# Murburn concept explains the acutely lethal effect of cyanide

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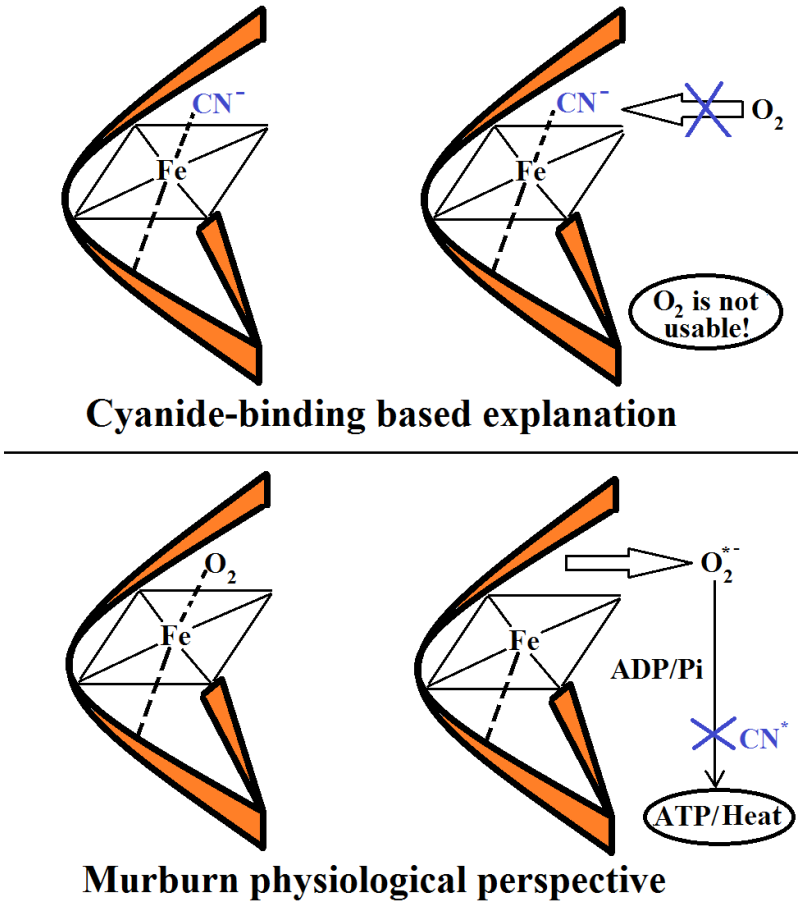
**Abstract**: Cyanide (CN) is long seen as a binder of heme-Fe centers, disrupting mitochondrial cytochrome oxidase function and oxygen transport by hemoglobin. Recently, a diffusible reactive oxygen species (DROS) mediated reaction mechanism called murburn concept was proposed to explain mitochondrial ATP-synthesis and heat generation. In this purview, CN ion-radical equilibrium dissipates the vital DROS into futile cycles, producing water. In the current study, a comparative/quantitative assessment of the two explanations is made for- (i) lethal dosage or concentrations of CN, (ii) thermodynamics and kinetics of the binding/reaction, and (iii) correlation of CN with the binding data and reaction chemistry of $H_2S$/CO. The quantitative findings suggest that the hemeFe binding-based toxicity explanation is untenable. The acute lethal effect of CN on cellular respiration is explained by the catalytic interaction of CN ion-radical equilibrium with DROS in matrix. Experimental modalities and predictable outcomes are further detailed to validate the new theory.

**Keywords:** cyanide-poisoning, murburn concept, cellular respiration, ATP-synthesis, hemoglobin, cytochrome oxidase, mitochondria, reactive oxygen species (ROS).

## Introduction

Cyanide caught human attention three centuries back, with the synthesis/discovery of the dye Prussian blue (ferric ferrocyanide, $Fe_7[CN]_{18}$) and prussic acid (hydrogen cyanide, HCN). Owing to its lethality, cyanide salts and acid (hereon termed CN) have been regulated by governments. Therefore, it has captured the imagination of common-folk and erudite alike, across cultures and continents. Though CN-based lethality is well studied, the exact reason for cell/organism mortality is not yet nailed. The traditional view for explaining CN-toxicity entails a binding-based mechanism wherein important heme-Fe centers are blocked by a highly efficient cyanide ligation.[1-2] Recently, an oxygen-centric mechanism called murburn (from 'mured burning') concept was proposed to explain the physiology of xenobiotic metabolism and cellular respiration.[3-7] The new proposal states that diffusible reactive oxygen species (DROS) generated at the mitochondrial complexes directly serve as the coupling agents of oxidative phosphorylation. It was proposed that cyanide ion-radical mediated catalysis, and not cyanide binding to heme (as currently held), is the primary rationale for cyanide lethality. This manuscript critically explores the theoretical and quantitative premises of the two explanations (as shown in Figure 1) for cyanide-induced acute toxicity.

***Figure 1. A scheme of the two mechanistic explanations for the toxicity of cyanide:*** *In the textbook explanation, CN ligates to heme-center, thereby preventing oxygen's accessibility, transport or reduction thereafter. This is a 1:1 reaction outcome, resulting out of a physical binding. In the new hypothesis, cyanide (radical) reacts with the catalytic DROS generated/stabilized at heme-center, thereby disrupting ATP-synthesis. This is a mechanism wherein cyanide acts as a recyclable catalyst.*



## Materials and Methods

The available literature was analyzed for the variables/parameters involved in heme-binding by small molecules/ions. Thereafter, the manuscript presents the quantitative aspects of stoichiometric, thermodynamic, and kinetic parameters involved for binding-based explanation of various gases with hemoglobin (Hb) and cytochrome oxidase (Cox or Complex IV). Further, the chemical and theoretical premises that account for the acute molecular toxicity of cyanide are detailed and further experimental modalities to verify the predictions are listed.

## Results & Analyses

***Dosage calculations based on stoichiometric CN binding:*** Following is the dosage analysis of cyanide, based on human blood's oxygen-transporting capacity (A) and Cox's oxygen-binding ability (B).

**A**. Blood's oxygen transporting capacity: Each RBC contains ~$10^9$ CN-binding sites (= 2.5 x $10^8$ Hb x 4 sites per Hb) and since one liter of blood contains ~ 5 x $10^{12}$ RBC, 5 liters of blood in a human has ~ 2.5 x $10^{22}$ binding sites of CN. As blood levels of ~3 mg/L cyanide is accepted to be lethal, the threshold blood load is 15 mg of cyanide.[8] Since 26 g (ionic mass) of cyanide contains 6.023 x $10^{23}$ cyanide ions, 15 mg contains [(6.023 x $10^{23}$) x (15/26)/1000] = 3.5 x $10^{20}$ cyanide ions. Granting the best-case scenario of 100 % irreversible binding efficiency, the CN lethal load is only a minute fraction (1.4 %) of the total binding sites in blood. This leads to the inference that cyanide-lethality is not brought about by the disruption of Hb-$O_2$ transport.

**B.** CN-binding to the heme-center of Cox: An average human has >$10^{13}$ cells. Considering ~$10^7$ Cox per cell (= $10^4$ Complex IV per mitochondrion x ~$10^3$ mitochondria per cell),[9] the estimate for the total Cox binding sites is ~$10^{20}$. Across several organisms/animals (including humans), KCN is lethal when administered (orally) at ~1 mg/Kg.[10] Assuming complete absorption and equi-distribution within a body mass (of equal density as water), the toxic physiological concentration of CN comes to ≤ $10^{-6}$ M (mg/Kg or mg/L). *In vivo* or *in situ*, the actual and effective concentration of CN would be far lower than what is administered, owing to loss through non-absorption (in the gut) and metabolism/excretion (by say, kidneys). This means that the ~$10^{20}$ CN present in the mg/Kg administration (calculated in point A above) would be inadequate for lethality even if the binding to Cox is 100 % irreversible and efficient.

***CN binding in physiological regimes:*** However, cyanide-binding to heme-centers is neither irreversible nor efficient in physiological realms. The thermodynamic equilibrium dissociation constant $K_d$ is defined as the concentration of CN at which half of the total available heme-centers is populated by the ligand (CN). Usually, when micromolar levels of protein is taken, at least 10X $K_d$ concentration of ligand is minimally required to get >90% of the total active sites occupied. Even in such conditions, binding is via a non-covalent and coordination scheme of 'off-now, on-now' kind of interaction, and it is not an 'always bound' scenario.[11] As measured by non-invasive spectroscopic assays in pure solutions, cyanide binding to micromolar levels of hemeproteins show $K_d$ values ~$10^{-3}$ M.[12-14] In particular, the affinity-based $K_d$ of cyanide for Cox approaches the mM ranges (quite similar to that of oxygen) and cyanide has relatively low affinities for the oxidized form of Cox.[14] Further, binding of small species to membrane-bound proteins with deep-seated active sites would be relatively slow, owing to constraints imposed by diffusion.[13] Such a reality translates to the conservative requirement of g/Kg or g/L dosage administration for achieving Cox hemeFe binding-based lethal effects (confirming the calculations in point B above). Thus, binding-based calculations fall short of explaining the theoretically required dosage outcomes by several orders of magnitude. [A counter-argument could be made (regarding the dosage and binding calculations made above) that cyanide is not necessarily distributed across the whole body, and therefore, higher concentration could be effectively presented in some vital

tissues/organs. This is countered by the fact not all of the cyanide presented is assimilated by the body and therefore, the calculations are only a conservative approximate, with benefit of doubt given to the binding-based explanation. Also, if a small amount of cyanide is injected (say, in the toe), then it should only render the foot as "reversibly dead". However, the whole animal dies rather quickly (and irreversibly!). Further, cyanide is acutely lethal when presented at very low dosages via various modalities (breathing, oral, contact, injection, etc.) to the vast majority of life forms of diverse masses and volume/surface area ratios. Most importantly, cyanide brings functional inhibition of certain heme enzymes' activities even in simple *in vitro* setups at micromolar levels.[12]]

***Assessment of the competitive process of physiological binding of low concentrations of CN:*** Physiologically, at the lethal cyanide concentration of ≤ $10^{-6}$ M, the symmetric diatomic oxygen molecule should out-compete the asymmetric triatomic HCN or diatomic ionic cyanide for binding Cox. This is because at the lethal CN concentration of ~ 1 µM, the hydrophobic oxygen is present at >$10^{-4}$ M in the aqueous milieu and oxygen is almost five times more soluble in the membrane milieu than in water. Experimentally, when 1 µM Cox is taken along with $10^{-4}$ M cyanide, and then aerated buffer is mixed in a stopped flow apparatus, one may not really note any spectral changes in Cox heme within seconds.[15] This is because at these "unrealistically high cyanide" scenarios, Fe-CN binding may in fact prevail! Such an observation cannot ratify the physiological competing ability of low doses of CN vis a vis oxygen. When high levels of the efficient ligand CO bound to blood haemoglobin is easily reversed (by aerating the intoxicated patients), the competitive binding efficiency of CN would only be lower. Further, the ligation of cyanide to heme-Fe finally results via the carbon atom of the $CN^-$ anion. It is known that the $pK_a$ of HCN is 9.4, two logarithmic units higher than the physiological pH. Even if an active site amino acid residue can aid HCN binding, this function would be efficient in pH range closer to the $pK_a$ of HCN. This implies that only a small fraction the low amounts of CN presented would effectively bind to Fe-centers at physiological regimes. This analysis further cripples the binding-based explanation for the acute toxicity of cyanide.

***Comparison of equilibrium and kinetic constants of CN with other small toxic heme ligands like CO and $H_2S$:*** The heme-ligand interactions for Hb or Cox interaction with oxygen can be theorized as a minimal scheme of two steps 1 and 2, as given in Box 1. The superscript of $^*$ and $^\pm$ connote a change in charge/electronic states from the initial state. For example, heme can have Fe(II) or Fe(III) states and the ligand could be $O_2$ or $O_2^{*-}$, respectively. The original heme concentration is [Heme], the formed complex is [Heme-L] and the free ligand in milieu is [L]. Then, for [Heme] < [L] (that is, the ligand in milieu is in far excess of the hemeprotein) and assuming a 1:1 stoichiometry of Heme:L binding (a single ligand binding site per hemeprotein), the laws of equilibrium and steady-state approximations afford the correlations (the famous Hill / Michaelis-Menten / Cheng-Prusoff equations) given in Box 1. The italicized small letter *k* is the pertinent rate constant (with units of $s^{-1}$ or $M^{-1}$ $S^{-1}$, depending on whether the reaction is first order or second order) whereas the capital K is the relevant equilibrium constant (in M). The forward reaction rate constants are $k_1$ and $k_2$ whereas the backward rate constants are $k_{-1}$ and $k_{-2}$ (the latter, $k_{-2}$, is deemed irrelevant for the current discussion now). The first step is the equilibrium interaction characterizing the $K_d$ (dissociation constant) or $K_s$ (association constant). The second step is a chemical reaction, which plays a significant factor in the determination of the value of the functional interaction constant $K_M$.

Now, from literature, it is evident that the functional $K_M$ of oxygen for cytochrome oxidase is ~0.3 mM, which is several orders lower than the experimental $K_d$ of 0.1 to 1.0 µM![16-18] From Box 1, it can be seen that this is a theoretical impossibility (as $K_M = K_d + k_2/k_1$). That is, $K_M$ can only be equal to or greater than $K_d$. This simple quantitative analysis dictates that "oxygen remains bound at heme-active site of Cox to

make water" is not a functional catalytic mechanism for aerobic respiration. In the various hemeperoxiases and mixed oxidase cytochrome P450 systems, similar prevailing fallacies were pointed out and the kinetic/mechanistic phenomena were explained with murburn concept.[3,4,12,13,20,21]

***Box 1: The scheme for interactive equilibrium and kinetic constants for heme-ligand binding.***

$$L^{\pm} + Heme^{\pm} \overset{1}{\leftrightarrow} Heme\text{-}L \overset{2}{\leftrightarrow} Heme^{*} \sim L^{*}$$

$$[Heme\text{-}L] = ([Heme] \times [L]) / (K_d + [L])$$

$$K_d = k_{-1}/k_1 = 1/K_s$$
$$K_M = (k_{-1} + k_2) / k_1 = K_d + (k_2/k_1)$$

If $k_{-1} >> k_2$ OR $(k_2/k_1) \approx 0$ OR $k_1 >> k_2$, then
$$K_M \approx K_d$$

If [Heme-L] = ½ [Heme], then
$$K_M = [L]$$

If I is a competitive inhibitor at step 1, then
$$K_{Mapp} = K_M \{1 + ([I]/K_i)\}$$
$$IC_{50} = K_i \{1 + ([L]/K_M)\}$$

If $[L] << K_M$, then
$$IC_{50} \approx K_i$$

Regardless of the proven "non-applicability of binding-based" kinetics (as seen from the murburn perspective), let us analyze the affinity based equilibrium constants for Cox hemeFe. Table 1 is a compilation of original experimental data from various reliable sources, along with some calculated $K_d$ values ($K_d = k_{-1} / k_1$) for corroboration.[14,22-27] The practical determination of the backward reaction rate constant $k_{-1}$ is fraught with errors (particularly more so for Cox!) and therefore, it is not given high importance here.

***Table 1: The equilibrium constants and the forward binding rate constants for Cox.***[14,22-27]

| Constants | $O_2$ | CO (Comp.) | HCN (Non-comp.) | $H_2S$ (Non-comp.) |
|---|---|---|---|---|
| **Exp. $K_d$ [M]** | $2.8 \times 10^{-4}$ | $4 \times 10^{-7}$ | $5.5 \times 10^{-4}$ | $4 \times 10^{-5}$ |
| **$K_d = (k_{-1} / k_1)$ [M]** | ($2.1 \times 10^{-4}$) | ($1.7 \times 10^{-7}$) | ($5 \times 10^{-7}$) | ($4 \times 10^{-8}$) |
| **$K_M$ or $K_i$ [M]** | $<1 \times 10^{-7}$ | $3 \times 10^{-7}$ | $2 \times 10^{-7}$ | $2 \times 10^{-7}$ |
| **$IC_{50}$ [M]** | NA | $4 \times 10^{-5}$ | $5 \times 10^{-5}$ | $5.3 \times 10^{-5}$ |
| **$k_1$ [$M^{-1}$ $s^{-1}$]** | $1 \times 10^{8}$ | $8 \times 10^{4}$ | $1.3 \times 10^{2}$ | $1.5 \times 10^{4}$ |

It is known that ferro-porphyrin hemes demonstrate similar binding profiles for diatomic ligands that induce a strong field splitting of the Fe atom's *d*-orbitals, like CO and CN. Both CN and CO are sigma-donors and pi-acceptors. The way a ligand binds, i.e. front on or side on, depends on the steric hindrances in the active site and on the molecular orbitals' overlaps. The diatomic gas of CO is

isoelectronic to oxygen but more soluble, and is known to be a much stronger ligand of ferrohemes (than oxygen). The longer Fe-C lengths in Fe-CN complexes indicate that CN is a poorer pi-acceptor than CO. Thus, it is an accepted scientific consensus that the Fe-CN ligation is weaker than Fe-CO bonding, for most heme-Fe systems. In fact, CO can effectively replace CN in several ferro-porphyrin complexes.[28] The triatomic gas $H_2S$ is symmetric, smaller and more hydrophobic than HCN, making it readily accessible across biological lipid membranes. Further, $H_2S$ has a $pK_a$ of 7.04, whereby significant amounts of $SH^-$ is also present in aqueous physiological milieu. Now, glancing across the experimental equilibrium binding constants of the four gases for Cox (the first data row of Table 1), it is noted that only CO has a markedly higher association with the respiratory protein. The other three gases have comparable affinities. When the equilibrium binding $K_d$ is extrapolated for the toxic gases from the kinetic constants listed by Cooper & Brown,[11] $H_2S$ is marked out to be the major inhibitor. This is an inference which has very little physiological relevance because it is a known fact that cyanide is a far more potent toxic principle than CO or $H_2S$. (The error-prone determination of $k_{-1}$ is seen here as the calculated $K_d$ for the non-competitive inhibitors do not agree with the experimental $K_d$.) Further, $H_2S$ & CO present identical $K_i$ and $IC_{50}$ as CN. The natural questions to be asked are- How can the binding-based explanations help us understand the toxicities? If the binding/interaction constants are comparable, then why is cyanide immensely more toxic? If we take the forward kinetics-based data alone independently, then also $H_2S$ and CO should be more potent toxic principles for aerobic organisms, which we know is not the case. Most animals can take in several breathes of $H_2S$ and CO without getting significantly affected whereas a few whiffs of HCN would knock them off. Exposure to CO is common place in modern life, and the physiologic background is ~1% CO-Hb in normal people (per point A, this approaches the equivalent load of cyanide needed to kill a human!). Even ~ 10 % CO-Hb does not cause any appreciable short term adverse effects in humans. Only ~50 % CO-Hb leads to a loss of consciousness and >80 % CO-Hb is acutely fatal.[29] Now, the fallacy of the binding-based explanation is clearly evident. Since CO (or $H_2S$) is a better binder, it should be more toxic, which is not the physiological case! Therefore, by comparing with the gaseous ligands of CO and $H_2S$, it is beyond reasonable doubt that the old binding-based hypothesis is unviable for explaining the disruptive efficacy of cyanide. Other traditional explanations that lead to specific organ or tissue failures and death thereafter stem from the molecular mechanisms related to events that follow hemeFe-CN binding. Therefore, it is concluded that heme-Fe binding-based outcomes cannot explain the acute physiological toxicity of CN.

**Discussion**

***Explaining the reaction chemistry at Complex IV and the effect of toxic gases:*** While the mechanism of toxicity of CO is understood to be related to decrease in energy levels and change in redox state owing to metabolic acidosis,[30] the acutely toxic effect of small doses of CN is rather unknown. The research in this area is fraught with ethical issues and want of literature. Only conjectures exist that the CN toxicity results owing to its ability to bind Cox. Unlike CO, however, hypoxia is not the reason for the lethal toxic effects of CN.[30]

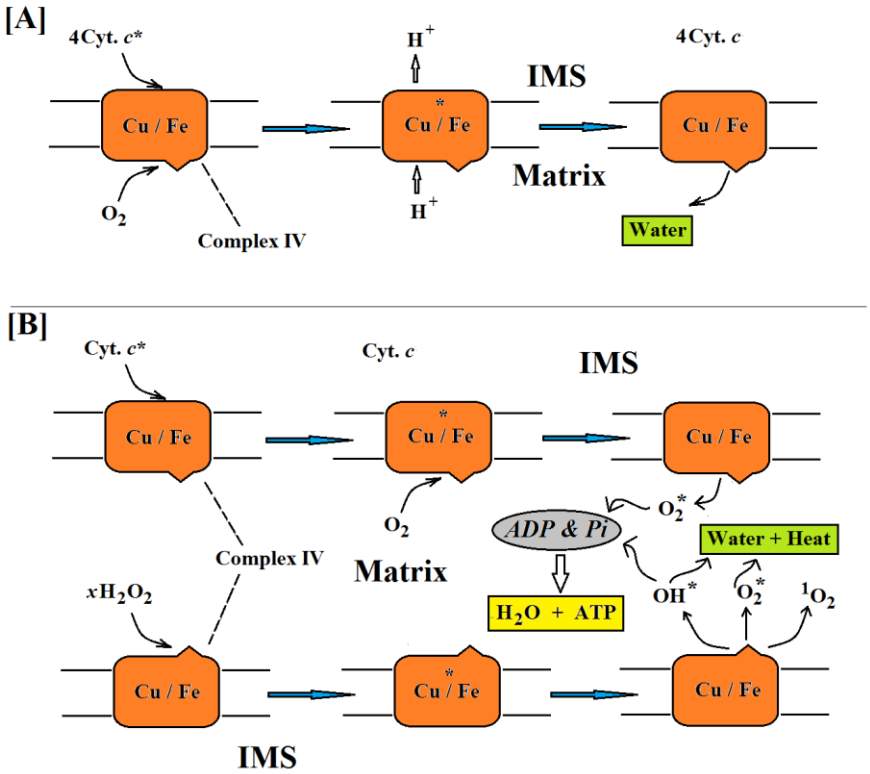
Wharton and Gibson had compared the oxidation of *Pseudomonad* cytochrome oxidase heme by $O_2$ and CO binding processes.[15] They found that the on rate was $5.7 \times 10^4$ $M^{-1}$ $s^{-1}$ for $O_2$, thrice the rate for CO (in similar ranges). The off rate for $O_2$ was less than four folds the same for CO (once again, in similar ranges). Other researchers also noted similar results.[15] Therefore, unless the protein's active site provides great distinction abilities to choose amongst the ligands, stark demarcations are not expected. Since there is little evolutionary pressure on Complex IV or hemoglobin to preferentially bind cyanide, the biological outcome observed with these ligands must be explained differently. If binding-based effects were the determinants, then the greater toxicity of CN (when compared to CO or $H_2S$) cannot be

explained. On the other hand, if the proteins evolved for a fast binding of oxygen/DROS and efficient utilization thereafter, the equilibrium and kinetic constants of Cox with $O_2$ would make better physiological sense. Further, if the toxicity of HCN was associated with its metabolism, then we could have a suitable explanation.

In our recent works with (sub)micromolar levels of heme-enzymes and several toxic small molecules and ions (N-heterocyclics, cyanide/azide, etc.), we had shown that the latter are more likely to act as pseudo-substrates in milieu.[12,13,31-33] This implies that only at higher heme:ligand ratios and high enzyme concentrations, an ion like cyanide serves as an efficient active-site ligand. The *in situ* or *in vitro* assays show a low functional $IC_{50}$ or pseudo-$K_i$ or pseudo-$K_M$ (~$10^{-6}$ M) for certain enzyme reactions, [12,13,31-33] which cannot be explained by binding-based effects and which captures the essence of physiological toxicities. We have also provided several evidence based arguments that DROS generated *in milieu* are obligatorily involved and utilized in several heme-enzyme catalyzed specific/selective reactions. It was recently proposed that DROS are ultimately and obligatorily required as catalytic agents for metabolic activities and electron transfer (not just as molecular messengers); particularly for ATP-synthesis and heat generation in mitochondria.[3-7,13,32,33] Now, the thought lines that the toxicity difference must primarily lie in the reaction/interaction dynamics with DROS makes better rationale, particularly under the light of the structure-function correlations of mitochondrial proteins. For, the mitochondrial respiratory proteins (Complexes I through IV) show ADP binding sites, $O_2$ accessible redox centers/channels, and the ability to produce DROS.[6] Therefore, the relevant scheme for various protagonists' interaction with Cox is projected in Figure 2. In the physiological schemes (at micromolar levels of cyanide), Cox (or other respiratory proteins) are rather unperturbed in their activity for directly interacting with $O_2$/DROS. (It can also be seen from Table 1 that $k_{on}$ is the highest for $O_2$!)

We have repeatedly cautioned that binding-based conventional kinetic/equilibrium constants do not explain reaction outcomes and are not of immense utility in many heme enzyme systems that work via murburn concept. [3,4,12,13,20,21,32-34] This is because the kinetic outcome is a reflection of the diffusible intermediates' reaction with the final substrate. It is in this final step where CN comes to play, wherein it interrupts the DROS-aided synthesis of ATP within the matrix (shown in yellow box). In the physiological scenarios, the role of small amounts of cyanide is proposed in the reaction scheme of Box 2. As seen, CN can effectively inhibit DROS utilization, forming non-productive two-electron sinks (peroxide or water). In short, within the old binding-based purview, low amount of CN is a **SUICIDE INHIBITOR**, getting consumed in the process. In the new murburn explanation, low levels of CN (radical) can effectively serve as a **CATALYTIC INHIBITOR**, which does not get consumed. ***Further, CN is only involved with Complex IV in the erstwhile perception, whereas the murburn concept vouches that CN inhibits the ATP synthesis mediated by DROS at/around the respiratory Complexes I-IV. Therefore, CN is a global and potent inhibitor of mitochondrial ATP synthesis.***[6] At low levels (≤ $10^{-6}$ M), via fast reactions occurring in the matrix, "cyanide anion-cyanyl radical" equilibrium effectively dissipates the functional DROS. With latency and at higher cyanide levels, clinical cyanosis could result. This is due to the production of the basic cyanate ion, which increases pH, enabling cyanide ion ligation to heme-Fe. (Systemic lactic acidosis could result thereafter, owing to higher order cellular machinery's efforts to generate energy currency via anoxic modality.) Therefore, death and cyanosis owes more to the production of cyanide radical and not owing to the original binding affinity of cyanide ion or HCN, per se. In heme-enzyme systems, EPR spectroscopy has evidenced the presence of cyanide radicals and the formation of cyanate (from cyanide) has also been reported.[35,36] Further, the lower toxicity of $H_2S$ is explained by the new proposal because of the facile oxidation of $H_2S$ to $SO_2$ (which could be further oxidized to sulphate, as sulfur moieties are efficient nucleophiles) via a radical process that still leaves catalytically useful DROS in milieu (Box 1).

***Figure 2: Complex IV's proposed interaction with cytochrome c, oxygen and peroxide: [A]*** *In the erstwhile mechanism, oxygen merely bound to Complex IV to get reduced by four electrons from four cytochrome c (Cyt. c) molecules and pump out protons on the way to making water at the active site.* ***[B]*** *In the new scheme as shown, interaction of both Cyt. c and peroxide is to aid a one-electron scheme within the matrix. The DROS produced attack the ADP (bound on the enzyme) and Pi in milieu to make ATP. The DROS that do not get to react with ADP/Pi react among themselves to make water and heat. A small amount of water formation is possible to be formed via Complex IV, but copper may be present to prevent this and to facilitate singlet oxygen formation (which can better activate flavins). Details of stoichiometry, electron and proton involvement are not shown. [IMS: inter-membrane space]*




***Box 2: Reaction-based inhibitory roles of HCN and $H_2S$ in physiological/mitochondrial milieu.***

| ***At low levels, HCN acts as a catalyst, dissipating DROS via futile cycles.*** | ***At higher levels, HCN reacts to give cyanate, also setting in a pseudo-chain reaction, raising pH and enabling CN-binding.*** | ***$H_2S$ is easily oxidized, while leaving behind useful DROS and forming products that sink the mobile electrons as pairs.*** |
|---|---|---|
| $HCN + OH^* \rightarrow CN^* + H_2O$<br>$CN^* + O_2^{*-} \rightarrow CN^- + O_2$<br>---<br>$\mathbf{OH^* + O_2^{*-} \rightarrow H_2O + O_2}$ | $HCN + O_2^{*-} \rightarrow CN^* + HO_2^-$<br>$2HCN + 2OH^* \rightarrow 2CN^* + 2OH^- + 2H^+$<br>$2CN^* \rightarrow (CN)_2$<br>$(CN)_2 + 2OH^- + 2H^+ \rightarrow HCNO + HCN + H_2O$<br>---<br>$\mathbf{2HCN + O_2^{*-} + 2OH^* \rightarrow CNO^- + CN^* + H_2O + H_2O_2}$ | $H_2S + O_2^{*-} \rightarrow SH^* + HO_2^-$<br>$H_2S + OH^* \rightarrow SH^* + H_2O$<br>$2SH^* + 2O_2 \rightarrow 2OH^* + 2SO$<br>$2SO + 2O_2 \rightarrow 2SO_2 + O_2$<br>---<br>$\mathbf{2H_2S + O_2^{*-} + 3O_2 \rightarrow 2SO_2 + OH^* + H_2O + HO_2^-}$ |

The new proposal is supported by the fact that HCN and $H_2S$ are metabolized in animal and plant systems.[37] So, the lethal dose and acute toxicity predicted by the DROS-chemistry scheme would be CN > CO > $H_2S$, which is in fact the physiological case. Very importantly, unlike CO, CN and $H_2S$ do not qualify as competitive inhibitors and are seen/known as non-competitive inhibitors[11]. The $K_d$ of HCN and $H_2S$ are are much higher than their $K_i$, which is an inexplicable theoretical premise. Further, non-competitive inhibition is one in which the inhibitor is supposed to bind at an alternative site, other than the substrate. In the current scenario, there is little scope to envisage for an "allosteric binding" based conformation or mechanistic change. Such an evolutionary pressure does not exist. This is yet another solid support for the DROS-based metabolic reaction mechanism that occurs outside the active site. ***Very importantly, DROS-radical reactions are fast, with rate constant k values of $10^8$ to $10^{10}$ $M^{-1}$ $s^{-1}$ (about six orders higher than CN's heme-binding rates!),[7] which could potentially explain the rapid/acute toxicity of cyanide (radical). The rate constant $k_{on}$ for cyanide interaction with hemeproteins falls in the consensus of only $10^2$ to $10^3$ $M^{-1}$ $s^{-1}$. Multiplying this second order constant with sub-micromolar concentrations of CN gives a process with rates of <$10^{-3}$ $s^{-1}$. That is, the binding-based activity should take at least tens of minutes to hours (depending on the variables involved), to result in cell/organism death. Therefore, such a slow and reversible binding of CN cannot explain the fast/acute lethality of cyanide that results in seconds to minutes. Radical formation and their reactions in mitochondria are thermodynamically facile.[6] Further the low $K_M$ for $O_2$ and low $K_i$ for the inhibitors HCN and $H_2S$ are the result of practically diffusion limited and zeroth order reaction of DROS with the various ions and molecules (that serve as pseudo-substrates).***

**Predictability and verifiability of the hypotheses:** We have experimentally and theoretically consolidated the idea already that DROS are obligatorily required in cellular metabolic schemes. We have also shown that DROS-modulating species can have potentially high impact in heme/flavin-enzyme systems' reaction outcomes and that these effects are mediated via one-electron (radical) chemistry. [3-7,12,13,19-21,31-34,38-41] The following experiments are suggested to further ratify the proposals/deductions of the current work on the impact of cyanide on cellular respiration and toxicity.

* In respiring cells or mitochondrial suspension, (sub)micromolar levels of cyanide should be presented and consumption of oxygen and formation of DROS noted. While the binding-based perspective projects cell death and cessation of respiration, the new proposal predicts oxygen consumption leading to DROS formation (albeit at low levels).

* The catalytic role of cyanide radical/DROS can be traced by the incorporation of radio-labeled carbon in cyanide or radio-labeled oxygen. After exposing an experimental system to the molecular probes, the radio-labeled cyanate formation can be traced to confirm the radical pathway of CN/oxygen interaction. On the other hand, if CN binding to heme is efficient and irreversible, cyanate should not be formed.

* Similarly, if the efficient competitive ligand CO bound that effectively, one should not have DROS production and thereafter, a dissipation reaction of the equation scheme- $\mathbf{2CO + OH^* + O_2^{*-} \rightarrow HCO_3^- + CO_2}$. Confirmation of the radical chemistry can be done with $H_2S$ and CO, by tracing the radio-labeling of C/S and O atom within $SO_2$/sulfate and $CO_2$/carbonate, respectively. It is expected that while $H_2S$ would be highly metabolized, CO would be much lesser converted (as it is a better binder and poorer substrate). This could also be because the Hb in the blood would take up significant amounts of CO, and they do not have mitochondria to metabolize via DROS. (This would also explain the evolutionary reason for the higher affinities of Hb for CO, in comparison to Cox.) But per the murburn perspective, a

mitochondrial suspension would surely oxidize CO to $CO_2$ and carbonate. Else, we cannot explain the reversal of toxicity in CO-exposed animals and individuals.

* Per the erstwhile purview, since cyanide has no bearing with Complexes I, II, & III (and since the purported ETC works without push or pull),[34] these proteins should assist Complex V produce ATP via the "proton-based machinery", if the old mechanisms were operative. So, at $K_d$ (mM) concentrations of cyanide taken experimentally, only 50% of Complex IV is 'bound by cyanide'. That would still leave 1:1-2:2-3:2-3:4-6:5-9 ratio of the complexes (I:II:III:IV:V:Cyt.*c*) in the mitochondrial suspension, and only a minimal or stoichiometric loss of activity should be noted.[5,9] The new scheme predicts an overwhelming loss (practical cessation) in the ATP production ability of the system.

* A 'cyanide-blocked' system could be subjected to a favorable external pH gradient (say, 6.5 out to 9.5 in), quite similar to the demonstration by Mitchell's group or a rhodopsin assisted ATP-synthesis via Racker-Stoeckinius type setups.[42,43] While the erstwhile hypothesis does not seek inhibitions, the new perspective advocates cessation of fresh ATP synthesis.

* In any experimental system above, at concentrations around or lesser than nM levels of cyanide, murburn concept may result in an interesting observation that ATP synthesis could even be enhanced by a small fraction (say, 5 to 10%). (An example was seen in the Cayman Chemicals brochure.[27]) This outcome is not expected in the erstwhile view.

**Conclusion**

The CN-Fe binding-based explanations fail short for explaining the dosage regimes. Also, thermodynamics/kinetics of hemeFe-CN binding-based outcomes do not account for the physiological toxicity. Further, the comparison with $H_2S$ and CO is also instrumental, leading to the inference that a more fundamental and directional catalytic reaction logic (and not a localized/isolated reversible effect) is the likely causation for the acute toxicity of cyanide. It is now an established idea that unusual *in vitro*, *in situ* and physiological dose responses are seen in heme-enzyme systems. These are mediated by low concentrations of diverse additives (like redox active small molecules, vitamins, phenolics, N-heterocyclics, azide, etc.), via the formation of catalytic radicals.[20] Quite analogously, it is proposed that acute lethality of cyanide results due to an ion-radical equilibrium/reaction chemistry. Lethality can be defined as a status where a cell "fails to do the works needed to sustain life". ATP is the known chemical powering molecule of the cell, and in the absence of DROS-assisted synthesis of ATP (resulting due to CN mediated catalysis), Complex V's high ATPase activity depletes the cellular reserves of energy. As a result, even micromolar level of cyanide is lethal within seconds. The ideas proposed herein may potentially aid cyanide-poisoning therapy and also qualify research efforts in redox biochemistry/medicine.

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