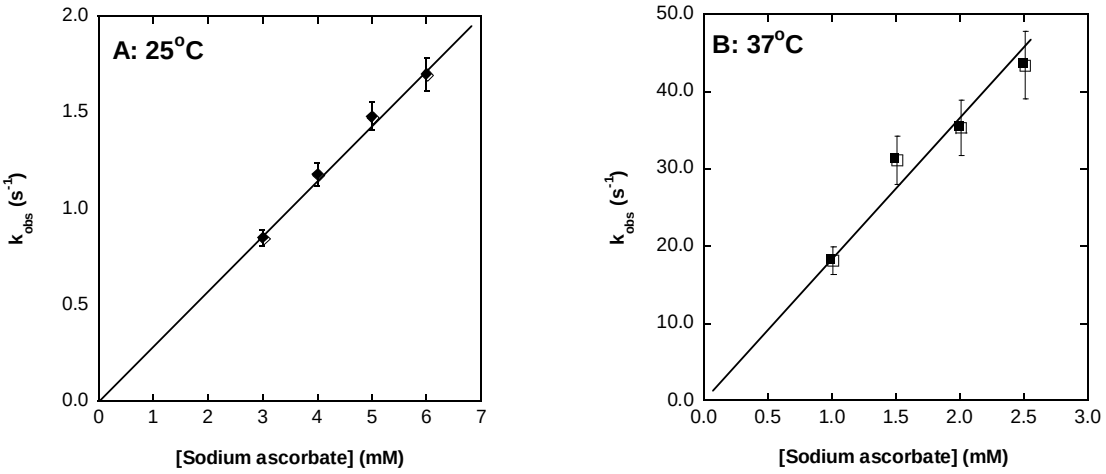

Figure 13: Kinetics of the reaction of ascorbate with Co^{III}TMPyP(OH)(H_2O) under pseudo first order conditions.

The reaction was followed at 427 nm in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA , at 25°C and 37°C under conditions of excess ascorbate (0 to 10 mM).

Cyclic Voltammogram of CoTMPyP in the presence of cyanide: Mosseri *et al.*[82] observed that after the reduction of Co^{III}TMPyP by γ-radiolysis, addition of KCN resulted in the disproportionation of Co^{II}TMPyP to $[Co^{III}TMPyP(CN)_2]^{2-}$, that is the Co^{III}-phorin species (where an electron resides on the porphyrin ring).[83] Using cyclic voltammetry (CV), Mosseri *et al.* found that the addition of excess cyanide to Co^{III}TMPyP resulted in a more negative reduction

potential.[82] CV performed on Co^{III}TMPyP in the presence of excess cyanide resulted in a single i_{pc} at 160 mV, which had no corresponding wave (i_{pa}) during a positive potential scan (Figure 14A). Under similar conditions, cobinamide (Co^{III}-corrin) exhibited a virtually identical CV with a slightly more negative potential, 125 mV (Figure 14B). These results suggest that perhaps there is an alternative mechanism for the oxidation of Co^{II}TMPyP to Co^{III}TMPyP in the presence of cyanide, such as the disproportionation mechanism suggested by Chan *et al.* in their γ-radiation reduction experiments. Certainly, in the case of neither CoTMPyP, nor cobinamide, is there any simple reversible Co^{III}/Co^{II} one-electron redox process in the presence of cyanide.

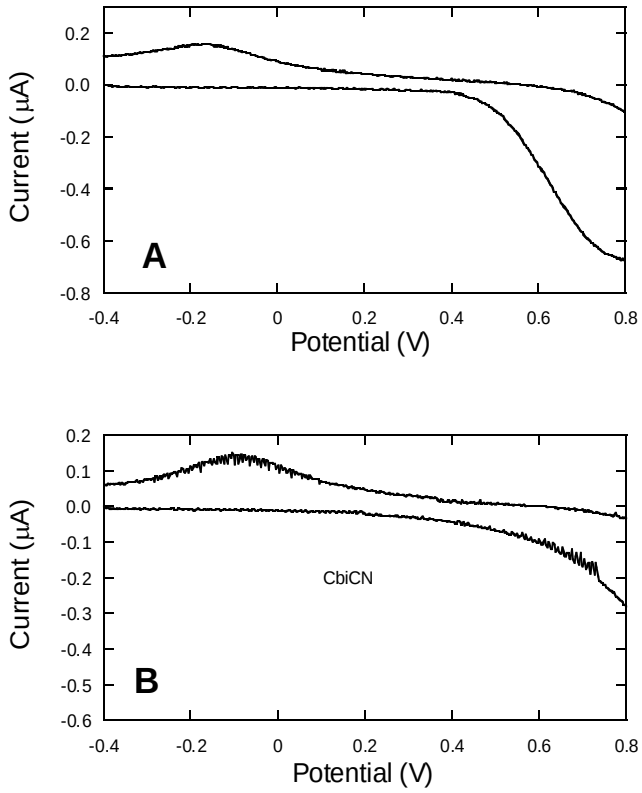

Figure 14: Cyclic Voltammograms of Co^{III}TMPyP(OH)(H_2O) and cobinamide with cyanide at pH 7.4 and temperature of 25°C.

Samples of Co^{III}TMPyP and $Cbi(OH)^+$ (200 μM) were prepared anaerobically in 0.1 M $NaNO_3$, 50 mM potassium phosphate buffer, pH 7.4, at room temperature, reduced with excess sodium ascorbate (20 mM), then excess cyanide (20 mM) was added.

Electronic paramagnetic resonance studies: Reduction of Co^{III}TMPyP by ascorbate results in the Co^{II}TMPyP species exhibiting an x-band electron paramagnetic resonance (EPR) spectrum[84] quite unlike that obtained following subsequent addition of excess cyanide (Figure 15A). The observed hyperfine results from the interaction of the unpaired electron ($Co^{II} = d^7$) with the ^{59}Co nucleus (I=7/2) having $A_{parallel}$ values of 104 gauss (Fig, 15A). For comparison, the EPR spectrum in the absence of cyanide, has an $A_{parallel}$ of 96 gauss (in the unliganded reduced form).[84] These EPR signals were only transiently observed; that is, they were completely absent in samples maintained at ambient temperature for a minute after the addition of cyanide before

freezing. These observations strongly suggest that cyanide binds to the reduced form of Co^{II}TMPyP and that the cyanide then catalyzes the oxidation of Co^{II}TMPyP back to its EPR-silent Co^{III} form. Very similar EPR spectral changes have also been observed following cyanide addition to cobinamide[85,86] – indicating an analogous set of reactions. For comparison, the EPR spectrum of the reduced Co^{II}TMPyP species in the presence of excess pyridine is shown (Fig. 15B) where three line super hyperfine due to ^{14}N can be observed. Note that this spectrum is indicative of a 5-coordinate mono(pyridine) adduct, since a bis(pyridine) structure would be expected to exhibit nine-line superhyperfine features. The lack of superhyperfine coupling in the cyanide bound form of Co^{II}TMPyP indicates that the binding of cyanide anion appears to be through the carbon atom.

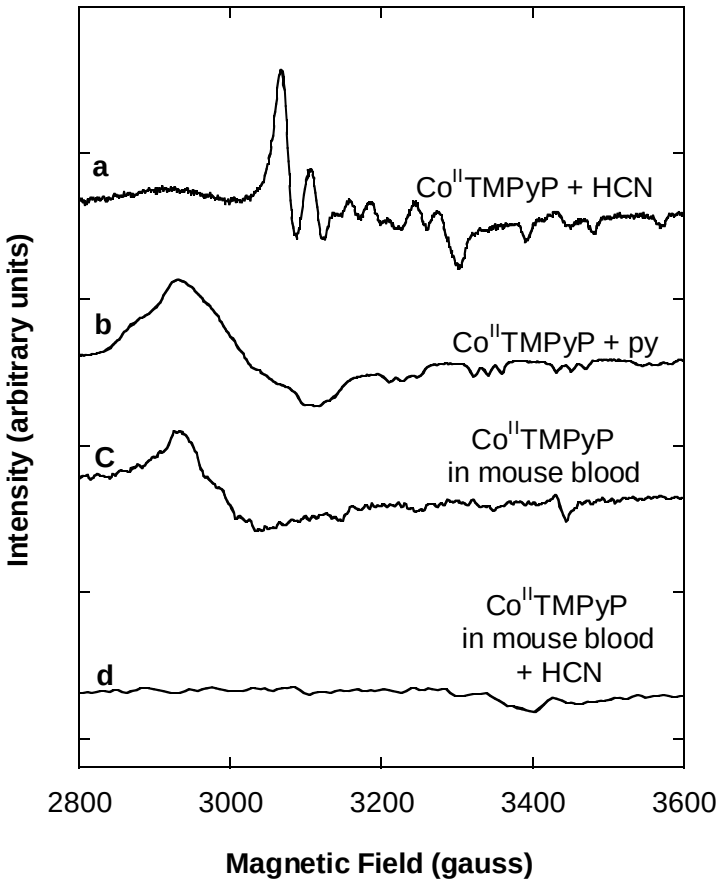

Figure 15: X-band EPR spectra of ascorbate-reduced Co^{II}TMPyP and mouse blood at 10 K.

Samples of Co^{III}TMPyP (1 mM) were prepared anaerobically in 50 mM potassium phosphate buffer, pH 7.4, at room temperature, reduced with excess sodium ascorbate (20 mM), then excess cyanide (20 mM) was added, and rapidly frozen in liquid nitrogen for subsequent recording of spectra. Samples of mouse blood were obtained by heart puncture to which Co^{III}TMPyP (1 mM) and then excess cyanide (20 mM) was added, and rapidly frozen in liquid nitrogen for subsequent recording of spectra. EPR conditions: 9.8 G modulation amplitude, 63.2 μW microwave power, 10 K. **a:** Co^{II}TMPyP-CN. **b:** Co^{II}TMPyP-py **c:** Mouse blood and Co^{III}TMPyP (1 mM) **d:** Sample **c** with excess cyanide (20 mM)

Mouse blood electron paramagnetic resonance studies: One way of assessing the biological reactions of CoTMPyP after adding it to the blood of mice is by EPR spectroscopy. While the absorbance of the normal porphyrin in blood would obscure the absorption spectrum of CoTMPyP, the EPR spectrum of the reduced form should be readily observable. When the blood of mice to which Co^{III}TMPyP had been added was examined, it was determined that a

portion of the CoTMPyP was reduced (see Fig. 15C) by the appearance of a signal at ~3000 gauss. This signal persisted for up to 30 minutes and no signals, which could be associated with the oxygen bound form of CoTMPyP were observed. The EPR signal observed in mouse blood had some features in common with the Co^{II}TMPyP(py) species (Fig. 15B); however, while some hyperfine was visible, no superhyperfine features are apparent. There is frequently "g-strain" associated with the EPR spectra of biological samples – a microvariability in the structure of metal-ion sites leading to line broadening and resulting elimination of hyperfine structure. Consequently, the failure to observe any superhyperfine features does not necessarily mean the absence of nitrogen donor ligands. In the blood samples, the added Co^{II}TMPyP is probably present as a mixture of five-coordinate structures in which the axial ligand is either a sulfur donor such as a cysteine thiol, or a nitrogen donor such as a histidine imidazole or a lysine amino group. Addition of an excess of cyanide resulted in the complete disappearance of these signals (Figure 15D), consistent with cyanide binding and subsequent oxidation to Co^{III}TMPyP$(CN)_2$. While an intermediate Co^{II}TMPyP(CN) complex could not be observed, this species rapidly oxidizes (see Electron paramagnetic resonance studies) and is difficult to trap.

Cyanide binding to Co^{II}TMPyP: In order to better understand the reaction of cyanide with the reduced porphyrin, Co^{II}TMPyP (prepared by ascorbate reduction) was anaerobically titrated with cyanide in 50 mM phosphate buffer, 1 mM EDTA, pH=7.4, using a spectrophotometric method. Co^{II}TMPyP in a sealed cuvette with little to no headspace was monitored spectrophotometrically as known amounts of cyanide (kept in borate buffer to prevent HCN loss) were added using a gas tight syringe and a time interval of 10 minutes was observed between additions for the solution to equilibrate (see Chapter 2.4.2). The electronic

absorption spectra obtained during of the titration of $Co^{II}TMPyP$ with cyanide at 25°C do not initially display tightly maintained isosbestic points (Figure 16A). After the addition of less than 1/2 of an equivalent of cyanide, the observed electronic absorption spectrum becomes, however, identical to that obtained during titrations starting from the oxidized $Co^{III}TMPyP(OH)(H_2O)$ species (Fig. 16A, dashed trace). The subsequent spectra do maintain isosbestic points and the final spectrum is that of the $Co^{III}TMPyP(CN)_2$ complex (Fig. 16A, dotted trace). Thus a very similar result to Chan *et al*. was observed, the presence of cyanide leads to generation of the oxidized $Co^{III}TMPyP(CN)_2$ form. Furthermore, because the reaction proceeds (to complete oxidation) in the presence of substoichiometric amounts of cyanide relative to the metalloporphyrin, it appears that the cyanide has a catalytic action. As a physiologically relevant reaction is desirable, an effective formation constant (K'_β) can be defined as $K'_\beta = [Co^{III}TMPyP(CN)_2] / ([Co^{II}TMPyP][HCN]^2)$ at pH 7.4 where the hydrogen ion concentration is ignored. From the spectra (at 454 nm) the fraction of the $Co^{III}TMPyp(CN)_2$ per total porphyrin (fractional saturation, Y) was determined, and thus the free cyanide concentration (protonated and unprotonated) could be calculated. In Figure 16B the free cyanide is plotted versus the fractional saturation (Y) and the data was fit using a nonlinear regression and the Hill equation:

$$Y= [(CN)\alpha_H/K'_\beta]/[1+(L)\alpha_H/K'_\beta]$$

Using $\alpha_H = 2$ gave the best fits of the data confirming the cooperativity of the cyanide binding and K'_β was found to be 2.1 (±0.1) x 10^{10} (Figure 16B) very similar to the binding of cyanide to the oxidized species, $Co^{III}TMPyP(OH)(H_2O)$ (2.1 (±0.2) x 10^{11}, Chapter 3).

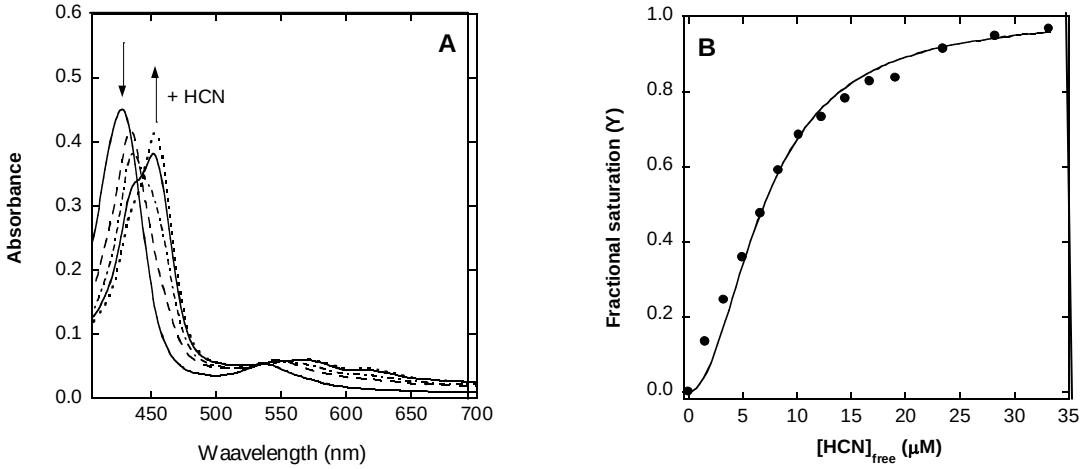

Figure 16: Titrations of Co^{II}TMPyP with cyanide at pH 7.4 and 25°C.

Small aliquots of sodium cyanide solution in 5 mM sodium tetraborate buffer (pH 11) were titrated into a solution of Co^{II}TMPyP (3.48 μM in 50 mM sodium phosphate buffer, 1 mM EDTA pH 7.4) using gas-tight syringes and a 1.00 cm pathlength septum-sealed cuvette at 25°C (see Chapter 2.4.2 for further details). **(A)** Electronic absorption spectra of Co^{II}TMPyP species titrated with NaCN, 1.00 cm pathlength. **(B)** Titration of 3.48 μM Co^{II}TMPyP with cyanide following the absorbance changes at 454 nm. The solid line represents a nonlinear least squares fit to the data using the Hill equation.

Rate of reaction of Co^{II}TMPyP with cyanide to form Co^{III}TMPYP$(CN)_2$: The reaction of Co^{II}TMPyP with excess cyanide under pseudo first order conditions was studied at 25 and 37°C. Interestingly, the following reaction phases did not seem to be sensitive to the presence or absence of oxygen. Co^{III}TMPyP$(OH)(H_2O)$ was reduced with an excess of ascorbate, rapidly mixed with excess cyanide, the reaction was observed at 427 nm (absorption maxima of the reduced species) and at 454 nm (absorption maxima of the oxidized dicyano species). The reaction was complicated with four phases, all of which were dependent on the cyanide concentration. The last two phases, with rate constants designated k_3 and k_4, were found to coincide, identically, with the last two phases previously observed for the reaction of cyanide with Co^{III}TMPyP$(OH)(H_2O)$ (Chapter 3). The first two phases (with rate constants designated as k_1 and k_2, shown in Table 2) were both dependent upon cyanide concentration at 25 and 37°C

(Fig. 17). These rate constants, however, were both much faster than those observed during the reaction of cyanide with $Co^{III}TMPyP(OH)(H_2O)$ (Chapter 3).

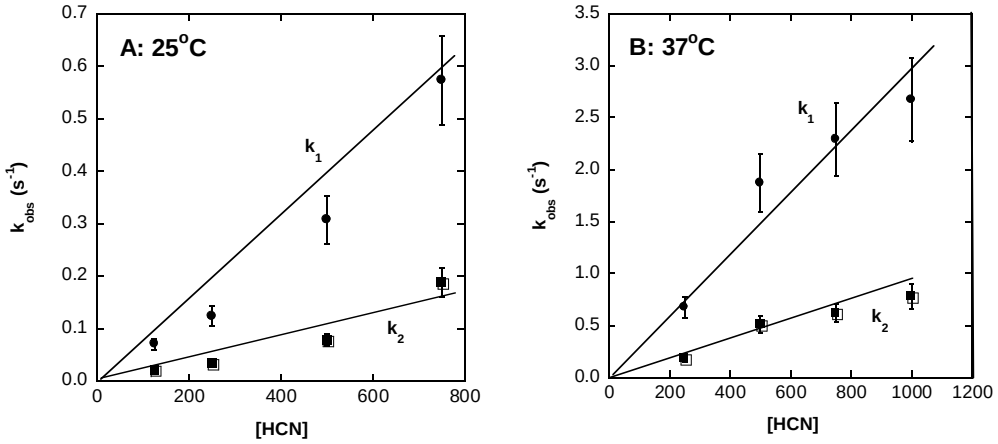

Figure 17: Kinetics of the reaction of cyanide with $Co^{II}TMPyP$ under pseudo first order conditions.

The reaction was followed at 454 nm in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, at 25°C and 37°C under conditions of excess cyanide. Cyanide solutions in the drive syringe were in 5 mM sodium tetraborate buffer (pH 11); cyanide concentrations after mixing were 20 to 2500 μM; upon completion of reactions, discharged solutions were verified to be pH < 7.45. The observed rates associated with the two fast rate constants (k_1 and k_2) plotted versus the cyanide concentration at **(A)** 25°C **(B)** 37°C. Rate constants (see Table 2) were then obtained from the slopes of the plots.

Table 2: Second order rate constants and absorbance amplitudes for the formation of $Co^{III}TMPyP(CN)_2$ from $Co^{II}TMPyP$ at 25-37°C, pH 7.4 in 0.1 M sodium phosphate buffers.

	Phase 1		Phase 2		Phase 3		Phase 4	
Conditions	k_1 ($M^{-1}s^{-1}$)	A%	k_2 ($M^{-1}s^{-1}$)	A%	k_3 ($M^{-1}s^{-1}$)	A%	k_4 ($M^{-1}s^{-1}$)	A%
pH 7.4, 25 °C	800 (±90)	22	263 (±52)	23	9 (±1)	8	0.35 (±.05)	46
pH 7.4, 37 °C	2500 (±600)	18	800 (±100)	27	20 (±3)	7	0.7(±.05)	48
pH 7.4, 25 °C*	111 (±7)	6	29 (±1)	40	9 (±1)	8	0.35(±.05)	46

The observed kinetics could be resolved into four exponential phases. Rate constants are calculated from linear fits of observed rates to the cyanide concentration as shown in Figure 17 (cyanide was in at least 20-fold excess over porphyrin). The percent absorbance change (amplitude) for each phase is also given. Uncertainties shown in parentheses are standard deviations. The last row contains the rate constants for the binding of cyanide to the oxidized form of the porphyrin ($Co^{III}TMPyP$) for comparison(*)).

When the kinetics of excess cyanide binding to $Co^{II}TMPyP$ were compared in the presence and absence of bovine serum albumin (BSA), a component of plasma, the initial rates were found to be indistinguishable for the first 15 minutes (Fig. 18). After 20 minutes, however, where essentially 80% of the reaction is complete, the cyanide binding in the presence of BSA is somewhat slower. It was previously shown that the last phase (described by k_4) of cyanide binding to $Co^{III}TMPyP$ is dependent upon ionic strength and therefore it might be expected to observe some changes in cyanide binding with BSA present. Whatever the complexity of the overall reaction, the key findings in relation to the kinetics of the reaction between $Co^{II}TMPyP$ and cyanide are: (i) cyanide binding is facile (Figure 17); (ii) the reaction is little affected by the presence of either oxygen (not shown) or the serum component protein BSA (Figure 18); and (iii) it occurs in blood (Figures 15C and 15D).

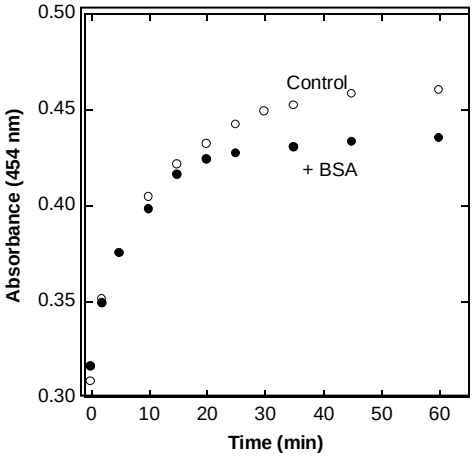

Figure 18: The rate of cyanide binding in the presence (solid circles) and absence (open circles) of BSA.

Conditions: 3.48 μM Co^{II}TMPyP in 50 mM sodium phosphate buffer, pH 7.4, 1 mM in EDTA and 0.1 mM in NaCN, 25°C. Reaction was followed spectrophotometrically at 454 nm.

4.4 DISCUSSION

The reduction of Co^{III}TMPyP(OH)(H_2O) is facile and easily carried out by ascorbate, with a rapid pseudo-first order rate constant of 1.4 x 10^5 M^{-1} s^{-1} at physiological pH and temperature (Figure 13). The appearance of an EPR signal with characteristics corresponding to the reduced form of Co^{II}TMPyP in mouse blood (Figure 15), where the ascorbate concentration is near 60 μM, also lends evidence to this occurring under physiological circumstances. Therefore, if CoTMPyP is used as an antidote to cyanide toxicity, its ability to bind cyanide in the reduced form is important. Many of the results presented in this paper led to the conclusion, however, that cyanide rapidly catalyses the conversion of the reduced Co^{II}TMPyP to the oxidized (Co^{III}) forms, leading to CoTMPyP exhibiting the same binding affinity for cyanide irrespective of the initial oxidation state (Co^{II}, or Co^{III}). The cyclic voltammetry experiments demonstrated that CoTMPyP in the presence of cyanide had a cathodic wave but no corresponding anodic

wave (Figure 14), indicating that another, quite rapid mechanism, exists to convert Co^{II}TMPyP to the Co^{III} analog. When cyanide is added to Co^{II}TMPyP, only a transient EPR signal corresponding to Co^{II}TMPyP(CN) is observed, again suggesting oxidation, as Co^{III}TMPyP is EPR silent (Figure 15). Frequently oxygenation, oxidation and reduction of Co^{II}TMPyP leads to the formation of μ-peroxo bridged dimers, which are EPR silent. These take many hours to form, however, which is certainly not the case in these studies, as the disappearance of the Co^{II} EPR signal is rapid – also the present reaction is not oxygen sensitive. Furthermore, when cyanide was added to Co^{II}TMPyP in substoichiometric amounts, Co^{II}TMPyP was converted to Co^{III}TMPyP(OH)(H_2O) as shown in the electronic absorption spectra shown in Figure 16A. In addition, the binding constant calculated from titrations of CoTMPyP in ascorbate is very similar to that obtained for the oxidized form of CoTMPyP (K_β = 2.0 x 10^{10}, Fig.16B). All of these results add compelling support to the argument that cyanide catalyses the oxidation of Co^{II}TMPyP.

The precise mechanism of cyanide induced oxidation of Co^{II}TMPyP remains unclear. The results of kinetic studies of the reaction of Co^{II}TMPyP with excess cyanide proved to be quite complicated (Table 2). Chan *et al.*[77] in their γ–radiation reduction experiments found the porphyrin ring contained a π-anion radical. No evidence for a π-anion radical in either the electronic absorption or EPR spectral data of the present samples were found. If the π-anion radical was formed, however, it may be very unstable in aqueous solution and rapidly react with water or protons. An attractive notion for a disproportionation mechanism is having cyanide act as a bridging ligand in order to facilitate an electron flow, for example:

$$TMPyPCo^{II}\text{-}CN\text{-}Co^{II}TMPyP \leftrightarrows TMPyPCo^{I}\text{-}CN\text{-}Co^{III}TMPyP$$

There are many known examples of cyanide forming bridges between metallo-macrocycles[87,88] and other metal complexes.[89-91] If Co^{II} disproportionates in this manner, however, it is not clear how the Co^{I} form is subsequently converted to Co^{III} as indicated by the data. When the reaction of Co^{II}TMPyP was conducted with cyanide anaerobically, no difference in the rate of reaction was found, indicating that oxygen was not involved. Therefore, the mechanism cannot involve the formation of a μ-peroxo-bridged dimer intermediate as observed in other cases.[87-91] Thus, by a process of elimination, the following reaction (that would remain undetected by cyclic voltammetry in solvent water) to explain the oxidation of Co^{I}TMPyP to Co^{III}TMPyP remains:

$$Co^{I}TMPyP\ (CN) + 2\ H^{+} \rightarrow Co^{III}TMPyP(CN) + H_2$$

Now, leaving aside the details of the oxidation-reduction components of the net reaction and restricting the discussion primarily to consideration of the ligand exchange processes, it seems that there are two broad types of plausible mechanism that fit these observations (Schemes I and II of Figure 19). It is inferred from the observed rate of Co^{III}TMPyP reduction by ascorbate (Figure 13) that oxidation-reduction reactions are faster than the ligand substitutions in these complexes and thus, only the latter should be rate determining. Scheme I is the simplest, where the parentheses enclosing one axial aquo ligand of the initial Co^{II}TMPyP complex should be taken to indicate uncertainty as to whether this species is 5- or 6-coordinate. Substitution of the aquo ligand(s) by the first cyanide anion, with the associated rate constant k_1, leads to the 5-coordinate Co^{II}TMPyP(CN) intermediate detected by EPR spectroscopy. Substitution of the second cyanide anion is characterized by the rate constant k_2, accompanied by rapid oxidation to the final product $Co^{III}TMPyP(CN)_2$. The alternate possibility, Scheme II, involves the initial rapid formation of the EPR-detectable Co^{II}TMPyP(CN) intermediate, followed by two discrete rate-determining pathways. One involved substitution of the second cyanide and accompanying

oxidation as in Scheme I. but now characterized as k_1 rather than k_2. The other pathway involves first oxidation of Co^{II} to Co^{III} followed by the second cyanide substitution with associated rate constant k_2. There are two obvious problems with Scheme II. The electron transfer reaction must be slow enough to be comparable with k_1, or only k_2 would be apparent in the kinetic traces. Also, the present k_2 should be the same rate constant as k_2 in the reaction starting from the oxidized $Co^{III}TMPyP(OH)(H_2O)$ (Chapter 3) but the numerical results for these two constants differ by a factor of about 9 and, consequentially, Scheme I is favored.

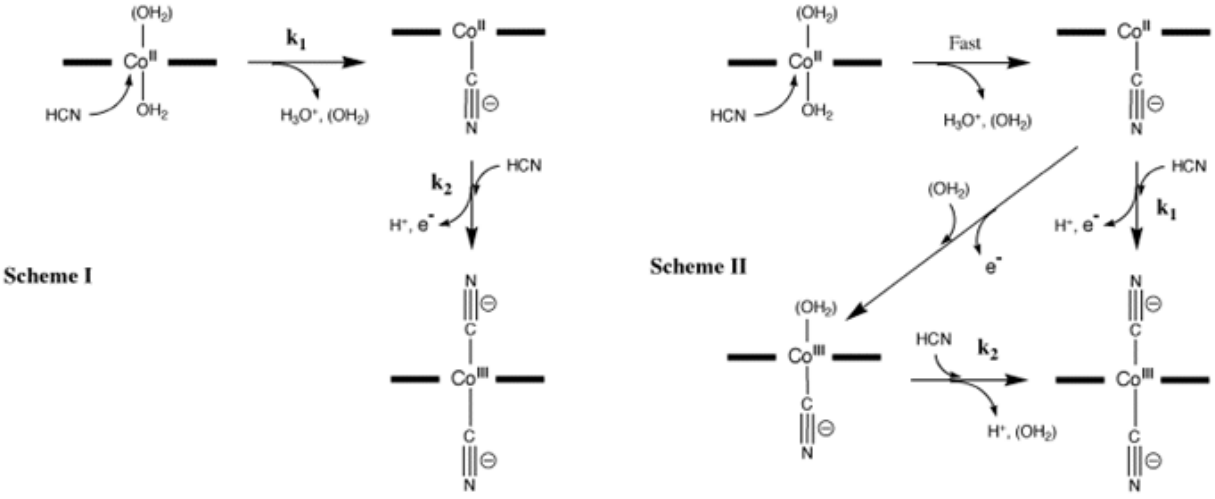

Figure 19: Plausible schemes for the two fastest phases of the reaction between $Co^{II}TMPyP$ and HCN at pH 7.4.

While most of the results in this work revolve around the cobalt porphyrin, CoTMPyP, the cyclic voltammetry of cobinamide has also been included for comparison (Figure 14B). Cobinamide is also reduced rapidly by ascorbate ($k \sim 1.4 \times 10^2$ $M^{-1}s^{-1}$) and its cyclic voltammogram exhibits similar characteristics to that of CoTMPyP (Figure 14A); that is, in neither case is an anodic wave detected. While the titration behavior (data not shown) is more

complicated, cobinamide also appears to undergo a cyanide catalyzed disproportionation reaction. Therefore, it is expected that cobinamide and CoTMPyP will be reduced *in vivo* but that upon exposure to cyanide, these compounds will rapidly be converted to their Co^{III} forms and bind cyanide with almost the same affinity as if the starting material were the oxidized species. This is fortuitously advantageous, since one obtains the more rapid substitution kinetics of the Co^{II} forms along with the higher binding affinities of the Co^{III} forms.

5.0 CONCLUSIONS

Cyanide Antidotes: In the U.S. cyanide antidotes are required for treating victims of potential occupational accidents, infrequent ingestions (attempted murder/suicide) and fire smoke inhalation cases. Ingestion of cyanogenic plants by livestock is a veterinary concern, particularly associated with post-drought new growth and, thus, is likely to increase in frequency and severity under the influence of global warming. In addition to the FDA approved use of cobalamin and the nitrite-thiosulfate combination, there are a few other potential cyanide antidotes in various stages of development, including cobinamide, sulfanegen sodium, and α-ketoglutarate.[30,35,38] This is almost certainly too few. Cobalamin is expensive and has only modest affinity for cyanide, hence the interest in cobinamide.[63] In the case of the nitrite-thiosulfate combination there are emerging toxicity issues.[20] For reasons of cost effectiveness, inappropriate pharmacokinetics, or toxicity, more than 90% of candidate compounds having promising therapeutic properties are, ultimately, never approved and marketed. Consequently, it is desirable to have dozens of candidate compounds under development to ensure a reasonable chance of success in the search for new cyanide antidotes.

Rational Development Strategy: Nowadays, there is a good deal of effort put into combinatorial approaches and high-throughput screening of libraries in the search for pharmaceuticals. This can certainly sometimes work, particularly in the search for compounds such as noncompetitive inhibitors of enzyme function, but relies on good luck. Screening can

both miss suitable compounds and give false positives; moreover, one can only discover the best candidate leads in the available libraries, which might be far from ideal or optimal. The task at hand is to find compounds that either bind or convert cyanide to less toxic forms. This is a chemically tractable problem and should be amenable to rational design – that is, take compounds with the desired activity, identify any undesirable characteristics of their chemistry, then modify the molecules in such a way as to suppress those undesirable characteristics while retaining and/or improving the desired activity. Herein, it has been shown that any cobalt–containing macrocyclic compound in which the Co^{III} form is more stable than the Co^{II} form probably has potential as an antidote to cyanide toxicity and the task becomes one of selecting one or more such lead compounds to modify in order to eliminate any undesired characteristics. For example, Busch and co-workers have synthesized many macrocycles into which cobalt can be inserted.[44]

Amido-linked Macrocycles: Initially, it was considered that structures derived from 4-nitrophenylenediamine and isophthaloyl dichloride (Figure 2) would be near ideal cyanide scavenger compounds. Stable molecules straightforwardly synthesized in one or two steps from readily available and inexpensive starting materials (*e.g.*, Figure 2) appeared particularly good candidates for stockpiling. Some considerable effort to develop these as candidates was expended before these endeavors were suspended due to their low solubility and consequent practical difficulty of purifying such compounds in reasonable amounts. Macrocyclic amido-linked structures are, however, known to stabilize higher oxidation states of chelated metal ions.[41] At the outset this looked like an attractive property, but is no longer considered a desirable characteristic. Now it is suspected that the ability of the functioning scavengers to bind

cyanide in their Co^{II} forms before becoming oxidized to stable Co^{III} forms to be mechanistically important (Chapter 4).

Corrinoids: Cobalt-corrins like cobalamin and cobinamide certainly work, but are less than ideal. In particular, they are expensive to produce and not especially stable, rendering them unsuitable for stockpiling. Less expensive alternatives, even of similar activity, would be beneficial.

Phthalocyanines: Cobalt-phthalocyanines do not work, binding cyanide only very slowly if at all (Chapter 1.3.2) The tendency of the phthalocyanine ring to stabilize the Co^{II} forms of these complexes may contribute to the low reactivity, but these structures were eliminated from further consideration as potential candidates without any more detailed investigation of their mechanistic properties.

Porphyrins: Herein, it has been shown that, despite any earlier suggestions to the contrary,[54] cobalt-porphyrins do work as cyanide antidotes (Chapter 3). This was, however, a proof-of-concept study, as CoTMPyP is not a practical candidate for further development. The antidotal activity was demonstrated at a dose of 20% of the LD_{50} for this compound (Figure 11) representing a therapeutic index (TI) of only 5. A TI of less than 10 is usually taken to indicate a "no go" decision should be made regarding further development of the compound in question. For this reason, additional studies of the pharmacokinetics of CoTMPyP in mice were not undertaken. There was, however, no unusual discoloration of the urine noticed in experimental animals within 1-2 hours of receiving CoTMPyP doses (*i.p.*) suggesting there was no significant excretion of the compound on the timescale of the experiment, since the kidneys are the predominant excretion pathway for water-soluble porphyrins and phthalocyanines.[44,45]

The failure of CoTMPyP to work as a prophylactic cyanide antidote when given 15 minutes prior to the toxin[54] remains unexplained. The idea that this may be due to inactivation of the administered Co^{III}TMPyP by reduction to Co^{II}TMPyP has been shown to be unfounded (Chapter 4). In fact, it is possible that the CoTMPyP does not become inactivated at all within 15 minutes. An undesirable characteristic of the structure is that its solubility in water is achieved by the introduction of four positively charged groups on the periphery of the ring. This is likely to render the molecule susceptible to sequestration into mitochondria where it may well have toxic consequences (Lopez, Benz *et al.*, manuscript in preparation). It follows that if sequestration into mitochondria is significant within 15 minutes, the previously observed "failure" to observe any prophylactic benefits of CoTMPyP in cyanide intoxication of mice[54] might in fact have been due to a synergistic toxicity on CNS mitochondria of cyanide plus CoTMPyP.

Schiff Base-linked Macrocycles: Busch and co-workers have reported the synthesis numerous macrocycles into which cobalt can be inserted in a one-step template synthesis followed by purification in a single recrystallization.[44] Typically, these compounds readily exhibit reversible Co^{II}/Co^{III} redox chemistry – a characteristic now considered to be important (Chapter 4). Many of these compounds are soluble in water and zwitterionic forms are accessible to prevent/suppress any tendency to partition into mitochondria. The structures often contain one or more six-membered aromatic rings (Figure 20). Starting materials with different aromatic ring substituents provide a convenient means to fine tuning the characteristics of the macrocyclic products. In general, the molecular masses of the Schiff base-linked macrocycles are about half those of the porphyrins and naturally-occurring corrinoids. One or more of these

cobalt-containing Schiff base-linked macrocycles would appear to be highly suitable candidates as lead compounds for future studies in the search for improved cyanide antidotes.

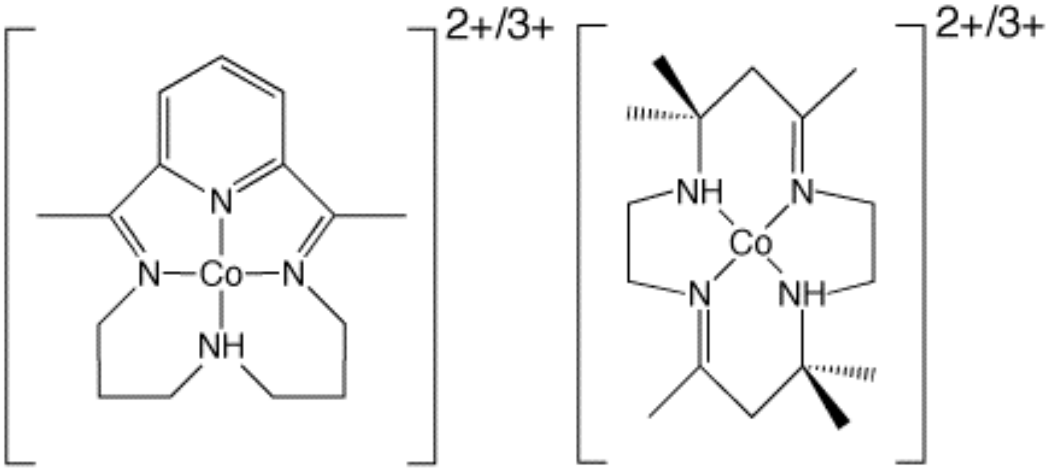

Figure 20: Representative Schiff base-linked macrocycles that are hypothetically suitable as cyanide antidotes.
Busch[96] synthesized the compound at left while Curtis[97] produced the compound at right.

Future Direction: The driving force (and funding) behind this work was the threat of chemical terrorism involving cyanide and the projected need to develop effective treatment options for mass casualties. During the course of the project, many of the leaders of the terrorist organization at the center of the perceived threat have been eliminated and the level of concern has abated. From a Public Health perspective there are two principal remaining issues with regard to cyanide toxicity. HCN is a significant toxic component of modern fires and smoke inhalation victims are probably the most frequent patients exhibiting symptoms of acute cyanide toxicity presenting at emergency rooms in Europe and the U.S.[1,92] In relation to the underdeveloped world, ingestion of cyanides and consequent chronic toxicity from food and possibly the water supply is an ongoing matter of international concern amongst policy makers.[93-95] Surprisingly, despite the obvious high level of worry, the relationships between relatively low-level chronic cyanide toxicity and its possible resulting human health issues are

very poorly documented and even less well understood. Improved understanding of the mechanism(s) of chronic cyanide toxicity at the molecular level, the long-term sequelae stemming from such exposures and the development of effect medical interventions/behavioral practices to prevent/treat these conditions are the areas where more research efforts need to be directed in the future.

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