MECHANISMS OF CYANIDE AND AZIDE BINDING TO COBALT COMPLEXES RELEVANT TO THEIR ANTIDOTAL ACTION

by

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ABSTRACT

In recent years, the possibility of mass casualties resulting from acts of chemical terrorism has emerged as a growing public health issue. Due in part to their wide availability and potential simplicity of deployment, cyanide and azide are two toxic agents that have already been nefariously employed to a limited extent and, moreover, are regarded as substances of concern in relation to possible acts of terrorism by the Department of Homeland Security. In the US, the two approved cyanide therapies, nitrite in combination with thiosulfate and the FDA-labeled hydroxocobalamin, are not suitable for use in mass casualty situations; while, no antidote is available for azide.

Recently, the cobalt Schiff-base complex 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1] heptadeca-1(17)2,11,13,15-pentaenyl Co(II/III) di/tribromide (CoN_4[11.3.1]) has been shown to have antidotal effectiveness toward cyanide and azide in mice. The reaction mechanism(s) by which CoN_4[11.3.1] detoxifies cyanide and azide, however, has(have) remained speculative until now – presenting something of a barrier to the rational development of the next generation of antidotes.

In this dissertation, the kinetics of the binding of cyanide and azide to the Co(II)N_4[11.3.1] compound studied under anaerobic conditions using stopped-flow

spectrophotometry are reported. In addition, the reduction kinetics of Co(III)N_4[11.3.1] by ascorbate have been examined as well as the oxidation of Co(II)N_4[11.3.1], the dicyano-Co(II)N_4[11.3.1] and the diazido-Co(II)N_4[11.3.1] by molecular oxygen. Mechanisms of cyanide and azide binding to CoN_4[11.3.1] are discussed as well as the possible utility of CoN_4[11.3.1] as a cyanide or azide antidote.

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1.0 INTRODUCTION

For centuries, certain chemical agents have been used for nefarious activities. In the Ancient Greek era, numerous toxins from poisonous plants and heavy metals (*e.g.* arsenic, lead, mercury) were examined. Some of these (notably hemlock – a tremendously poisonous plant) were used for suicide by individuals and for administering the death penalty by the government [1]. Chemical agents have also been employed as lethal weapons. In China, arsenic was used for fight during the period 960 through 1279 AD (the Song dynasty) [2]. In World War I (1914-1918) several toxic chemicals, such as chlorine, phosgene, hydrogen cyanide, or sulfur mustard, were employed in combat between the western allies and the Germans [2, 3].

Generally, not all toxic chemicals are equally likely to be chosen by the terrorist or others intent on any criminal wrongdoing; only particular types of compounds with particular characteristics: (1) high toxicity; (2) difficulty of detection; and (3) efficient distribution *in vivo* [4].

1.1 CYANIDE OVERVIEW: CHEMICAL PROPERTIES AND HUMAN EXPOSURE

According to the US Centers for Disease Control and Prevention (CDC) types and categories of hazardous chemicals [5], cyanide, along with arsine, carbon monoxide and sodium monofluoroacetate, are classified as “blood agents – Poisons that affect the body by being

absorbed into the blood" [5]. With regard to toxicological mechanism, once inside the human body, cyanide acts as an inhibitor of complex IV (cytochrome *c* oxidase) of the mitochondrial electron transport system. The binding of cyanide to heme a_3 of complex IV alters cellular respiration by preventing electron transfer to the terminal acceptor, molecular oxygen (O_2). Consequently, ATP production comes to a halt, leading to histotoxic hypoxia and eventually, death of cells.

Regarding chemical properties, cyanide refers to an anion with a one-negative charge consisting of a carbon atom forming a triple bond to a nitrogen atom. Cyanide in aqueous solution is present as two species: the acid form, HCN; and the anionic form, CN^-; the amount of each species being dependent on the pH. Since hydrocyanic acid (HCN) has a pK_a of 9.2 [6], at the optimal pH of human blood of 7.4, the vast majority (>98%) of total cyanide circulates in the blood as HCN. Gaseous HCN has a faint smell of bitter almond and seems to be pale-blue to colorless in hue. A substantial minority (10-20%) of the population cannot smell the gas and so, could not perceive their exposure to cyanide [7, 8].

Cyanide has been recognized as a dangerous toxicant for decades. In the past, cyanide was deployed as a chemical weapon by the allied forces in World War I, but failed to cause mass destruction of the Germans troops [9]. In World War II, cyanide was adopted by the Nazi as an unspeakably cruel method to slaughter Jewish and Romani people in gas chambers [9]. In the late 1980's, a chemical agent widely believed to be cyanide was also employed in a civil war in the Middle East region [9]. Moreover, in the 1990's, cyanide was used for terroristic purposes at the World Trade Center attack in 1993 [10], and the Tokyo subway attack in 1995 [11]. At present, the possible release of cyanide in locations with re-cycled air handling, such as modern buildings or mass transport systems, remains a public health concern. Apart from criminal or

terroristic activities, people could be exposed to cyanide through occupational tasks, particularly firefighting. Especially fires associated with modern structures and aircraft can generate smoke that is composed of several toxic chemicals including hydrogen cyanide. Besides asphyxia by smoke inhalation, cyanide in the smoke can play a major role as a silent killer among the firefighters [12, 13]. Other occupations that are prone to cyanide exposure include electroplating, mining, certain pesticide applications, metal cleaning, and gold extraction [7]. Moreover, cigarette smokers, including secondhand smokers, are at risk since cyanide is produced as one of the poisonous byproducts of tobacco smoke [14]. In an environmental setting, diets derived from cyanogenic plants like cassava, lima beans, or bitter almonds [15, 16], can be a rich source of cyanide, leading to poisoning in humans after being consumed. Interestingly, chronic exposure of cyanide due to consumption of cyanogenic plants, especially cassava, can cause epidemic outbreaks of permanent muscle weakness of the lower extremities called “spastic paraparesis” or “konzo” in West Africa [16].

1.2 AZIDE OVERVIEW: CHEMICAL PROPERTIES AND HUMAN EXPOSURE

Azide is a singularly-charged anion consisting of three nitrogen atoms forming bonds in linear molecular geometry. Azide in aqueous solution is composed of two species: hydrazoic acid (HN_3) and azide anion (N_3^-). The pK_a of HN_3 is 4.7 [17] and, so, in aqueous solution at pH close to normal blood pH of 7.35-7.45, over 99% of the total azide is present as N_3^-.

Formerly, azide was known for certain to be a potent vasodilator; therefore, it had been studied in relation to its anti-hypertensive effect in both normotensive and hypertensive patients in 1954 [18]. Nowadays, although azide is no longer used as therapeutic, its compounds are used

for other purposes [19]. In automobile airbags, in particular, sodium azide is one of the leading chemical components for generating nitrogen gas, immediately after a collision occurs. Azide is also commonly used as a preservative in the medical laboratory, and due to its toxicity, azide could potentially be used as a pesticide or herbicide. Furthermore, azide compounds play various roles in some manufacture of rubber, latex, wine, and Japanese beer [19].

Like cyanide, azide is known to inhibit cytochrome *c* oxidase (Complex IV) of the electron transport system in mitochondria [20]. Undoubtedly, azide has been used as a poisoning agent for decades, but so far, only in a few locations. In Kyoto, Japan, four azide-related poisoning incidents occurred within three months (August-October 1998) [21]. A total of 28 victims including 8 physicians, 10 laboratory workers, and other 10 people, were poisoned by sodium azide in hot beverages made with azide-contaminated water. Fortunately, no one developed any life-threatening symptoms in these incidents. In the US in 2009, six medical researchers were reported to be deliberately poisoned by sodium azide laced coffee at Harvard University [22]. One year later in 2010, following an investigation by US CDC, five people were found to be poisoned by sodium azide in iced tea at a local restaurant in Dallas, TX [23, 24]. Recently, the occurrence of azide poisoning in coffee repeated itself in late February 2017 at Yale School of Medicine [25]. In addition, some people, particularly laboratory personnel or health care workers, sporadically use sodium azide for suicide attempt [26-29] maybe because they are well-informed regarding azide toxicity and can easily acquire the poison from the laboratory or hospital inventory.

Despite being a less potent complex IV inhibitor, as compared to cyanide [30], azide is formally recognized by the US CDC as one of the hazardous chemicals that could cause a severe emergency [31]. Sodium azide, the most common form available, is a white, odorless powder.

Unlike cyanide or other blood agents, after dissolution in water, azide exists principally in its anionic form (N_3^-) at neutral pH [32] and is not quickly lost to the atmosphere. Since the aqueous solution of azide is likely to be colorless and has an unnoticeable odor, it is an effective toxicant with which to lace food/beverages to poison unsuspecting victims. As another possibility, public water systems could be targeted if enough sodium azide were available.

1.3 TOXICOLOGICAL MECHANISM(S) OF CYANIDE AND AZIDE

So far, many studies have provided ample and compelling evidence to indicate that cyanide itself is responsible for its toxicity, through inhibitory binding in the anionic form to cytochrome *c* oxidase (complex IV). On the other hand, the clear toxicological mechanism(s) of azide toxicity remain(s) inconclusive and require(s) further investigation. There are three plausible hypotheses that have been proposed to explain azide toxicity.

The first hypothesis is that after entering the human body, azide is converted to nitric oxide (NO), and NO is the principal toxic metabolite. Several attempts have been made to support this idea. In 1987, Kruszyna et al. [33] demonstrated that under optimal temperature and pH conditions, azide was found to be transformed to NO in human erythrocytes. In another study [34], tonic convulsion following intracerebroventricular administration of sodium azide in rats could be explained by the effect of NO derived from azide. Moreover, hypotension caused by azide poisoning could be explained by its conversion to NO [33] since the production of NO leads to the dilatation of blood vessels, decreasing blood pressure [35].

The second hypothesis is that azide could be transformed to cyanide, and cyanide acts as the principal toxicant. As previously mentioned, both cyanide and azide can inhibit mitochondrial

cytochrome *c* oxidase (complex IV) and, in fact, they share some similarities in clinical signs developed by patients. Persuasive evidence to support this proposition is based on the detection of a substantial amount of cyanide in the plasma of two azide-intoxicated patients. As reported by Klein-Schwartz et al. [28], the first case was a 52-year-old male who attempted to commit suicide by ingesting 1.2-2 grams of sodium azide. At the hospital, cyanide level in his plasma was found to be 1.6 mg/L, which considerably exceeded the minimum concentration of 0.5 mg/L; while, the substance from the powder ingested by the patient was tested negative for cyanide. According to Lambert et al. [29], the second case was a 25-year-old female who was presumed dead before arrival by azide poisoning. Upon post-mortem examination, a tremendous amount of azide remained in many samples, such as blood (262 mg/L), bile (1,283 mg/L), and stomach contents (745 mg/L); while, cyanide was also detected in the blood (9 mg/L). Additionally, Lambert et al. [29] demonstrated that cyanide was produced during *in vitro* incubation of azide with whole blood; however, the mechanism by which cyanide was present under these conditions remains unknown.

Lastly, the third hypothesis is that the azide moiety itself is responsible for the effects of the toxicant. Lack of clarity with regard to the mechanism of toxicity is clearly a barrier to logical discovery of antidotes and, consequently, distinguishing between these three hypotheses is of some importance and was an objective of the current work.

1.4 CURRENT CYANIDE AND AZIDE ANTIDOTES IN THE US

1.4.1 Cyanide antagonism

Several chemical substances have been reported to antagonize cyanide toxicity by various mechanisms. According to a recent review by Petrikovics et al. [36], nitrites, cobalt compounds, and carbonyl compounds (such as pyruvate or α-ketoglutarate) act as direct/indirect cyanide scavengers, subsequently lowering the circulating free cyanide. Some sulfur compounds (such as thiosulfate or sulfane sulfurs) play a role in the conversion of cyanide to a less toxic metabolite, thiocyanate (SCN^-). Other cyanide therapies, such as oxygen or chlorpromazine, have been suggested although mechanisms of antidotal action for these therapies remain in question. In the US, there are currently two antidote kits available for cyanide poisoning: Nithiodote™ and Cyanokit®

1.4.1.1 Nithiodote™

Nithiodote™, supplied by HOPE pharmaceuticals [37], is a combination cyanide therapy consisting of two compounds – sodium nitrite (300 mg/10 mL) and sodium thiosulfate (12.5 g/50 mL). Intravenous administration is the recommended route for cyanide treatment. As proved by a study in 1952 [38], due to the synergistic effect, the combination therapy of these two sodium salts was more effectively antidotal toward cyanide (at several times the LD_{50} dose) than administration of either sodium nitrite or sodium thiosulfate alone. As demonstrated by Leininger and Westley [39], thiosulfate functions as a sulfur donor substrate for the enzyme rhodanese which catalyzes the conversion of cyanide to the considerably less toxic thiocyanate anion (SCN^-). Thiosulfate is probably only helpful, however, if the dose of cyanide ingested by

patients is more than 4 x oral LD_{50} [40]. Furthermore, a recent study has proved that sulfide, a potent complex IV inhibitor like cyanide, is found in blood following the administration of thiosulfate. Instead of yielding a benefit, it turns out thiosulfate could aggravate cyanide toxicity by producing sulfide in the blood [41].

Previously, the mechanistic explanation by which sodium nitrite neutralized cyanide was based on the fact that sodium nitrite causes the oxidation of deoxyhemoglobin to form methemoglobin in the bloodstream; subsequently, the methemoglobin formed scavenges any free cyanide [42]. This will ameliorate the harmful effect of cyanide [43]. However, recent literature offered new findings contradicting the idea that the antidotal mechanism related to methemoglobin might not be entirely correct. As discussed by Way [44], the process by which nitrite produces methemoglobin is slower than any ameliorative effect of nitrite. Under conditions that methemoglobin formation is antagonized by methylene blue, nitrite is still effectively antidotal toward cyanide. Moreover, nitrite exhibits protection against cyanide toxicity in cell cultures derived from chick embryonic neurons [45] or rat brains [46] despite the fact that these cultured cells are unable to produce methemoglobin due to lack of hemoglobin.

Important discoveries of the mitochondrial NO synthase and the auxiliary role of NO in the mitochondrial electron transport system lead to a more convincing explanation for the cyanide antidotal action of nitrite. As demonstrated by Cambal et al. [41], the delivery of nitrite results in rapid production of NO in circulating blood. NO, derived from nitrite, can effectively alter cyanide-related inhibition of cytochrome *c* oxidase, by displacing cyanide from the substrate/inhibitor binding site; subsequently, restoring its O_2 binding/reducing capability [47, 48].

1.4.1.2 Hydroxocobalamin (Cyanokit®)

Cyanokit®, supplied by Meridian Medical Technologies [49], is the trade name of hydroxocobalamin, which is the US FDA approved cobalt-containing antidote for cyanide poisoning. Hydroxocobalamin is a form of vitamin B_{12} (cobalamin), which has a macrocyclic structure (molecular weight = 1,329 g/mol) with cobalt(III) surrounded by a corrin ring (Figure 1). In keeping with most transition-metal-ion complexes, the cobalt ion at the center of the macrocycle can bind ligands such as cyanide anion. Hydroxocobalamin has essentially one available site to substitute hydroxide with cyanide, subsequently, forming cyanocobalamin, which may be excreted via the renal system [50].

Hydroxocobalamin has been proved its effectiveness to detoxify cyanide in numerous animal and human studies since 1952 [36]. It was demonstrated that hydroxocobalamin, delivered by either intravenous or intraperitoneal injection, shows protection from cyanide poisoning through various routes of exposure such as inhalation, enteral or parenteral routes [51, 52]. According to the treatment guideline for cyanide poisoning in adults [53], 5 gram of hydroxocobalamin needs to be infused intravenously over 15 minutes; an additional dose may be applied, depending on the patient's clinical condition at that moment. Hydroxocobalamin can also be administered via intramuscular injection (IM); however, the IM protocol is recommended only for a case with vitamin B_{12} deficiency [54], not cyanide poisoning.

Even though the antidotal efficacy of both cyanide antidote kits (Nithiodote™ and Cyanokit®) have been well documented in cyanide-intoxicated patients, they are both far from practical for use in mass casualty situation, which a short administration time and fast ameliorative effects of the drug are essential.

1.4.2 Azide antogonism

Unlike cyanide, there is no currently approved antidote for azide poisoning. Several case reports suggest that patients who had ingested a low dose of sodium azide usually developed mild symptoms and eventually survived by receiving just supportive treatment (such as intravenous fluid and gastric lavage) without any antidotes [23, 55, 56]. However, in severe cases, specific antidotal treatment to detoxify the harmful effect of azide remains necessary.

Due to the close similarity in the characteristic inhibition of cytochrome *c* oxidase by azide and cyanide, it could be rationally argued that traditional remedies for cyanide poisoning (nitrite/thiosulfate and hydroxocobalamin) could be favorable for azide toxicity. Nevertheless, experience with the azide antidotes in humans is limited mainly to case reports, and none has rendered a conclusive, convincing, or consistent benefit of these two remedies. Based on several reports of azide cases, nitrites (either amyl nitrite or sodium nitrite) were given to several azide-intoxicated patients, but they did not show significant improvement and ended up dead afterward [28, 57]; while, only one case report from Austria has revealed that one patient had the satisfactory clinical outcome after receiving intravenous hydroxocobalamin [58]. Therefore, other cobalt complexes, may be a better choice than the nitrite compounds in the search for potential antidotes to azide toxicity.

1.5 SOME NEWLY DEVELOPED COBALT-CONTAINING PUTATIVE CYANIDE/AZIDE ANTIDOTES

Hydroxocobalamin has been proved to have an ameliorative effect on cyanide toxicity despite its limitations in mass casualty circumstances. In contrast, the clinical effectiveness of hydroxocobalamin for amelioration of azide poisoning remains questionable due to insufficient data. There has been some effort to discover more efficacious and/or less expensive cobalt-containing complexes for application as cyanide/azide antidotes, including the putative candidates: cobinamide, CoTMPyP, and $Co(II)N_4$[11.3.1] (Figures 1, 2 and 3 respectively).

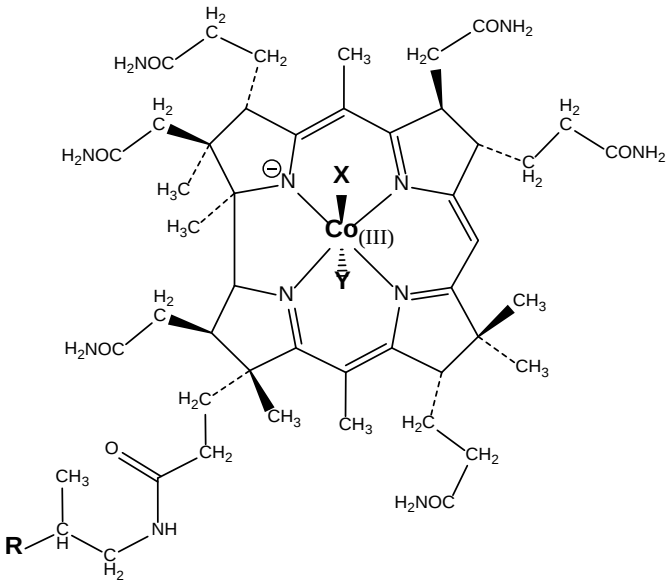
1.5.1 Cobinamide

Cobinamide is known to be the penultimate precursor of cobalamin in biosynthesis. Cobinamide (molecular weight = 990 g/mol) has a similar macrocycle structure as cobalamins, except the absence of the 5,6-dimethylbenzimidazole ribonucleotide tail linked to the cobalt atom in the lower axial ligand position (Figure 1) [59]. Thus, cobinamide has two axial ligand sites available to bind to nucleophiles such as cyanide or azide; while, cobalamin has only a single available axial position.

Numerous studies have indicated that cobinamide is effectively antidotal toward cyanide to a greater extent than cobalamin. For instance, cobinamide can significantly reverse complex IV inhibition by cyanide in mammalian cells [60], fruit flies (*Drosophila Melanogaster*) [60], and New Zealand white rabbits [61], better than hydroxocobalamin. A mouse study has suggested that cobinamide should be the best cyanide antidote, compared with other conventional cyanide therapies (hydroxocobalamin, sodium thiosulfate, sodium nitrite, and

nitrite/thiosulfate) because cobinamide is found to be 3-11 times more potent than hydroxocobalamin [62]. Another advantage of cobinamide over cobalamin has been pointed out by Bebarta et al. [63] that intravenous cobinamide requires only one-fifth of the cobalamin dosage in order to reverse cyanide-induced apnea in swine.

Cobinamide is not simple to manufacture, however, leading to a high cost of commercial production. The estimated cost of cobinamide production is sixty times more than that of hydroxocobalamin [64]. Moreover, while there has been considerable research on the benefit of cobinamide for cyanide poisoning, its ameliorative effect toward azide remains obscure and awaits exploration.



Cobinamide: R = $^{-}$OH

Cobalamins: R =

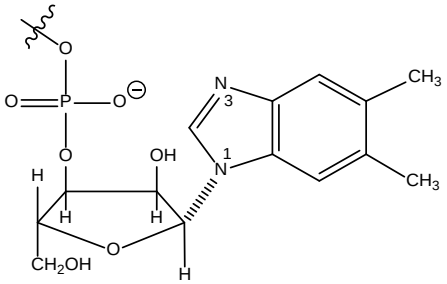


Figure 1. Chemical structures of cobalamins and its biological precursor, cobinamide.

Several attempts have been made to find alternative cobalt-containing macrocycle molecules that can efficaciously neutralize the toxicity of cyanide. According to Benz's dissertation [65], several kinds of cobalt-containing complexes have been examined, such as cobalt(III) tetraamido macrocycles, cobalt(II) tetrasulfophthalocyanine or cobalt(II) tetraaminophthalocyanine; none of which proved suitable for various reasons.

1.5.2 Cobalt(III) meso-tetra(4-N-methylpyridyl)porphyrin (Co(III)TMPyP)

Recently, Benz et al. [66] have suggested that Co(III)TMPyP (Figure 2) could be a potential candidate for cyanide poisoning. In mice, Co(III)TMPyP, administered one-minute post cyanide by intraperitoneal injection, significantly increased percent survival, and decreased the recovery time after "knockdown" (loss of consciousness) compared to controls. Due to square-planar geometry (Figure 2), Co(III)TMPyP has two available ligand positions to bind nucleophiles like cyanide and azide, similar to cobinamide. Preliminary indications are that Co(III)TMPyP could be a better cyanide antidote than hydroxocobalamin [66], comparable to cobinamide but less expensive [64].

So far, however, there has been little consideration of the reaction of Co(III)TMPyP with azide. According to Benz et al. [66], Co(III)TMPyP certainly binds azide, but no previous study has investigated the azide-antidotal effect of Co(III)TMPyP in animals.

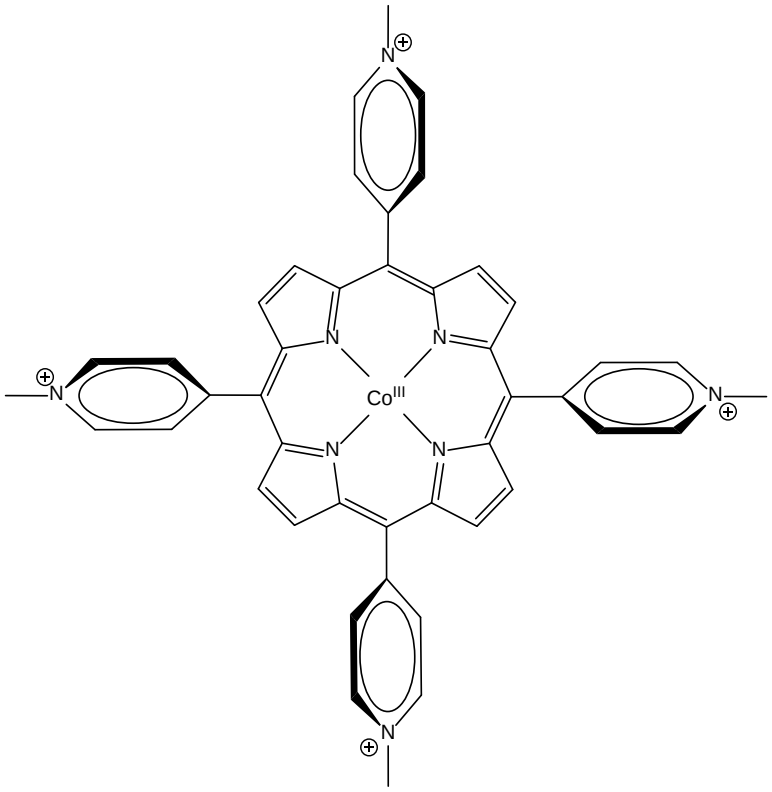


Figure 2. Chemical structure of cobalt(III) meso-tetra(4-N-methylpyridyl)porphyrin (Co(III)TMPyP).

1.5.3 Cobalt(II) 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17)2,11,13,15-pentaene (Co(II)N_4[11.3.1])

In an attempt to find new alternative antidotes for cyanide and azide, we hypothesized that cobalt Schiff-base macrocycles could be a viable option. The chemical structure of Co(II)N_4[11.3.1] is illustrated in Figure 3. According to Long and Busch [67], some transitional metals like Co(II) (the "reduced" form) or Co(III) (the "oxidized" form) can be complexed with Schiff-base macrocyclic ligands in a single step (the synthesis of Co(II/III)N_4[11.3.1] is explained in detail in Chapter 2.2). It has been shown that after delivery into the bloodstream, Co(III)N_4[11.3.1], in keeping with other cobalt macrocycles, is generally converted to the reduced Co(II) complex by ascorbate – the principal reductant present in human blood [68]. This reduction process will

clearly affect the association reaction between the Co(III) complex and toxicants; while, there should be no impact if the compound is administered in the Co(II) form. It follows that recent research has focused on the capabilities of toxicant binding to the reduced cobalt macrocycle, Co(II)N_4[11.3.1], rather than Co(III)N_4[11.3.1]. Accordingly, the investigations described in the present Dissertation follow this trend.

Regarding the cyanide-antidotal effect of Co(II)N_4[11.3.1] in a sub-lethal mouse model [68], Co(II)N_4[11.3.1] was shown to exhibit an impressive cyanide scavenging effect without evidence of long-term sequelae up to one week later. Co(II)N_4[11.3.1], administered prophylactically to cyanide-intoxicated mice, significantly decrease the recovery time after "knockdown" from 22 minutes (for the control group) to 3 minutes. The azide-antidotal effect of Co(II)N_4[11.3.1] has not previously been reported.

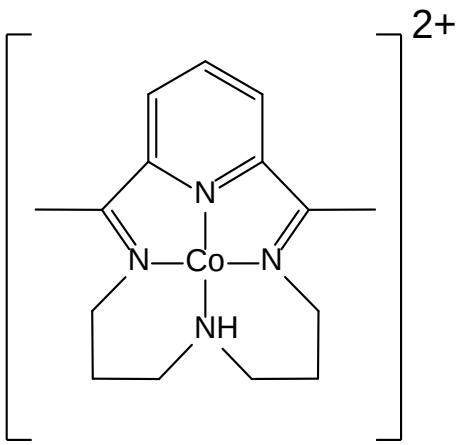


Figure 3. Chemical structure of cobalt(II) 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17)2,11,13,15-pentaene (Co(II)N_4[11.3.1]).

1.6 SCOPE OF THE DISSERTATION

It is undeniable that two currently approved cyanide antidotes in the US, nitrite/thiosulfate and hydroxocobalamin, can be beneficial in the treatment of acute cyanide poisoning, especially individual cases. Despite their safety and efficacy, both therapies suffer from the major drawback that they are seemingly not suitable for some urgency situations such as mass casualties, which requires short times for drug administration and fast antidotal action of the drugs. In case of azide poisoning, it seems that use of nitrites as an antidote has proved ineffective [28, 57]; while, the beneficial effect of hydroxocobalamin for azide toxicity remains in doubt due to lack of convincing scientific evidence.

Recently, several attempts have been made to develop new cobalt-containing antidotes for both cyanide and azide poisoning. Evidence based on literature review of some selected alternative cobalt-containing compounds suggests that Co(II)N_4[11.3.1] is arguably the best choice for cyanide poisoning, as compared to others at various stages of development (cobinamide, CoTMPyP). As it is unethical to test cyanide antidotes on humans, Federal Drug Administration (FDA) approval for such compounds must be through demonstration of efficacy in animals (the “Animal Rule”). Amongst other stipulations, the Animal Rule requires that the mechanism of action be understood well enough to have evidence-based expectation of similar efficacy in humans. The chemical reaction mechanism(s) by which Co(II)N_4[11.3.1] neutralizes the toxicants (cyanide/azide) remain(s) speculative. In addition, no previous study has investigated the azide-ameliorative effect of Co(II)N_4[11.3.1] in an animal model before. The key toxicant causing azide toxicity (azide itself or its secondary metabolites, such as NO or cyanide) is still questionable. This Dissertation seeks to remedy these problems by (1) demonstrating the cyanide/azide-antidotal properties of Co(II)N_4[11.3.1] by virtue of (a) binding

activity and (b) chemical kinetics and reaction mechanism, and (2) assessing the effectiveness of Co(II)N_4[11.3.1] in the amelioration of azide toxicity in mice.

The overall structure of the Dissertation is composed of five chapters including this introduction (Chapter 1). The second chapter is concerned with materials and methods used for these studies. Chapters 3 and 4 present the findings of two peer-reviewed papers on, respectively, the antidotal action of Co(II)N_4[11.3.1] towards cyanide and azide toxicity. The final chapter (Chapter 5) provides a summary of the entire dissertation, public health implications, and some recommendations for future research.

2.0 MATERIALS AND METHODS

2.1 MATERIALS

All reagents were ACS grade, or better, used without further purification, and unless stated to the contrary, were obtained from Fisher or Sigma-Aldrich. Sodium dithionite (87% minimum assay) was purchased from EMD Chemicals Inc. Argon gas (99.998%) and oxygen gas (grade 4.7, 99.997%) were acquired from Matheson Incorporated. 0.1 M sodium phosphate buffer was prepared by mixing an appropriate volume of 0.1 M sodium phosphate monobasic (NaH_2PO_4) and 0.1 M sodium phosphate dibasic (Na_2HPO_4) to make buffers at desired pH ranging from 4 to 8. CHES (2-[N-Cyclohexylamino]-Ethanesulfonic Acid) at a concentration of 0.1 M was used as a buffer at pH > 8, and borate buffer (0.1 M) was used at pH ≥ 9. The desired pH of CHES and borate buffers were adjusted by adding 0.1 M NaOH or HCl solution. Cyanide solutions were prepared by dissolving KCN salt in a pH 11.6 water solution adjusted with NaOH in septum-sealed vials with limited head volume. Gastight syringes were used for transferring KCN solutions. To prepare NaN_3 solutions, the salt was dissolved in 0.1 M sodium phosphate buffer at desired pH in septum-sealed vials with minimal headspace. In order to maintain ionic strength, sodium nitrate or sodium chloride were added to 0.1 M sodium phosphate buffer.

2.2 SYNTHESIS OF Co(II)N_4[11.3.1]

The synthesis of cobalt(II) 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17)2,11,13,15-pentaene dibromide (Co(II)N_4[11.3.1]) was done anaerobically based upon the method of Long and Busch [67], adapted by Lacy et al. [69]. Briefly, $CoBr_2$ (1.35 g, 6.17 mmol) and 2,6-diacetylpyridine (1.00 g, 6.13 mmol) were dissolved in 20 mL of deoxygenated ethanol, followed by the addition of 0.5 mL of water and then degassed 3,3'-diaminodipropylamine (0.857 mL, 6.13 mmol) under argon at room temperature over the course of several minutes. The blue-green color of $CoBr_2$ and 2,6-diacetylpyridine gradually turned to dark red. Next, a small amount of glacial acetic (1 µL) was added before letting the solution stir at 50°C for 12 hours. After that, the solution was taken to a glovebox equipped with purified argon (with < 0.5 ppm O_2, ~4 ppm H_2O) and then filtered using a fritted-glass funnel after cooling to room temperature. The filtration product, which was a purple solid, was then washed with ethyl acetate and left to dry over phosphorus pentoxide (P_2O_5) for 24 hours. Elemental analyses for $C_{15}H_{22}N_4Br_2Co$, performed by Atlantic Microlab Inc., gave the following results. Calcd: 37.76% C, 4.65% H, 11.74% N, and 33.50% Br; Found: 37.66% C, 4.67% H, 11.62% N, and 33.16% Br (see cation structure in Figure 3). Co(III)N_4[11.3.1] was prepared by allowing the reduced compound to oxidize overnight under ambient conditions and used without any further purification.

2.3 INSTRUMENTATIONS

2.3.1 Stopped-flow spectrometry

The rapid reaction kinetics were performed using an SX.18MV-R stopped-flow spectrometer, supplied and manufactured by Applied Photophysics Limited [70] (Figure 4). The operation of the stopped-flow apparatus is illustrated on the diagram below (Figure 5). Briefly, the drive syringes are filled with the solutions of two reagents. After the pneumatically controlled drive ram moves forward rapidly, the reagents will pass into the mixer, and immediately into the optical observation cell. The reaction mixture then passes into the stop syringe. Eventually, the stop syringe plate strikes the stopping block. This will concomitantly stop the flow of the reaction mixture and commence data acquisition. This machine is also equipped with a xenon arc lamp as a light source, monochromator that allow us to select the particular wavelength of interest, and absorbance detector. The signal received from the detector is then processed as an electronic spectrum, and the signal can be recorded on millisecond to second timescales.

A major limitation of stopped-flow spectrometry is that the reaction must not be too fast (faster than one millisecond of the exact age, also known as the dead time [70]) in order to be detectable by the machine. In addition, to be able to measure the rate of reaction, the concentration of the reagent has to be high enough to see changes in the optical density.

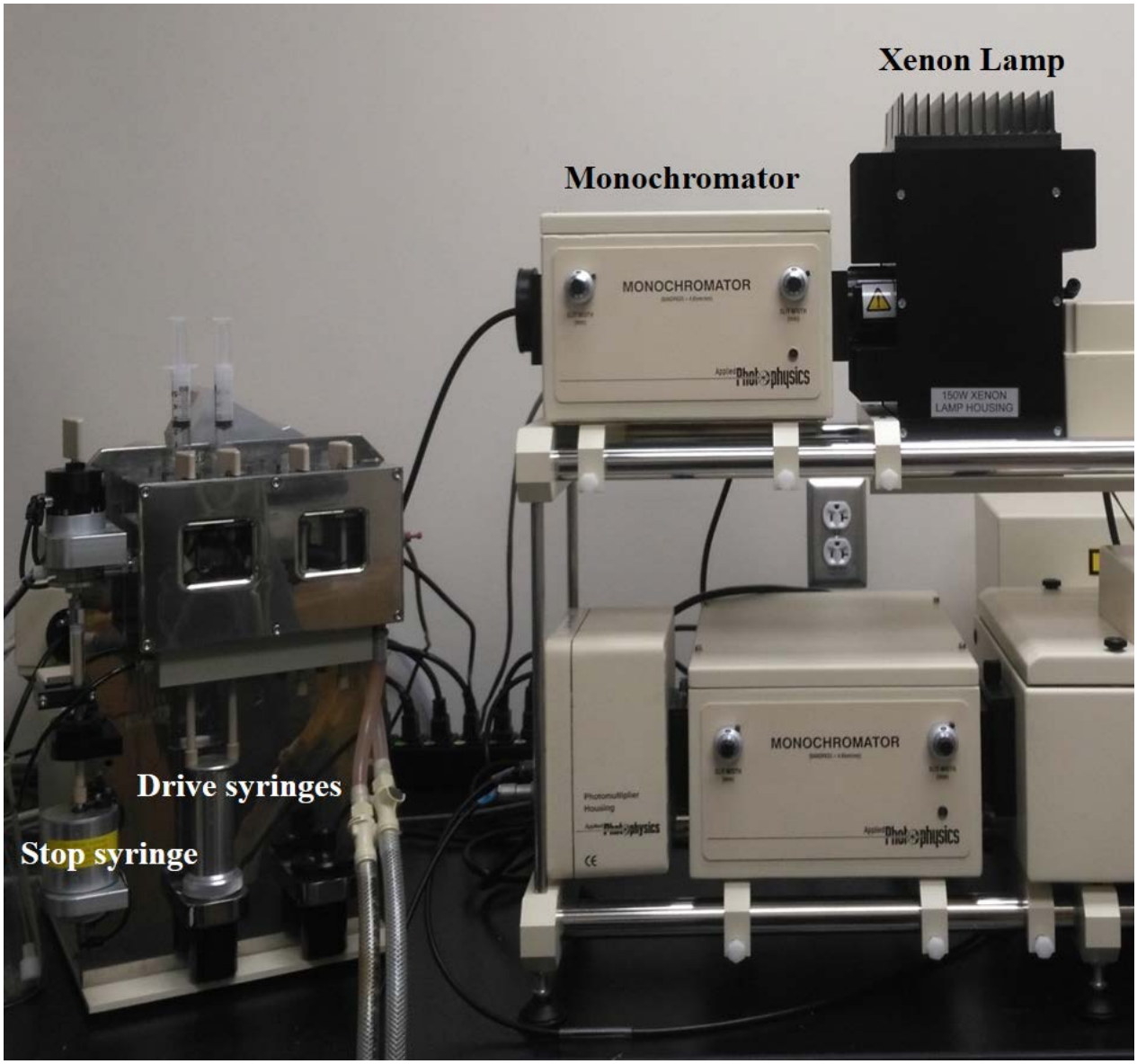


Figure 4. An SX.18MV-R stopped-flow spectrometer, manufactured by Applied Photophysics Limited.

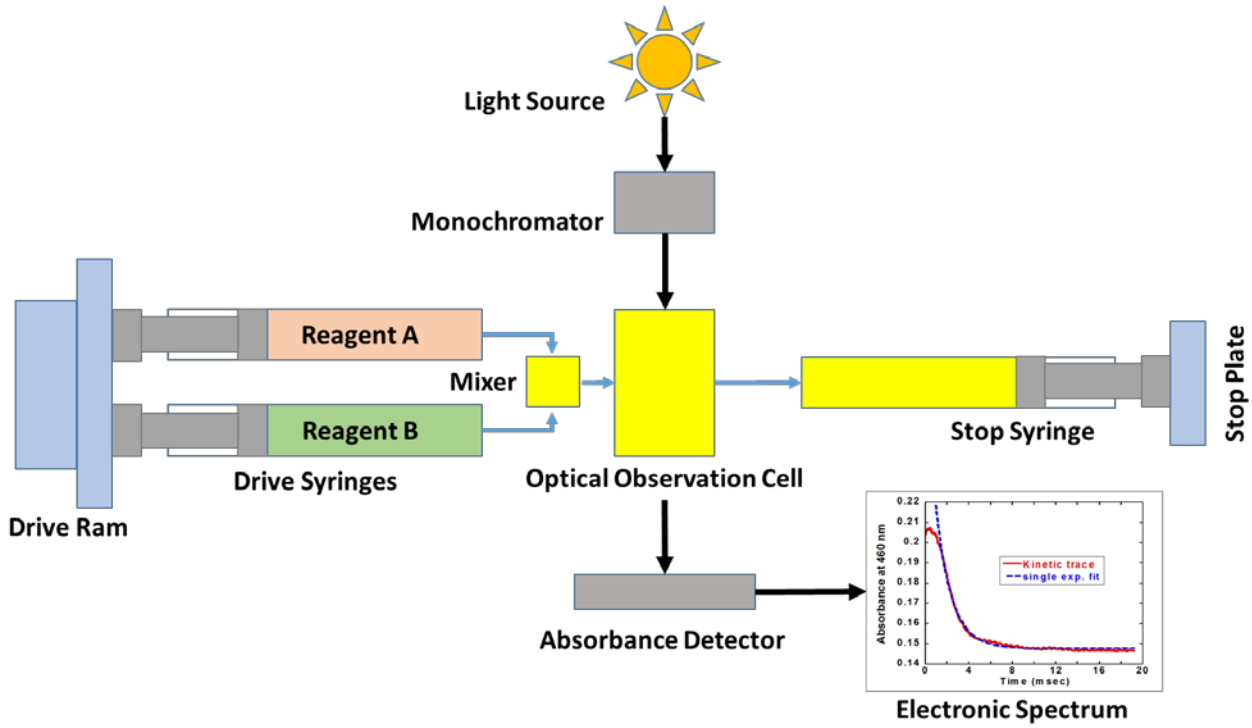


Figure 5. Operation of the SX.18MV-R stopped-flow (single mixing) apparatus.

Adapted from Applied Photophysics SX.18MV-R Stopped-Flow Reaction Analyser User Handbook [71].

2.3.2 Other instrumentations

The measurement of electronic absorption spectra was carried out using Shimadzu UV-1650PC and UV-2501PC spectrophotometers. During the experiment, temperature levels were controlled using a thermostat reaction chamber. A Corning Pinnacle 555 pH/ion meter was used to measure pH. The concentrations of oxygen solutions in 0.1 M sodium phosphate buffer, pH 7.4, were generally calculated from Henry's law. In a limited number of cases, the oxygen concentration was verified by measurement with a Clark-type electrode housed in an Oxygraph O2k polarographic instrument (Oroboros, Innsbruck, Austria).

2.4 METHODS

2.4.1 Anaerobiosis

Most deoxygenated solutions were generally prepared using an Omni-Lab glovebox, manufactured by Vacuum Atmospheres Company; however, a Schlenk line (or vacuum gas manifold), was employed when necessary. Argon gas was used as an inert gas for both devices. To be certain that oxygen was eliminated from the stopped-flow spectrometer, the reaction chamber including drive and stop syringes were washed at least three times with 1 M $Na_2S_2O_4$ solution and left overnight with the argon gas flowing through the machine before starting the anaerobic experiment.

2.4.2 Kinetic experiments

The stopped-flow kinetic experiments were carried out in both multiple (photodiode array, PDA) and single wavelength absorbance (photomultiplier tube, PMT) modes. At least three data sets were collected for each set of kinetic conditions and subsequently averaged. The stopped-flow instrument was flushed out at least five times to ensure that the previous chemicals remaining in the reaction chamber were completely removed before starting the next set of reactions.

In the concept of chemical kinetics [72, 73], a rate constant, denoted by *k*, is used for the determination of how fast the reaction proceeds. A large value for k indicates that the chemical reaction is fast. For a reaction between compounds A and B to form a product C (A + B → C), the rate of reaction, which represents the rate of disappearance of compound A or B, can be written as the equation below.

$$-\frac{d[A]}{dt} = -\frac{d[B]}{dt} = k[A][B] \quad (1)$$

After integration technique is applied to (1), the rate equation with respect to time can be shown below (Equation 2).

$$\frac{1}{[B]_0 - [A]_0} ln \frac{[B][A]_0}{[B]_0[A]} = kt \quad (2)$$

In this study, the pseudo-first order reaction (flooding technique) was used to obtain the observed rates (k_{obs}). Under pseudo-first order conditions, the concentration of compound B is seemingly in very large excess, compared to the concentration of compound A ($[B]_0 >>> [A]_0$). Therefore, the concentration of B remains approximately constant throughout the entire reaction, and the concentration of B at the end of the reaction would be similar to the initial concentration of B ($[B] \approx [B]_0$). Based on the underlying assumptions made from the flooding technique, the rate equation as shown in (2) can be considerably simplified as the equations demonstrate below.

$$\frac{1}{[B]_0} ln \frac{[A]_0}{[A]} = kt \quad (3)$$

$$A = A_0 e^{-[B]kt} \quad (4)$$

Or $$A = A_0 e^{-k_{obs}t} \text{ where } k_{obs} = k[B] \quad (5)$$

According to (5), a rate constant (k) can be determined from the slope of the linear relationship between the observed rates (k_{obs}) and the different concentrations of compound B as shown in (6). In our stopped-flow experiments, compound B represents various substrates including NaN_3, KCN, CoN_4[11.3.1], oxygen, or sodium L-ascorbate.

$$Rate\ constant\ (k) = \frac{k_{obs}}{[B]} \quad (6)$$

All kinetic data were analyzed and modeled using Pro-K and SX.18MV software programs, supplied by the manufacturer. Pro-K software was performed a global analysis; while, SX.18MV was used to fit both single and double exponential floating endpoint models ($A = ae^{-k_{obs1}t} + c,\ A = ae^{-k_{obs1}t} + be^{-k_{obs2}t} + c$) for a single wavelength.

Additionally, the temperature dependence of reaction rates was demonstrated in our experiment based on the linear form of the Eyring Equation [74, 75] as shown below (Equation 7).

$$ln\frac{k}{T} = \frac{-\Delta H^{\ddagger}}{R}\frac{1}{T} + ln\frac{k_B}{h} + \frac{\Delta S^{\ddagger}}{R} \quad (7)$$

where k is the rate constant

T is the absolute temperature in Kelvin (K)

h is the Planck's constant (6.626 x 10^{-34} J sec)

R is the gas constant (8.3145 J mol^{-1} K^{-1})

k_B is the Boltzmann's constant (1.381 x 10^{-23} J K^{-1})

The activation parameters can be determined from an Eyring plot, a plot of natural log for the observed rates over the absolute temperature ($ln\ k_{obs}/T$) against the reciprocal temperature in Kelvin ($1/T$). The enthalpy of activation ($\Delta H^{\ddagger}$) was calculated from the slope of the Eyring equation (slope = $-\Delta H^{\ddagger}/R$); whereas, the entropy of the entropy of activation ($\Delta S^{\ddagger}$) was calculated from the y-intercept (y-intercept = $ln\ k_B/h + \Delta S^{\ddagger}/R$).

Stopped-flow experiment with oxygen employed sodium phosphate buffer solutions saturated with 99% oxygen (exposed to 1 atm oxygen for 2 hours) resulting in solutions with oxygen concentrations of 1 mM [76]. Anaerobic experiments were performed under buffers flushed with argon and drive syringes were filled with reagents in the glove box.

All graphs were drawn using Kaleida-graph software v.4.5.1. All chemical structures were created using ChemDraw Professional v.15.1.

3.0 KINETICS AND ANTIDOTAL MECHANISMS OF THE REACTION OF A COBALT SCHIFF-BASE MACROCYCLE WITH CYANIDE

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Keywords: ascorbate, cyanide, cobalt macrocycle, reaction kinetics, mechanisms

3.1 INTRODUCTION

While many cobalt complexes have been shown to exhibit cyanide-scavenging properties, only hydroxocobalamin has been FDA-approved [77] for use in ameliorating the harmful effects of cyanide toxicity in patients without causing life-threatening side effects [51, 78]. Despite its safety and clinical success, hydroxocobalamin requires the intravenous administration of large volumes [79]. Finding more effective and less expensive cyanide antidotes would be beneficial to both patients and public health. Recently, a cobalt-containing Schiff-base macrocycle compound, cobalt 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17)2,11,13,15-

pentaene dibromide (CoN_4[11.3.1]), was found to be a viable option as a cyanide antidote [68]. CoN_4[11.3.1] is a low molecular weight compound that binds two molecules of cyanide (hydroxocobalamin binds only a single cyanide) and is synthesized in a single step with affordable starting materials. In a sub-lethal mouse model, CoN_4[11.3.1] was more effective at ameliorating cyanide toxicity when compared to hydroxocobalamin, cobinamide, or a cobalt-containing porphyrin [64, 66], and no long-term sequelae were observed in these mice more than a week later [68]. Due to its relatively low molecular mass (~4-times smaller than hydroxocobalamin) [64, 68], CoN_4[11.3.1] may be more soluble than other larger cobalt complexes, leading to an enhancement of the ameliorative effect. The working hypothesis for the mechanism of cyanide detoxification by many of these cobalt macrocycles is that the cobalt(II) forms are substitution-labile and more easily bind cyanide; after which they are easily oxidized to their cobalt(III) forms (due to changes in redox potential), become substitution-inert, and therefore retain the bound cyanide. It is also important to determine if ascorbate, the primary reductant in the human body, can reduce Co(III)N_4[11.3.1] and how rapidly this reaction takes place. In addition, determining the kinetics and thermodynamics of cyanide binding to the reduced form of CoN_4[11.3.1] may give us further insight into the mechanism of the cyanide association and aid in the development of this and other cyanide antidotes.

3.2 RESULTS

3.2.1 Kinetics of the reduction of Co(III)N_4[11.3.1] with ascorbate

We have previously determined that Co(III)N_4[11.3.1] can be reduced by ascorbate and the reduced compound was also observed in mouse blood [68]. The electronic absorption spectrum of the oxidized form of CoN_4[11.3.1] is shown in Figure 6 (dotted trace) and upon an addition of a 50-fold excess of sodium L-ascorbate, the compound was reduced to the Co(II) form (solid trace) which exhibits an absorption maximum at 460 nm. Under pseudo-first order conditions (10 to 50-fold excess of sodium ascorbate), the rate of Co(III)N_4[11.3.1] reduction was followed by observing an increase in absorbance at 460 nm using a stopped-flow spectrometer. A single phase was observed and a linear fit of the observed pseudo-first order rate constants versus the sodium L-ascorbate concentrations was obtained to determine a second-order rate constant of 11.7 (± 0.4) M^{-1} s^{-1} at 25°C and pH 7.4 (Figure 7A). To determine the activation parameters of the reaction, the observed rates were measured at six different temperatures between 10 and 37°C. The enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation was then be calculated based on a linear fit of the data, a plot of $ln\ k_{obs}/T$ vs. $1/T$ (Figure 7B). The enthalpy of activation ($\Delta H^{\ddagger}$), obtained from the slope of the line in Figure 7B, was found to be 53.9 (± 0.8) kJ mol^{-1}; while, the entropy of activation ($\Delta S^{\ddagger}$), determined from the y-intercept of the linear line, was determined to be -79 (± 3) J mol^{-1} K^{-1}.

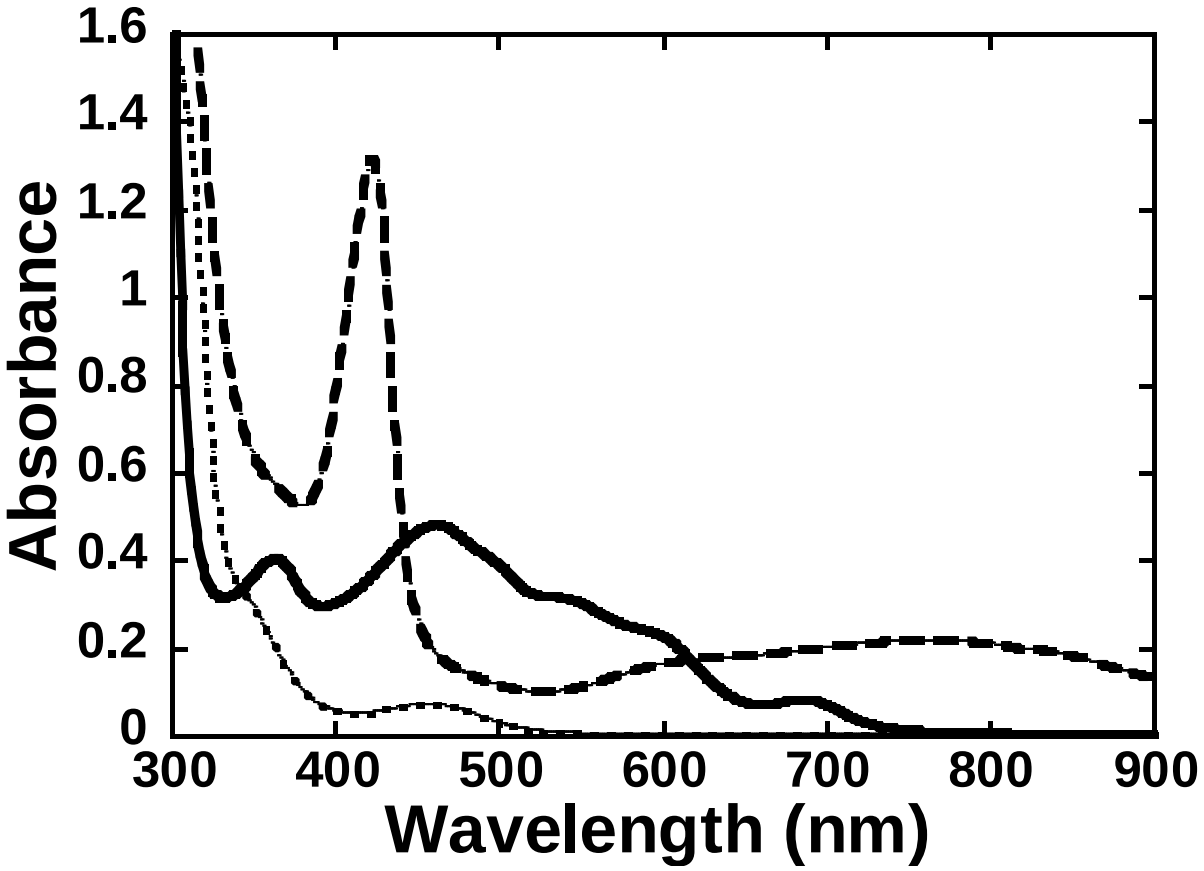

Figure 6. Electronic absorption spectra of CoN_4[11.3.1] species in the absence and presence of cyanide.

Electronic absorption spectra of Co(II)N_4[11.3.1] (solid trace), Co(III)N_4[11.3.1] (dotted trace) and Co(III)N_4[11.3.1]$(CN)_2$ (dashed trace). Co(II)N_4[11.3.1] (0.3 mM) was prepared anaerobically in sodium phosphate buffer (0.1 M, pH 7.4). KCN (15 mM) was prepared anaerobically in a pH 11.6 water solution adjusted with NaOH. Co(III)N_4[11.3.1] (0.3 mM) was prepared by allowing the reduced compound to oxidize overnight under ambient conditions.

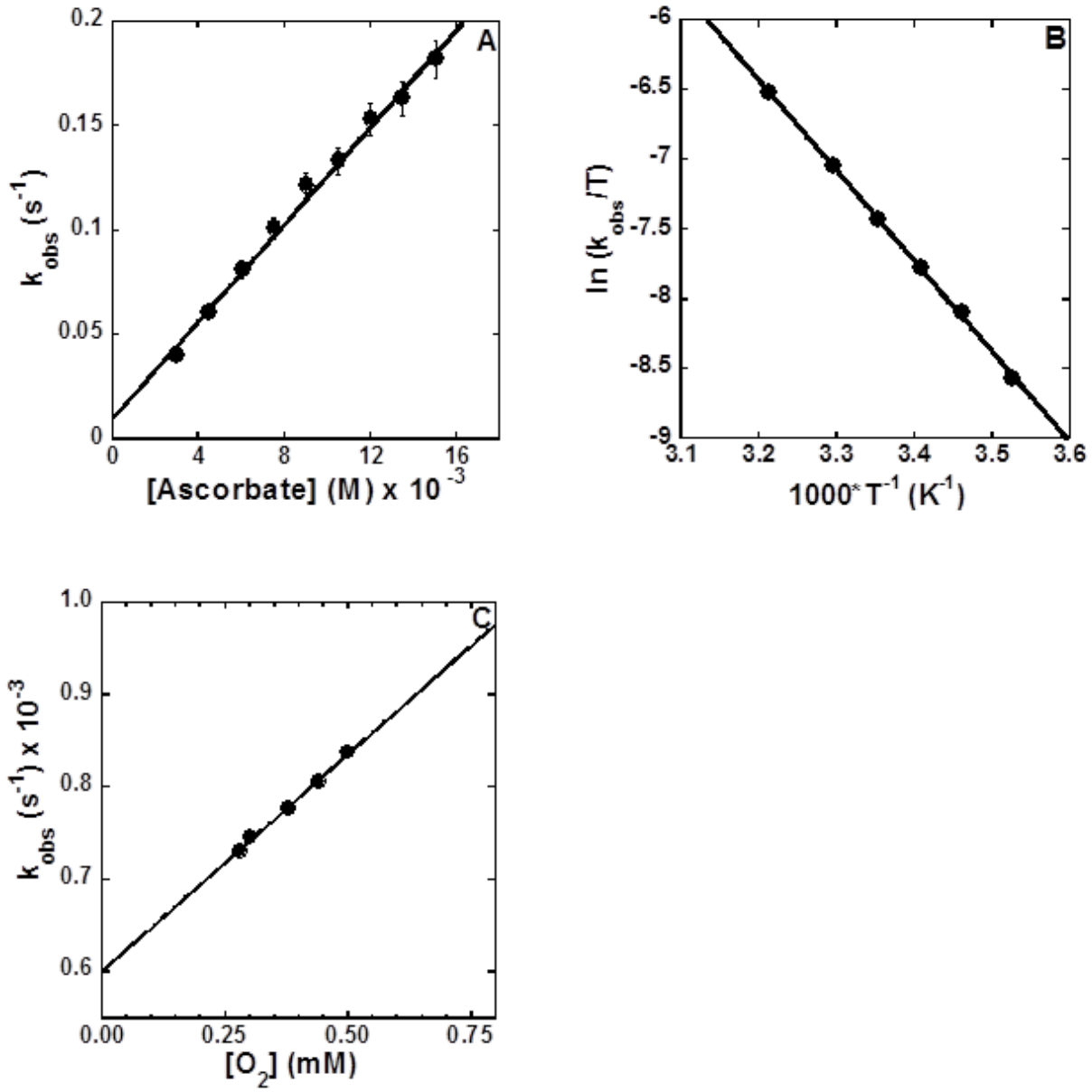

Figure 7. Kinetics of the reaction of CoN_4[11.3.1] with ascorbate or oxygen.

(A) Stopped-flow kinetics of the reaction of Co(III)N_4[11.3.1] (0.3 mM) with an excess of sodium L-ascorbate (3-15 mM) followed at 460 nm in 0.1 M sodium phosphate buffer, pH 7.4 at 25°C. The observed rates are then plotted against the sodium ascorbate concentrations. The rate constant was determined from the slope of the line. **(B)** Eyring plot for the reduction of Co(III)N_4[11.3.1] by sodium L-ascorbate. Reactions between sodium ascorbate (15 mM) and Co(III)N_4[11.3.1] (0.3 mM) were carried out at 6 different temperatures ranging from 10-37°C. The natural log of the observed rates over temperatures in Kelvin were plotted versus the reciprocal temperatures in Kelvin. The activation parameters were obtained from the slope and y-intercept of the plot. **(C)** Stopped-flow kinetics of the reaction of oxygen with Co(II)N_4[11.3.1] (0.05 mM) under pseudo-first order conditions at 25°C. Sodium phosphate

buffer solutions (0.1 M, pH 7.4) at different oxygen concentrations, prepared by mixing between deoxygenated buffer and 100% oxygen saturated buffer, were rapidly mixed with Co(II)N_4[11.3.1] and followed at 460 nm. The observed rates were plotted versus the final oxygen concentrations (0.28-0.5 mM).

As it had previously been observed that leaving a solution of the Co(II)N_4[11.3.1] on the benchtop overnight resulted in the complete conversion of the compound to its oxidized form, it seemed important to determine the rate of oxidation of the reduced compound if only to insure that it was not faster than the reduction by ascorbate. The oxidation of Co(II)N_4[11.3.1] was performed under pseudo-first order conditions using an excess of oxygen in solution made by the use and dilution of oxygen-saturated buffer. The second order rate constant, determined from the linear fit of the data, was found to be 0.5 (± 0.02) M^{-1} s^{-1} at 25°C (Figure 7C). The reaction may be somewhat complicated as the y intercept is non-zero; an indication that some other process is involved in the reaction. The oxidation has a second order rate constant ~20-fold slower, however, than the reduction of Co(III)N_4[11.3.1] by ascorbate. Since physiological ascorbate levels are ~60 μM [80] and if oxygen levels are not above that, then CoN_4[11.3.1] is most likely to be found in its reduced in the circulation when used as an antidote. Therefore, it is important to examine the binding of cyanide to the reduced form of CoN_4[11.3.1].

3.2.2 Kinetics of cyanide binding to Co(II)N_4[11.3.1] under anaerobic conditions

The electronic absorption spectra of Co(II)N_4[11.3.1] (solid trace) and the dicyano-Co(III) complex (dashed trace) are shown in Figure 6 at 25°C, pH 7.4. A new absorption peak appeared at 420 nm (see dashed trace) after the addition of an excess of cyanide, while the previous absorption peak at 460 nm disappeared. Lopez-Manzano et al. [68] had previously shown that the binding of two molecules of cyanide to the reduced cobalt compound, even under anaerobic

conditions, generated an oxidized dicyano-cobalt(III) complex. The kinetics of this reaction were examined by following spectral changes at both 420 nm (peak of dicyano-Co(III) compound) and 460 nm (the peak associated with the Co(II) parent complex) using a stopped-flow spectrometer. At 25°C, the reaction of Co(II)N_4[11.3.1] with cyanide under anaerobic conditions was too fast to be observed. In order to slow the reaction, both the temperature (to 10°C) and the cyanide concentrations (only a 10 to 20-fold excess) were lowered as far as possible while still maintaining pseudo-first order conditions. The rate of reaction, monitoring the kinetic traces at both 460 and 420 nm, was found to be very rapid and reached equilibrium within 20 ms. A plot of normalized absorption spectrum changes over time for kinetic traces at 460 and 420 nm (Figure 8A) exhibited a mirror image. This suggested that the single phase observed from the rate of the disappearance of the absorption maximum at 460 nm (due to Co(II)N_4[11.3.1]) was identical to the rate of the appearance of the peak at 420 nm (due to Co(III)N_4[11.3.1]$(CN)_2$). The kinetic traces generated at both 460 and 420 nm were best fit with a single exponential model. A plot of the observed rate constants ($k_{obs\text{-}420\ nm\ \&\ 460\ nm}$) versus varying cyanide concentrations was linear and second-order rate constants (k_f) were obtained from the slopes of fits: 8.5 (± 0.5) x 10^4 and 8.0 (± 0.5) x 10^4 M^{-1} s^{-1} (pH 7.6, 10°C) for data obtained at 420 and 460 nm, respectively (Figure 8B). However, the y-intercepts of linear fits to the data were non zero, suggesting that either a portion of the reaction was missed, a back reaction occurred or a pre-equilibrium existed in the reaction of cyanide binding to the cobalt complex. The y-intercept (k_r ~400 s^{-1}) may also represent the rate constant of a reaction that does not depend upon the concentration of cyanide, such as a reverse reaction: Co(II)N_4[11.3.1]$(CN)_2$ → Co(II)N_4[11.3.1](CN) + CN^-.

The reaction of cyanide binding to Co(II)N_4[11.3.1] was found to be temperature dependent over the range of 10 to 25°C. Using the Eyring equation, the reciprocal of the temperature was plotted versus $ln\ k_{obs}/T$ (Figure 8C). The enthalpy of activation ($\Delta H^{\ddagger}$) was calculated from the slope of a linear fit to the data; a value of 10.7 (± 0.3) kJ mol^{-1} was determined. The entropy of activation ($\Delta S^{\ddagger}$) was calculated from the y-intercept of the line and found to be -153 (± 1) J mol^{-1} K^{-1}. The small $\Delta H^{\ddagger}$ value we determined indicates a low enthalpic barrier to the reaction, typically found in the range of 20-150 kJ mol^{-1} for bimolecular reactions and is certainly consistent with the large rate constant value observed (k_f of ~10^5 M^{-1} s^{-1}).

Additionally, reactions between cyanide and the cobalt complex were performed at a range of hydrogen ion concentrations (pH 6.3 to 9.0, see Figure 8D) and we observed an increasing rate of reaction with increasing pH. Indeed, we found that the reaction rate above pH 9.0 was so fast that we were not able to measure it. These results are in contrast to some reactions of cyanide with cobalt complexes in which the rate of the reaction decreases with increasing pH, indicating HCN as the attacking nucleophile, although other reactions clearly depend on both on species [81]. Since our observations showed an increasing rate with increasing pH, this suggests that the anionic form of cyanide (CN^-) is the attacking species during the reaction and that the reaction with HCN must be very much slower. The pK_a of HCN is 9.3 at 25°C and thus the concentrations of cyanide anion (CN^-) increases under more basic conditions (at pH ~9.3 the ratio of HCN/CN^- is 1:1). The percent cyanide anion calculated at various hydrogen ion concentrations (using the Henderson-Hasselbalch equation) is plotted alongside the observed rates of cyanide binding to the cobalt complex (Figure 8D). The rates correspond quite well with the amount of cyanide anion present during the reactions, thus lending additional support to the idea of cyanide anion as the attacking species.

While the pH dependence of the reaction fits with the idea that the cyanide anion is the predominant attacking species, there is another reagent involved in the reaction, Co(II)N_4[11.3.1]. Many other cobalt compounds with coordination to 4 nitrogen atoms have a square-planar arrangement (see Figure 3 in Chapter 1), with 2 other available sites for ligand binding. Frequently these other ligands are water molecules which when bound to metals undergo a change their pK_a. A bound water molecule may lose a proton to become a bound hydroxide, for example, which can cause a change in the reaction kinetics. In addition these changes in the axial ligands' protonation state may be reflected by changes in the electronic absorption spectrum of the compounds. Therefore, Co(II)N_4[11.3.1] (0.6 mM) samples in either 0.1 M sodium phosphate (pH 4.5-8.5) or borate (pH 9-11) buffers, with 0.3 M NaCl added to maintain ionic strength, were prepared anaerobically and the electronic absorption spectra of these solutions were recorded at pH levels (measured after mixing), ranging from 4.9 to 10.6. All of these spectra were shown to be essentially identical (see Figure 6, solid trace, as an example). However, we found that the intensity of the Co(II)N_4[11.3.1] spectrum recorded below pH 5 decreased over time, probably due to some degradation of the Schiff-base macrocycle structure [82]. Thus, in the range pH 5 to 10, we can find no evidence that the axial positions of the square-planar structure of Co(II)N_4[11.3.1] in aqueous solution are perturbed due to changing pH and are more convinced that the kinetic evidence supports the cyanide anion as the attacking species in the reaction of cyanide with Co(II)N_4[11.3.1]. It has also been suggested that the CoN_4[11.3.1] complex has a square planar geometry, rather than octahedral, and that there are no axial ligands bound to the complex [83]. The lack of any change in the absorption spectrum with pH, while hardly definitive, does seem to support this notion.

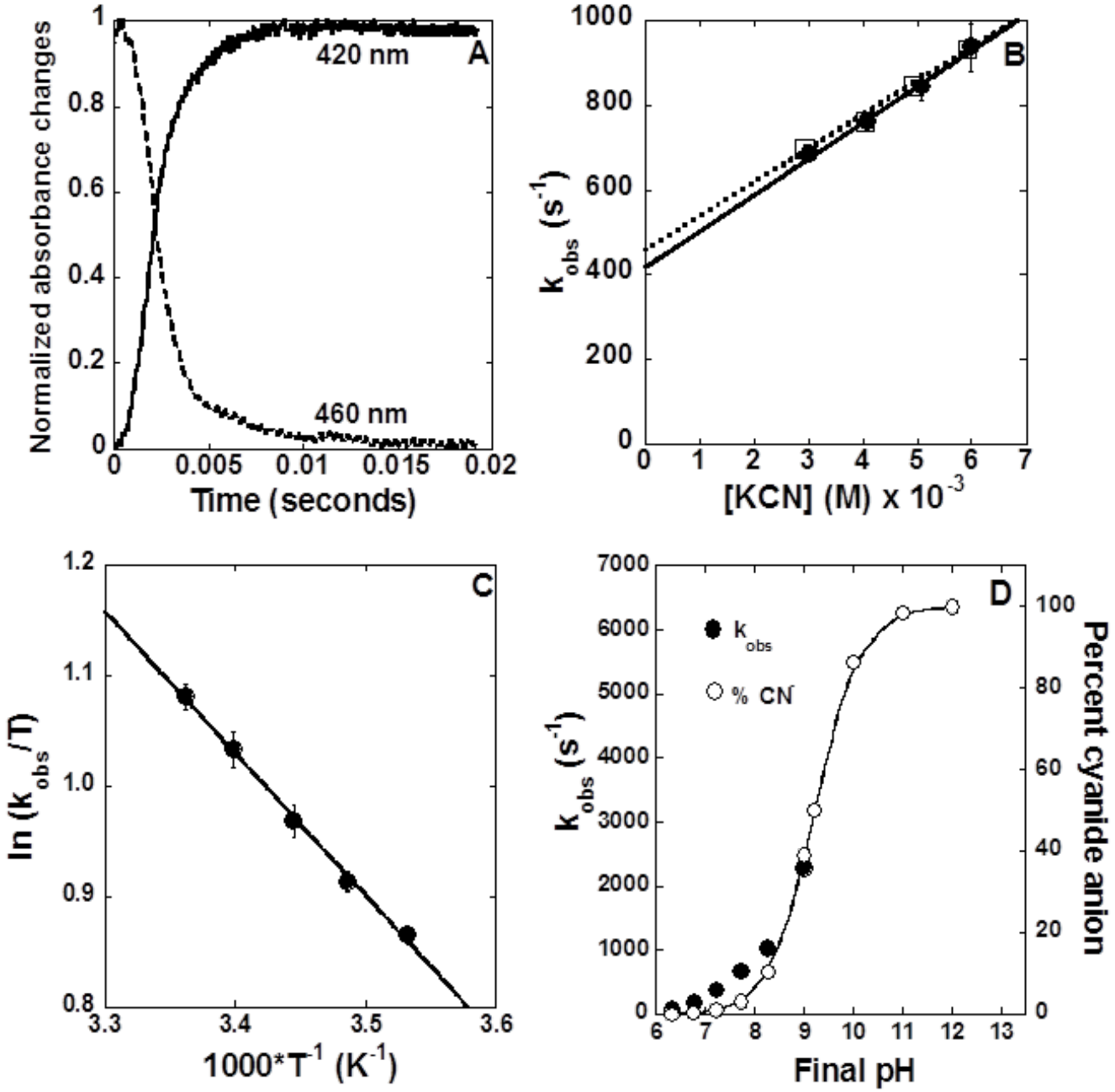

Figure 8. Kinetics of the reaction of Co(II)N_4[11.3.1] with cyanide under pseudo-first order conditions.

(A) Representative stopped-flow kinetics of the reaction between Co(II)N_4[11.3.1] and KCN under pseudo-first order conditions at 10 °C, pH 7.6 (after mixing). The reactions were followed anaerobically at 420 (solid line) and 460 (dashed line) nm under the following circumstances: 0.3 mM Co(II)N_4[11.3.1] in 0.05 M sodium phosphate buffer, pH 7.4, 3 mM KCN (10-fold excess, prepared in a pH 11.6 water solution adjusted with NaOH) at 10 °C. Absorbance changes were followed at both 420 and 460 nm and normalized. **(B)** Stopped-flow kinetics of the reaction of Co(II)N_4[11.3.1] (0.3 mM) with KCN (3-6 mM) under anaerobic conditions. The reaction was followed

under pseudo-first order conditions at both 420 and 460 nm at pH 7.6 (after mixing), 10°C. The observed rates obtained from both kinetic traces at 420 (closed circles) and 460 (open squares) nm were then plotted against the KCN concentrations. Rate constants were determined from the slope of the lines. **(C)** Eyring plot determining the activation parameters for the reaction kinetics of Co(II)N_4[11.3.1] (0.3 mM) with KCN (3 mM) were followed as in (B) at 420 nm under anaerobic conditions at 5 different temperatures between 10 and 25°C. The natural log of the observed rates over temperatures in Kelvin were then plotted against the reciprocal temperatures in Kelvin. The enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation were determined from the slope and y-intercept of the plot, respectively. **(D)** Stopped-flow kinetics of the reaction of Co(II)N_4[11.3.1] (0.3 mM) with KCN (3 mM) under anaerobic conditions at 10°C. Kinetic traces were monitored at 420 nm under pseudo-first order conditions at different pH (measured after mixing) ranging from 6.3 to 9.0. The observed rates were plotted versus the final pH values (closed circles). The right axis represents the percentage of cyanide anion plotted versus the pH (open circles).

3.2.3 Kinetics of cyanide binding to Co(II)N_4[11.3.1] under non-pseudo first order conditions

As observed in the pseudo-first order reactions of Co(II)N_4[11.3.1] with excess cyanide, the intercept was non-zero indicating that a portion of the reaction was either due to a pre-equilibrium or that we were missing part of the reaction. In order to investigate the potential portion of the reaction represented by the non-zero intercept (see Figure 8B), the reaction of cyanide with Co(II)N_4[11.3.1] was carried out under non-pseudo first order conditions. The kinetics of this reaction were performed under anaerobic conditions (10°C, pH 7.6) using a 1:1 ratio of [cyanide]:[Co(II)N_4[11.3.1]] (0.3 mM each) and observed that the absorption spectrum in the 400-500 nm region initially increased during a 27 millisecond time-frame, then decreased slightly (Figure 9A). Under the same reaction conditions, but in the presence of oxygen, we found an initial small increase in absorbance (over 27 ms), followed by a decrease (over 500 ms) in the absorption spectrum of 400-500 nm (Figure 9B). However, neither of these reactions for

the 1:1 ratio of reagents, showed a peak at 420 nm, which, when present, indicates the dicyano-Co(III) complex. Therefore, it appears that under these conditions we are observing the formation of a monocyano cobalt complex.

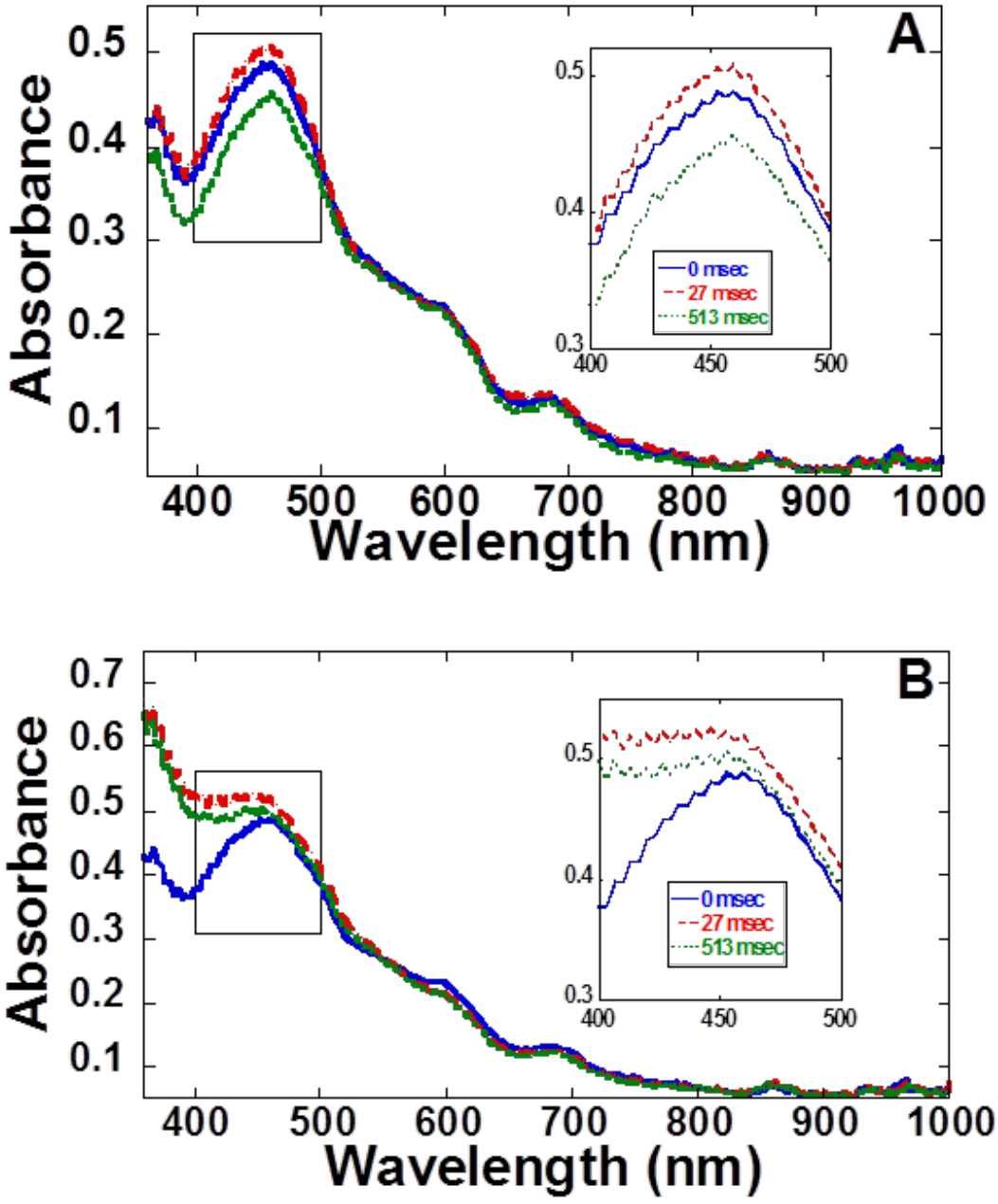

Figure 9. Kinetics of the reaction of Co(II)N$_4$[11.3.1] with cyanide at a 1:1 ratio in the absence and presence of oxygen at 10°C, pH 7.6.

Reactions between Co(II)N_4[11.3.1] (0.3 mM) in 0.05 M sodium phosphate buffer, pH 7.4 and KCN (0.3 mM) in a pH 11.6 water solution adjusted with NaOH were performed at 10°C, pH 7.6 (after mixing). Stopped-flow kinetic data were recorded using photodiode array mode at 3 different time points; **Main panel:** Electronic absorption spectra of the reaction of Co(II)N_4[11.3.1] with KCN at t = 0 ms (solid trace), 27 ms (dashed trace) and 513 ms (dotted trace). **Inset:** Enlarged electronic absorption spectra between 400 and 500 nm. The experiments were carried out in the **(A)** absence and **(B)** presence of oxygen.

In addition, reactions of Co(II)N_4[11.3.1] with cyanide were carried out with a 1:2 ratio of reactants (0.3 mM Co(II)N_4[11.3.1]:0.6 mM KCN) (Figure 10) under anaerobic conditions, at 10°C, pH 7.6. In this reaction, an increase in absorption at 420 nm was observed, while during the same time the 460-nm absorption band decreased. This behavior resembled the results observed under pseudo-first order conditions (Figure 6, dashed trace), suggesting the formation of the dicyano-Co(III)N_4[11.3.1] species. Interestingly, in the presence of oxygen, the absorption at 420 nm also initially increased, but the absorbance increase did not reach the same absorbance level as the reaction performed in the absence of oxygen. This suggested that a dicyano-Co(III)N_4[11.3.1] species was formed; while, the decrease in 420-nm absorption band indicated that there could be another additional pathway(s) to produce the final product. Taken together, the data from the non-pseudo first order reactions of cyanide binding to CoN_4[11.3.1] suggest that the first cyanide binding is extremely rapid followed by association of the second cyanide which is then the rate-determining step that we observe (see Figure 8B).

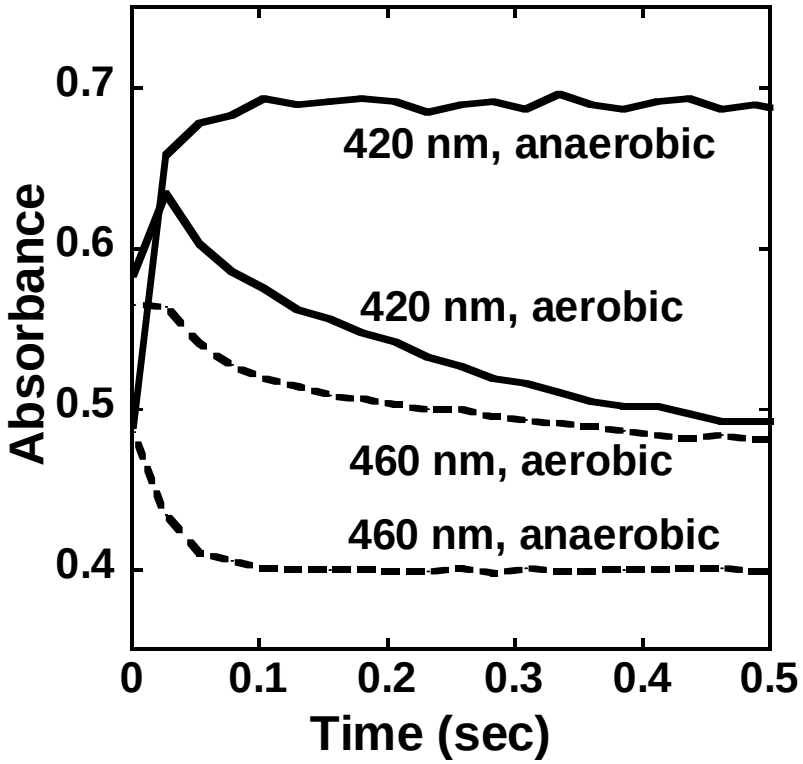

Figure 10. Kinetics of the reaction of Co(II)N$_4$[11.3.1] with cyanide at a 1:2 ratio in the absence and presence of oxygen at 10°C, pH 7.6.

Reactions between Co(II)N$_4$[11.3.1] (0.3 mM) in 0.05 M sodium phosphate buffer, pH 7.4 and KCN (0.6 mM) in a pH 11.6 water solution adjusted with NaOH were carried out in the absence and presence of oxygen at 10°C, pH 7.6 (after mixing). Stopped-flow kinetic data were recorded using photodiode array mode and followed at both 420 (solid traces) and 460 nm (dashed traces) over a 500 ms time frame.

3.2.4 Cyanide binding to Co(II)N$_4$[11.3.1] in the presence of oxygen

As we are interested in the potential antidotal activity of Co(II)N$_4$[11.3.1] under physiological conditions, the binding of cyanide to the reduced cobalt complex was carried out under aerobic conditions. While most of the oxygen in mammals is found bound to hemoglobin, the circulating levels of oxygen may increase during cyanide poisoning as cytochrome *c* oxidase will no longer

be able to turnover the available oxygen with cyanide bound to its active site. Cyanide binding to Co(II)N_4[11.3.1] in the presence of oxygen was initially assessed using electronic absorption spectrophotometry. KCN solutions (6 mM) were prepared aerobically, [O_2] ~250 μM, whereas Co(II)N_4[11.3.1] (0.3 mM) was prepared anaerobically in 0.1 M sodium phosphate buffer, pH 7.4. The equal volume of the two solutions were then mixed in a cuvette, leading to the disappearance of the absorption peak at 460 nm associated with reduced cobalt species (solid trace, Figure 6). The resultant spectrum closely resembled the spectrum of Co(III)N_4[11.3.1] (dotted trace, Figure 6). When this reaction was attempted under the similar conditions ([KCN] = 6 mM, [Co(II)N_4[11.3.1]] = 0.3 mM) but in the stopped-flow spectrophotometer, even at 10°C the reaction occurred within the dead time of the instrument and, thus, no rate constant for the reaction under oxygen could be determined due to an extremely rapid reaction.

3.3 DISCUSSION

Co(III) complexes are famously kinetically inert to substitutions. Consequently, a good deal of effort has gone into understanding the apparent ease with which Co(III) corrinoids (*e.g.* cobalamin) bind cyanide. We have recently argued, however, that one does not necessarily need to invoke phenomena such as favorable kinetic trans effects to explain facile cyanide substitutions in these systems. The net reducing conditions *in vivo* favor reduction to Co(II) forms and, therefore, the available cobalt based cyanide scavengers almost certainly work by binding cyanide to their Co(II) forms, followed by oxidation to kinetically stable Co(III) cyanide adducts [59, 66, 68]. This ensures the cyanide can be excreted rather than systemically redistributed. In the current experiments, we have examined the physicochemical properties of

CoN_4[11.3.1] to ensure that it fits this model and seek to explore why it may have improved decorporation characteristics compared to other cobalt scavengers.

3.3.1 *In vivo* oxidation/reduction considerations

Ascorbate is deemed to be one of the main reductants in body fluids and tissues in humans with the estimated ascorbate concentration in blood plasma of ~60 μM [80]. The reduction of Co(III)N_4[11.3.1] by ascorbate, was found to be reasonably facile with a rate constant of 11.7 (± 0.4) M^{-1} s^{-1} at pH 7.4 and 25°C. This rate constant is comparable to those observed for other cobalt amine (N_4) complexes: Co(Me_6[14]4,11-dieneN_4)(H_2O), 3.4 M^{-1} s^{-1}; Co(Me_6[14]tetraeneN_4)(H_2O), 42 M^{-1} s^{-1}; Co(ms-Me_6[14]aneN_4)(H_2O), 42 M^{-1} s^{-1}; and Co([14]aneN_4)(OH), 29 M^{-1} s^{-1} in their reactions with ascorbate [84]. The rate constant for the oxidation of Co(II)N_4[11.3.1] was found to be 0.5 (± 0.02) M^{-1} s^{-1}, almost 20-fold slower than the rate constant observed for the ascorbate reduction of the complex. Estimates of the effective free oxygen concentration in mammals are ~30 μM [85], but the level of oxygen might be expected to be a little higher under circumstances where the consumption of oxygen is inhibited (*e.g.* during cyanide intoxication). So assuming that the level of oxygen could be double that of normal physiologic concentration, *i.e.* equal to the circulating level of ascorbate and, therefore, the relative rate constants would ensure that the reduction of the cobalt complex would dominate by more than an order of magnitude. In short, whatever form administered, the CoN_4[11.3.1] will quickly be reduced in circulating blood and remain so with cyanide bound.

3.3.2 Kinetics and plausible antidotal mechanisms of cyanide binding to Co(II)N_4[11.3.1]

It follows that the binding of cyanide to Co(II)N_4[11.3.1] is the functionally significant issue. In this study, the overall reaction under anaerobic conditions, leading to formation of a dicyano final product, was found to be extremely rapid with a second order rate constant of 8 x 10^4 M^{-1} s^{-1} (pH 7.6) when measured at 10°C. While we could not measure the second order rate constant of cyanide binding at 37°C, it can be estimated from the activation parameters at greater than 10^5 M^{-1} s^{-1}. In addition, this rate constant is consistent with describing the binding of the second cyanide (the rate determining step) since the reaction of the first cyanide with Co(II)N_4[11.3.1], leading to a monocyano intermediate, was not observable (see Figure 11). As we alluded to in the beginning of this discussion, there is a dearth of data concerning the binding of cyanide to the reduced forms of several potential antidotes containing cobalt. While we can only estimate the rates of some of these reactions, it is clear that the binding of cyanide to Co(II)N_4[11.3.1] is extremely fast. We also note that the pH behavior of the rates of cyanide binding to a cobalt porphyrin (*e.g.* CoTMPyP) [66] and a cobalt corrin (cobalamins) [86] indicated that the protonated form of cyanide, HCN, was one of the attacking species in these reactions. In contrast, the pH behavior of the reaction of cyanide with Co(II)N_4[11.3.1] (Figure 8D) indicates that the cyanide anion (CN^-) is the major attacking species. It might be expected that the cyanide anion (1-) would combine with the Co(II)N_4[11.3.1] (2+) at an increased rate over HCN (0) binding to other cobalt complexes.

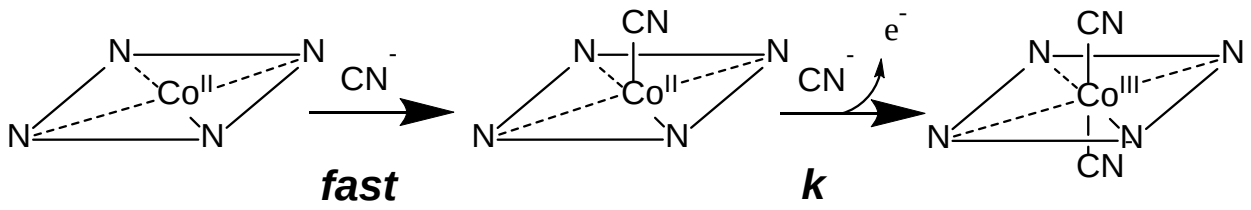

Figure 11. A plausible mechanism for the reaction of Co(II)N_4[11.3.1] with excess of cyanide in the absence of oxygen at pH 7.6.

The step with the second order rate constant (*k*) is the rate determining step (experimentally accessible).

3.3.3 Effect of oxygen on the dicyano cobalt complex

When cyanide was added to Co(II)N_4[11.3.1] in the presence of oxygen, the absorption spectrum generated was essentially identical to the spectrum of Co(III)N_4[11.3.1] (dotted trace, Figure 6). This suggested that cyanide could be lost from the "final" Co(III)N_4[11.3.1]$(CN)_2$ product. The overall reaction under aerobic conditions was so fast that we could neither identify the exact pathways nor determine the rate constant(s). Since the rate of the first cyanide binding to the cobalt complex was faster than that for the second cyanide, it is likely that cyanide is released from the monocyano-Co(III)N_4[11.3.1] by an inner sphere reaction with oxygen before the second cyanide can be bound to the complex (see Figure 12). Reactions of Co(II)N_4[11.3.1] with cyanide at a 1:2 ratio under aerobic conditions, however, showed that a small amount of dicyano-Co(III)N_4[11.3.1] complexes could also be formed as a minor product. The possible mechanism for the loss of cyanide from monocyano-Co(II)N_4[11.3.1] is currently under investigation. A plausible series of reactions for the reaction of Co(II)N_4[11.3.1] with excess of cyanide in the presence of oxygen is shown in Figure 12.

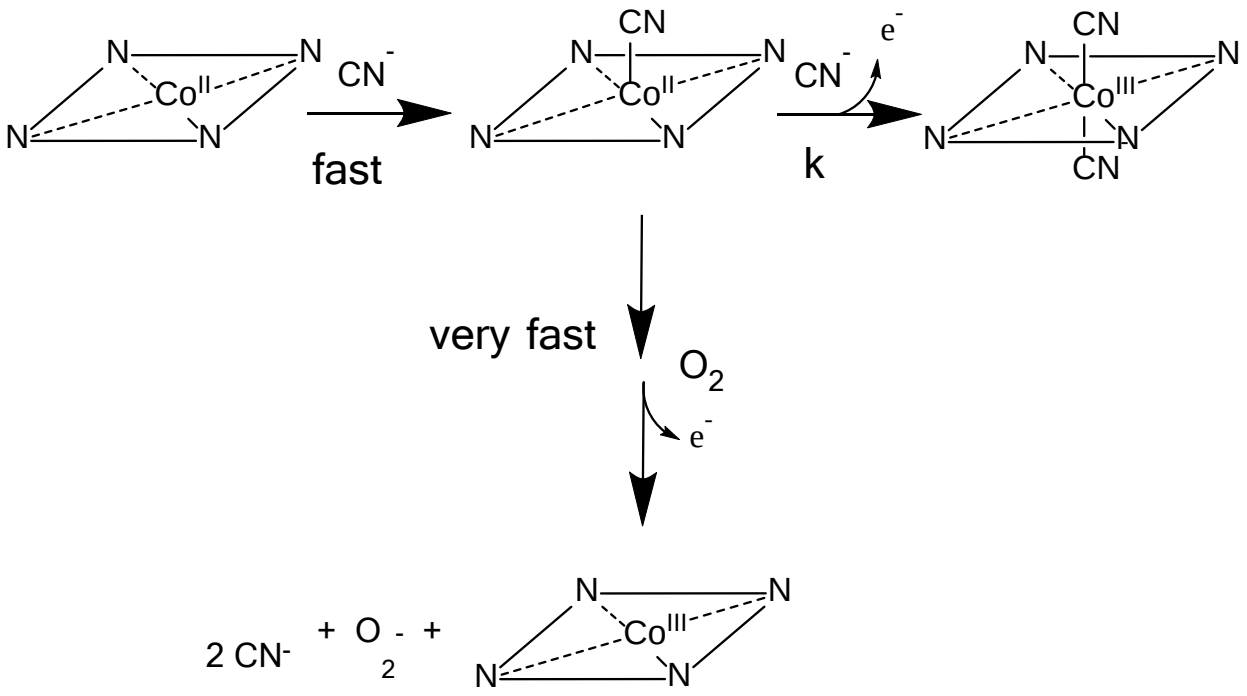

Figure 12. A plausible mechanism for the reaction of Co(II)N_4[11.3.1] with excess of cyanide in the presence of oxygen at pH 7.6.

The step with the second order rate constant (*k*) is the rate determining step (experimentally accessible).

The release of both cyano ligands from the cobalt complex in the presence of oxygen would certainly suggest that Co(II)N_4[11.3.1] might *not* be an effective antidote against cyanide toxicity, but we have certainly demonstrated that it does work effectively *in vivo* [64, 68]. How can these two observations be reconciled? We have also previously demonstrated that in the presence of excess ascorbate, oxygen is turned over (possibly to H_2O_2), in a catalytic fashion, by Co(II)N_4[11.3.1] [68]. In addition, when cyanide was added to the aforementioned reaction, the oxygen turnover was effectively halted. The available evidence supports either that the concentration of reductants (ascorbate and other reducing compounds) must be higher than the normal physiologic level of free oxygen *in vivo*, or that the oxygen concentration can be efficiently lowered by the turnover reaction of CoN_4[11.3.1].

3.3.4 Activation parameters

The enthalpy ($\Delta H^{\ddagger}$) of activation for the Co(III)N_4[11.3.1] reduction was found to be 53.9 (± 0.8) kJ mol^{-1}; while, the $\Delta H^{\ddagger}$ value for cyanide binding to the cobalt complex (10.7 (± 0.3) kJ mol^{-1}) is even lower than the typical $\Delta H^{\ddagger}$ values ranging from 20-150 kJ mol^{-1} [75]. This small $\Delta H^{\ddagger}$ value is relevant to the large rate constant value (k of ~10^5 M^{-1} s^{-1}), indicating that the cyanide binding process to Co(II)N_4[11.3.1] was undoubtedly very rapid.

Interestingly, the entropy ($\Delta S^{\ddagger}$) of activation of both reactions (ascorbate reduction and cyanide binding) showed large negative values: -79 (± 3) J mol^{-1} K^{-1} for the Co(III)N_4[11.3.1] reduction and -153 (± 1) J mol^{-1} K^{-1} for cyanide binding to Co(II)N_4[11.3.1]). Generally, entropies of activation are subject to much more speculation concerning their values, as opposed to enthalpies of activation. Differing $\Delta S^{\ddagger}$ values for rather similar reactions are often attributed to changes in solvation between ground and transition states. These large negative values, however, do suggest much more ordered transition states compared to their ground states. Large negative values of $\Delta S^{\ddagger}$ for bimolecular reactions have been observed in cases of ionic reactions between similarly charged ions, for example, CH_2BrCOO^- + $S_2O_3^{2-}$ ($\Delta S^{\ddagger}$ = -80 J mol^{-1} K^{-1}), $S_2O_3^{2-}$ + $S_2O_3^{2-}$ ($\Delta S^{\ddagger}$ = -160 J mol^{-1} K^{-1}), $[Co(NH_3)_5Br]^{2+}$ + Hg^{2+} ($\Delta S^{\ddagger}$ = -160 J mol^{-1} K^{-1}) [87]. In our study, however, the reactions were between positively charged ions (Co(II/III)N_4[11.3.1]) and negatively charged ions (ascorbate$^-$ and CN^-) and, thus, this is not an adequate explanation. One would, however, expect negative activation entropies in associative reactions as two entities come together (associate), *e.g.* cyanide or ascorbate with cobalt ions. Therefore, the negative activation entropies seem to indicate that both of these reactions (ascorbate reduction and cyanide binding) are associative with the cyanide binding reaction being strongly so.

This study has gone some way towards enhancing our understanding the reaction of $Co(II)N_4$[11.3.1] with cyanide. The exceptionally fast rate of reaction of the complex with cyanide implies a high efficacy of its cyanide-scavenging property. These findings further support the conclusion, suggested by our previous work using a sub-lethal mouse model, that $Co(II)N_4$[11.3.1] could potentially be a more cost-effective and better cyanide antidote, rather than the conventional therapy, hydroxocobalamin.

4.0 A COBALT SCHIFF-BASE COMPLEX AS A PUTATIVE THERAPEUTIC FOR AZIDE POISONING

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Keywords: azide antidote, azide decorporation, cobalt macrocycle, cytochrome *c* oxidase

4.1 ABSTRACT

The administration of Co(II)N_4[11.3.1] at 5 minutes post sodium azide intraperitoneal injection to mice resulted in a substantial decrease of righting-recovery times, compared to controls. The percent survival for the mice receiving Co(II)N_4[11.3.1] also increased, compared to the controls. Sodium nitrite showed no ameliorative effect toward azide in mice. Anaerobically, two azide molecules cooperatively bind to one cobalt complex with a binding (association) constant of 2.8 x 10^8. However, in the presence of oxygen, oxidation of the Co(II)N_4[11.3.1]$(N_3)_2$ adduct occurs

with the concomitant loss of at least one azido ligand. The stopped-flow kinetics of cyanide binding to Co(II)N_4[11.3.1] in the absence of oxygen exhibited three experimentally observable phases; I (fast), II (intermediate) and III (slow). Phase II accounted for ~100% of the overall absorbance changes, representing the major process observed with a second-order rate constant of 29 (± 4) M^{-1} s^{-1} at 25°C. The data demonstrated pH independence of the reaction around neutrality, suggesting the unprotonated azide to be the attacking species. In the presence of excess oxygen, the oxidation of Co(II)N_4[11.3.1]$(N_3)_2$ to Co(III)N_4[11.3.1](N_3) exhibited biphasic kinetics. Both phases were oxygen dependent but the faster phase was linearly dependent on the square of the oxygen concentration. The mechanism of this oxidation is complicated and warrants further study. However, the simplest explanation would be that the oxidation of the substitution-labile Co(II)N_4[11.3.1]$(N_3)_2$ requires displacement of an azido ligand by oxygen, followed by inner-sphere electron transfer forming Co(III)N_4[11.3.1](N_3) and superoxide, resulting in the kinetically stable monoazido-Co(III) adduct.

4.2 INTRODUCTION

Most azide poisonings are accidental, or suicide attempts, but there also seem to have been at least half a dozen reported instances of malicious injury to others caused by spiking beverages with azide in Japan [21] and the US [23-25]. The symptoms of acute azide (N_3^-) intoxication are similar to those induced by cyanide (CN^-) [88, 89] and, analogous to cyanide, azide is known to be an inhibitor of the mitochondrial electron transport system, specifically at cytochrome *c* oxidase (complex IV) [30, 90, 91]. Unlike cyanide, however, there is no antidote for azide poisoning, either approved or off-label, currently available. When dissolved in aqueous solution,

azide is an odorless, colorless and highly toxic substance, clearly useful characteristics for nefarious purpose. Sodium azide has a number of commercial uses, most notably in the explosive deployment of automobile airbags, but it is also a widely used bactericidal agent. Consequently, there probably exists a significant quantity of sodium azide stored in poorly documented locations. Due to the similarity of action for azide and cyanide, conventional remedies for cyanide poisoning, such as nitrite/thiosulfate and/or hydroxocobalamin, might be expected to be of benefit for treating azide toxicity. None of these, however, has so far demonstrated a substantial or convincing amelioration of azide toxicity [92].

Recently, we have shown that a cobalt-containing Schiff-base macrocyclic compound, cobalt 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17)2,11,13,15-pentaene dibromide (CoN_4[11.3.1]), effectively scavenged cyanide in mice [64, 68]. Here, we explore whether CoN_4[11.3.1] might also be an effective antidote toward azide toxicity by virtue of its scavenging capability, which we demonstrate through the application of various physical methods and by restoration of righting recovery in sub-lethally intoxicated mice. In addition, some previous authors have questioned whether the toxic effects of azide are due to N_3^- itself, suggesting that secondary metabolites like cyanide [89] or nitric oxide [34] might be the principal toxic species. The present findings distinguish between these possibilities.

4.3 RESULTS

4.3.1 Potential antidote to azide toxicity in mice

The righting recovery behavior of mice has recently been employed to assess the ability of potential antidotes to ameliorate sub-lethal doses of toxicants (especially cyanide) [41, 68, 93]. In the present study, mice were injected intraperitoneally (IP) with sodium azide (t = 0) then, after "knockdown" (loss of consciousness), placed on their backs and the time until they "righted" themselves onto four feet recorded. Mice given sodium azide exhibited a rather steep response curve: 26 mg/kg was an LD_{20} (or knockdown) dose; 24 mg/kg (LD_0) showed minimal effect; 30 mg/kg was essentially a lethal dose. This is similar to cyanide but the molar doses of azide required to elicit a similar righting-recovery response to cyanide in mice were ~4 times the cyanide doses. When given 26 mg/kg sodium azide (IP), 12 week-old mice demonstrated knockdowns at 7-8 min post injection, with the majority of mice surviving (88%) and exhibiting righting-recovery times of 40 (± 8) min (Table 1).

Table 1. Antidotal activity of CoN_4[11.3.1] against azide toxicity in mice.

Experimental conditions	Number of knockdowns	Recovery Time (min)	% survivors
26 mg/kg NaN_3	8 of 8	40 (± 8)	88
26 mg/kg NaN_3 + 24 mg/kg $NaNO_2$ (5 min later)	6 of 6	38 (± 8)	83
26 mg/kg NaN_3 + 37 mg/kg CoN_4[11.3.1] (5 min later)	2 of 7	12 (± 4)	100

Since sodium azide exhibits a similar toxicological profile to cyanide, with presumably the same biological target (cytochrome *c* oxidase), two antidotes that were previously found to be effective against cyanide toxicity in mice were investigated. Sodium nitrite (12 mg/kg) has been shown to ameliorate cyanide toxicity in mice up to 20 min after the toxicant dose (5 mg/kg NaCN, LD_{75}) but when sodium nitrite (24 mg/kg, IP) was given to mice 5 min after the azide dose, it had virtually no effect, with mice exhibiting a righting recovery at 38 (± 8) min (see Table 1). Previously the protective effect of CoN_4[11.3.1] against cyanide toxicity has been demonstrated in a mouse model [64, 68]. When CoN_4[11.3.1]Br_2 (37 mg/kg) was given at 5 min post sodium azide, all of the mice recovered, the majority of the mice did not knockdown (71%) and those that did (29%) recovered by 12 min (Table 1). These results clearly demonstrate that CoN_4[11.3.1] is a potential antidote to azide toxicity, while sodium nitrite is apparently not.

4.3.2 Azide binding to CoN_4[11.3.1]

The addition of Co(III)N_4[11.3.1] to mouse blood was previously shown to result in its reduction to the Co(II) form [68]. We, therefore, studied the stoichiometry and determined the equilibrium constant for the binding of azide to Co(II)N_4[11.3.1]. The electronic absorption spectrum of Co(II)N_4[11.3.1] exhibits a broad band in the visible region with a maximum at 460 nm (Figure 13, solid trace). Following the anaerobic addition of excess sodium azide (0.1 M sodium phosphate buffer, pH 7.4) the absorption maximum shifts to ~475 nm with slight loss of intensity (Figure 13, dashed trace). These spectra are consistent with the observed transitions being largely d-d in origin and, perhaps surprisingly, their similarity is not suggestive of much change in net ligand geometry around the central Co(II) ion upon binding azide. However, we found that no azide bound when this experiment was carried out in low or red light. Thus we conclude

that the binding of azide to Co(II)N_4[11.3.1] must be photo-catalyzed and subsequently all other experiments were carried out under ambient light. When excess azide was added to Co(II)N_4[11.3.1] in the presence of oxygen, the spectrum obtained displayed significantly reduced absorption intensity throughout most of the visible region (Figure 13, dot-dashed trace) in keeping with the typically weak d-d spectra of Co(III) compounds. The absorption spectrum of azide-free Co(III)N_4[11.3.1] demonstrating a similar lack of distinct features in the visible region is shown for comparison (Figure 13, dotted trace). The 5-fold more intense band at 350 nm in the azide adduct formed under aerobic conditions (Figure 13, dot-dashed trace) is presumably more charge-transfer (ligand-to-metal) in nature than the visible-region bands in these spectra. Overall, these data suggest that in the presence of oxygen, the binding of azide to Co(II)N_4[11.3.1] results in oxidation of the cobalt to a Co(III) form. The conversion of an initial Co(II) species to a Co(III)-containing azide adduct under aerobic conditions has subsequently been confirmed by EPR measurements (see below).

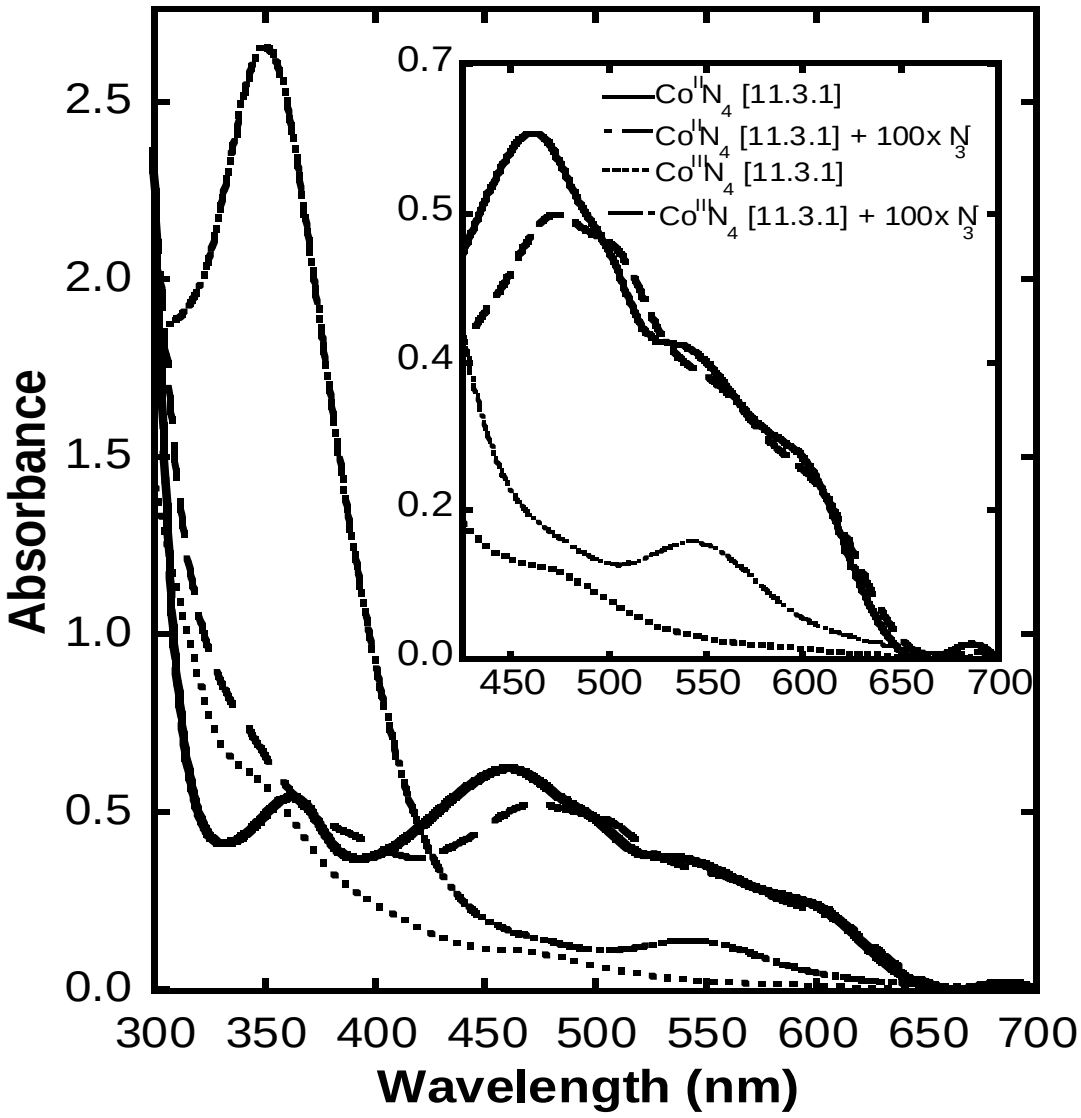


Figure 13. Electronic absorption spectra of Co(II/III)N_4[11.3.1] binding to azide.

Sodium azide was titrated into Co(II)N_4[11.3.1] (0.5 mM) under anaerobic conditions and into Co(III)N_4[11.3.1] (0.5 mM) under aerobic conditions at 25ºC and pH 7.4 (0.1 M sodium phosphate buffer) in septum-sealed cuvettes (1 cm path length) using gastight syringes. **Main panel:** Electronic absorption spectra of Co(II)N_4[11.3.1] alone (solid trace) and in the presence of 100-fold excess of sodium azide (dashed trace) and Co(III)N_4[11.3.1] alone (dotted trace) and in the presence of 100-fold excess of sodium azide (dot-dashed trace). **Inset:** Enlarged electronic absorbance spectra of Co(II)N_4[11.3.1] and Co(III)N_4[11.3.1] with a 100-fold excess of sodium azide.

While the electronic spectral changes indicated that azide bound to Co(II)N_4[11.3.1], the stoichiometry remained unresolved. Job's method has been used for over a century to determine molecular associations such as acid-base equilibria, transition metal coordination, host-guest association, *etc*. Briefly, the mole fraction of a single component of a two-component system can be plotted versus some physical property, commonly absorbance, which linearly relates to the formation of a complex. This presentation is often called a Job plot [94-96]. As a positive control, we applied a minor variation of the method to a system containing similarly weak chromophores to the cobalt complexes we intended to investigate further. Formation of the Cu(II)EDTA complex was monitored by absorption difference spectroscopy monitoring the peak-to-trough changes at 723 and 500 nm (Figure 14A). This well-known 1:1 complex gave a maximal absorption difference at the volume fraction of 0.5, confirming the expected 1 Cu(II):1 EDTA stoichiometry. In addition, the triangle-like plot (rather something more curved in the vicinity of the maximum) indicates a large binding constant [94-96]. It is known that EDTA binds Cu(II) with a K_{eq} of ~10^{18} and thus the triangular plot (Figure 14A) is consistent with this large binding constant [94]. When the same approach was applied to the anaerobic binding of azide to Co(II)N_4[11.3.1], monitoring the peak-to-trough changes at 360 and 490 nm, the maximum was clearly not found to be at a volume fraction of 0.5, but at 0.33 (Figure 14B). This correlates with a 1 Co(II)N_4[11.3.1]:2 azide ratio similar to the stoichiometry previously observed for the binding of cyanide [68]. Interestingly, if an otherwise analogous experiment was carried out in the presence of oxygen, the resulting Job plot was found to exhibit a maximum at a volume fraction of 0.6 (Figure 14C). This result fits a 1:1 stoichiometry better than the 1:2 ratio found for the Co(II)N_4[11.3.1]-azide complex in the anaerobic experiment, but may also indicate the presence of some additional minority species with alternate stoichiometry (*e.g.* 2

cobalt:1 azide). Whatever the correct explanation for this perplexing result, we can be reasonably certain that oxidation of the $Co(II)N_4[11.3.1](N_3)_2$ adduct occurs with the concomitant loss of at least one azido ligand.

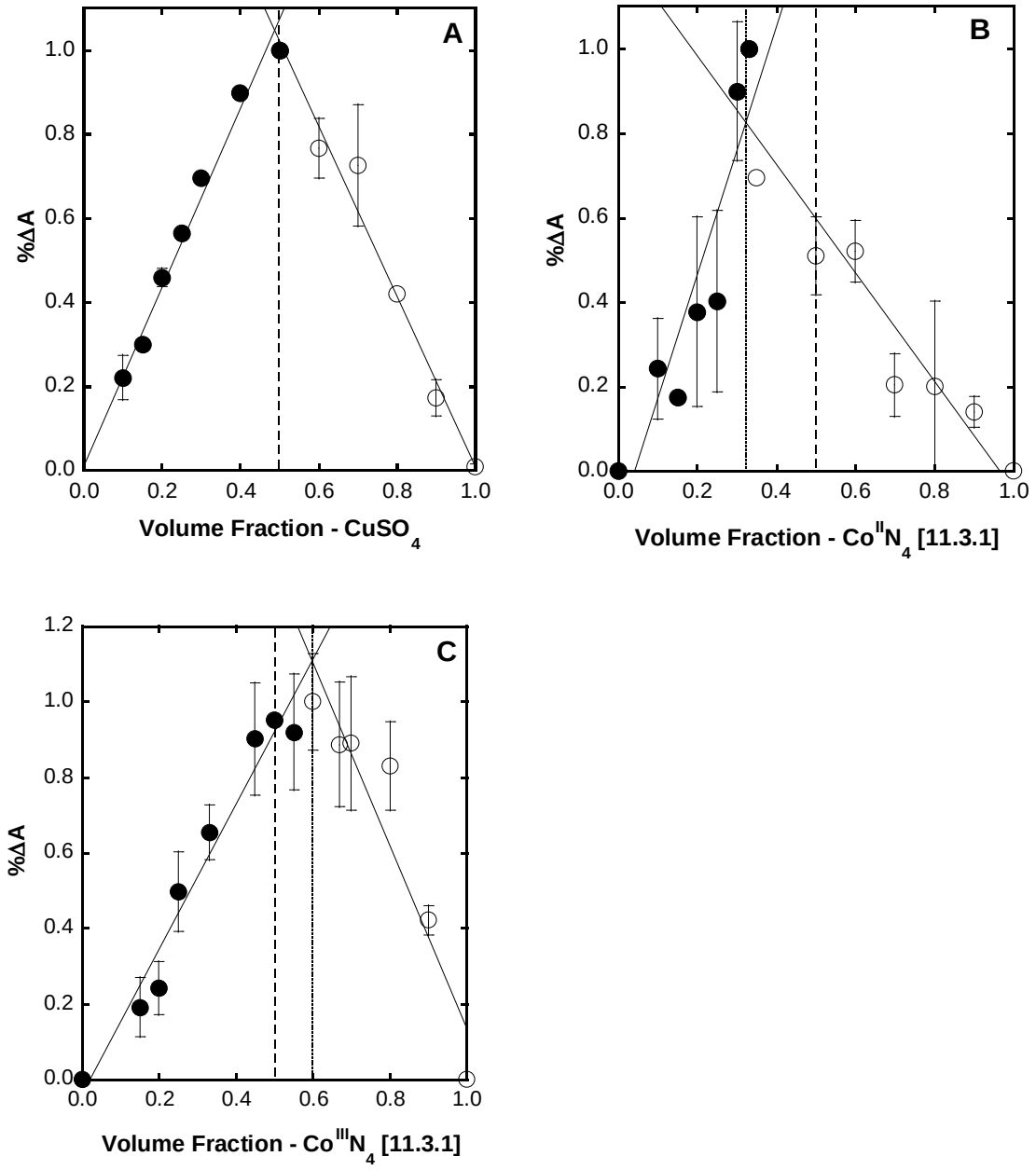


Figure 14. Job plots resulting from the titrations of $Cu(II)SO_4$ to Na_2EDTA and $Co(II/III)N_4[11.3.1]$ to azide.

The titrations of **(A)** $Cu(II)SO_4$ to Na_2EDTA (0.025 M acetate buffer, pH 4.7), **(B)** $Co(II)N_4[11.3.1]$ to sodium azide (0.1 M sodium phosphate buffer, pH 7.4), and **(C)** $Co(III)N_4[11.3.1]$ to sodium azide (0.1 M sodium phosphate

buffer, pH 7.4) by the method of continuous variations. The intersection of linear fits represents the volume fraction (X_A = [A]/([A]+[B])) of host corresponding to its binding ratio with the ligand. Open and closed circles represent absorbance change values associated with each linear fit within each plot.

Co(II)N_4[11.3.1] has an EPR spectrum typical of S = 1/2 Co(II) complexes (d^7) which has an axis of symmetry with $g_{||} < g_{\perp}$ and exhibits signals near 3300 G (Figure 15, A). We can observe a series of signals on top of the main signal (crossover at 3085 G) which consists of 8 hyperfine lines resulting from the interaction of the unpaired electron (d^7) with the ^{59}Co nucleus (I = 7/2). Following the addition of increasing amounts of azide to the initial sample, a new EPR signal begins to become apparent (Figure 15, B-D) finally resulting in a spectrum with the main crossover feature at 3121 gauss (Figure 15, E) – a small but highly reproducible change. Even when a 100-fold excess of azide was added to Co(II)N_4[11.3.1], the EPR signal obtained did not significantly change from that observed with a 10-fold excess of sodium azide (Figure 15, E). When this sample was exposed to oxygen, the signal disappeared, demonstrating the formation of an EPR-silent Co(III)N_4[11.3.1] azide compound. The concentration of the Co(II)N_4[11.3.1] sample and the fully formed Co(II)N_4[11.3.1]$(N_3)_2$ were determined by double integration of the signals with reference to a Cu(II)EDTA standard using the program SpinCount and found to be within 90% of the calculated concentrations, *i.e.* 63 μM. The concentrations of the azide-free and azide-bound signals could be determined by combining the two EPR signals arising from the fully formed Co(II)N_4[11.3.1]$(N_3)_2$ and Co(II)N_4[11.3.1] in the samples and matching to the intermediary spectra (Figure 15, B-D). This procedure enabled us to quantify concentrations of the Co(II) species present without any interference from small amounts of Co(III). This overcame a significant interference that prevented using electronic absorption data to evaluate the azide binding constant. The K_{β}' was determined using the following equation:

$$K_{\beta}^{'} = \frac{[Co(II)N_4[11.3.1](N_3)_2]}{[Co(II)N_4[11.3.1]][N_3]^2}$$

The concentrations of both cobalt complexes were determined by EPR and the free azide concentration was calculated by subtracting the amount of bound azide bound from the initial (total) concentration, assuming that 2 azide anions bound to each Co(II)N_4[11.3.1] (see Figure 14B). The overall binding (association) constant, K_{β}', for the binding of azide to Co(II)N_4[11.3.1] was then calculated to be 2.8 x 10^8.

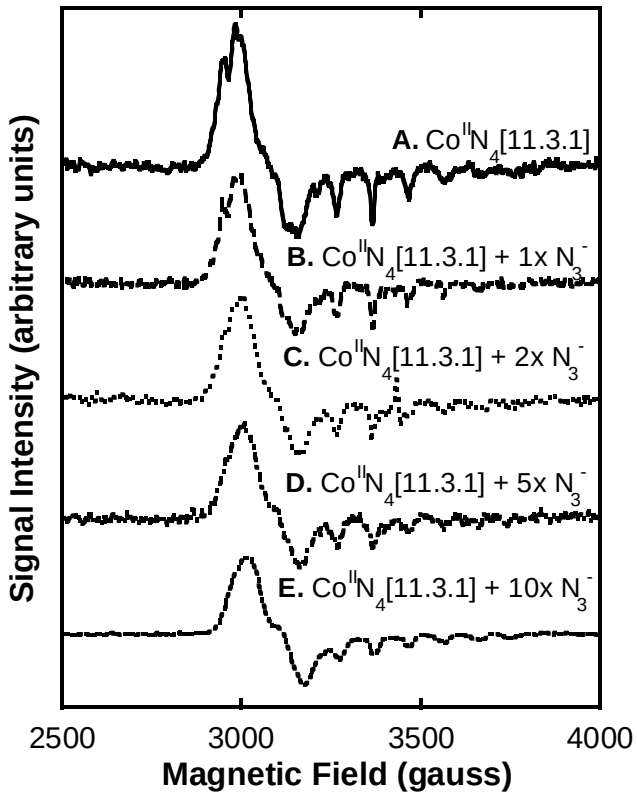


Figure 15. X-band EPR spectra at 20 K of Co(II)N_4[11.3.1] in 0.1 M sodium phosphate buffer (pH 7.4), 20% glycerol and a 10-fold excess sodium ascorbate and with azide.

Samples of 0.063 mM Co(II)N_4[11.3.1] were prepared anaerobically at room temperature **(A)** and with anaerobic aqueous sodium azide in increasing excess (up to 10-fold) over the cobalt complex concentration **(B-E)**. EPR

conditions: 9.8 G modulation amplitude, 63.2 μW microwave power. Samples were frozen and stored in liquid nitrogen quickly after preparation for later analysis.

Since we found that in the presence of oxygen the Co(II)N_4[11.3.1]$(N_3)_2$ loses an azide anion, we determined the overall equilibrium under these conditions. A titration of Co(II)N_4[11.3.1] in the presence of oxygen with azide resulted in a K_β' of 816.

4.3.3 Kinetics

Previously, the reduction of Co(III)N_4[11.3.1] by ascorbate (~60 μM in the circulating blood) was shown to be facile and thus, in relation to the question of antidotal mechanism, it seemed most important to determine the rate of binding of azide to the reduced cobalt complex rather than the oxidized form. The kinetics of azide binding to Co(II)N_4[11.3.1] were conducted under anaerobic conditions, at pH 7.4 in 0.1 M sodium phosphate buffer, by following the absorbance changes at 460 nm (absorption maximum of Co(II)N_4[11.3.1]) using stopped-flow spectrophotometry at both 25 and 37 °C. The resulting absorbance changes were indicative of three phases; I (fast, Figure 16A), II (intermediate, Figure 16B) and III (slow, not shown). The intermediate phase II consisted of ~100% of the absorption decrease at 460 nm and, therefore, represents the major process observed. Phase II was found to be linear with respect to both the concentration of azide and the cobalt complex (Figures 17A and 17B). Second-order rate constants (designated k_2) of 29 (± 4) M^{-1} s^{-1} at 25°C and 70 (± 10) M^{-1} s^{-1} at 37°C were found for this phase. In addition, reactions of azide and the cobalt complex were carried out at pH values of 6.5 (solid diamond) and 8.5 (open square) at 25°C (Figure 17C) demonstrating the absence of

any pH dependence of the reaction around neutrality. As the pK_a for azide is 4.7, greater than 98% of the total azide in solution is in the anionic form (N_3^-) at pH values above 6.4. Thus, as expected, the kinetic results are consistent with the unprotonated azide ion being the reacting species.

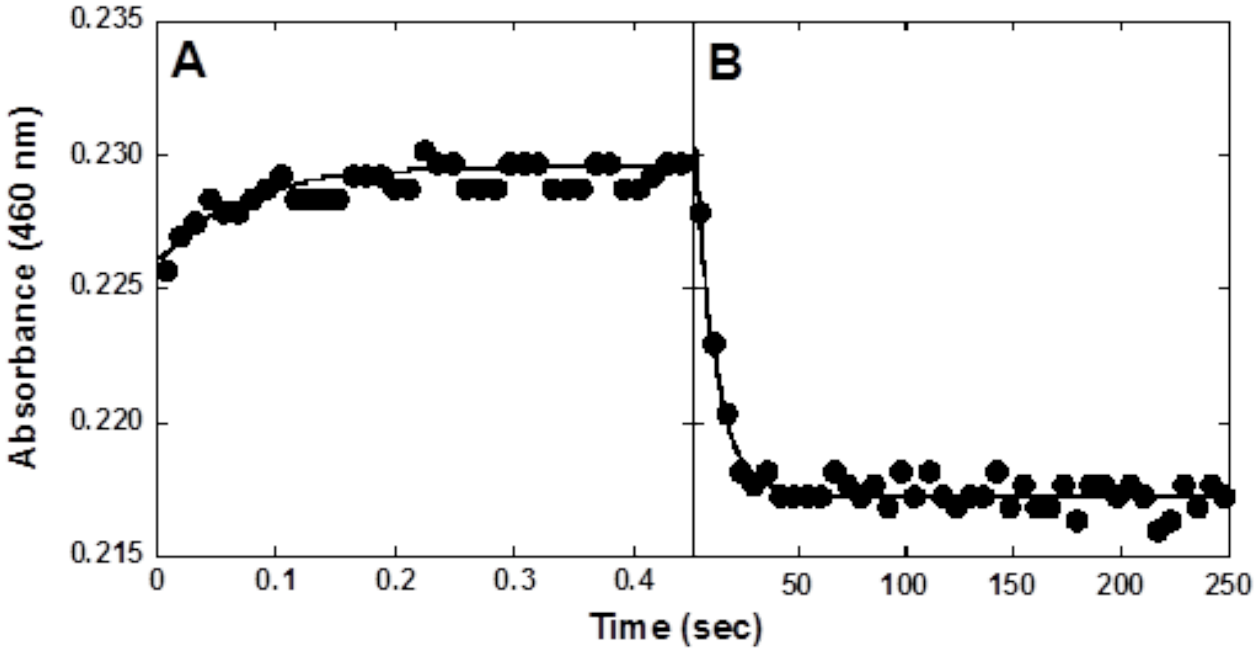


Figure 16. Representative stopped-flow kinetics of the reaction of Co(II)N_4[11.3.1] with azide under pseudo-first order conditions.

The reaction was followed anaerobically at 460 nm under the following conditions: 0.15 mM Co(II)N_4[11.3.1], 7.5 mM (50-fold excess) NaN_3, 0.1 M sodium phosphate buffer, pH 7.4, 25°C. The absorption at 460 nm was shown to **(A)** first increase over a 1 second time frame and then **(B)** decrease afterward over a 500-second runtime. Closed circles represent the collected data with solid lines showing the single exponential fits to the data. All concentrations given are those obtained after mixing.

The fast phase I coincided with an absorbance increase at 460 nm with k_1 = 2.6 (± 0.1) x 10^3 M^{-1} s^{-1} (25°C, 0.1 M sodium phosphate buffer, pH 7.4) and was dependent on both the azide concentration and the square of the cobalt complex concentration (Figures 17C and 17D). This initial phase was also independent of pH between 6.5 (closed diamond) and 8.5 (open squares). The slow phase III was evident from a very small decrease in absorbance at 460 nm (only a few percent change compared to phase II). Interestingly, this final phase was linearly dependent on the square root of the azide concentration (Figure 17E). When the reaction between azide and Co(II)N_4[11.3.1] was carried out aerobically, the presence of oxygen appeared to have no impact on phases I, or II. Phase III was masked by the relatively slow oxidation reactions described below, so it is unclear if it was affected.

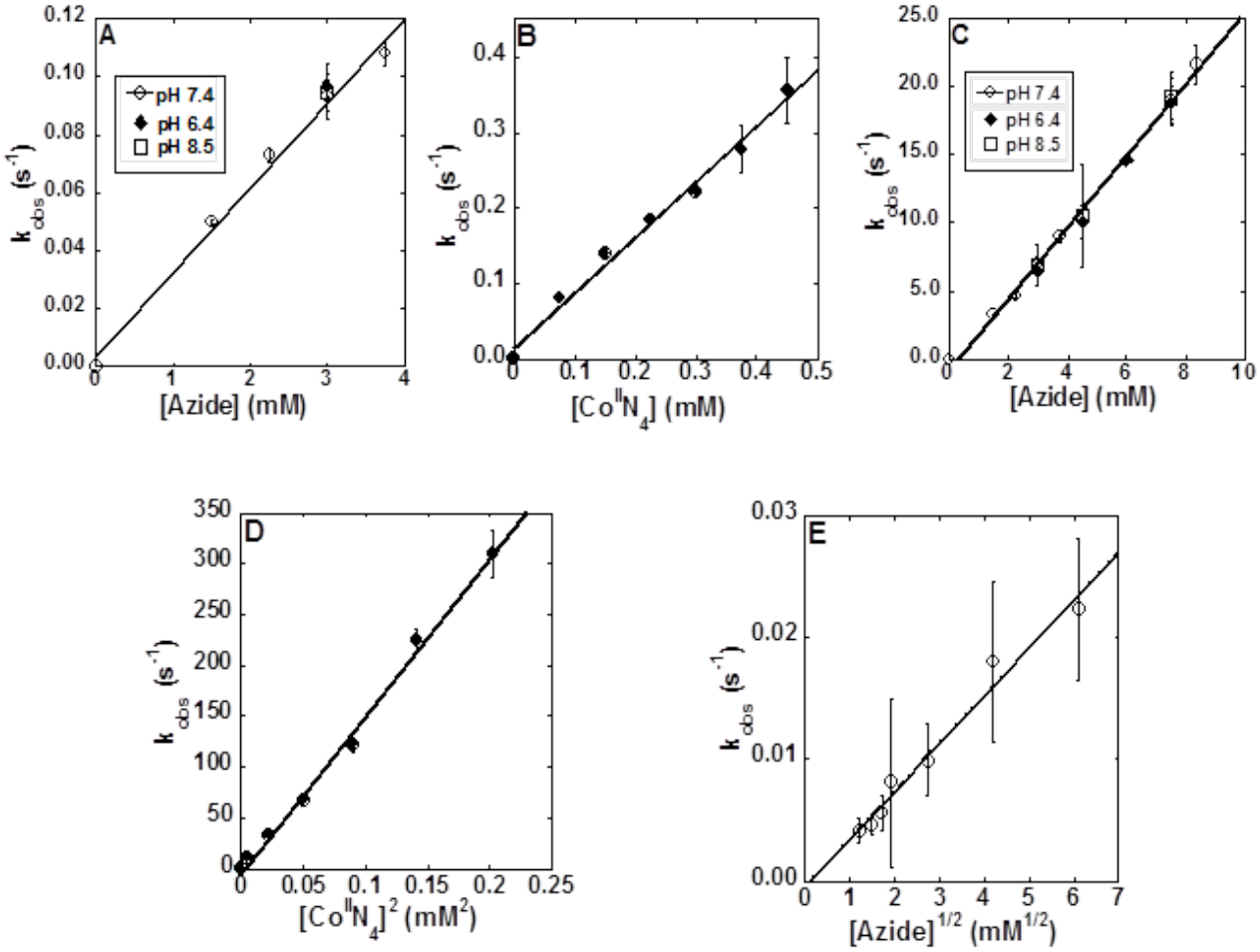


Figure 17. Kinetics of the reaction of Co(II)N$_4$[11.3.1] with azide under pseudo-first order conditions at pH 7.4, 25°C.

Three phases (fast, k_1; intermediate, k_2; and slow, k_3) were followed anaerobically under pseudo first-order conditions at 460 nm in 0.1 M sodium phosphate buffer, pH 7.4, at 25°C with excess sodium azide (1.5-15 mM) and Co(II)N$_4$[11.3.1] (0.1-0.5 mM). The observed rates were then plotted versus either the azide concentrations or the Co(II)N$_4$[11.3.1] concentrations. All concentrations given are those obtained after mixing. Plots shown are: **(A)** intermediate phase, k_2, varying azide concentrations with [Co(II)N$_4$[11.3.1]] = 0.15 mM, **(B)** intermediate phase, k_2, varying Co(II)N$_4$[11.3.1] concentrations with [NaN_3] = 15 mM, **(C)** fast phase, k_1, varying azide concentrations with [Co(II)N$_4$[11.3.1]] = 0.15 mM, **(D)** fast phase, k_1 varying Co(II)N$_4$[11.3.1] concentrations with [NaN_3] = 15 mM, and **(E)** slow phase, k_3, varying azide concentrations in the presence of 0.15 mM Co(II)N$_4$[11.3.1]. Rate constants were obtained from the slopes of the plots.

The oxidation of Co(II)N$_4$[11.3.1](N_3)$_2$ to Co(III)N$_4$[11.3.1](N_3), in the presence of excess oxygen (pseudo-first-order conditions) exhibited biphasic kinetics. Both phases were

oxygen dependent (Figures 18A and 18B) but the faster phase was linearly dependent on the square of the oxygen concentration (Figure 18A) with rate constants of 3.0 (± 0.1) x 10^4 M^{-2} s^{-1} and 130 (± 13) M^{-1} s^{-1}, respectively. The mechanism of this oxidation is complicated and still under investigation. In contrast, the reaction of azide-free Co(II)N_4[11.3.1] with oxygen was significantly slower, 0.5 (± 0.02) M^{-1} s^{-1}, with only one significant phase observed (see Chapter 3, Figure 7C). Both oxidation processes appear to be somewhat complicated and warrant further study. Most importantly, however, with regard to the present investigation, the oxidation of the cobalt-azide complex is at least two orders of magnitude faster than the oxidation of the azide-free cobalt complex.

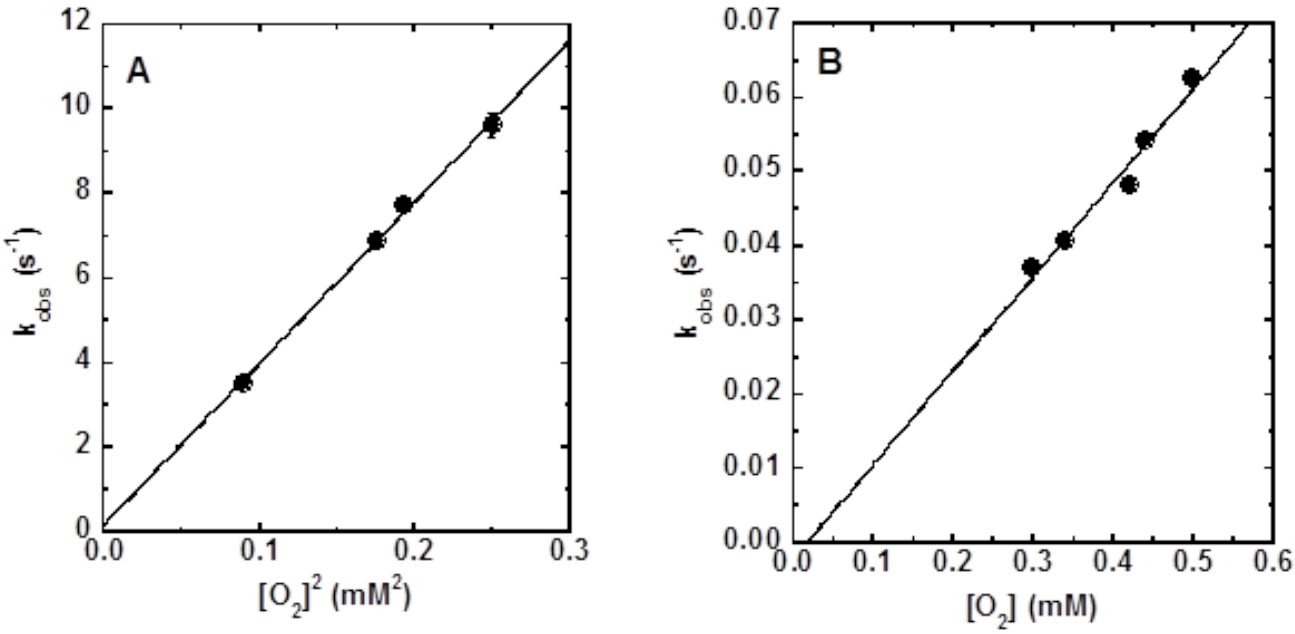


Figure 18. Kinetics of the reaction of Co(II)N_4[11.3.1]$(N_3)_2$ with oxygen under pseudo-first order conditions at pH 7.4, 25°C.

Sodium phosphate buffer solutions (0.1 M, pH 7.4) at different oxygen concentrations, prepared by mixing between deoxygenated buffer and 100% oxygen saturated buffer, were rapidly mixed with Co(II)N_4[11.3.1]$(N_3)_2$ and followed at 350 nm. The observed rates are plotted versus the final oxygen concentrations (0.28-0.5 mM). All concentrations given are those obtained after mixing. The oxygen dependence of the rates of **(A)**

$Co(II)N_4[11.3.1](N_3)_2$ (0.05 mM), fast phase, and **(B)** $Co(II)N_4[11.3.1](N_3)_2$ (0.05 mM), slow phase. Rate constants were obtained from the slopes of the plots.

4.4 DISCUSSION

4.4.1 The principal toxicant species

It has been suggested that the azide anion (N_3^-) might not be responsible for the observed toxicity of azide, with secondary metabolites, namely, cyanide [89] and nitric oxide [34], proposed as the principal species involved. The present findings firmly refute these alternative hypotheses. We have previously shown [41, 93] that (i) administration of sodium nitrite to mice (at similar dose to that employed here) results in the appearance of > 0.1 mM nitric oxide in the bloodstream, starting at less than 5 min and continuing up to about 20 min following the nitrite dose; and (ii) the administration of sodium nitrite/nitric oxide is markedly antidotal toward cyanide intoxication. Consequently, the current observation that sodium nitrite has no measurable effect on azide toxicity in mice (Table 1) argues strongly against any role for either nitric oxide or cyanide in azide poisoning. If nitric oxide were the key toxicant involved, then sodium nitrite would be expected to exacerbate azide toxicity and, if cyanide were important, then sodium nitrite should ameliorate azide toxicity. Of course, we cannot categorically state that some other, presently unidentified, secondary metabolite of azide might not be involved, but this would only be erroneous speculation. At the present time, so far as we are aware, there is no other unambiguous data or rational and well-formulated hypothesis to suggest that the toxicity of azide is due to anything except the N_3^- anion itself. Therefore, in the absence of any information

to the contrary, it behooves us to continue working under the extremely reasonable assumption that N_3^- alone is responsible for the observed acute toxicity.

The lack of any measurable effect stemming from the addition of sodium nitrite is noteworthy for another reason relating to the vasodilatory action of nitric oxide generated from nitrite. Hypotension is a well-documented symptom of azide poisoning [34, 88, 97, 98] and, thus, if nitrite were to be administered at higher levels than employed here, or at longer times following the azide dose at onset of the toxic symptoms, a net deleterious hypotensive effect may become apparent.

The observed duration of knockdown and time until knockdown following administration of toxicant were certainly longer for azide (40 and 8 min) compared to those previously found for cyanide (24 and 2 min) [68]. This slower onset and longer duration of knockdown in the case of azide can probably be explained by consideration of the two relevant acid dissociation constants (K_as). The pK_a for hydrazoic acid is 4.7, indicating that at physiological pH > 98% of the azide will be present as the unprotonated anion (N_3^-). On the other hand, the pK_a for hydrocyanic acid is 9.2, indicating that at physiological pH > 98% of the cyanide will be present as the molecular acid (HCN). HCN is free to diffuse across biological membranes and also quite soluble in aqueous media, while N_3^- must locate channels to efficiently cross membranes. It follows that diffusion of HCN to its mitochondrial site of inhibition from the bloodstream should be faster and quantitatively transferred in less time than N_3^- is transported.

4.4.2 Antidotal activity

In our previous studies concerning the amelioration of cyanide toxicity by CoN_4[11.3.1] in mice we found the complex to be an efficacious antidote when given either 5 min before or 2 min after

an LD_{40} dose of cyanide (5 mg/kg NaCN) [64, 68]. The cobalt macrocycle was shown to bind two molecules of cyanide cooperatively [68] and extensive studies in mice showed it to be at least as good an antidote as cobinamide while measurably better than hydroxocobalamin [64]. Consequently, because azide is a slower acting toxicant, it is perhaps not surprising that preliminary experiments with CoN_4[11.3.1] in mice indicate that it will function as an azide antidote when given at least 5 min after the toxicant (Table 1). More remarkable is the apparent failure of established cyanide antidotes to display any ameliorative effect toward azide toxicity [41, 68]. If we accept the consensual viewpoint that the target for both poisons is primarily cytochrome *c* oxidase, then it is reasonable to expect similarities in behavior of azide and cyanide, both in terms of their toxic mechanisms and responses to antidotes. That is, it might be possible to understand any quantitative difference in the manner in which these two toxicants behave based on knowledge of how each reacts with isolated cytochrome *c* oxidase. In oxygen turnover experiments, isolated cytochome *c* oxidase has been reported to be inhibited by azide and cyanide with $K_i s$ of 33 μM and 0.2 μM, respectively [99]; indicating cyanide to be 150-fold more inhibitory than azide toward the enzyme. Intriguingly, the sodium azide dose used in the present study (400 μmol/kg) is only 4 times the sodium cyanide dose (100 μmol/kg) used in our previous studies also monitoring knockdown and righting recovery.

The amount of CoN_4[11.3.1] employed to ameliorate azide toxicity was ~80 μmol/kg (37 mg/kg, Table 1) or approximately 20% of the azide dose (400 μmol/kg). Previously, in order to ameliorate cyanide toxicity, the most efficacious dose in mice was found to be approximately one half the cyanide dose, consistent with CoN_4[11.3.1] binding 2 molecules of cyanide [64, 68]. The present dose of CoN_4[11.3.1] found to ameliorate azide toxicity would have maximally bound about 40% of the azide dose (based on 1:2 stoichiometry) and approximately 20% if

oxidation results (based on a 1:1 stoichiometry, see below). This is low compared to the amount of antidote necessary to bind all the azide, but would have the effect of lowering the residual azide dose from 26 mg/kg to ~20 mg/kg (*i.e.* less than the 24 mg/kg at which we able to observe minimal effects). Consequently, the findings are in keeping with the steepness of the dose-response curve for azide toxicity in mice.

4.4.3 Mechanism of decorporation

The detoxification of cyanide by cobalt complexes has recently been suggested to consist of the toxicant binding to the reduced (substitution labile) Co(II) forms, resulting in lowering of their oxidation potentials, this in turn facilitating oxidation to the (substitution inert) Co(III) cyanide adducts, that may be then excreted [59, 66, 68, 79]. That is, bound cyanide becomes trapped in kinetically stable forms from which it is slow to dissociate, preventing systemic redistribution of the toxicant and, thereby, nullifying its toxicity [64]. From this perspective, the oxidation-state change upon binding the toxicant is an essential component of the net mechanism of decorporation. Unlike the cyanide reaction, we observed no oxidation of Co(II) to Co(III) under anaerobic conditions following azide binding, the kinetics of oxygen dependence extrapolate through the origin in this case (Figures 18A and 18B). However, upon exposure to air Co(II)N_4[11.3.1]$(N_3)_2$ is oxidized to a Co(III) complex (Figure 13, dash-dot trace). While the oxidation of the Co(II)N_4[11.3.1]$(N_3)_2$ is perhaps not as facile as Co(II)N_4[11.3.1]$(CN)_2$, it may be assumed that inhibition of cytochrome *c* oxidase must lead to tissue hyperoxia and, therefore, a kinetically-stable Co(III)N_4[11.3.1]-azide complex will be formed. In addition, we note that the oxidation of Co(II)N_4[11.3.1]$(N_3)_2$ by oxygen is still significantly faster than oxidation of the parent compound (see Chapter 3, Figure 7C). The Job plots (Figures 14B and 14C) indicate

something more akin to a 1:1 ratio of Co(III):azide in the oxidized form (Figure 14C) rather than the 1:2 ratio of Co(II):azide found in the reduced form (Figure 14B). The simplest explanation for these observations appears to be that the oxidation of the substitution-labile $Co(II)N_4[11.3.1](N_3)_2$ requires displacement of an azido ligand by oxygen, followed by inner-sphere electron transfer forming $Co(III)N_4[11.3.1](N_3)$ and superoxide, resulting in the kinetically stable monoazido-Co(III) adduct (Figure 19). We offer this interpretation of the oxidation process as our working model, but have yet to investigate its validity further, since the overall mechanism of decorporation merely requires an oxidation takes place, the details of exactly how being less important.

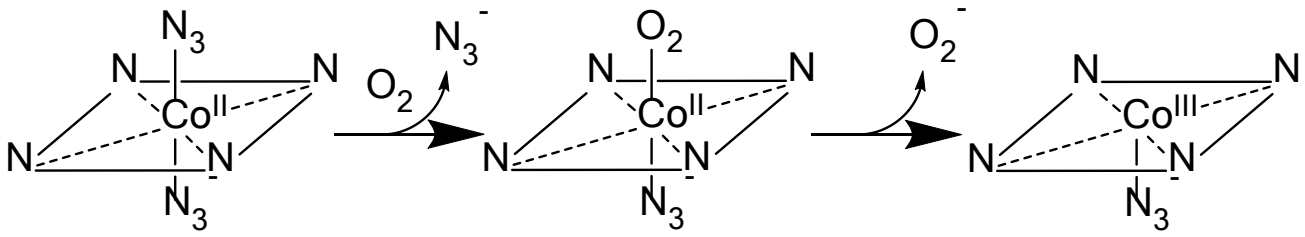


Figure 19. A plausible scheme for the oxidation of $Co(II)N_4[11.3.1](N_3)_2$ by oxygen.

5.0 CONCLUSIONS

The purpose of these studies was to further develop Co(II)N_4[11.3.1] as a possible decorporating agent for cyanide (HCN/CN^-) and azide (N_3^-) and so it might be advantageous to consider the characteristics and properties of a typical agent. Decorporation is defined as the therapeutic removal of a radioactive material that has been adsorbed by the body. It can, however, be used to describe the removal of a number of heavy metals (*e.g* lead) and indeed, here the term is used to describe the removal of cyanide from the body. This method of cyanide detoxification is in contrast to those that, for example, convert cyanide to a less toxic substance, *e.g.* thiosulfate.

A decorporation agent should be nontoxic, specifically bind the potential toxicant tightly and excrete the agent-toxicant complex rapidly from the body. Ethylenediaminetetraacetate ion (or EDTA) was proposed as a decorporation agent for the removal of lead from the body in 1942. EDTA is multi-dentate ligand that binds very strongly to metal ions, especially the transition metals. For example, the binding constant of EDTA for lead (Pb^{2+}) is 10^{18} [100, 101]; the high value indicates the reverse reaction must be slow and/or equilibrium must lie to the right to form a stable complex, a very desirable characteristic. The formation of the [Pb-EDTA]$^{2-}$ complex reduces free Pb^{2+} circulating in blood; subsequently, being eliminated through the kidneys. EDTA has been proved to be safe to use in patients with lead poisoning; however, EDTA can also chelate other essential cations, especially calcium ion. Consequently, the patient treated with EDTA could develop a low level of calcium in the blood (hypocalcemia) if not adequately

supplemented with calcium during the decorporation therapy. While EDTA is a very useful decorporating agent, it is nonspecific in its binding to a large number of metal ions and care must be taken in its use. EDTA is a good example of the potential pitfalls associated with the use of decorporating agents.

5.1 THE ROLE OF Co(II)N_4[11.3.1] IN CYANIDE DECORPORATION

Our previous work has shown that 1 molecule of Co(II)N_4[11.3.1] cooperatively binds 2 cyanide anions, and then, undergoes an oxidation process, *in the absence of oxygen*, to become the kinetically-inert dicyano-Co(III)N_4[11.3.1] adduct, subsequently being excreted via the urine [68]. The kinetic data from the current study demonstrates that the binding of the second cyanide anion is the rate-determining step with the rate constant (k) of 2 x 10^5 M^{-1} s^{-1} at 37°C indicating that the detoxification of cyanide might be very rapid. Co(II)N_4[11.3.1] binds with strong ligands such as CN^- and N_3^- in aqueous media, but does not bind weak ligands such as chloride or bicarbonate. Thus Co(II)N_4[11.3.1] is relatively specific in its ligand binding, unlike EDTA, and therefore, we should not have to worry about the complex lowering the concentrations of important physiological anions such as chloride. It is possible, however, that Co(II)N_4[11.3.1] and/or its final product, dicyano-Co(III)N_4[11.3.1], may bind to some biomolecular sites (*e.g.* proteins or other receptor molecules), leading to some change in physiology. Co(II)N_4[11.3.1] is not itself naturally found in the body, therefore, it may be less likely that there are some specific binding sites for the cobalt molecule. Thus, we think that Co(II)N_4[11.3.1] may be safe to use, however, more study must be done to determine the pharmacokinetics and the toxicity of this compound.

According to the previous mouse studies [64, 68], Co(II)N_4[11.3.1] is found to exhibit a significant ameliorative effect on cyanide toxicity. In the *in vivo* experiment, 100 µmol/kg of NaCN and 50 µmol/kg of Co(II)N_4[11.3.1] were given to the 40-gram mice. The concentrations of cyanide and Co(II)N_4[11.3.1] in mice's blood circulation can be calculated as 1 x 10^{-3} M and 0.5 x 10^{-3} M, respectively. Based on the rate law equation and using the experimentally determined rate constant, the rate of cyanide binding to Co(II)N_4[11.3.1] can be calculated as 0.1 M/s. This suggests that Co(II)N_4[11.3.1], in these conditions, can very rapidly bind all the circulating cyanide in mouse blood within 0.01 seconds. Additionally, we observed that the dose of 52 µmol/kg of Co(II)N_4[11.3.1] used in the experiment did not cause any neurological sequelae [68]. Taken together, this data supports the potential use of Co(II)N_4[11.3.1] as a cyanide decorporating agent.

Another finding to emerge from this study is that, under aerobic conditions, oxygen can remove cyanide from Co(II)N_4[11.3.1]. The loss of cyanide from the cobalt complex in the presence of oxygen might lead one to think that Co(II)N_4[11.3.1] would *not* be an effective antidote against cyanide toxicity, but our previous studies have demonstrated that the cobalt compound indeed works effectively in the mouse model [64, 68]. At this moment, we do not understand how the release of cyanide occurs and determining the mechanism of cyanide removal in the presence of oxygen will require a great deal more study. The effect of oxygen on the dicyano complex, however, is a potential problem if the Co(II)N_4[11.3.1] complex were to be used as a cyanide antidote in a clinical setting. Typically, most cyanide-intoxicated patients present with respiratory distress and tend to receive oxygen therapy at the hospital. Since oxygen could potentially remove cyanide from the cobalt-containing antidote, the patients' conditions may not be improved or may even become worse after receiving the treatment.

5.2 THE ROLE OF Co(II)N_4[11.3.1] IN AZIDE DECORPORATION

The stoichiometry of azide binding to the cobalt complex under anaerobic conditions was found to be 2:1. The kinetic data has shown that the binding of the first azide ligand is the rate-determining step with a second-order rate constant of 71 M^{-1} s^{-1} at 37°C. Unlike cyanide, after two azide anions bind to Co(II)N_4[11.3.1], the diazido-Co(II)N_4[11.3.1] complex does not undergo oxidation to form the kinetically inert species (Co(III) complex) in the absence of oxygen. Based on the Job plot analysis, (see Chapter 4, Figure 14C) at least one azido ligand is lost upon oxidation.

However, the mouse study has shown that Co(II)N_4[11.3.1] is significantly antidotal toward azide in mice, compared to either controls or receiving another known cyanide antidote, sodium nitrite. In this experiment, 400 μmol/kg of NaN_3 and 77 μmol/kg of Co(II)N_4[11.3.1] were given to the 40-gram mice. The estimated concentrations of azide and Co(II)N_4[11.3.1] in mice's blood circulation would be about 4 x 10^{-3} M and 0.77 x 10^{-3} M, respectively. By applying the rate law, we can determine the rate of azide binding to Co(II)N_4[11.3.1] in the mouse as 2 x 10^{-4} M/s. This suggests that Co(II)N_4[11.3.1], under these conditions, can rapidly bind all the circulating free-azide molecules within 18 seconds. This calculation rationally suggests that Co(II)N_4[11.3.1] should work effectively for azide toxicity similar to the way that hydroxocobalamin efficaciously detoxifies cyanide. Not surprisingly, Co(II)N_4[11.3.1] was found to have an ameliorative effect in azide-intoxicated mice.

We have observed that there was some resemblance between the azide binding to Co(II)N_4[11.3.1] and the cyanide binding to hydroxocobalamin. For example, both reactions both bind one ligand in the oxidized form. Since Co(II)N_4[11.3.1] does work as an azide antidote, we think that hydroxocobalamin should probably work as an azide antidote too. There

is one case report from Austria revealing that an azide-intoxicated patient improved after receiving hydroxocobalamin [58]; nevertheless, some might argue that the patient could perhaps have recovered by the use of only supportive treatment without receiving any antidotes.

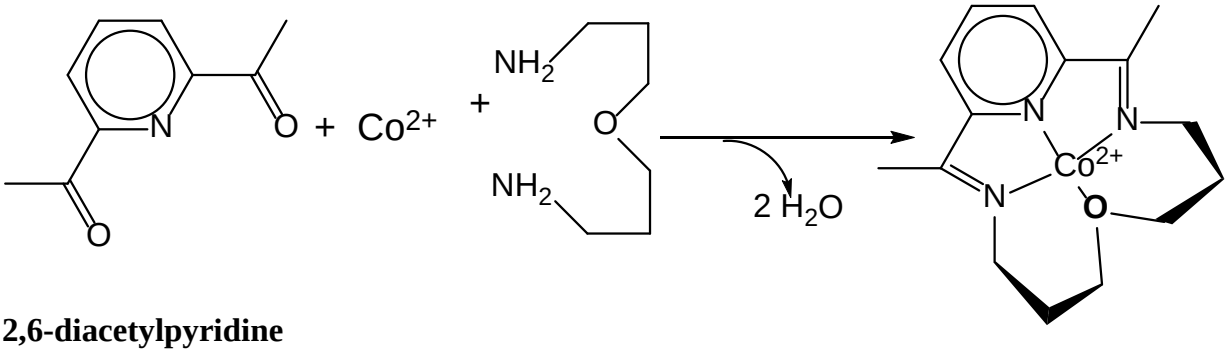
The findings from the kinetic study show that the rate of azide binding to Co(II)N_4[11.3.1] is 3 orders of magnitude slower than the cyanide binding to the complex despite the fact that Co(II)N_4[11.3.1] works effectively for both cyanide and azide toxicity in the mouse model [64, 68]. This suggests that the rate constant might not be a crucial factor in determining the effectiveness of the antidote. Determining values of the rate constants and the rate equations is still a useful exercise in order to provide insight into the reaction mechanisms and to find out if the reaction is not unacceptably slow, *i.e.* hours vs. minutes.

5.3 PUBLIC HEALTH IMPLICATIONS AND FUTURE DIRECTIONS

Since the emergence of modern terrorism, strategies to address mass casualty's scenarios resulting from chemical/biological terrorism have entered the realm of public health. In fact, the Department of Homeland Security has funded the CounterACT (Countermeasures Against Chemical Threats) program aiming to prepare for chemical emergencies by supporting research regarding the development of new or improved therapeutic countermeasures for chemical threats [102]. Being part of the CounterACT program, the present study was designed to assess the effectiveness of Co(II)N_4[11.3.1] as a potential cyanide/azide antidote. (*i.e.* identify as a lead compound to secure funding for further development). On the way to gaining approval for therapeutic use, any new compound requires years of supportive investigation in animals regarding effectiveness, pharmacokinetics, pharmacodynamics, toxicity and demonstration of

basic safety in healthy human volunteers. While Co(II)N_4[11.3.1] is at the very beginning of this process, the initial signs are encouraging in that the compound is clearly antidotal toward cyanide/azide in mice at doses where there has been no indication of any untoward toxicity to date. A characteristic orange color appear in the urine about 30 min after injecting the compound and clears after a couple of hours, so excretion seems to follow the pattern for water-soluble porphyrins and any problems stemming from long-term retention are not expected.

There is currently no available antidote for azide poisoning and, therefore, the successful amelioration of azide toxicity in mice by Co(II)N_4[11.3.1] (Chapter 4) is a highly significant result, perhaps pointing the way forward. Due to its tendency to lose an azido ligand upon oxidation, however, Co(II)N_4[11.3.1] itself may ultimately turn out to not be a sustainable lead compound in the search for an optimal azide antidote. The focus should be on seeking a cobalt-based azide scavenger that binds azido ligands in the Co(II) form, then becomes oxidized to substitution inert Co(III) product without any inner sphere reaction involving oxygen. Strategies for synthesizing the next generation of potential azide scavengers may include developing cobalt porphyrins, or other cobalt macrocycles with heteroatomic ligands (*e.g.* Figure 20).



2,6-diacetylpyridine

Figure 20. An example of the synthesis of a cobalt macrocycle with heteroatomic ligands.

With regards to its possible potential as a cyanide antidote, the findings (Chapter 3) continue to suggest that Co(II)N_4[11.3.1] might prove better than anything else available, or currently under development. Like Co(III)TMPyP and cobinamide, Co(II)N_4[11.3.1] has the comparable two axial ligand positions available to bind toxic anions [68], but when directly compared with the other cobalt-based scavengers in mice [64] (see also Appendix A), Co(II)N_4[11.3.1] has the edge. For purposes of stockpiling in anticipation of emergency events involving mass casualties and requiring rapid employment, this compound has several advantageous characteristics. First, due to its relatively low molecular mass of 318 g/mol (about 4-time smaller than hydroxocobalamin), Co(II)N_4[11.3.1] is likely to be more soluble and easily delivered than the larger cobalt complexes such as hydroxocobalamin or cobinamide. Therefore, rapid delivery by intramuscular/intraosseous injection will certainly be possible, but inhalation of aqueous vapor using something like a pneumatic inhaler might also be achievable. Second, Co(II)N_4[11.3.1] is significantly less expensive to synthesize than hydroxocobalamin and cobinamide [64]. Third, because of its purely synthetic nature and macrocyclic structure, Co(II)N_4[11.3.1] does not require refrigerated storage and is stable under ambient conditions for at least months (as monitored by spectroscopic measurements). The implications regarding any strategic plan for chemical terrorism preparedness and response are that the government could produce and reserve a substantial amount of antidote (whatever is needed) due to the low cost of medication and its storability.

There is a need for improved cyanide antidotes for reasons other than counter-terrorism. Modern fires almost invariably involve combustion of much nitrogen-containing plastic such as acrylonitriles and polyurethanes. Consequently, while frequently misdiagnosed as carbon monoxide poisoning, HCN toxicity secondary to smoke inhalation is almost certainly the most

prevalent form of cyanide poisoning experienced today, particularly in occupational settings. Inexpensive cyanide antidotes, safe enough for self-administration, that could be routinely carried on emergency vehicles without the need for special storage or frequent replacement, could prove to be of broad significance in firefighting. Thus far, $Co(II)N_4$[11.3.1] appears to have possible application in this area that might surpass any more narrow role in countermeasures to terrorism. Therefore, $Co(II)N_4$[11.3.1] certainly warrants further drug-development research regarding its potential application as a cyanide antidote.

APPENDIX

A COMPARISON OF THE CYANIDE-SCAVENGING CAPABILITIES OF SOME COBALT-CONTAINING COMPLEXES IN MICE

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Keywords: Cobalamin; cobinamide; complex IV; cyanide; cytochrome *c* oxidase; electron transport; mitochondria; macrocycle; porphyrin; respiratory poison; righting recovery; RotaRod

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A.1 ABSTRACT

Four cobalt-containing macrocyclic compounds previously shown to ameliorate cyanide toxicity have been comparatively evaluated with an acute sub-lethal toxicity model in conscious (un-anesthetized) adult male Swiss-Webster mice. All of the compounds (the cobalt-corrins cobalamin and cobinamide, a cobalt-porphyrin, plus a cobalt-Schiff base macrocycle) given 5 min prior to the toxicant dose significantly decreased the righting-recovery time of cyanide-intoxicated mice, but the doses required for maximal antidotal effect varied. Additionally, all of the compounds tested significantly reduced the righting-recovery time when administered at either 1 or 2 min after cyanide intoxication, but none of the compounds tested significantly reduced the righting-recovery time when delivered 5 min after the toxicant dose. Using the lowest effective dose of each compound determined during the first (prophylactic) set of experiments, neuromuscular recovery following cyanide intoxication in the presence/absence of the cobalt-based antidotes was assessed by RotaRod® testing. All the compounds tested accelerated recovery of neuromuscular coordination and no persistent impairment in any group, including those animals that received toxicant and no antidote, was apparent up to 2 weeks post-exposures. The relative effectiveness of the cobalt compounds as cyanide antidotes are discussed and rationalized based upon the cyanide-binding stoichiometries and stability constants of the Co(III) cyano adducts, together with consideration of the rate constants for axial ligand substitutions by cyanide in the Co(II) forms.

A.2 INTRODUCTION

Other compounds, including dicobalt ethylenediaminetetraacetate (Kelocyanor®) [103] and 4-dimethylaminophenol [104], remain in use worldwide, but there are currently only two acceptable antidotes to cyanide poisoning available in the United States [105, 106]. The first, Nithiodote®, is a combination treatment of sodium nitrite and sodium thiosulfate [107]; in which (i) the nitrite anion probably acts as a nitric oxide (NO) donor leading to the removal of cyanide bound to cytochrome *c* oxidase [41, 93] rather than simply being a methemoglobin generator and (ii) the thiosulfate reacts with free cyanide in a reaction catalyzed by the enzyme rhodanese leading to formation of the considerably less toxic thiocyanate anion (SCN^-) [107]. Intravenous infusion of sodium nitrite in the pre-hospital setting must be undertaken with caution, due to the likelihood of induced hypotension and methemoglobinemia, the latter being of particular concern if there has been any concomitant carbon monoxide poisoning through smoke inhalation [106, 108]. The second antidote, Cyanokit®, contains hydroxocobalamin (Cb), a vitamin B_{12} derivative (Figure 21A) [109]. Cb binds a single cyanide anion to its central cobalt(III) cation, with high affinity thereby acting as a scavenger of the toxicant in the bloodstream. Unfortunately, while Cb appears to be efficacious and the safest available option for treating cyanide intoxication [110, 111], it is still a less than ideal antidote because it must first be dissolved (5 g solid in ~200 mL saline) before it can be intravenously infused (~15 mL per min for almost 15 min in adults) [109]. Particularly with regard to acute-poisonings/mass-casualty situations, this slow administration is a significant problem as cyanide is such a quick-acting toxicant.

Cobinamide (Cbi) lacks the dimethylbenzimidazole nucleotide tail of Cb (Figure 21B) allowing for the binding of two cyanide anions to the cobalt ion and has been shown to be a

potentially better cyanide scavenger [61, 62]. We have recently demonstrated the cyanide scavenging ability of cobalt(III) meso-tetra(4-N-methylpyridinyl)porphine (CoTMPyP) (Figure 21C) a water soluble metalloporphyrin complex [66, 79]. In addition, we have begun to investigate the cyanide binding and scavenging activities of Schiff-base macrocyclic compounds, including cobalt 2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17)2,11,13,15-pentaene (CoN_4[11.3.1]) (Figure 21D) [68]. These smaller cobalt cations, CoTMPyP and CoN_4[11.3.1] or similar, which are also able to bind two cyanide anions per cobalt, may be soluble at higher concentrations in the bloodstream than Cb and Cbi, resulting in improved cyanide-scavenging capabilities. In this paper, to directly assess the relative merits of such cobalt-containing compounds (Table 2) as cyanide antidotes, we have undertaken a series of head-to-head comparative assays in mice, in which experiments with Cb were included as a benchmark.

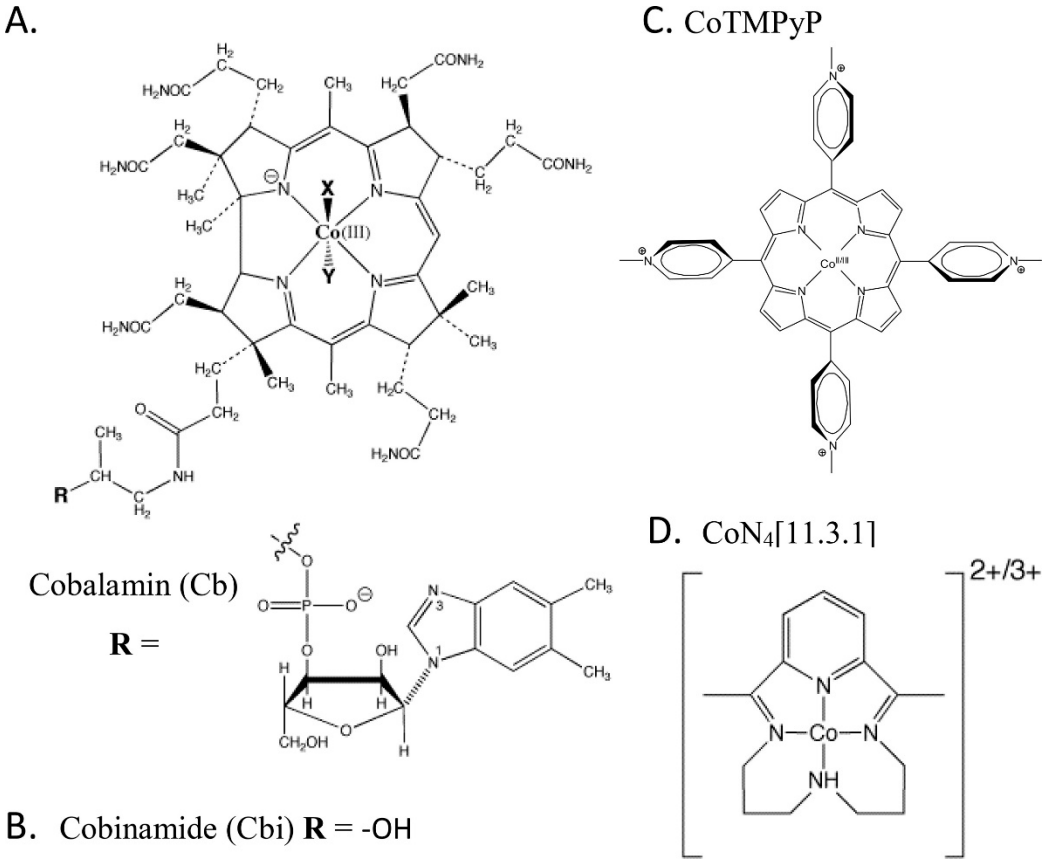

Figure 21. Structures of cobalt-containing compounds selected for comparison of cyanide scavenging abilities in mice.

(A) Cobalamin (Vitamin B_{12}), a FDA approved cyanide antidote **(B)** Cobinamide, the biological precursor to cobalamin **(C)** CoTMPyP **(**Cobalt(III) meso-tetra(4-N-methylpyridinyl)porphine) and **(D)** CoN_4[11.3.1] (Cobalt 2,12-dimethyl-3,7,11,17-tetraazabicyclo-[11.3.1]heptadeca-1(17)2,11,13,15-pentaene) [66, 68, 112].

Table 2. Selected properties of the cobalt-containing trial compounds.

Property	Hydroxocobalamin (Cb)	Cobinamide (Cbi)	CoTMPyP	CoN_4[11.3.1]
Molecular masses (of cations)	1329	990	678	317
Comparative (estimated) costs[a]	1[b]	63[c]	~0.8[d]	< 0.2[e]
Available sites for exogenous ligands	1	2	2	2
Stability constants[f]	10^5 M^{-1}	10^9-10^{10} M^{-2}	2 x 10^{11} M^{-2} [66]	> 10^7 M^{-2} [68] [g]
Rate constants[h]	> 10^2 M^{-1} s^{-1} [86] [i]	> 10^3 M^{-1} s^{-1} [j]	10^2 - 10^3 M^{-1} s^{-1} [79] [k]	~10^5 M^{-1} s^{-1} [l]

[a]Based on the Sigma-Aldrich catalog (accessed on line October 2017) molar comparisons. [b]Hydroxocobalamin hydrochloride $165/g (equating to $825 for a single adult dose). [c]Comparing prices of cyanocobalamin and dicyanocobinamide, the sole derivative of the latter available at the time. The Cbi was only available in mg quantities, so there is likely to be reduced cost associated with scaling up production; however, Cbi will remain many-fold more expensive than its precursor Cb as additional manufacturing steps (chemical modification, purification, recovery) are necessary. [d]CoTMPyP was not available, so this estimate is based on the mean of the pricing for cobalt(II)-5,10,15,20-tetraphenylporphine and cobalt(II)-5,10,15,20-tetrakis(4-methoxyphenyl)porphine, two similar metalloporphyrins. [e]CoN_4[11.3.1] is, so far as we are aware, not commercially available at this time (except by custom synthesis). This estimate is based on the catalog prices of the starting materials multiplied by 10. [f]Stability constants for cyano adducts in terms of total cyanide concentrations [HCN + CN^-] for Co(III) forms at pH 7.4 and 25°C. [g]The stability constant for the bis(cyano)Co(II) adduct was actually determined and found to be 3 x 10^5. There are remarkably few studies reporting the stability constants of cyano complexes of transition-metal ions in different oxidation states. However, based on available reliable data for (i) the hexacyanoferrate(II) and hexacyanoferrate(III) pair, plus (ii) the stepwise formation constants for some other polycyano complexes [113], we estimate the increase in the net stability constant for the Co(III) form of CoN_4[11.3.1] to be at least an order of magnitude per cyano ligand: 3 x 10^5 multiplied by 10^2 = 3 x 10^7, or greater. [h]Rate constants for cyanide binding to the Co(II) forms at pH 7.4 and 25°C. Where dicyano complexes are formed, it is often the case that the rate for only one ligand association can be observed, the other presumably being too fast to measure. [i]The reported rate constant

is for the Co(III) form (80 M^{-1} s^{-1}) hence that for the Co(II) form must be greater as Co(III) complexes are typically more substitution inert than their Co(II) counterparts [32, 114, 115]. [j]The rate constant for the largest (~90%) of two [cyanide]-dependent phases observed for the Co(III) form is 3 x 10^3 M^{-1} s^{-1}, hence that for the Co(II) form must be greater. [k]Two [cyanide]-dependent phases of comparable extent were observed. [l]Praekunatham et al., manuscript in preparation.

For this investigation, we chose an acute-toxicity model using intraperitoneal (IP) injections of NaCN (saline solutions) in adult male Swiss-Webster mice. In this paradigm, which has previously been well-validated [41, 93, 116, 117] the mice are conscious and freely moving at the time of toxicant injection, avoiding the confounding influence of anesthesia present in some other animal protocols [93, 118]. The ameliorative capabilities of the compounds in question given both prophylactically and therapeutically have been compared. Antidotal effects in the short term (< 30 min) have been quantitatively assessed by observing shortening of recovery times following cyanide-induced unconsciousness ("righting recovery"). Antidotal effects in the longer term (24 hr - 2 weeks) have been quantitatively assessed by measuring the duration that the animals were able to remain in position on a rotating cylinder (RotaRod®) a test of neuromuscular coordination). The results suggest that the trial compounds all work by similar mechanisms and identify potential strengths/weaknesses of each in the pursuit of cobalt-containing compounds as antidotes to cyanide poisoning.

A.3 EXPERIMENTAL SECTION

A.3.1 Chemicals

All non-gaseous reagents, obtained from Fisher or Sigma-Aldrich, were ACS grade or better and used without further purification. Argon and nitrogen gases were purchased from Matheson Incorporated. Previously described procedures were employed to prepare Cbi [59], CoTMPyP [66] and CoN_4[11.3.1] [68]. Solutions of sodium cyanide in saline were prepared immediately prior to use in septum-sealed vials with minimized headspaces and volumetric transfers made with gastight syringes.

A.3.2 Animal Exposures

The University of Pittsburgh Institutional Animal Care and Use Committee (Protocol Number 13092637) approved all animal produces used in these experiments. The Division of Laboratory Animal Research of the University of Pittsburgh provided all veterinary care during this study. With the exception of most animals exposed to sodium nitrite (see below), male Swiss-Webster mice weighing 35-40 g (6-7 weeks old) were purchased from Taconic, Hudson, NY, housed four per cage and allowed access to food and water *ad libitum*. Animals were allowed to adapt to their new environment for one week prior to carrying out experiments. All animals were randomly assigned to experimental groups of predetermined size. All solutions administered to mice were prepared by dilutions into sterilized saline in septum-sealed vials using gastight syringes and were given by ~0.1 mL intraperitoneal (IP) injections.

Similar procedures (Protocol Numbers 0808101 and 1008725) approved by the University of Pittsburgh Institutional Animal Care and Use Committee were employed in the case of the majority of mice treated with sodium nitrite. Veterinary care was provided by the Division of Laboratory Animal Research of the University of Pittsburgh. Male Swiss-Webster mice 16-20 weeks old, weighing 40-45 g were purchased from Charles River Laboratories, Wilmington, MA. All solutions were prepared by dilutions into sterilized saline and administered through ~0.1 mL intraperitoneal (IP) injections. In general, a group of at least 4 mice were tested for each experimental point. Efficacy was tested through the recovery of righting ability after $NaNO_2$ (12 mg/kg) was injected 2, 4, 8, 12, 16 or 20 min following the administration of NaCN (100 μmol/kg).

A.3.3 Righting Recovery

Following cyanide administration, the duration of time required for the recovery of righting ability in mice was measured following a simplification [41] of the procedure originally used by Crankshaw et al. [116] The toxicant (5 mg/kg NaCN) was administered to mice (IP) and they were then placed in a transparent but dark green-colored plastic tube (Kaytee CritterTrail, available from pet stores) in a supine position. The time it took from the initial administration of the toxicant until the mouse flipped from the supine to a prone position in the plastic tube was taken as the endpoint (righting-recovery time).

A.3.4 Prophylactic Dose-Response

The established antidote (Cb) and potential prophylactic antidotes (Cbi, CoTMPyP or CoN_4[11.3.1]) were injected (IP) into mice (n = 6-8 per dose) at levels of 30, 40, 45, 50 or 70 µmol/kg, 5 minutes before the administration (IP) of 100 µmol/kg NaCN. Control animals received cyanide alone. The righting-recovery times were recorded for mice that survived the cyanide intoxication. A single injection of cyanide administered at this sub-lethal dose typically results in a persistent state of unconsciousness within 1-2 min that can last for more than 25 min [41, 93] allowing for multiple measures of trial-compound efficacy given both prophylactically and therapeutically. The lowest dose of each putative antidote (Cbi, CoTMPyP or CoN_4[11.3.1]) having the maximal ameliorative response following prophylactic administration was determined. Based on these results we then selected a single dose ("lowest dose having maximal antidotal effect") for subsequently testing the therapeutic powers of the trial compounds. For comparison, Cb was given at 70 µmol/kg in these subsequent experiments (see below) as there was no maximal antidotal effect apparent for this compound in the experimental range.

A.3.5 Therapeutic Time Response

After determining the lowest doses having maximal antidotal effect for each putative antidote, mice (n = 6-8 per dose) were given 100 µmol/kg NaCN and subsequently (1, 2, or 5 min later) injected with either 50 µmol/kg CoN_4[11.3.1] or, alternately, 70 µmol/kg Cb, Cbi, or CoTMPyP. Righting-recovery times were recorded for mice that survived the toxicant injections and, later, the same animals were used in the RotaRod assessments described below.

A.3.6 RotaRod Testing

The accelerating RotaRod® (Coulbourn Instruments, Whitehall, PA), a rotating cylindrical apparatus, was used to assess motor skill, learning and recovery subsequent to cyanide intoxication. Well-established experimental protocols were followed [119, 120]. An individual trial was started by placing a mouse on the RotaRod device turning at 4 rpm. Subsequently, acceleration was varied linearly from 4 to 22 rpm over the course of 60 s. Trials ended when the mouse either fell off, or had remained on the rotating cylinder for 60 s. Latency to fall, and highest speed reached were recorded for each trial. The animals were evaluated over a period of three consecutive days. On the first day, each mouse was trained in a series of 8 sequential trials on the RotaRod device. The baseline motor performance was established on the second day by determining the max speed (rpm) reached before each animal fell off the RotaRod apparatus, averaged over three trials. On the second day, animals were tested in sets of 3 trials at 15 min intervals (with one set of trials made prior to any injections) over a period of 2.5 hrs. On the third day, 24 hr after the previous experiments, mice were tested again for a single set of 3 trials to determine whether any latent longer-term differences between experimental groups had emerged. A subset of the animals were further tested on the RotaRod apparatus up to 2 weeks following their injections.

Data were analyzed using 2-way ANOVA to determine the main effect of treatment and time with Tukey's multiple-comparison test to determine the differences between groups.

A.4 RESULTS

A.4.1 Comparison of the prophylactic effects of Cb, Cbi, CoTMPyP and CoN_4[11.3.1] on acute (sub-lethal) cyanide toxicity

All of the cobalt-containing compounds selected for comparison in this paper have been reported to be protective against acute cyanide poisoning [66, 68, 79, 105, 109], but they have not previously all been tested in a head-to-head fashion in the same model system. We chose to use the prophylactic administration of the cyanide scavenging compounds in mice as a starting point for comparing the efficacy of the four chosen compounds (Cb, Cbi, CoTMPyP and CoN_4[11.3.1]) against cyanide toxicity. In order to determine the lowest dose having maximal antidotal effect in each case, Swiss-Webster mice (age 7-8 weeks) were injected intraperitoneally (IP) with either 30, 40, 45, 50, or 70 µmol/kg of each cobalt compound to be tested, followed 5 min later by injection (IP) of NaCN (100 µmol /kg). Control animals that received NaCN alone were "knocked down" (*i.e.* became unconscious) within 1-2 minutes, 20% (16 of 82) died within 4 min of the toxicant delivery, and those that survived exhibited a mean righting recovery time of 24 ± 7 min (n = 66).

All of the compounds employed in this prophylactic paradigm significantly decreased the righting recovery time of the cyanide-intoxicated mice, but the dose of each test compound required for the maximal effect (*i.e.* minimal recovery time) varied (Figure 22). While the righting recovery time seemed to decrease as the Cb administered was increased from 30 to 50 µmol/kg, the only significantly effective dose was 70 µmol/kg (Figure 22A). The righting recovery time was 9 ± 5 min (compared to 24 min for controls) and, in addition, 100% of the mice receiving this Cb dose (15 of 15) survived. Interestingly, Cb was the only antidotal

compound of the four for which a majority of the mice (14 of 15) still experienced knockdown, even at the highest dose tested. Lower doses of Cbi (45, 50 and 70 μmol/kg) compared to Cb significantly reduced the righting-recovery times of cyanide-intoxicated mice to 8 ± 8 min, 12 ± 10 min, and 5 ± 6 min, respectively (Figure 22B) – all the animals survived (16 of 16) and half experienced knockdown (8 of 16). However, there was noticeably more variation within the Cbi-treated groups of mice compared to all the other groups, particularly in mice administered 45 or 50 μmol/kg Cbi, and the response was not strictly linear. The most effective dose of Cb (70 μmol/kg), against cyanide intoxication, was ~1.6 times a similarly effective dose of Cbi (45 μmol/kg). The lower effective dose of Cbi can be rationalized by consideration of its structure (Figure 21B) in which the loss of the dimethylbenzimidazole ribonucleotide tail of the Cb structure (Figure 21A) allows Cbi to bind two exogenous cyanide anions rather than only one.

The other two compounds forming dicyano adducts, CoTMPyP and CoN_4[11.3.1], were then tested. CoTMPyP significantly reduced the righting recovery time at all doses tested (Figure 22C) with righting recovery times of 15 (± 10) min, 9 (± 7) min, 9 (± 5) min, 5 (± 4) min, and 1 (± 3) min for doses of 30, 40, 45, 50, and 70 μmol/kg, respectively. The dose of Cb (70 μmol/kg) most effective against cyanide intoxication, was ~1.8 times a similarly effective dose of CoTMPyP (40 μmol/kg). No deaths (100% survival) were observed when mice were administered either 45, 50 or 70 μmol/kg CoTMPyP before cyanide intoxication. Out of 13 mice that were administered 70 μmol/kg of CoTMPyP, only two knocked-down.

Finally, dose-response testing demonstrated that CoN_4[11.3.1] administered at 45, 50, or 70 μmol/kg significantly reduced the righting recovery time versus control mice to 5 (± 7), 3 (± 4), 3 (± 3) min, respectively (Figure 22D) – all the animals survived (15 of 15) and about half experienced knockdown (7 of 15). Since, we found that the average time of righting recovery is

unchanged when the dose administered was increased from 50 to 70 μmol/kg, the lower dose (50 μmol/kg) was chosen for the therapeutic experiments (see below). The dose of Cb (70 μmol/kg) most effective against cyanide intoxication, was at least 1.6 times a similarly effective dose of CoN_4[11.3.1] (45 μmol/kg) – this value could be up to 1.8 times (70 μmol/kg Cb:40 μmol/kg CoN_4[11.3.1]) but for a single outlying point in the data (Figure 22D).

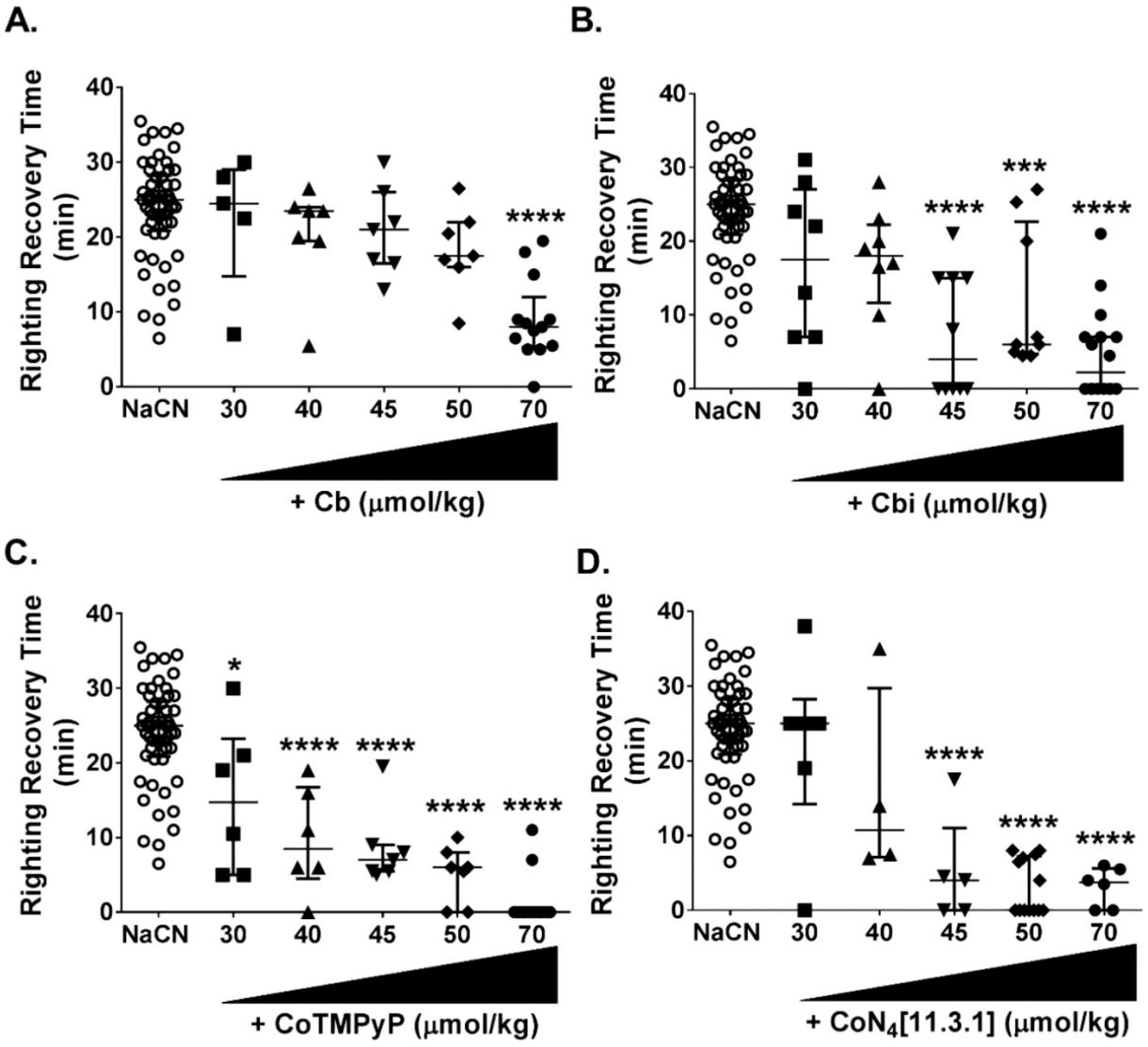

Figure 22. Dose-response profiles for prophylactically administered Cb, Cbi, CoTMPyP and CoN_4[11.3.1] in cyanide-intoxicated male mice as determined by righting-recovery times.

Swiss-Webster male mice (7-8 weeks of age) were injected (IP) with either Cb **(A)**, Cbi **(B)**, CoTMPyP **(C)** or CoN_4[11.3.1] **(D)** 30, 40, 45, 50, and 70 μmol/kg in saline, 5 min before the administration of 100 μmol/kg NaCN (in saline). Control animals received only 100 μmol/kg NaCN injections (open circles). Righting recovery times were recorded and the medians and interquartile ranges are shown. One-way ANOVA with Tukey's multiple comparisons post-test was performed for each compound tested to determine the significance of the righting-recovery time as compared to controls. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$.

A.4.2 Comparison of the therapeutic effects of Cb, Cbi, CoTMPyP and CoN_4[11.3.1] on acute (sub-lethal) cyanide toxicity

To examine the ability of the cobalt-containing compounds to ameliorate the effects of cyanide intoxication when given after the toxicant, Cb, Cbi, CoTMPyP, or CoN_4[11.3.1] were administered at the maximally effective doses (as described above) to male Swiss-Webster mice (7-8 weeks of age) at either 1, 2, or 5 min after injection of NaCN (100 μmol/kg). That is, all the cobalt compounds were administered at a dose of 70 μmol/kg, except for CoN_4[11.3.1], which was given at a dose of 50 μmol/kg. The righting-recovery times following the cyanide injections were recorded and compared with the controls given NaCN only (Figure 23).

The mean recovery times for animals given 70 μmol/kg Cb at 1 or 2 min after cyanide injections were found to be 11 ± 8 min and 14 ± 3 min, respectively (Figure 23A), longer than that observed when the same dose was prophylactically administered (9 ± 5 min, Figure 22A). Mice receiving 70 μmol/kg Cbi at 1 or 2 min post-cyanide intoxication had righting-recovery times of, respectively, 7 ± 4 min and 11 ± 2 min, again longer than for the prophylactically administered dose (5 ± 6 min, Figure 22B). CoTMPyP performed no better than Cb or Cbi in this therapeutic test (Figure 22C) exhibiting recovery times of 10 ± 7 min and 11 ± 6 min, for delivery of 70 μmol/kg CoTMPyP at 1 and 2 min, respectively, after cyanide intoxication

compared to prophylaxis (1 ± 3 min, Figure 22C). Additionally, the majority (6/7) of mice tested knocked-down when CoTMPyP was administered at 1 min after cyanide injections, comparable to the effect seen with both Cb and Cbi. However, CoN_4[11.3.1] performed better than the other compounds when given 1 min after cyanide intoxication (Figure 23D) with a righting recovery time of 4 ± 3 min, comparable to its prophylaxis (3 ± 3 min, Figure 22D) even though a lower dose of the antidote (50 μmol/kg) was used. When given 2 min after the toxicant, the mean righting-recovery time for CoN_4[11.3.1] was 13 ± 6 min, no better than the other cobalt compounds at the same time point. Additionally, when the CoN_4[11.3.1] had been administered at 1 min after the cyanide injections, 4 of the 12 mice tested did not knockdown, a noteworthy improvement compared to the effects seen with the other compounds.

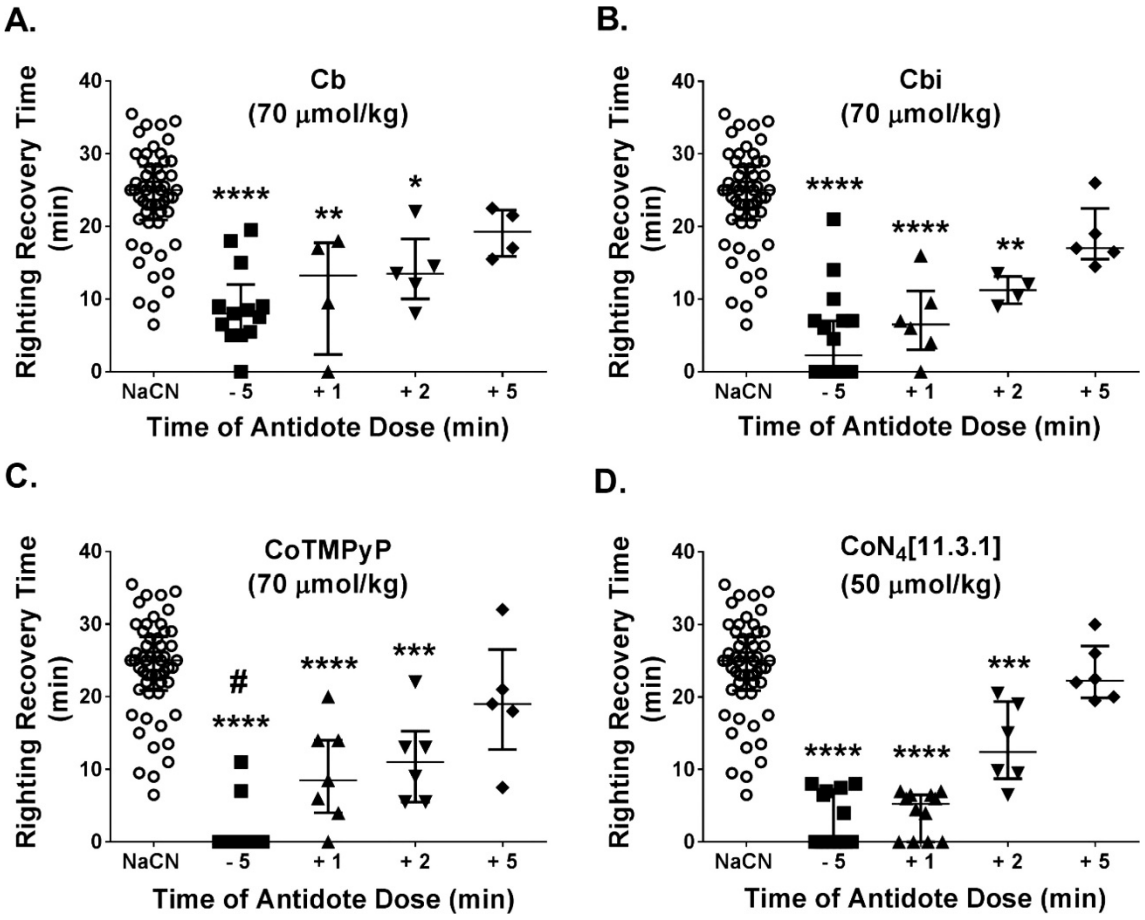

Figure 23. Therapeutic effects of cobalt-containing compounds in male mice after cyanide intoxication.

Swiss-Webster mice were injected with either 70 μmol/kg of Cb **(A)**, Cbi **(B)**, CoTMPyP **(C)**, or with 50 μmol/kg of CoN_4[11.3.1] **(D)** at 1, 2, or 5 min post cyanide intoxication. NaCN given alone (100 μmol/kg) and with the test compounds (at the doses above) injected prophylactically at -5 min are included in each plot for comparison purposes. Righting-recovery times were recorded for each set of injections; the median and interquartile range are shown. One-way ANOVA with Tukey's multiple comparisons post-test was performed for each compound tested to determine the significance of the righting-recovery time as compared to controls. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$. #median and interquartile range are both equal to 0.

In summary, all of the cobalt compounds tested significantly reduced the righting-recovery time when administered at either 1 or 2 min after cyanide intoxications, but none of the compounds tested had any measurable impact on the righting-recovery time when delivered 5 min after the cyanide administration (Figure 23). Using exactly the same righting-recovery

approach in sub-lethally intoxicated mice (16-20 weeks of age) we have previously shown that sodium nitrite clearly is an effective cyanide antidote when given therapeutically more than 5 min after the toxicant dose [41]. Such is the significance of this earlier result that we present data collected from the younger mice (7-8 weeks, closed diamonds) in this study along with the data previously published for the older mice here (Figure 24, solid circles) both for emphasis and as a positive control. In terms of preventing knockdown and the shortest righting recovery observed, prophylactically administered CoTMPyP appeared to be the most effective of the cobalt compounds (Table 3). Otherwise, CoN_4[11.3.1] was the most efficacious of the cyanide scavengers tested by several criteria, not the least of which was that it performed comparably, or slightly better than the others, at lower relative dose.

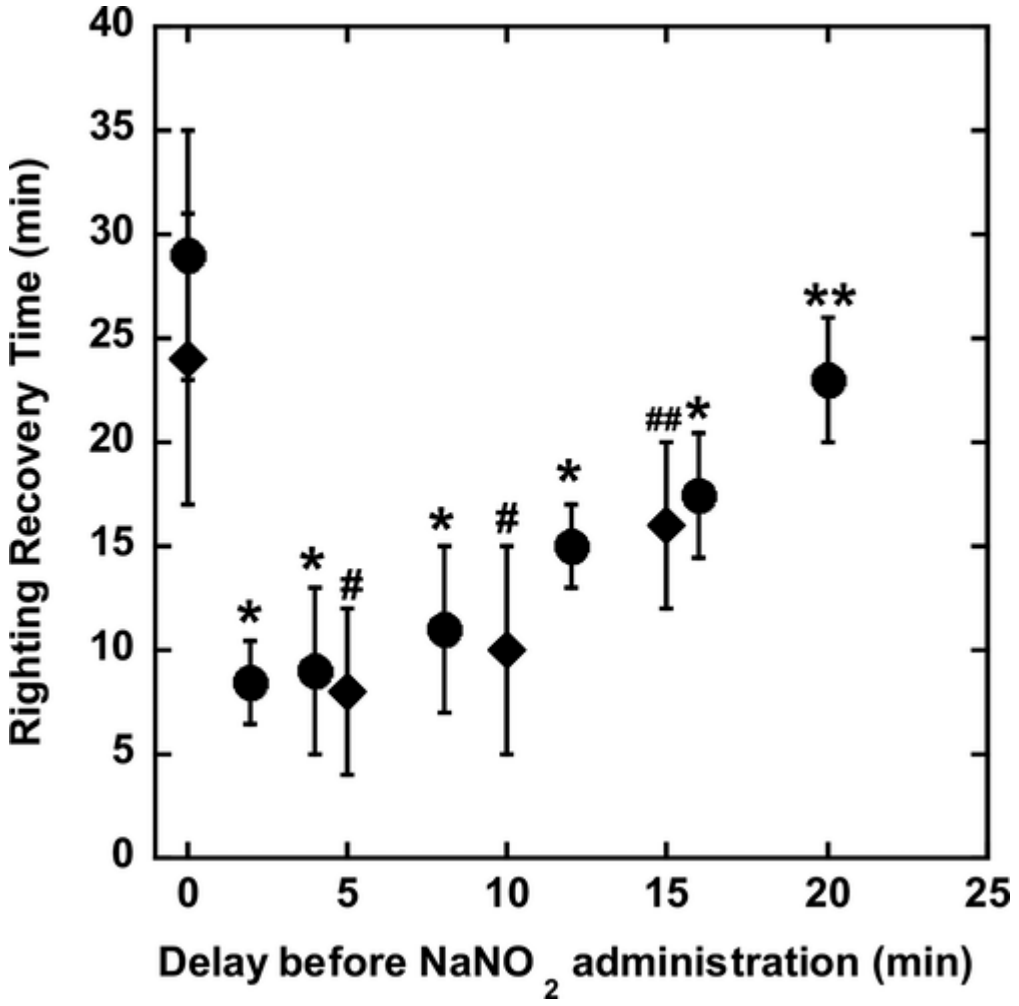

Figure 24. The ameliorative effect of $NaNO_2$ on cyanide intoxication.

Swiss-Webster mice (males, 16-20 weeks of age, solid circles and males 7-8 weeks of age, solid diamonds) injected with NaCN (100 µmol/kg, IP) and then administered $NaNO_2$ (12 mg/kg, IP) 2 to 20 min after cyanide. Control animals received NaCN only. Values represent means and standard deviations. In general, at least 4 animals per point were used, except for control (n = 17 for 16-20 weeks of age and n = 66 for mice of 7-8 weeks of age). One-way ANOVA with Tukey's multiple comparisons post-test was performed to determine the significance of the righting-recovery time as compared to controls.*p ≤0.0001, **p ≤ 0.05 (16-20 week old mice) #p ≤0 .0001. ##p ≤ 0.01 (mice of 7-8 weeks of age). Solid circles (mice of 16-20 weeks of age) are reformatted data from Cambal et al. [41]

Table 3. Distinguishing animal data for the cobalt-containing trial compounds

Assessment	Controls (100 µmol/kg NaCN)	Hydroxocobalamin (NaCN + Cb, 70 µmol/kg)	Cobinamide (NaCN + Cbi, 70 µmol/kg)	CoTMPyP (NaCN + 70 µmol/kg)	CoN_4[11.3.1] (NaCN + 50 µmol/kg)
Prophylaxis[a]: Death/group	16/66	0/15	0/16	0/13	0/15
Prophylaxis: knockdowns/group	66/66 (100%)	14/15 (93%)	8/16 (50%)	2/13 (15%)	7/15 (47%)
Prophylaxis(5 min pre-NaCN): righting recovery time (min)	24	9	5	1	3
Prophylaxis: effectiveness ratio[b]	N/A	1.0	1.6	1.8	1.7
Therapeutic[c]: knockdowns/group[d]	N/A	4/4 (100%)	5/6 (83%)	6/7 (86%)	8/12 (67%)
Therapeutic: righting recovery time (min)[d]	N/A	11	7	10	4

[a]Putative antidotes given 5 min before NaCN.

[b] Relative to Cb (see text for explanation).

[c]Putative andidotes given 1 min post NaCN administration

[d]For antidotal doses delivered at 1 min time points.

A.4.3 Comparison of neuromuscular recovery in mice administered acute (sub-lethal) cyanide doses (controls) or saline (shams)

The RotaRod testing paradigm employed (Figure 25A, see Experimental Methods for further details) involves measuring the duration that individual animals can remain in position walking on a rotating cylinder; any shortening of the observed duration following some experimental insult being taken as evidence of impairment. While primarily a measurement of neuromuscular coordination [119, 120] the technique also routinely provides some assessment of learning

capability and memory. During the training periods (day 1, 1-8 min; Figures 25B, 25C and 25D) the performance of all the animal groups increased steadily, indicating the mice were adapting to (*i.e.* learning) the test. The trained performance was essentially maintained by 24 hr later with a very slight loss in the previous day's level (pre-IP data sets, Figures 25B, 25C and 25D) and sham mice receiving saline solution without toxicant continued to adapt until reaching a plateau in their performance (open circles, day 2, 15-150 min; Figure 25B). Control mice receiving only 100 μmol/kg NaCN were still completely incapacited 15 min after the toxicant dose, unable to remain on the rotating cylinder for any time at all, but then steadily improved over the next 2 hr (filled squares, day 2, 15-150 min; Figure 25B). The performances of the sham and control animals remained indistinguishable 24 hr later (day 3, 24 hr points; Figure 25B). A subset of these animals were evaluated at longer times post injections and, in fact, the performances of the sham and control animals subjected to training regimens remained indistinguishable at 48 hr, 1 week and 2 weeks after the intoxications (see Supporting Information).

A.4.4 Comparison of neuromuscular recovery in mice administered Cb, Cbi, CoTMPyP and CoN_4[11.3.1] prior to acute (sub-lethal) cyanide exposures

Before testing any ameliorative capabilities, an initial set of experiments was performed in which the cobalt compounds were administered in the absence of cyanide to investigate the presence of possible undesirable side effects (Figure 25C). Interestingly, the performance of mice on the RotaRod device was enhanced at 15 and 30 min after the administration of Cb (filled circles, Figure 25C, $p < 0.01$ compared to saline shams in Figure 25B); the effect being quite small, but reproducible over multiple days of testing. In contrast, Cbi-treated mice had a significantly decreased rate of performance on the RotaRod device at 15 and 30 min post-cyanide

administration (open squares, Figure 25C, $p < 0.01$ compared to saline shams in Figure 25B). From about 90 min onwards, the data obtained for the animals treated with Cb, Cbi and the saline shams were indistinguishable (Figure 25C). There were no significant differences in the entire experimental range from 15 min - 24 hr between the performances of either CoTMPyP- or CoN_4[11.3.1]-treated animals (filled squares and open circles, Figure 25C) when compared to the performance of the sham mice injected with saline (Figure 25B).

In a second set of experiments, animals were injected with the cobalt cyanide-scavenging compounds 5 min prior to the toxicant dose, then tested every 15 min for 2.5 hr and again at 24 hr (Figure 25D). Mice administered 70 μmol/kg Cb (5 min before the toxicant dose) had significantly improved performance up to 105 min after the cyanide injection (filled circles, Figure 25D) when compared to mice receiving cyanide alone (filled squares, Figure 25B). Interestingly, when comparing the performance of cyanide-challenged mice given the different antidotal compounds, the performance of the mice administered Cb was significantly improved compared to the others at the earliest time (15 min) after the cyanide intoxication (filled circles, Figure 25D). Otherwise, the performances of the cyanide-challenged mice administered Cb, Cbi, CoTMPyP, or CoN_4[11.3.1] were not significantly different (Figure 25D). Compared to the results for control animals given cyanide only (filled squares, Figure 25B) all the test compounds appeared to be significantly antidotal in mice in the 15 min to ~90 min window. Consistent with a previous report [68] there appears to be no persistent impairment of neuromuscular coordination detectable as judged by RotaRod testing at 24 hr in any group that received cyanide, irrespective of whether antidote was also given, or not (Figures 25B, 25C and 25D). In fact, no impairment was observed at 2 weeks time post toxicant and/or antidote for any mice using the RotaRod testing (see Supporting Information).

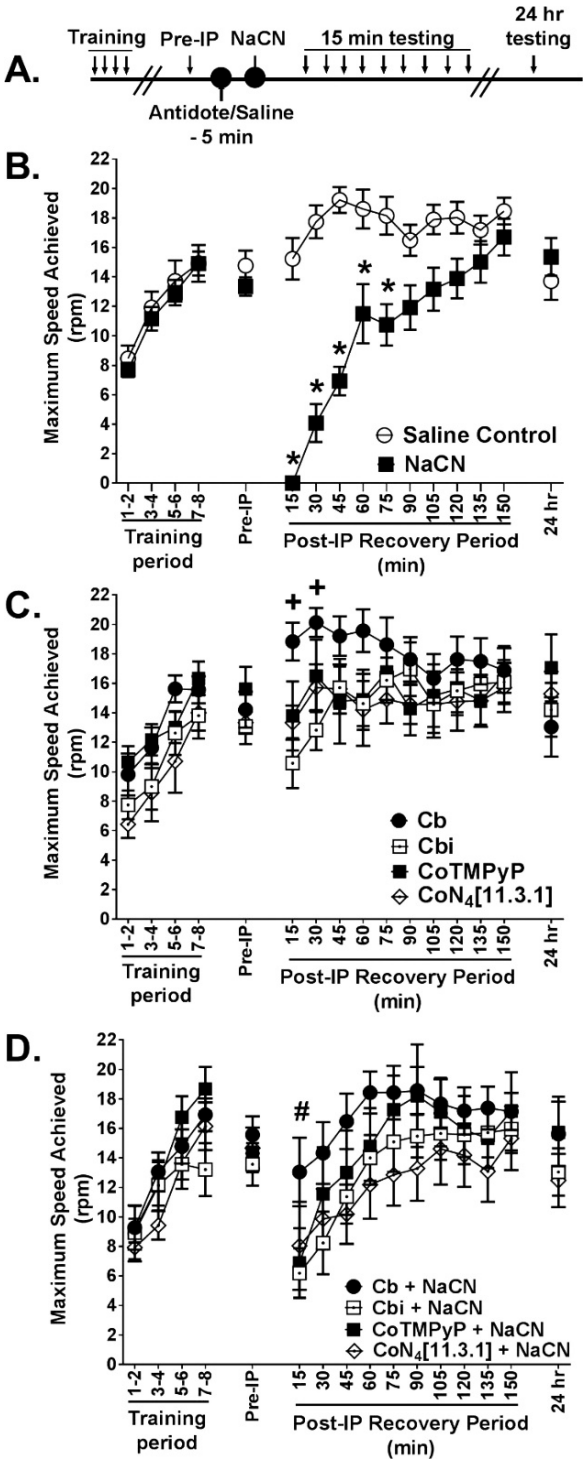

Figure 25. Neuromuscular coordination comparison of Cb, Cbi, CoTMPyP and CoN_4[11.3.1] amelioration following NaCN administration in mice.

(A) RotaRod testing paradigm: arrows indicate RotaRod testing times; lines with circles indicate injection times (all IP). Mice were trained on the RotaRod 24 hr before injection, and a baseline performance was obtained 1 hr before

injection (Pre-IP). Mice were tested every 15 min after injections for 2.5 hr (up to 150 min) and again at 24 hr to assess recovery. One-way ANOVA with Tukey's multiple comparisons post-tests were performed to determine the significance between controls and a particular compound tested or between compounds tested as noted. **(B)** Comparison of performance (maximum speed achieved) for injections of 100 μmol/kg NaCN (closed square) and saline control (open circle). ***p** ≤ 0.001 vs. saline control. **(C)** Comparison of performance (maximum speed achieved) for mice injected (IP) with 70 μmol/kg of Cb (closed circle), Cbi (open square), CoTMPyP (closed square) or 50 μmol/kg CoN_4[11.3.1] (open diamond). +p ≤ 0.01 vs. Cbi. **(D)** Comparison of performance of mice (maximum speed achieved) injected with either 70 μmol/kg of Cb (closed circle), Cbi (open square), CoTMPyP (closed square) or 50 μmol/kg CoN_4[11.3.1] (open diamond) and 100 μmol/kg cyanide. #p ≤ 0.05 for Cbi & NaCN vs. Cbl & NaCN. Numbers of animals (in parentheses) used in each set of experiments are as follows: NaCN (16), saline (13), Cb (8), Cbi (8), CoTMPyP (6), CoN_4[11.3.1] (7), Cb & NaCN (7), Cbi & NaCN (7), CoTMPyP & NaCN (6) and CoN_4[11.3.1] & NaCN (7).

A.4.5 Importance of the essentially irreversible kinetics of antidotal cyanide-scavenging compounds

We have now repeatedly argued [68, 79] that the cobalt-based compounds Cb, Cbi, CoTMPyP and CoN_4[11.3.1], even if administered in their Co(III) forms, are all quickly converted to Co(II) forms in circulating blood due to the presence of endogenous reductants such as ascorbate. Following cyanide binding, however, the reduction potentials of the central cobalt ions become lowered to the extent that the cyanide adducts revert to oxidized forms. This is crucially important because Co(III) complexes are typically more substitution inert than their Co(II) counterparts [32, 114, 115] and the cyanide forms are thus stabilized, so that they may be excreted rather than assist in systemic redistribution of the toxicant. This point of view is, seemingly, reinforced by the observation that assimilation of the cobalamin cofactor in B_{12}-dependent enzymes requires reductive decyanation of cyanocobalamin catalyzed by its chaperone [121]. As further demonstration of this principal, we undertook a set of experiments

employing gallium nitrate as a trial antidote to both cyanide and sulfide (*i.e.* H_2S/HS^-) intoxication in mice. Ga(III) is known to be a relatively safe ion when introduced into mammals, including humans, for other purposes [122, 123]. It is also well-known to form a stable complex with cyanide, but this is substitution labile rather than inert [124, 125]. Accordingly, when attempts were made to investigate Ga(III) as a cyanide antidote in mice using the righting-recovery procedure, no effect was observed, beneficial or otherwise (Table 4). On the other hand, the similarly acting mitochondrial poison sulfide [117, 126] forms a precipitate with Ga(III) [127]. This is not a readily reversible process and, consequently, Ga(III) was clearly efficacious in ameliorating sulfide intoxication (Table 4) in keeping with the suggestion that a degree of irreversibility associated with the final adduct is a highly desirable characteristic for effective scavenging.

Table 4. Effects of $Ga(NO_3)_3$ on cyanide and sulfide toxicity in Swiss-Webster mice.

	Survivors/group (%)	**Time until deaths of non-survivors (min)**
5 mg/kg NaCN only (control)	50/66 (76%)	~3
50 mg/kg $Ga(NO_3)_3$ given 1 min after NaCN	6/8 (75%)	~3
18 mg/kg NaHS only (control)	16/24 (67%)	< 4
50 mg/kg $Ga(NO_3)_3$ given 1 min after NaHS	8/8 (100%)	—

A.5 DISCUSSION

When given prophylactically, Cb, Cbi, CoTMPyP and CoN_4[11.3.1] all clearly ameliorate the toxic effects of cyanide as assessed by righting recovery (Figure 22) and restoration of neuromuscular coordination (Figure 25D compared to filled squares in Figure 25B). In all cases, including mice given only the toxicant, or only one of the cobalt compounds, recovery of normal neuromuscular function at 24 hr appeared complete (Figures 25B, 25C and 25D). That is, there was no sign of any long-term impairment (up to 2 weeks), providing encouragement for the continued development of the latter three compounds as potential cyanide antidotes. The slightly toxic effect detected in the case of Cbi at 15 and 30 min (open squares, Figure 25C) is, however, of some minor concern. The value of these behavioral assessments of toxicity and antidote-dependent recovery with conscious mice should be fully appreciated – in addition to providing information not necessarily accessible with unconscious animals, such experiments avoid the well-known confounding complications of anesthesia [93, 118].

In experiments where the putative antidotes were administered after the toxicant, Cbi, CoTMPyP and CoN_4[11.3.1] (Figures 23B, 23C and 23D) all performed better than Cb (Figure 23A). Particularly if the lower dose is considered, CoN_4[11.3.1] (Figure 23D, 50 μmol/kg) was measurably better than Cbi and CoTMPyP (Figures 23B and 23C, each 70 μmol/kg) as a therapeutic. This lower effective dose of CoN_4[11.3.1] could, of course, be of importance for treating higher levels of cyanide intoxication than would be possible with the other compounds. In relation to management of public health emergencies, there is currently some interest in stockpiling cyanide antidotes and, consequently, other factors may become important for ranking these cobalt compounds. The relatively low cost of CoN_4[11.3.1] (Table 2) should not be overlooked in this regard. Also, however, the storage requirements for Cb and Cbi (biological

materials) are that they be refrigerated (-20°C) whereas we have stored CoTMPyP, CoN_4[11.3.1] and similar complexes in darkened, screw-top vials at room temperature for months (years in some cases) without noticeable decomposition (assessed by mass spectrometry and spectral analysis) beyond some slow oxidation of Co(II) to Co(III).

The stability constants (*K*) of all four cyano adducts (Table 2) are large enough to ensure that the compounds are efficient cyanide scavengers. For example, if Cb and cyanide are present in approximately equal quantities, $K = 10^5$ implies that > 99.99% of the cyanide will be bound to the Co(III) center; alternately, if CoN_4[11.3.1] and cyanide are present in approximately equal amounts, $K = 3 \times 10^5$ (the lower end of the possible range – see footnote g to Table 2) implies that > 99.9% of the cyanide will be bound to the Co(II) center; in the other two cases, the stability constants are larger and even more of the cyanide will be scavenged. Furthermore, since the rate constants for cyanide binding by the other three compounds are all similar or larger than that for Cb, we may argue that equilibrium is attainable in each case at fast enough rates that the relative efficacies of these compounds as antidotes should simply be given by the number of exogenous cyanide anions they can bind. In other words, the antidotal capabilities of Cbi, CoTMPyP and CoN_4[11.3.1] (all binding 2 CN^- per Co(III)) should be comparable on a molar basis and they should be better than Cb (binding 1 CN^- per Co(III)) by no more than a factor of approximately 2. In the present investigation, this is exactly what we find, the "effectiveness ratios" of Cbi, CoTMPyP and CoN_4[11.3.1] compared to Cb are all approaching 2 (Table 3). It requires comment that prophylactically administered Cbi has previously been reported to be 3-fold to 11-fold more efficient than Cb as an *in vivo* cyanide scavenger in mice [62]. Certainly, the anesthesia used in this previous study may have influenced the results, but when the toxicant was given as NaCN solutions (IP injections) the 3-fold increased effectiveness of Cbi compared

to Cb observed is not very different from the 2-fold increase we suggest here to be limiting. Much more surprising is the finding that Cbi was 11-fold more effective than Cb when the toxicant dose was delivered as inhaled HCN. This simply does not appear possible if the only relevant activity of Cbi is straightforward complexation of 2 CN^- per Co(III); strongly suggesting that there must be some presently unrecognized aspect to the antidotal action of Cbi particularly associated with toxicant inhalation. In the earlier study [62] the animals were given the antidotes 15 min before starting the HCN dose which was then continued for a period of 30 min, so there was plenty of time for other processes to become involved.

In the therapeutic experiments (Figure 23) where antidotes were given after the toxicant, the CoN_4[11.3.1] performed best of the four when given 1 min after the cyanide (Table 3) perhaps reflecting its significantly faster reaction rate than the others (Table 2). All the compounds were comparably effective when given up to 2 min after the cyanide, but if a delay of 5 min was allowed before giving antidote, none were effective (Figure 23). We interpret this observation to indicate that within 5 min of receiving a cyanide dose the toxicant has bound to its molecular target, namely, cytochrome c oxidase in the mitochondria, and the cobalt-based scavengers are not able to reverse the inhibition of the enzyme as we have unambiguously demonstrated for at least Cb and Cbi [59]. To the contrary, sodium nitrite clearly does work therapeutically if given as an antidote at times longer than 5 min after the cyanide dose (Figure 24). It should be noted that the majority of these nitrite data were obtained employing older mice and from a different supplier compared to the other animal experiments. The older control animals given no antidote exhibited a mean righting recovery time of 29 min, enabling effectiveness to be demonstrated if the nitrite was given up to 20 min following the cyanide dose. On the other hand, younger control animals given no antidote exhibited a mean righting recovery

time of 24 min, enabling effectiveness to be demonstrated if the nitrite was given up to only about 15 min following the cyanide dose. It is to be understood that the 15-20 window of effectiveness represents a limitation of the method, it does not necessarily mean that the nitrite would be ineffective if the therapeutic doses were to be delayed for more than 20 min following a toxicant dose in some other poisoning scenario. We have previously shown that nitric oxide (NO) is able to reverse cyanide inhibition of cytochrome *c* oxidase [47, 48] and used this observation to infer a plausible mechanism by which nitrite anion (acting as an NO donor) may be antidotal toward cyanide intoxication independent of methemoglobin formation [41, 93]. Therefore, unlike the cobalt-based scavengers, nitrite anion can reverse the toxic effect of cyanide at its principal molecular target by reversing the inhibition of cytochrome *c* oxidase; hence its broader window of action. It follows that there are numerous experimental protocols that could inadvertently be undertaken that might misleadingly suggest cobalt-based scavengers (or any other cyanide-complexing compounds) can be of therapeutic benefit 5 min or more after the toxicant dose has ceased. For instance, if a putative cyanide scavenger is given as a nitrite salt, or NO complex, then this really represents a combination therapy, not an unambiguous assessment of the scavenger. Less obviously, any animal model in which cyanide scavengers are to be tested, but where there may be inflammation (with accompanying upregulation of inducible nitric oxide synthase), or where analgesics/anesthetics are employed (at least some of which are known [93] to behave like stimulators of endogenous NO production), a combination therapy is probably, if unintentionally, being investigated.

We have previously shown [59, 68, 79] that the cobalt-based compounds Cb, Cbi, CoTMPyP and CoN_4[11.3.1] are all quickly converted to Co(II) forms by reductants at the levels they are present in circulating blood, facilitating suitably rapid cyanide binding. Upon binding

cyanide, however, the reduction potentials of the central cobalt ions become lowered to the extent that the cyanide adducts become oxidized to stable forms that may be excreted. Here, we have described a set of experiments (Table 4) employing gallium nitrate as a trial antidote to both cyanide and sulfide (*i.e.* H_2S/HS^-) providing further conformation of the importance that the final adduct is essentially substitution inert – otherwise, the intended scavenger will only assist in the systemic redistribution of the toxicant. The overall similarity in the relevant physicochemical properties (Table 2) of the cobalt compounds able to bind two cyanide anions per metal ion, namely Cbi, CoTMPyP and CoN_4[11.3.1] and the comparability of their performances in the antidotal trials (Table 3) suggests that many cobalt complexes with four approximately equatorial nitrogen donors should display the necessary oxidation-reduction chemistry and ligand substitution characteristics suitable for application as cyanide scavengers. Such compounds may, however, differ considerably the type of macromolecular biomolecules with which they interact. That is, their relative toxicities will likely depend to some extent on the peripheral structures of their chelating ligands.

A.6 SUPPORTING INFORMATION

Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/acs.chemres-tox.7b00234.

Cyanide binding kinetics of Co(II)N_4[11.3.1] and raw data for animal experiments presented in Figures 22, 23 and 25

A.7 ACKNOWLEDGEMENTS

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