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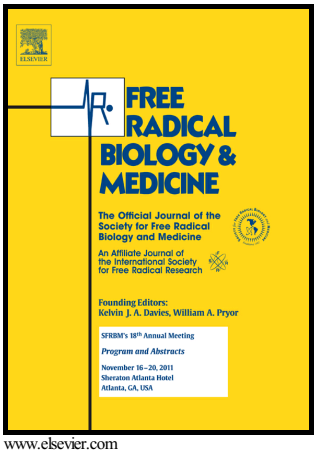

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Jason P. Eiserich[a,b,1], Sean P. Ott[a], Tamara Kadir[a], Brian M. Morrissey[a], Keri A. Hayakawa[a], Michele A. La Merrill[c] and Carroll E. Cross[a,b,*]

[a]Department of Internal Medicine, Division of Pulmonary/Critical Care and Sleep Medicine, University of California, Davis, CA 95616

[b]Department of Physiology and Membrane Biology, University of California, Davis, CA 95616

[c]Department of Environmental Toxicology, University of California, Davis, CA 95616

[*]**Corresponding Author**: Carroll E. Cross, M.D. Phone: +1-916-734-3564, Fax: +1-916-734-7924, cecross@ucdavis.edu

ABSTRACT

Rationale:

Cystic fibrosis (CF) patients are known to produce cyanide (CN^-) although challenges exist in determinations of total levels, the precise bioactive levels, and specificity of its production by CF microflora, especially *P. aeruginosa*. Our objective was to measure total CN^- levels in CF sputa by a simple and novel technique in *P. aeruginosa* positive and negative adult patients, to review respiratory tract (RT) mechanisms for the production and degradation of CN^-, and to interrogate sputa for post-transcriptal protein modification by CN^- metabolites.

Methods:

[1] Current affiliation: Senior Environmental Scientist (Supervisory), California Environmental Protection Agency, Department of Pesticide Regulation, Sacramento, CA, 98517.

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Sputa CN^- concentrations were determined by using a commercially available CN^- electrode, measuring levels before and after addition of cobinamide, a compound with extremely high affinity for CN^-. Detection of protein carbamoylation was measured by Western blot.

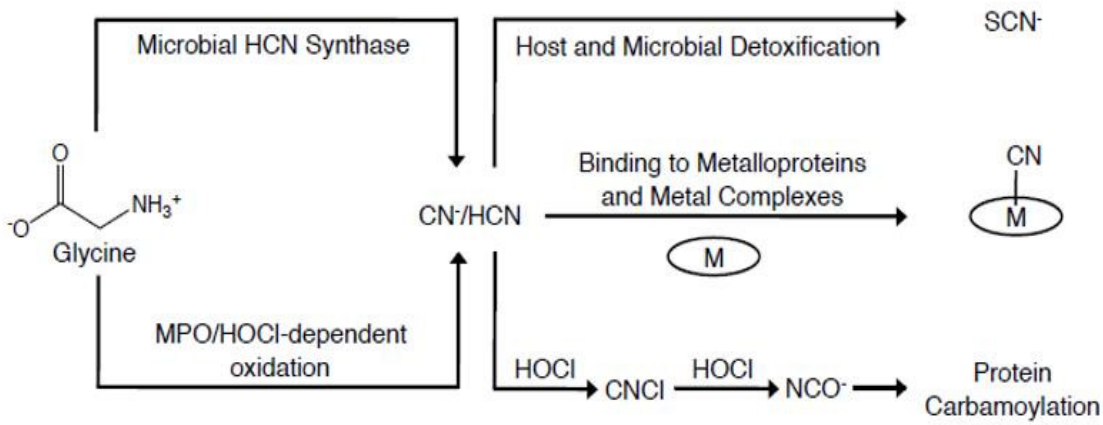
Measurements and Main Results:

The commercial CN^- electrode was found to overestimate CN^- levels in CF sputum in a highly variable manner; cobinamide addition rectified this analytical issue. Although *P. aeruginosa* positive patients tended to have higher total CN^- values, no significant differences in CN^- levels were found between positive and negative sputa. The inflammatory oxidant hypochlorous acid (HOCl) was shown to rapidly decompose CN^-, forming cyanogen chloride (CNCl) and the carbamoylating species cyanate (NCO^-). Carbamoylated proteins were found in CF sputa, analogous to reported findings in asthma.

Conclusions:

Our studies indicate that CN^- is a transient species in the inflamed CF airway due to multiple biosynthetic and metabolic processes. Stable metabolites of CN^-, such as cyanate, or carbamoylated proteins, may be suitable biomarkers of overall CN^- production in CF airways.

PICTORIAL ABSTRACT



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Key words:

cystic fibrosis, cyanide, ion-specific electrode, cyanogen chloride, cyanate, P. aeruginosa, neutrophils, myeloperoxidase, hypochlorous acid, carbamylated protein

Abbreviations:

Cyanide (CN^-), Cystic Fibrosis (CF), carbamoylating species cyanate (NCO^-), hypochlorous acid (HOCl), cyanogen chloride (CNCl), respiratory tract (RT), *Pseudomonas aeruginosa* (*P. aeruginosa*)

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is the most persistent respiratory tract (RT) pathogen, eventually dominating cultures obtained from over 80% of adult Cystic Fibrosis (CF) patients [1]. *P. aeruginosa* releases numerous metabolites and virulence factors that likely contribute to RT deterioration in the lungs of CF patients [2]. These species include hydrogen cyanide/cyanide (CN^-), which binds with high affinity to multiple metalloproteins (i.e. iron and copper) to varying degrees and particularly inhibits cytochrome c oxidase in mammalian cell mitochondria [3]. Of particular interest, *Bacillus pyocyaneus* (later determined to be what is today regarded as *P. aeruginosa*), was found to produce CN^- as early as 1913 [4]. The heavily infected inflammatory hypoxic conditions of the CF RT represent a fertile milieu for cyanogenic pathogens. CN^- is presumably produced to gain a survival advantage against the competing growth of CN^--sensitive microorganisms and/or possibly aggravating metabolic conditions of the host RT) [2, 5, 6]. Biosynthesis of CN^- is carried out by cyanogenic bacteria using an enzyme known as hydrogen cyanide synthase, using the amino acid glycine, abundant in CF sputa (range 0.27 to 1.73μM) as a substrate [2, 7]. The reaction proceeds as shown in Scheme 1, with an unstable imino acetic acid intermediate, with ultimately the stoichiometric liberation of HCN and CO_2.

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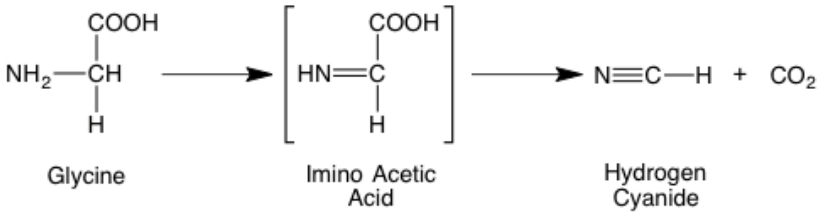

Scheme 1: Enzymatic production of hydrogen cyanide (HCN) by the Pseudomonas HCN synthase enzyme system

CN^- levels of up to 200 μM have been reported in the sputa of CF patients, whereas much lower to nil amounts have been measured in non-CF patients with bronchiectasis and in CF patients not culturing *P. aeruginosa* [2, 6-10]. While the commercial CN^- electrode provides a convenient method for quantifying CN^-, the complex nature of CF sputum presents significant challenges including non-specificity. Moreover, sputa also have pigments and other substances that interfere with spectrophotometric determination of CN^- complexed with cobinamide, a compound with very high affinity for CN^-. Cobinamide itself has been used as a method for measuring cyanide and as a therapeutic agent for CN^- poisoning [11, 12].

In the present study CN^- concentration was quantified in the sputa of adult CF patients infected with *P. aeruginosa* and/or non - *P. aeruginosa* bacterial strains using a combination of an ion-specific electrode system and cobinamide to correct for non-specific background interference. The refined method is simple, convenient and accurate and would be particularly useful in the clinical setting for routine analyses.

Methods

Subjects:

Twelve adult CF-outpatients attending the UC Davis Adult CF Center clinic were recruited for this study. The study was approved by the Office of Human Research/Institutional Review

Board (IRB) of the University of California, Davis. All subjects provided informed consent. All subjects recruited were confirmed CF patients based on the published diagnosis criteria given by the CF Foundation. Subjects were asked to void their mouths of saliva prior to producing a spontaneous sputum sample. Sputum samples were expectorated into sterile containers and immediately frozen on dry ice and subsequently stored at -80°C for analyses.

Sputum processing*:*

The -80°C frozen sputum samples were processed within two weeks of collection. The samples were homogenized with ice cold PBS (10 μM phosphate, pH 7.4, containing 154 μM NaCl; 1:1 w/v) using a dounce homogenizer. Homogenized samples were then incubated with DNase (0.2 mg/mL) for 10 min at room temperature and made alkaline with NaOH to prevent escape of HCN by volatilization.

Cobinamide synthesis:

Hydroxoaquocobinamide (cobinamide) was synthesized using a modified method by Armitage et al. [13]. Briefly, 20 mg hydroxocobalamin was incubated with 150 μL concentrated HCl for 8 min at 65 °C. The reaction was stopped by placing the mixture on ice. The pH was increased to 5 by the slow addition of 6 M NaOH. Cobinamide was isolated using a PrepSep C_{18} solid phase extraction column. The column was rinsed with double deionized water to remove excess salt and then 10% acetone to remove unreacted hydroxocobalamin. Cobinamide was eluted from the column with 20% acetone. The cobinamide solution was then concentrated under N_2 gas. Cobinamide was quantified by converting it to diaquocobinamide with 0.1 M HCl and absorbance read with a spectrophotometer at 348 nm. Diaquocobinamide has an extinction coefficient of ε_{348} = 2.8 x 10^4 $M^{-1} \cdot cm^{-1}$[14]. Purity of cobinamide synthesis was assessed by thin layer chromatography. Reverse-phase silica plates were developed in

isopropanol:ammonium hydroxide:H_2O (7:1:2) and 2 mM CN^-. Purity of cobinamide product was also assessed by converting it to dihydroxocobinamide in 0.1 M NaOH and reading the absorbance at 344 nm and 356 nm. If the ratio of A_{344}/A_{356} is between 1.05 and 1.11 the cobinamide sample is considered pure [15].

Cyanide measurement in sputum*:*

CN^- concentrations were measured with a micro-cyanide ion-selective electrode (ISE) (Lazar Research Laboratories, Los Angeles, CA). All data were collected using a PowerLab/16SP recording unit and PowerLab Chart v3.6.5 data acquisition software package (ADInstruments Inc., Colorado Springs, CO). Sputum samples were brought to a concentration of 0.1 M NaOH by a 1:2 dilution in a 96-well plate to trap CN^- in its non-volatile ionic form (CN^-) and to bring the sample within optimal ISE working conditions. To determine non-specific electrode responses cobinamide (100 μM final concentration) was added to scavenge any CN^- in the sputum sample. The mixture was incubated 5 min at room temperature and re-measured with the electrode. A second addition of the cobinamide solution was added to confirm that no additional signal change occurred. The resultant non-specific response was then subtracted from the sample's original measurement to obtain the corrected CN^- concentration in the sputum sample. A linear standard CN^- curve (0 – 250 μM) was made utilizing this method of cobinamide addition. The lower limit of CN^- detection of the ISE with cobinamide background correction was 2.5 μM.

Intra-assay variabilities were determined by making HCN/CN^- measurements on 3 aliquots of the same sample. Inter-assay varabilities were determined by HCN/ CN^- measurements on aliquots of the same sample on 3 different days. Several samples were spiked with known concentrations of HCN/ CN^- to estimate recoveries of HCN/ CN^- when added to sputa. To validate and assess the precision and accuracy of our method, we determined the intra-assay and inter-assay variability. This new method has an intra-assay variability of 7.2%, and an inter-assay variability is 10.2%. To further define recovery yields of CN- by this method

in sputum, we spiked samples with known concentrations of CN- and achieved a recovery of 109.5% ± 6.8%.

Reactions of CN^- with HOCl*:*

CN^- (100μM) was reacted with various concentrations of HOCl (0-200μM) in phosphate buffer (50 mM), pH 7.4) at room temperature using a vortex mixer. After 15 min of incubation, an aliquot of the reaction mixture was made alkaline for determination of CN^- concentration using the CN^- electrode (described above), and a second aliquot was used for analysis of CNCl as described below. Stock solutions of both HOCl (pH 12) and CN^- (pH 7.4) were freshly prepared prior to each experiment. The concentration of HOCl (as the deprotonated form, OCl^-) was determined spectrophotometrically at 290 nm using an extinction coefficient of 362 $M^{-1}cm^{-1}$.

Analysis of cyanogen chloride (CNCl)*:*

CNCl was detected using the pyridine-1,3-dimethyl barbituric acid reagent as previously described [16,17]. CNCl reacts with pyridine to form glutacon dialdehyde, that then reacts with 1,3-dimethyl barbituric acid and condenses to form a violet-colored polymethine complex. Coloring reagent (100 µl) was added to the HOCl/CN^- reaction mixture (100 µl) and was incubated for 15 min at room temperature. The absorbance of the resulting violet-colored solution was measured at 587 nm. The concentration of CNCl was calculated using the extinction coefficient of 1.03 x 10^5 $M^{-1}cm^{-1}$.

Western blot analyses for carbamoylated proteins.

Aliquots of sputum samples containing 20 µg of total protein were separated by SDS-PAGE on 12% gels under reducing conditions. Bovine serum albumin (BSA, 10 mg/ml) was reacted with sodium cyanate (NCO^-, 1 mM) for 12 hrs at room temperature in PBS (pH 7.4) to serve as a

positive control for carbamoylated proteins. Native BSA or carbamoylated BSA (20 µg) was separated by SDS-PAGE as noted above. Proteins were transferred to nitrocellulose membranes and incubated overnight in PBS containing 0.05% Tween-20 and 5% non-fat dry milk. Membranes were incubated with anti-carbamyllysine (CBL) antibody (rabbit polyclonal antibody against carbamoylated Keyhole Limpet hemocyanin; Academy Biomedical Company, Inc.; Catalog #CBL30S-R1a) at a dilution of 1:5,000. A horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody (diluted 1:3,000), followed by detection using enhanced chemiluminescence.

Statistical analyses.

Data were analyzed in general linear models (PROC GLM, SAS version 9.4). Sputum cyanide was modeled as a function of Pseudomonas status, with lsmeans statement to generate least square means, standard error, and p-values for their differences. Lung function (FEV1) was modeled as a function of either apparent or corrected cyanide concentration. Data are presented using Graphpad Prism.

Results

Demographics*:*

Demographic data of the 12 subjects are summarized in **Table 1.** All sputum samples collected cultured positive for common CF microbes. Eight subjects cultured one or more strains of *P. aeruginosa*.

Effect of cobinamide on the detection of CN^-:

The CN^- electrode is known to respond non-specifically in RT secretions [8,9]. We first sought to use a simple model buffer system to establish whether cobinamide can be used to bind CN^- and

prevent its detection from the CN^- electrode. The structure of cobinamide is shown in **Fig 1A**, and illustrates the cobolt atom in the center of the molecule. Cobinamide is capable of forming complexes with two molecules of CN^- sequentially with very high affinity, as illustrated in **Fig 1C** [18]. As shown in **Fig 1B**, a linear standard curve is obtained with increasing amounts of CN^- (5 - 80 μM in 0.1 M NaOH) in the absence of cobinamide. With the addition of cobinamide (25μM), the electrode does not detect CN^- until concentrations exceed 50 μM. This confirms that cobinamide can bind 2 CN^- ions and the cobinamide – CN^- complex does not produce a reading from the electrode. When the stoichiometry exceeds 2:1, a linear electrode response is observed (**Fig 1B**).

Measurement of CN^- in sputum*:*

When measuring the sputum samples without the addition of cobinamide, we observed that there was an average CN^- concentration of 104.4 ± 10.4 μM (Mean ± SEM) (**Table 2**). To correct for non-specific interference of sputum components with the electrode, we repeated the measurements for each sputum sample in the presence of cobinamide. The electrode signal that was observed in the presence of cobinamide was considered non-specific detection due to inference. This value ('non-specific background') was then subtracted from the initially obtained measurement ('apparent') to provide a 'corrected' concentration value of CN^-. When using this approach, the average CN^- concentration was significantly lower at 37.0 ± 6.8 μM (Mean ± SEM, p-value ≤ 0.0001, paired t-test), a value nearly 3 times lower than the uncorrected (apparent) CN^- concentration average. The degree of sputum background interference also varied extremely widely between sputum samples (**Table 2 and Fig. 2**), indicating that the degree of non-specific interference is vastly different with sputum taken from individual patients.

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Cyanide concentration in sputum obtained from *P. aeruginosa* positive and negative patients*:*

Sputum from 8 patients cultured positive for *P. aeruginosa* and 4 subjects cultured negative for *P. aeruginosa*, but were positive for other microbes (**Table 1**). As shown in **Fig 3**, both *P. aeruginosa* positive and *P. aeruginosa* negative patients displayed significant levels of CN^-, both utilizing uncorrected electrode measurements (**Fig 3A**), as well as cobinamide corrected CN^- levels **(Fig 3B).** There were a general trend indicating decreased levels of CN^- in the *P. aeruginosa* negative sputum samples. Values of CN^- in the *P. aeruginosa* negative patients were 23.1 ± 11.1 μM whereas *P. aeruginosa* positive patients were 43.9 ± 7.8 μM **(Fig 3B).**

Correlation of FEV-1 (% predicted) with sputum CN^- concentrations*:*

In an attempt to determine whether our measured CN^- levels could reflect the severity of lung injury in CF patients, we plotted FEV-1 (% predicted) versus CN^- concentrations using both apparent and corrected measurement methods. As shown in **Fig 4**, when apparent (**A**) or corrected (**B**) concentrations of CN^- in sputa are plotted against FEV-1 (% predicted) it is clear that there is no statistically significant correlation.

Reaction of HOCl with CN^-: As myeloperidase (MPO)-mediated HOCl generation is known to be high in CF [19] we asked whether HOCl could alter the stability of CN^-. As shown in **Fig 5**, we found that HOCl reacts with and decreases CN^- in a near 1:1 stoichiometry. In parallel with the loss of CN^-, we observed the formation of cyanogen chloride (CNCl). However, quantitatively, we could only attribute approximately 35-40% of the lost CN^- to CNCl. When the ratio of HOCl:CN^- exceeded 1:1 stoichiometry, CNCl was completely consumed. The further reaction of CNCl with HOCl has been shown to result in the formation of cyanate (NCO^-) [20]. These data clearly indicate that in the presence of HOCl, CN^- is an unstable species and may be only transiently present in the highly inflamed CF RT, and thus may not be a biomarker that

adequately reflects the overall degree of RT bacterial CN^- production or lung function (i.e. FEV - 1, **Fig 4**).

Analysis of carbamoylated proteins in CF sputum:

We next determined whether carbamoylated proteins could be detected in CF sputum. As shown in **Fig 6B**, BSA incubated with NCO^-, but not unreacted native BSA, showed immunoreactivity in Western blots against antibodies that recognize carbamoylated proteins. When sputum proteins from adult CF patients were subjected to Western blot analysis using the same anti-carbamyl lysine antibodies, immunoreactive bands from 15-200 kD were detected (**Fig 6C**). Interestingly, CF sputum from *Pseudomonas*-positive patients showed more robust staining for carbamoylated proteins compared to a *Pseudomonas*-negative patient. Whereas a comprehensive quantitative assessment of carbamoylated protein in CF patient sputum was beyond the scope of this investigation, the data provide a proof of concept and illustrate the potential for carbamoylated proteins to serve as biomarkers of NCO^--dependent protein modification in the airway of CF patients.

Discussion

The present data confirms the findings of many others that RT secretions contains considerable levels of CN^- (14.1-98.1 μM), that a combined CN^- electrode/cobinamide methodology can conveniently be used as a simplified methodology for sputum total CN^- determinations and that the presence of CN^- is not specific for the presence of *Pseudomonas*. The rapid reactions of the CN^- with the myeloperoxidase derived oxidant HOCl indicate that CN^- is not likely to be stable species in the CF airway and is likely to be involved in the production of NCO^-, a compound capable of generating carbaml-lysine, homocitruline). Findings are

discussed from the perspective of technological advances and challenges, issues related to RT CN^- metabolism and chemical reactions as well as some pathobiological perspectives.

Technological advances and specificity of CN^- presence in infected CF patients:

The present methodology makes use of a commercially available cyanide ion-selective electrode and cobinamide, a substrate with a very high affinity for CN^-. Our data demonstrate that caution should be exercised in assuming that CN^- is the only substance detected by the electrode in the complex matrix of CF sputum. The method presented here utilizes cobinamide to correct for the non-specificity of the CN^- electrode.

Limitations of the currently described method include that the non-CN^- species detected by the electrode remain uncharacterized. Uncertainties also exist regarding the magnitude of the CN^- species remaining sequestered in non-solubilized portions of RT secretions after processing. Common ions at concentrations found in CF RT secretions (thiocyanate, nitrate and nitrite) were not detected by the CN^- electrode (data not shown). It is also likely that some limited amount of HCN escapes as a volatile gas prior to alkalization of the collected RT secretions. HCN has a pK_a of 9.3 and is detected in low concentrations in the collected breath of control non-CF subjects (≈ 5 ppb) [21] and in the breath of CF patients in higher concentrations (≈ 13.5 ppb) [22].

Is CN^- a specific marker of P. aeruginosa?

There are considerable discordant data in the literature related to the specificity of RT CF CN^- levels to reflect the presence of *P. aeruginosa*. Interestingly, one study reports that exhaled breath HCN levels in *P. aeruginosa* positive CF patients (~ 7.95 ppb) and in *P. aeruginosa* negative patients (~ 6.95 ppb) are not dramatically different [23]. Recently *Staphylococcus aureus*, a prominent CF pathogen, has also been shown to produce CN^- in both *S. aureus*

cultures and breath from *S. aureus* infected CF patients, albeit in lower quantities than CF patients infected with *P. aeruginosa* [24]. Whereas a previous study [8] concluded that CN^- was detected only in *P. aeruginosa* positive patients, our present study and those of others [9,24] reveal that CN^- is detected in CF patients who culture either positive or negative for the bacteria (**Fig 3**) and thus is not a specific marker for *P. aeruginosa*.

Mechanisms of airway CN^- formation:

There are multiple potential mechanisms (summarized in **Fig 7**) that may explain why *P. aeruginosa* negative patients still have significant quantities of CN^- in sputum and exhaled breath: 1) There are multiple other microorganisms that are cyanogenic and could contribute in the absence of *P. aeruginosa* [25]. For instance, in addition to *S. aureus, Burkholderia cepacia* appears to be cyanogenic under biofilm colonial growth conditions [26] although there are controversial data [27]. 2) The microbiome colonizing the CF lung is highly diverse [28] and they are only partially culturable in routine clinical assays; thus it could be reasonable to speculate that microbes previously not recognized as being cyanogenic may be participants in biosynthesis of CN^- in the CF airway. 3) There are multiple potential mechanisms for microorganism-independent formation of CN^- in the CF airway. It has been demonstrated that neutrophil-derived myeloperoxidase (MPO), which is highly abundant in the CF airway of all adult CF patients (even in *P. aeruginosa negative patients*) [19], is capable of producing CN^-. In fact, the reaction is analogous to some degree with that of the bacterial HNC synthase. MPO-derived hypochlorous acid (HOCl) is capable of reacting with glycine to form the *N*-dichloroglycine, which spontaneously rearranges to liberate HCN and CO_2 [29]; the same products formed by the HCN synthase enzyme of cyanogenic bacteria.

Analogous reactions have also been demonstrated with the amino acid serine undergoing reaction with HOCl [17]. Additionally, HOCl and/or *N*-chloramines can react with thiocyanate (SCN^-) [30, 31] or uric acid [32] to form CN^-. Both of these substrates are present in

high concentrations in CF sputum [33-35] and hence may be additional chemically-mediated pathways for the synthesis of CN^- in the inflamed CF airway. The contribution of MPO/HOCl to CN^- formation in the CF airway remains to be fully characterized and is worthy of further investigation. As shown in **Fig 7B** there are multiple potential biological and chemical potential pathways for the synthesis of CN^- in the CF RT.

Mechanisms of airway CN^- metabolism:

Similar to the multiple mechanisms of CN^- synthesis in the CF airway, a number of both enzymatic and chemical pathways for CN^- metabolism/catabolism potentially exist, as simplistically illustrated in **Fig 7A** (and in mechanistic detail in **Fig 7B**). The major CN^- metabolizing enzyme in most living organisms, rhodanese (thiosulfate cyanide sulfur transferase), is present in vertebrate lung mucosal tissues [36]. Rhodanese enzymatically converts CN^- into thiocyanate (SCN^-), which itself has been demonstrated to be present in CF airway fluids [33] and has the potential to alter a number of biological pathways including antimicrobial activity and modulating reactive oxidant pathways [37]. It is worth noting that there is considerable functional polymorphism of this major human CN^- detoxification enzyme. This should be taken into consideration when interpreting CN^- levels in RT secretions [38]. In addition, selected microbial species, including *P. aeruginosa*, express enzyme systems capable of metabolizing/detoxifying CN^- and utilizing CN^- for biosynthetic pathways [2, 39].

In addition to enzymatic pathways of CN^- metabolism, there also exist a number of potential chemical mechanisms for the catabolism of CN^-. First, CN^- reacts with MPO-derived HOCl at near-diffusion limited rates ($k=10^9\ M^{-1}s^{-1}$) [40] to produce CNCl as an intermediate, which is then further converted by HOCl to cyanate (NCO^-) [41]. Moreover, CN^- reacts with peroxidase-derived hypothiocyanous acid (HOSCN) to form thiocyanate (SCN^-) and NCO^- via intermediate formation of dicyanosulfide (NCSCN) [42]. The formation of NCO^- by these two independent pathways can lead to carbamoylation of proteins that could potentially be useful as

biomarkers of CN^- and could also impact protein structure and function [43, 44]. One final pathway of CN^- "metabolism" is the high affinity sequestration of CN^- by metalloproteins and metal complexes such as metal-containing siderophores [45].

Carbamoylation reactions in CF airways:

As illustrated in **Fig 7B**, metabolism of CN^-/HCN in the presence of the abundant MPO/HOCl in inflamed CF airways can be expected to yield cyanate (NCO^-), a potent carbamoylating species [20,46]. In fact, our data in **Fig 6** revealing the presence of carbamoylated proteins in CF sputum serves as a proof-of-concept for this pathway in the CF airway. Carbamoylated proteins are increasingly being identified from sites of chronic inflammatory processes, most notably in rheumatoid arthritis [47,48], asthma [44], kidney disease [4,46-51], atherosclerosis [43,49] and aging [50]. It is likely that protein carbamoylation results from the chemical and enzymatic metabolism of both CN^- and SCN^- (**Fig 7B**). The posttranslational carbamoylation of proteins is likely to not only serve as a marker for CN^-/SCN^-, but is also likely to alter the structure and function of proteins and potentially exert biological pro-inflammatory effects [44,46,51].

Biological implications of CN^- in the CF airway:

It is important to address potential hypotheses regarding the consequences of the increased CN^- produced by cyanogenic microbes (largely but not exclusively *P. aeruginosa*) in CF airways [2,8,9,18,52-54]. The fact that CN^- is produced only under hypoxic conditions suggests that the CN^- synthase is likely to be highly active in the adjunct mucus and host mucosal cell region of the microbial biofilm [2,55]. Like the case for the strong *P. aeruginosa* virulence factor pyocyanin [56], the CN^- can be expected to have multiple pathobiologic effects including those on respiration, metabolism and mucociliary clearance [57].

CN^- would be expected to augment the already hypoxic microenvironment of the biofilm thus having synergistic effects on mitochondrial reactive oxygen species activations of hypoxia-

induced transcription factor (HIF-1) and presumably impacting transcription nuclear factor erythroid 2-like2 (Nrf-2). Of interest, both HIF-1 activation and Nrf-2 activation have been reported to be protective against CN^- toxicity in select model systems [58,59]. Further insights will be needed to more fully understand the effects of airway CN^- concentrations of the magnitude being generated in CF airways on juxtapositioned cellular actors of signaling transduction networks.

A major understudied issue in the CF-related CN^- community relates to the levels of bioavailable CN^- in the complex matrix of CF secretions. Our study and those of others do not address this important consideration. For instance, it is not currently known how much of the CN^- in the CF airway is free vs. bound to metalloproteins. Thus, it is difficult to estimate the precise bioavailable CN^- levels impacting the biology of the extracellular and cellular host milieu in the proximity of the CN^- producing biofilms [60]. Depending on such factors as binding affinity, equilibrium kinetics and pH, sputa CN^- sequestrations are likely to depend on a wide spectrum of species including metalloproteins and metal complexes such as siderophores which are capable of binding with CN^- [45]. The presence of measurable amounts of HCN in breath validates the presence of sizable amounts of free CN^- in CF secretions [52-54].

Conclusions:

Bacterial cyanogenesis in the CF RT is likely to have broad-ranging implications related to not only the CF RT microbiome, but also to CF RT pathophysiology [2, 6]. Simplified and more accurate assay methods, such as the one presented here, and including newer clinic adapted breath analysis methodologies [2], should facilitate more in-depth studies of the significance of CN^- production and metabolism in the CF RT. The ratio of free bioavailable CN^- to sequestrated metal bound CN^- represents an important remaining challenge. Finally, therapeutic intervention strategies that decreases airway levels of CN^- (such as cobalamin/cobinamide or small

molecular inhibitors of bacterial HCN syntheses) should allow for clinical elucidation of the impact that CN^- plays in CF pathobiology.

Author contributions:

Conception and design: J.P.E., C.E.C.; Provided patient materials: B.M.M., C.E.C.; data analysis and interpretation: J.P.E., M.A.L.M., C.E.C.; manuscript preparation: J.P.E., S.P.O., M.A.L.M., C.E.C.; final revision and approval to be published as well as agreement to be accountable for all aspects of the work: J.P.E., S.P.O., T.K., B.M.M., K.A.H., M.A.L.M., C.E.C.

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Table 1. **Demographics of adult cystic fibrosis patients.** A: *Aspergillus*, Bcc: *Burkholderia cepecia* complex, MRSA: Methicillin – resistant *Staphylococcus aureus*, PA: *Pseudomonas aeruginosa*, SA: *Staphylococcus aureus*. Averages stated as average ± SD.

Subject	Age (years)	Sex	FEV_1 (% predicted)	Culture
1	25	M	28	PA, Bcc
2	51	F	91	SA, A
3	23	M	20	MRSA
4	43	M	47	PA
5	27	M	60	PA
6	40	M	55	PA, SA
7	44	M	18	Bcc, SA
8	44	F	35	PA, MRSA
9	26	F	30	PA, MRSA
10	20	F	58	SA
11	34	M	65	PA
12	26	M	35	PA
Average	**34 ± 10**		**45 ± 22**	

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Table 2. **Cyanide concentration detected in CF sputa.** CN^- was determined using an ISE. Apparent CN^- was the concentration of CN^- in sputum samples measured before cobinamide addition. Cobinamide was added to sputum samples to determine the background interference to calculate the corrected CN^- concentration. Values listed are the average ± SEM.

Subject	Apparent CN^- (μM)	Corrected CN^- (μM)	% Difference
1	133.5	21.1	632.7
2	74.8	18.2	411.0
3	68.8	44.7	153.9
4	49.7	30.0	165.7
5	105.7	32.3	327.2
6	179.1	98.1	182.6
7	80.6	14.1	571.6
8	112.4	25.9	434.0
9	113.0	44.4	254.5
10	78.3	15.7	498.7
11	147.1	58.8	250.2
12	109.2	41.0	266.3
Average	**104.4 ± 10.5**	**37.0 ± 6.8**	**345.7 ± 46.8**

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Figure 1. Cobinamide prevents the detection of CN^- by an Ion-Selective Electrode (ISE). **(A)** Structure of dihydroxocobinamide **(B)** Simplified structure of cobinamide indicating the location of cyanide binding and illustrating cobinamide binds CN^- with a 2:1 stoichiometry. **(C)** Standard curves of CN^- detection with the ISE in the absence (- Cobinamide) and presence (+ Cobinamide). ISE response (mV) to cyanide (5 - 80 μM) with (+ Cobinamide) and without (- Cobinamide) 25 μM cobinamide in 0.1 M NaOH. In the presence of cobinamide, the ISE only detects CN^- once the concentration exceeds the 2:1 binding stoichiometry, and then a linear detection curve is obtained. Thus, cobinamide is an effective method of removing cyanide from ISE detection.

Figure 2. **Utilization of cobinamide to correct for non-specific measurements of the CN^- electrode in CF sputum.** Homogenized sputum samples from CF patients (1:1 w/v PBS, 10 μM phosphate, 154 μM NaCl, pH 7.4) were brought to a concentration of 0.1 M NaOH. Using an ISE electrode apparent CN^- was the concentration of CN^- in sputum samples measured before cobinamide addition. Cobinamide was added to sputum samples (100 μM final concentration) to determine the background interference and calculate the corrected CN^- levels. Lines indicate individual CF patient sputum samples.

Figure 3. **Cyanide concentration in sputum between *P. aeruginosa*-positive and *P. aeruginosa*-negative CF patients.** Shown are data obtained directly from the CN^- electrode measurements (A) and data utilizing the corrected method presented herein (B). Neither sets of data are statistically significant (apparent measurement, $p = 0.049$; corrected measurement, $p = 0.158$). However, *P. aeruginosa* negative patients showed a trend of lower CN^- concentrations by both methods.

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Figure 4. Cyanide (CN^-) concentration in sputum does not correlate with lung function (FEV_1) in CF patients. Assessment of CN^- concentration using either (A) apparent CN^- concentrations or (B) corrected CN^- concentrations does not reveal a statistically significant correlation between CN^- concentration and lung function as assessed by FEV_1 (% predicted). Statistical p-values and R^2-values are provided in each figure panel.

Figure 5. Hypochlorous acid (HOCl)-dependent oxidative consumption of CN^-. Reactions of HOCl (0-200 μM) with CN^- (100 μM) were conducted in phosphate-buffered solutions (50 μM, pH 7.4) at room temperature. Quantification of CN^- and cyanogen chloride (CNCl) were performed 10 min after initiation of the reaction as described in the Materials and Methods section. CN^- was stoichiometrically consumed by HOCl to form CNCl, maximally at a 1:1 ratio. As the concentration of HOCl was increased beyond the 1:1 ratio with CN^-, CNCl concentrations were decreased and non-existent at a ratio of 2:1 (HOCl/CN^-), suggesting the formation of other chemical species, most likely cyanate (NCO^-).

Figure 6. Detection of carbamoylated proteins in CF sputum. (A) Schematic illustrating that cyanate (NCO^-) reacts with protein lysine residues to form carbamyl-lysine (homocitrulline). (B) Western blot indicating that bovine serum albumin (BSA) reacted with cyanate (NCO-), but not native BSA, is recognized by an antibody against protein carbamyl-lysine. (C) Western blot illustrating that proteins in CF sputum harbor carbamyl-lysine post-translational modifications. CF sputum from *Pseudomonas*-positive and *Pseudomonas*-negative patients are noted by (+) and (-), respectively. Western blots are representative of multiple experiments.

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Figure 7. Summary of the various potential biochemical pathways for the synthesis and metabolism of CN^- in the CF airway. (A) General scheme illustrating synthesis and metabolism of CN^-. Multiple chemical/biochemical pathways are potentially involved in the synthesis and metabolism/fate of CN^- in the CF airway and which illustrate its' dynamic and transient nature. (B) Detailed mechanistic scheme illustrating pathways involved in the synthesis and metabolism of CN^-. Pathways of CN^- synthesis: (1) Oxidative biosynthesis of CN^- by cyanogenic bacteria via the HCN Synthase enzyme, utilizing the amino acid glycine as a substrate. [2] (2) HOCl-dependent chlorination of glycine. [16,29] (3) HOCl-dependent chlorination of serine. [17] (4) HOCl- and *N*-chloramine-dependent oxidation of thiocyanate (SCN^-). [30,31] (5) HOCl-dependent chlorination/oxidation of uric acid. [30] (6) Metabolism of CN^- to thiocyanate (SCN^-) by both host and microbial enzymatic systems (ie. rhodanese, thiosulfate sulfurtransferase). [25,36-39] (7) Microbial utilization of CN^- as carbon and nitrogen source for biosynthesis. [39] (8-10) HOCl-dependent conversion of CN^- into CNCl and NCO^-, and its utilization in protein carbamoylation reactions. [41,43] (11) Reactions hypothiocyanous acid (HOSCN) with CN^- to form NCO^-, and its utilization in protein carbamoylation reactions. [42,43] (12) Sequestration of CN^- by high-affinity interactions with metaloproteins and/or metal complexes. [45]

HIGHLIGHTS

- **Ion-Specific electrode and cobinamide used to quantify CN- in CF sputum**
- **Sputum CN- levels do not correlate with infection or lung function**
- **Hypochlorous acid rapidly degrades CN- into cyanogen chloride and cyanate**
- **CF respiratory tract biosynthetic and catabolic pathways of CN- reviewed**
- **Carbomoylated proteins are present in CF sputum.**

Figure 1
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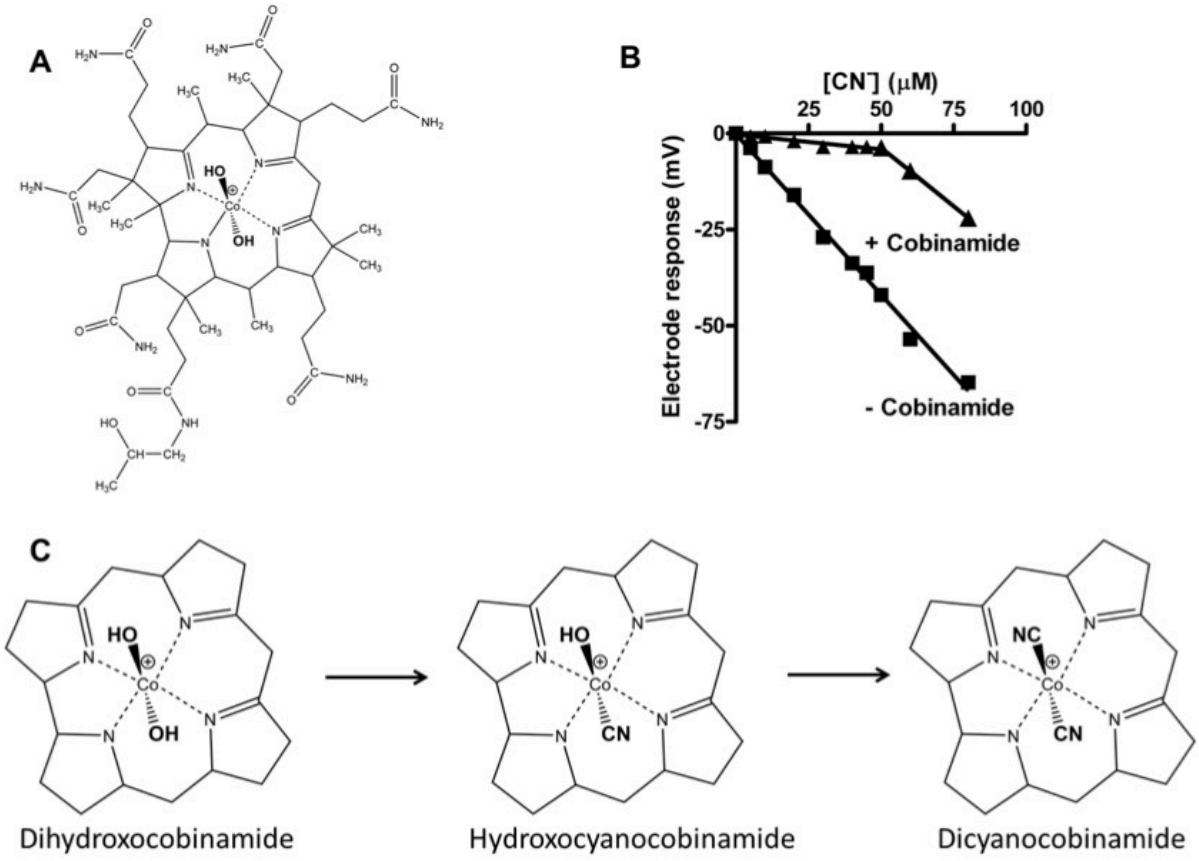

Figure 2
Eiserich *et al.*

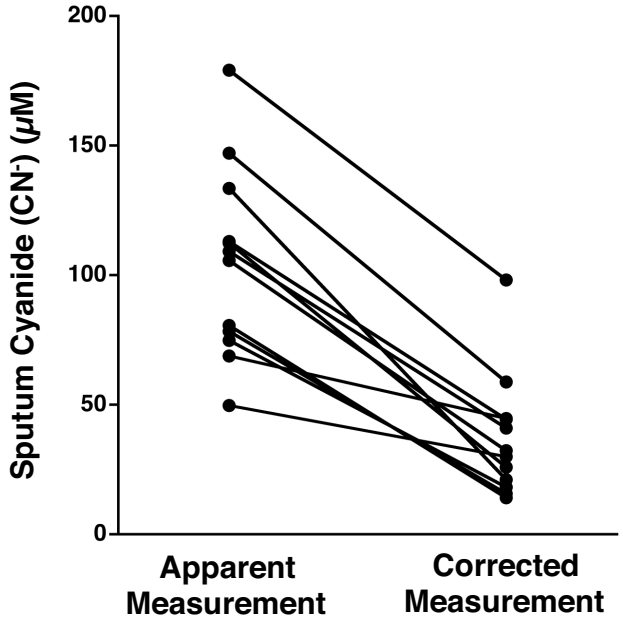

Figure 3
Eiserich *et al.*

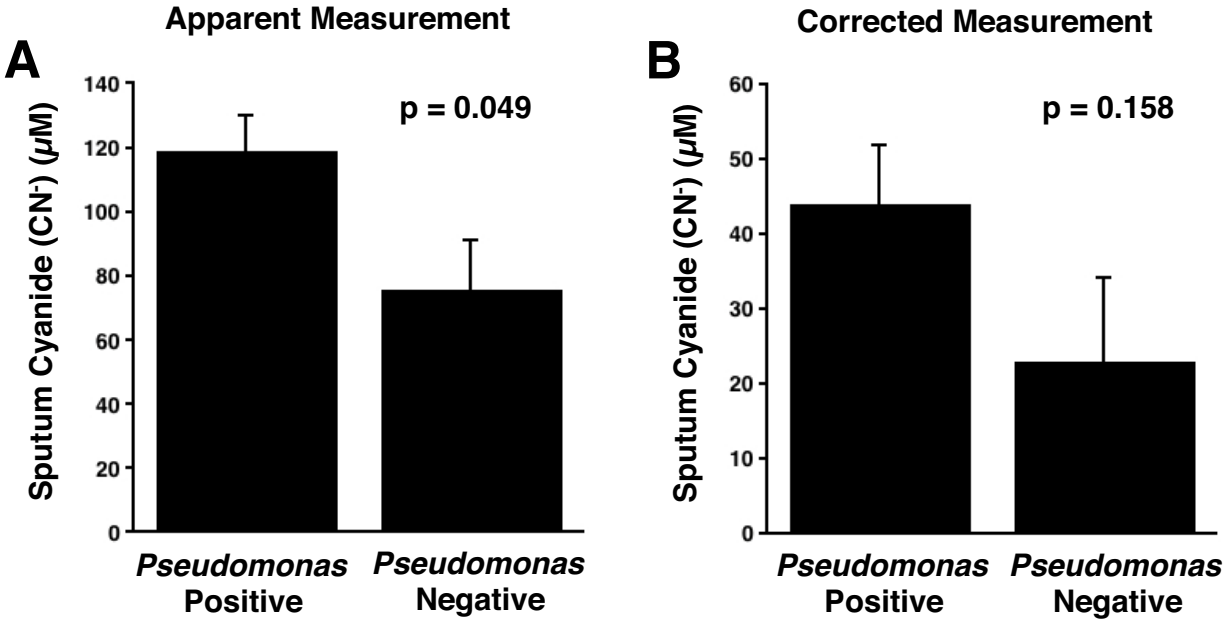

Figure 4
Eiserich *et al.*

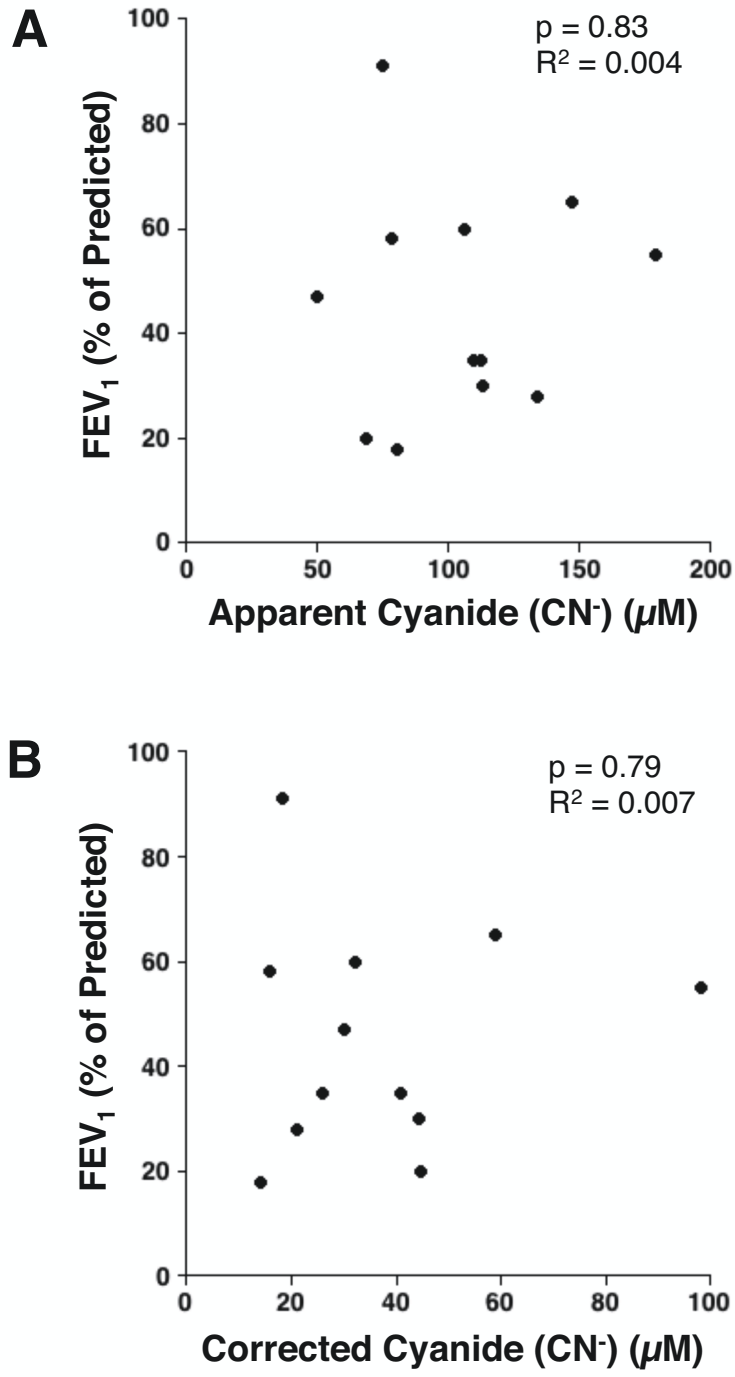

Figure 5
Eiserich *et al.*

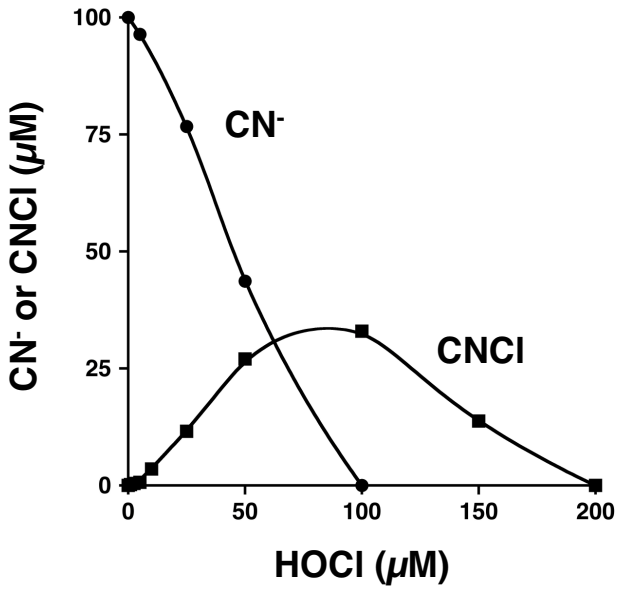

Figure 6
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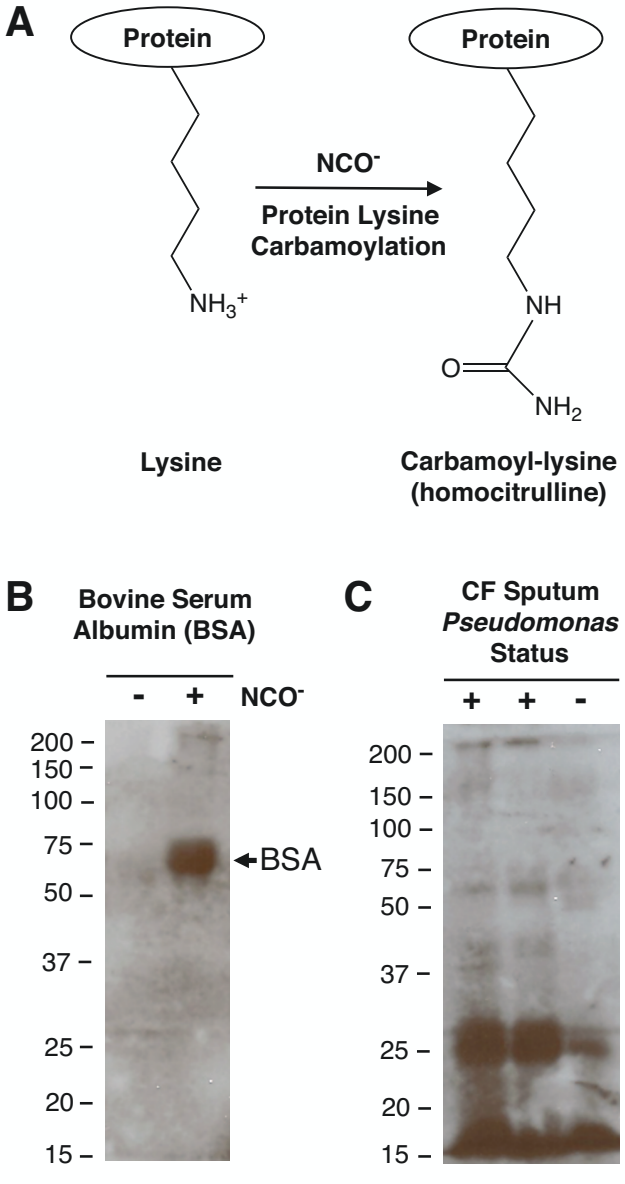

Figure 7A
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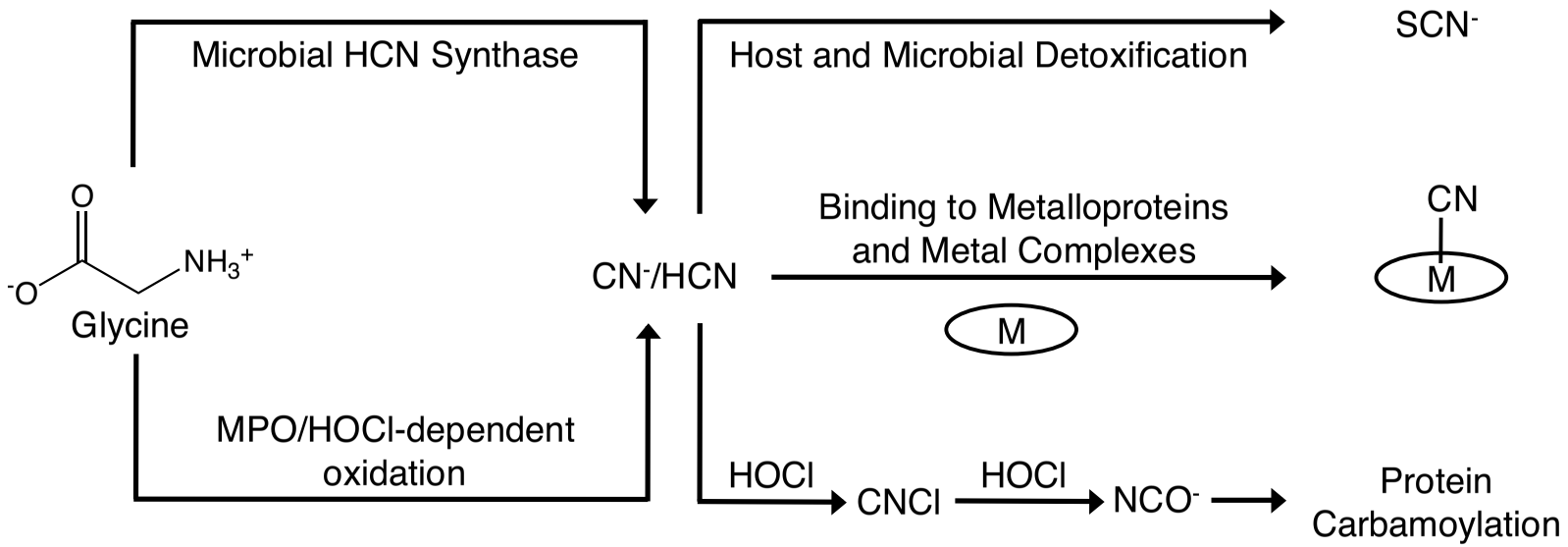

Figure 7B - Eiserich et al.