Discussion Letter

Pathobiochemistry of CO poisoning*

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A hypothesis for the pathobiochemical mechanism of CO poisoning, amenable to in vivo testing with optical reflectance spectrophotometry, is presented. It differs from the classical formulation in which loss of cytochrome *c* oxidase function is attributed entirely to O_2 depletion, by including ligation and oxygenation of CO as essential components of the inhibitory process.

CO toxicity; Cytochrome *c* oxidase; Haldane's laws

1. INTRODUCTION

Except for two recent refinements, the intravascular component of J.S. Haldane's hypothesis [1] has withstood the test of time. Thin-layer optical measurements [2] have shown that the carboxyhemoglobin (HbCO) and oxyhemoglobin (HbO_2) saturation curves are not parallel (see also [3,4]) and that binding of CO to Hb is more cooperative than for O_2. Thus, Haldane's laws $\{\%HbCO/\%HbO_2 = M$ (the Haldane constant) $\times p_{CO}/p_{O2}$ and $\%HbCO + \%HbO_2 = f(p_{O2} + M \times p_{CO})\}$ are valid only at total saturation (T = number of occupied binding sites = 4). Quantitation of incompletely saturated forms present during ligation of CO and O_2 to Hb [5] shows that the CO-induced left shift of the HbO_2 dissociation curve (seen but not explained by Haldane) is due to bound CO promoting O_2 ligation at low partial pressures of O_2. Although some controversy [6–9] surrounds the idea that the CO-induced left shift and funcational anemia (the latter a result of the slow dissociation of CO from HbCO [10]) produce cellular hypoxia, the weight of evidence favors this viewpoint. In contradistinction to Haldane, we believe that CO can bind to tissue cytochromes during CO intoxication, but do not subscribe the idea that this reaction is the sole reason for its toxicity.

J.B.S. Haldane was the first to provide evidence for an extravascular CO-binding site [11] when he observed that rats with virtually 100% HbCO could live on dissolved (hyperbaric) O_2, but died if CO (not N_2) was then added. This finding was confirmed by Drabkin et al. [12] and Goldbaum et al. [13,14], who found that increasing the HbCO by non-inhalatory means to 80% was non-lethal. Supporters of Haldane's thesis have attempted to discredit these data, but we believe their objections [15,16] lack compulsion. While anoxia prior to mixing inhaled CO throughout body CO stores [15] may have contributed to the cause of death in Goldbaum's control group, it does not explain why his experimental group (with levels of 65–80% HbCO maintained for at least 2 h) failed to succumb. Goldbaum's explanation for his data (the pulmonary exhalation of dissolved CO *prevents* its entry into the arterial circulation) was rejected [15], but this was based on an erroneous extrapolation of in vitro kinetic data [17] (neither the time frame nor the reactant concentrations match conditions in vivo (see also [18,19])). Another report [16] asserted that 70% HbCO was always non-lethal because: (i) animals given CO by peritoneal infusion survived indefinitely at 70% HbCO; and (ii) physiological parameters in anaesthetized animals that received CO by inhalation or infusion were equivalent for 150 min. The problem is that the maximum %HbCO monitored in the latter pair of experiments was only 60%. Given these numerical imprecisions, it is imperative that HbCO levels of up to 80% be examed before discarding Goldbaum's ideas.

2. A POSSIBLE DIFFERENCE BETWEEN INHALED AND INFUSED CO

Although as yet unproven, Goldbaum's hypothesis does serve as a useful vehicle to introduce the reader to some of the basic concepts surrounding current views on the mechanism of CO toxicity. A corollary of the

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hypothesis is that when CO uptake is by *inhalation*, blood in the pulmonary veins will contain more dissolved CO (CO not bound to Hb) than when uptake is by *infusion*. We will consider a situation in which 80% HbCO is attained over the course of several hours by inhalation of 0.24% CO in air or by periodic peritoneal infusion of 200 ml CO/kg [20]. Based on the %CO in the inhaled gas (and given that the amount of dissolved CO this is in equilibrium which is readily removed from an *infused* sample during a single passage through the lungs), the difference in dissolved CO between *inhaled* and *infused* samples is 1.98 μM (total dissolved O_2 is 141 μM for both).

Such a difference in CO levels could arise if during exhalation of *infused* CO there was insufficient time to achieve equilibrium between HbCO, dissolved CO and gaseous CO. This represents no violation of the laws of thermodynamics because it is a kinetic process arising from the combination of a relatively rapid passage of the erythrocyte through the pulmonary capillary bed with a slow release of CO from HbCO, an outwardly directed CO gradient and a requirement for Henry's law to be satisfied. When CO is inhaled, equilibrium between solution and gas phase CO is more nearly approached, but even here equilibration is imperfect [21–24]. The difference in CO levels could be maintained till the capillary beds of the heart and brain were reached, if after the blood left the capillary-alveolar interface there was no dissociation of CO from HbCO. Diffusion of CO into a gas phase [25] is impossible and given the rate constants for ligation of CO to Hb [6] ($k_{off}/k_{on} = 0.25\ s^{-1}/2.4\times10^7\ M^{-1}\ s^{-1}$) plus the short time for travel from the pulmonary vein to the coronary (< 1 s) or cerebral (3–5 s) arteries [26], there is good reason to believe that the CO level will not be changed.

3. THE *R* CONSTANT PROBLEM

But can 2 μM CO bind to cytochrome *c* oxidase (CcO) in vivo, if the intravascular $[O_2] = 141\ \mu M$? Proponents frequently quote the observation of Chance et al. [27] that the response (cytochrome *c* oxidation) of isolated mitochondria to a 17 μM O_2 pulse was 'greatly impaired' by just 100 ppm of gas phase CO (equivalent to 82 nM dissolved CO; CO/O_2 ratio = 0.0048). Aside from the fact that the response was not quantitated, the experimental protocol itself (preincubation of mitochondria with CO followed by addition of O_2) lessens the force of the finding, since with [CcO] = 275 nM [28] and K_d CO = 460 nM [29], 15% of the oxidase molecules would be CO-bound prior to the O_2 pulse.

The justification for the opposing viewpoint [30] is even less compelling. It has been claimed that since tissue CO/O_2 ratios are at the most 0.1, an oxidase *R* constant (K_d CO/K_d O_2 or the CO/O_2 ratio at either 50% ligation or inhibition of function) of 5–15, precludes CO inhibiting CcO in vivo. This proposition has at least three problems associated with it, e.g., the possibility of tissue O_2 gradients [31]; functional anaerobiosis facilitating CO ligation by CcO; and finally no measurement of the CcO K_d O_2 at 37°C. A value of 500 μM obtained at −91°C [32] was influenced by concomitant complexation of the active site copper with CO, while a *calculated* value of 0.4 nM at 25°C [29] reflects the rapid reduction of bound O_2 by reduced cytochrome a_3/Cu a_3. The latter is more correctly termed a K_m and in the discussion which follows use of this parameter to express oxygen affinity implies the presence of sufficient reducing equivalents to rapidly regenerate species that competently bind O_2.

As the first part of a plan to answer the question posed at the start of this section, rapid mixing experiments at different CO/O_2 ratios would be used to determine the initial rate of cytochrome *c* oxidation in isolated murine or canine brain mitochondria, followed by a series of steady-state measurements of the O_2 consumption rate and spectral form of cytochromes *b*, c_1, *c* and aa_3 with the various CO/O_2 ratios *held constant* during the course of the measurement. Along with appropriate controls using O_2 alone, studies like these would provide an in vitro data base for the in vivo studies to be elaborated on shortly. The in vitro experiments will permit all variables of consequence to be measured, which is critical because one of the problems in vivo is determining the intracellular O_2 and hence the CcO K_m O_2, i.e., how does one differentiate a K_m O_2 of 6 μM with an $[O_2]$ of 10 μM from a K_m O_2 of 0.6 μM with an $[O_2]$ of 1 μM? In vitro criteria can be established which will permit an in vivo determination, e.g., since $K_m = TN/4k_1$ (TN = turnover number; k_1 = second-order rate constant for the O_2 reaction with CcO [33]), the CcO kinetic profile (at different initial $[O_2]$ and/or [CcO]) during the transition from a constant O_2 flux steady-state to anaerobiosis can be used.

4. IN VIVO EVIDENCE OF AN INTRACELLULAR EFFECT OF CO

The brain is a good place to look for in vivo evidence of an inhibitory effect of CO on tissue cytochromes, since it lacks CO buffers (like myoglobin and cytochrome P-450). Indeed, it has long been claimed that as little as 3–5% HbCO significantly impairs performance of audiovisual discrimination tasks [34]. A more direct approach has been to probe the intact cortex with reflectance spectrophotometry, and two recent observations are of interest.

The first is that during spontaneous respiration on room air supplemented with 5% CO_2 a substantial fraction of reduced CcO (20%) was present in Hb-perfused cortex [35], which oxidized when the O_2 content was increased to 95%. With the presumption that 5% CO_2 eliminates perfusion effects, it was proposed that in vivo there was a subset of enzyme molecules which had

a higher K_m for O_2 than found in isolated mitochondria. However, it should be remembered that, although the level of oxidase reduction during in vitro State 3 respiration is typically 3–4%, under certain circumstances it can be as high as 20% [36,37]. The second finding of note concerns the response of the respiratory chain of fluorocarbon-perfused brain to inhalation of 1–5% CO in O_2 [38,39]. Ligation of CO to cytochrome a_2, reduction of cytochrome *b* and oxidation of cytochromes c_1, *c* and *a* were reported. The data were interpreted with a dual oxidase conformer model, but we have another explanation.

The best evidence to date indicates that there is no electron flow after ligation of CO to CcO (with or without CO oxygenation) [40] and thus we have discussed previously, the most likely reason for the redox state of respiratory chain components other than cytochrome a_3 is a mitochondrial branching reaction. With respect to the terminal cytochrome, when both CO and O_2 are present the spectral form of the oxidase will be dictated by the CO/O_2 ratio – a high ratio favors the ferrous carbonyl form, while a low one favors an oxidized species. At intermediate ratios, the CO-oxygenating form will be present [40–44]. Interestingly, the α-band characteristics of the in vitro, half-reduced, CO-oxygenating species [40] (as in the resting enzyme – low intensity with a broad maximum at 595–600 nm) are compatible with the features of a species which could be present during CO exposure in vivo, where the α-band difference spectrum of 90% O_2 + 1% CO vs 100% O_2 is essentially flat. The Soret spectrum of the in vitro species is unknown, but a related moiety, the *aerobic CO* complex of Nicholls [45], has one which differs little from that of the oxidized enzyme. While it could be argued that there is no effect of 1% CO, the level of cytochrome *b* reduction is increased in its presence. We do not claim that only CO-oxygenating complexes are found under these conditions and in fact more than one form of the enzyme is probably present.

The liquid phase CO/O_2 ratio in the in vitro spectroscopy study was 12, but CO oxygenation has been observed at a ratio of 3. On the basis of prior studies [40–45] and the analysis in sections 5 and 6, we believe that it will occur at ratios less than 3, although at present we cannot say what the lower limit is. The intravascular CO/O_2 ratio in the in vivo study was 0.008, but the intracellular ratio was not determined and is expected to be much higher than this (see below).

5. THE PROBLEM OF IN VIVO CO/O_2 RATIOS

We and others [1,5–8,15–22] believe that both the O_2-carrying and O_2-delivery capabilities of Hb are significantly diminished in the presence of CO, i.e., ligation of CO to Hb in vivo perturbs O_2 homeostatis to such an extent that the concentration of dissolved O_2 in the intracellular compartment is markedly different from that found at the same $\%HbO_2$ but with no CO present. As previously discussed, in vivo tissue p_{O2} measurements must be interpreted with caution, but suffice it to say that with continuum values of 5–40 mmHg [30] found intracellularly under normoxic conditions (arterial p_{O2} of 100 mmHg = 141 μgM O_2) a rapid lowering of the O_2 concentration occurs when the gas diffuses out of the capillary erythrocytes. During simple hypoxia, if the supply of reducing equivalents is adequate, O_2 turnover can be maintained by an increase in the blood flow. If an 'O_2-consumption buffer' existed, then even the effects of superimposed hypotension could be temporarily ameliorated, i.e., if hypotension reduced the time-dependent O_2 concentration by 50%, a recruitment of resting electron transport chains (the buffer) could maintain a basal O_2 consumption rate of 200/s by doubling the number of active units and increasing O_2 extraction. The lowest p_{O2} satisfying this basal requirement is synonymous with the so-called critical p_{O2}, i.e., that p_{O2} below which O_2 turnover is dependent on the p_{O2} – the 'critical threshold' [46].

If CO is present in the inspired gas, the anemia and most importantly the left shift of the HbO_2 dissociation curve make tissue hypoxia worse. Furthermore the tissue CO should theoretically continue to increase until its *free* concentration reaches the level dictated by Henry's law. For CO to be a competitor of O_2 consumption by CcO, a set of conditions must exist where the intracellular $[O_2]$ is low enough that 'significant cell damage' (vide infra) occurs only because the perimitochondrial CO/O_2 ratio is high enough for a quantitative, *competitive* binding of CO to CcO. If Goldbaum is correct, this same degree of hypoxia without CO will not seriously affect the cell.

Fictitious 'normoxic' CO/O_2 ratios of 0.035 to 0.28 can be calculated from the intracellular p_{O2} measurements mentioned previously. However, because of its effect on tissue p_{O2} we believe that ratios significantly greater than 0.28 will be present during hypoxia induced by CO. Proof of this requires accurate in vivo measurements of intracellular CO/O_2 ratios and O_2 concentrations which can be obtained using strategies like those employed in the in vitro studies, e.g., reflectance spectrophotometry would measure spectral and 'activity' parameters as a function of the intravascular O_2 level with and without CO. Then using the in vitro data set, one could compute the *effective* K_m O_2, CO/O_2 ratio and $[O_2]$ and also determine, vis à vis the Goldbaum-Haldane controversy, the extent of the similarity between hypoxic and carbonmonoxic (both inhaled and infused) hypoxia.

6. THE PR COEFFICIENT

Table I lists the results of a detailed quantitative assessment of our ideas using a series of O_2 'equili-

Table I

	K_mO_2[a]	CcO	O_2	CcO·O_2[b]	CcO·O_2[c]	PR	CcO·O_2	CcO·CO
1	0.1	0.275	10	0.272	0.272	0.025	0.268	0.007
2	0.7	0.275	10	0.257	0.257	0.175	0.234	0.041
3	6.0	0.275	10	0.172	0.171	1.50	0.110	0.165
4	0.1	0.275	1.0	0.250	0.243	0.25	0.220	0.055
5	0.7	0.275	1.0	0.162	0.151	1.75	0.100	0.175
6	6.0	0.275	1.0	0.039	0.038	15.0	0.017	0.258
7	0.1	2.75	10	2.72	2.71	0.025	2.68	0.07
8	0.7	2.75	10	2.57	2.51	0.175	2.34	0.41
9	6.0	2.75	10	1.72	1.60	1.50	1.10	1.65
10	0.1	2.75	1.0	2.50	0.95	0.25	2.20	0.55
11	0.7	2.75	1.0	1.62	0.74	1.75	1.00	1.75
12	6.0	2.75	1.0	0.39	0.29	15.0	0.170	2.58
	K_dCO	CcO	CO	CcO·CO				
13	0.4	0.275	1.0	0.196	-	-	-	-
14	0.4	0.275	0.1	0.055	-	-	-	-

[a] Except for the PR coefficient all entries in the table are in μM
[b] $[O_2]$ or [CO] is the free amount
[c] $[O_2]$ is the total amount

brium' (see section 3) calculations with and without CO. The PR coefficient (a normalized ratio of the perimitochondrial CO/O_2 ratio (P) to the oxidase R value (R)) has been used to determine whether CO will bind to CcO in the presence of O_2. A 50% level of inhibition (identical to the 'lethal threshold' [46]) has been selected as being sufficient to cause 'significant cell damage' (vide supra). With reservations concerning the data of Gothert et al. [47] (CO oxygenation was discounted), we have lowered the perimitochondrial [CO] to 1 μM. It has been assumed that the K_d CO (an average of four determinations [29,48-50]) has the same value (0.4 μM) in vivo as in vitro. Current in vitro measurements of the K_m O_2 range from 0.1 to 0.6 μM in uncoupled mitochondria [51,52] and from 0.7 to 6 μM in coupled mitochondria and intact cells [47,52-54] (associated critical p_{O2} values of 1-20 mmHg). Although it is a crude approximation, the effect of a transition from normotension to hypotension has been modelled by letting the $[O_2]$ represent *free* and *total* perimitochondrial $[O_2]$, respectively. The choices of a 50% inhibition of CcO oxido-reductase activity as the lethal threshold and the values for various components of the PR coefficient, while reasonable, are nonetheless somewhat arbitrary. The proposed experimental protocl can ascertain the truth or fiction of the estimates. Nothing will be said concerning functional consequences of violating critical or lethal thresholds. This is a complicated problem to address, due in part at least to the ability of the cell to reset priorities when its energy-producing apparatus is stressed.

We note the following: (i) During 'hypotensive hypoxia' if the K_m O_2 is 0.1 μM and the $[O_2] = 1.0$ μM, significant amounts of reduced CcO appear only in regions of relatively high CcO concentration (entry 10; entry 11 for K_m $O_2 = 0.7$ μM). (ii) If the K_m O_2 is 0.1 μM, there is no significant competition between 1 and 10 μM O_2 and 1 μM CO. (iii) If the K_m O_2 is 0.7 μM, entry 5 shows that 1 μM CO can act as a competitive inhibitor of 1 μM O_2, i.e., without CO there is 59% oxygenation while in its presence this decrease to 36% (should Gothert [47] be incorrect, the values would be 59% and 22%, respectively). (iv) Entries 6 and 12 show a 'deoxy CcO effect' in which non-competitive CO binding occurs because the K_m O_2 is so low compared to the K_m O_2 (as is true for comparable calculations with Hb, the PR coefficient somewhat overestimates %CcO·CO or %CcO·O_2 when the amount of deoxy-CcO is significant).

These observations can be applied to specific questions raised by our discussion as follows: (i) The distribution of 'poisoned' CcO species may vary from region to region within a particular organ as well as being organ-dependent per se. (ii) The effect of CO is solely one of hypoxia if the K_m O_2 is 0.1 μM but the tissue O_2 must decrease to a very low level (0.1 μM) before the lethal threshold is crossed. (iii) When the K_m O_2 is 0.7 μM, an O_2 concentration of 1.0 μM is non-lethal unless CO is present. This shows that Goldbaum's hypothesis is tenable at the molecular level. (iv) Our analysis indicates that when K_m $O_2 = 0.7$ μM, the mechanism of CO toxicity depends on the CO concentration, e.g. below 0.4 μM CO (44% HbCO or 58% HbCO if Gothert [47] is correct) hypoxia predominates, while above this concentration competitive inhibition of O_2 consumption occurs (CO oxygenation can take place anytime CO and O_2 are present together but is most efficient when CO is bound to CcO prior to O_2 [32,44, 45,55]). As the CcO K_m O_2 increases, the %HbCO at which the transition occurs, diminishes.

7. SUMMARY AND CONCLUSIONS

Our purpose has been not only to question dogma but to provide a testable alternative. We have examined the chemical-biochemical basis of the two current theories and believe that the ideas presented here support the alternative hypothesis that the toxicity of CO can be due not only to cellular O_2 starvation but also to ligation of CO by CcO (with or without CO oxygenation). Recent developments from the laboratories of Chance [56] and Jobsis-VanderVleit [57] should facilitate examination of CcO in the intact, Hb-perfused brain, since further investigation is needed if the debate is to be resolved. The technique of in vivo reflectance spectrophotometry can assess what merit our interpretation of the in vivo spectrophotometric studies has and determine more precisely the role of CO oxygenation in acute and chronic CO poisoning. We have previously presented the case for the latter [40] and though preliminary, both in vitro [40] and in vivo [58] evidence for the former exists, e.g., it appears that the rate of CO oxygenation in mitochondria and respiring humans is much faster than in the isolated enzyme (2/min per aa_3).

In closing, we would like to mention several medical implications of our hypothesis. We assert that the %HbCO remains the single most accurate and reliable indicator of the severity of acute inhalation of CO. Carbon monoxide is a toxic gas and, although it can be metabolized by man, this is done so at a cost, e.g., paresis of ATP production. Therefore, currently mandated environmental levels of exposure should not be tampered with. Finally, it seems fair to state that, whatever future experimentation tells us about the precise mechanisms of CO toxicity, the current treatment of severe acute exogenous CO poisoning (controlled ventilation with hyperbaric O_2) could be improved by incorporating exchange transfusions with O_2-saturated blood.

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