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The blood transfer conductance for CO and NO

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ABSTRACT

Nitric oxide was introduced over 30 years ago as a test gas for alveolar capillary diffusion. As for CO its transfer has been interpreted according to the Roughton Forster relationship: $1/DL = 1/DM + 1/\theta Vc$. There has been disagreement, since the first measurements of DLNO, over whether θ_{NO} is infinite and thus DLNO = DMNO. There is overwhelming *in vitro* evidence that θ_{NO} is finite yet several groups (Coffman et al., 2017; Tamhane et al., 2001) use an infinite value *in vivo*. They also assume that DMNO is greater than twice DMCO, making DMCO less than that predicted by the physical laws of diffusion. Finally some (Coffman et al., 2017) recommend use of Reeve and Park's value for θ_{CO} (Reeves and Park, 1992; Coffman et al., 2017) rather than Forster's (Forster, 1987). Their grounds for doing so are that the combination of an infinite theta NO, an empirical value for DMNO/DMCO (>2.0) and Reeve and Park's θ_{CO} gives a value of DMCO (using a combined DLNO–DLCO analysis) which agrees with the DMCO value calculated separately by the classical two-stage oxygen technique of Roughton and Forster. In this paper we examine whether there are physiological reasons for assuming that DMNO is over twice DMCO *in vivo*. We are critical of Reeves and Park's estimate for the $1/\theta_{CO}$–PO_2 relationship. We review *in vitro* estimates of θ_{CO} in the light of Guenard et al.'s recent *in vivo* estimate.

1. Introduction

According to Roughton and Forster's original model for carbon monoxide transfer from 1957

$$1/DL = 1/DM + 1/\theta Vc \quad (1)$$

where "DM is the diffusing capacity, based on molecular diffusion, of the membrane separating the alveolar air from the red cell membrane. DL is the overall diffusing capacity of the lung, Vc is the total volume in millilitres of blood exposed to alveolar air, θ is the number of millilitres of gas taken up by the red cells in 1 ml of blood per minute per 1 mmHg gradient of partial pressure of dissolved gas between plasma and red cell interior" (Roughton and Forster, 1957). θ was measured using a dilute solution of red cells in a rapid reaction apparatus *in vitro*. The reciprocals in Eq. (1) express transfer (DL is a conductance) in terms of two resistances in series (the membranes and the red cell). Later in a review in 1983 (Forster, 1983), Forster modified this view regarding $1/\theta Vc$ as "that part of the carbon monoxide transfer resistance which changes with alveolar PO_2 and 1/DM is the transfer resistance that is left over", *i.e.* that which remains at zero PO_2. This is a "functional" or "operational" definition whereas anatomically 1/DM includes the resistance of the surfactant layer, alveolar membrane, interstitium and plasma (both flowing and stagnant). Nitric oxide (NO) was first introduced 30 years ago as a test gas for alveolar capillary diffusion. NO is of similar molecular weight and approximately double the water solubility of CO. The Roughton–Forster has been applied to NO gas transfer. This was a logical step as between individuals correlation between DLNO and DLCO has been close suggesting transfer by a similar mechanism. Nevertheless, because the reaction of NO with *free haemoglobin* is two orders of magnitude faster than CO, there has always been disagreement as to whether θ_{NO} *is finite or, effectively, infinite.* In this paper we examine the Roughton–Forster equation in relation to NO and CO, specifically (i) whether θ_{NO} is finite or quasi-infinite, (ii) how DMNO/DMCO could be greater than twofold as predicted by water solubility divided by the square root of its molecular weight and (iii) the optimal value for θ_{CO}, focusing on our concerns with Reeves and Park's formula (Reeves and Park, 1992) and the possible advantages of the equation of Guénard et al. (2016).

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2. Calculation of θ_{NO} *in vitro*

The rate of reaction of NO with haemoglobin in aqueous solution is about 280 times greater than the equivalent reaction for CO (Borland and Higenbottam, 1989; Meyer et al., 1990). It has been postulated, therefore, that the red cell NO conductance (θ_{NO}) is effectively infinity (Guénard et al., 1987). Thus, from Eq. (1), $1/\theta Vc$ is removed and 1/DLNO = 1/DMNO.

θ_{CO}, at a particular oxygen concentration (or PO_2) is derived from the second order rate constant "l'_4" for the reaction of CO with haemoglobin in a rapid reaction apparatus:

$$\boldsymbol{\theta}_{NO} = \alpha_{NO} \times 0.2 \times 60 \quad (3)$$

where α_{CO} is the solubility of CO in blood in ml ml^{-1} $mmHg^{-1}$, 0.2 is the capacity of blood for CO in ml CO gas STPD ml^{-1} and 60 converts minutes to seconds (Roughton and Forster, 1957).

θ_{NO} can also readily be calculated from the second order rate constant for NO and Hb (j'_c) measured by Carlsen and Comroe (1958) in the same rapid reaction apparatus as the θ_{CO} measurements of Roughton and Forster (1957). Note that NO reacts directly with the haemoglobin molecule to form nitrate and (methaemoglobin), whereas CO competes with O_2 to combine with Hb:

$$-d(NO)/dt = j'_c(NO)(Hb) \quad (4)$$

$$\theta_{NO} = j'_c \times \alpha_{NO} \times (Hb) \times 60 \quad (5)$$

As none of the terms in the right of Eq. (5) are infinite, θ_{NO} cannot be infinite unless Carlsen and Comroe's value does not apply *in vivo*.

Accordingly there is no *in vitro* evidence for infinite blood conductance for NO; in other words there are no *in vitro* measurements to show that the reaction of NO with the red cell is as fast as the reaction with free haemoglobin. Abundant evidence has accumulated over the last 58 years since Carlsen and Comroe's original measurements that the rate of reaction of nitric oxide with the red cell is significantly slower than with free haemoglobin. θ_{NO}, *in vitro*, is much less than infinity. Eight independent *in vitro* experiments, from five different mammalian species, using six very different methods, all came to the same conclusion, as discussed by Borland et al. (2014). Inevitably, all these studies used conditions very different from those within the pulmonary capillary bed. How those differences might affect θ_{NO} are the subject of the next paragraph.

3. How could θ_{NO} (but not θ_{CO}) be finite *in vitro* but infinite *in vivo*?

The conditions under which θ_{NO} was measured *in vitro* in the Hartridge–Roughton continuous flow rapid reaction (CFRRA) apparatus (see Hughes and Bates, 2003) were necessarily very different from the rheological and anatomical conditions in pulmonary capillaries *in vivo* in terms of (a) pH, (b) PO_2, (c) velocity of blood flow (d) ligand concentration and (e) haemoglobin concentration (see Appendix A). If infinite blood conductance is indeed occurring *in vivo*, it might be due to differences in one of the physical factors (a–e) just mentioned. Taking each in turn: (a) pH is unlikely to account for differences between *in vitro* and *in vivo* since the reaction rate of NO with HbO_2 is so rapid that minor alterations with pH are unlikely to affect blood conductance, (b) for the same reason PO_2 would not affect conductance (anyway, DLNO has been shown to be independent of hyperoxia in man (Borland and Cox, 1991)), (c) we have shown that NO uptake in an oxygenator model is independent of blood flow (Borland et al., 2006) making reagent velocity an unlikely reason for differences in blood conductance *in vitro* and *in vivo*, (d) one difference of significance is the 1:40 ligand concentration which is approximately 10,000 times greater in the rapid reaction apparatus than in a single breath DLNO (see Appendix A), (e) likewise the substantial (1:40) dilution of blood used in the *in vitro* experiments with the CFRRA (Borland et al., 2014; Carlsen and Comroe, 1958; Hughes and Bates, 2003); this was done to increase the sensitivity of the spectrophotometric estimates of NOHb and COHb (see Appendix A). Its relevance is discussed in the next paragraph.

A companion paper (Borland et al., 2017) shows that the distance NO molecules penetrate on reaching the red cell falls exponentially, the rate of decline being determined by the rate of reaction of NO with Hb divided by the diffusion rate within the cell. *In vivo*, we calculate that the rate of reaction is so fast that NO only penetrates about 0.1 μM into the cell. With 1:40 dilution of blood, the concentrations of haemoglobin and NO (and CO) gas became approximately equimolar (see Appendix A). As blood and NO solutions proceed down the CFRRA reaction tube, an unreactive layer of NOHb builds up, impeding access of NO molecules to free Hb; this lowers the red cell conductance for NO (θ_{NO}) *in vitro* compared to *in vivo*; this could account for θ_{NO} being "quasi-infinite" *in vivo* and finite *in vitro*. For CO, the slower rate of reaction with Hb means less restricted access of CO to Hb molecules both *in vivo* and *in vitro*, and greater dependence on Hb concentration.

4. How could the DmNO/DmCO ratio be greater than 2?

Diffusion from gas to tissue is described by Krogh's diffusion constant which is the product of gas diffusivity ($\sim 1/\sqrt{MW}$) and tissue solubility (dC/dP or α), *i.e.* $\alpha/\sqrt{MW}$. For NO *versus* CO, the α ratio in water (and, by implication, plasma) is approximately 1.93–1.97 and the molecular weights (MW) are about the same. Thus, DMNO/DMCO is about 2.0. This ratio assumes that the geometric factors (surface area/thickness) are identical for NO and CO. Krogh's diffusion constant stands up well to modern chemical engineering diffusion theory (see Appendix A).

Surface area/thickness ratio differences for NO *versus* CO might exist, leading to DMNO/DMCO > 2.0 if the surface area for diffusion for NO was (i) greater or (ii) the thickness could be less or (iii) facilitated diffusion might occur. (i) Diffusion of NO into a greater area could be due to bronchial mucosal uptake. However, calculations based on the measured diffusing capacity of the airways indicate that this could only account for 0.02% of inhaled NO (Zavorsky et al., 2017). (ii) The diffusion path length for NO could be less because the penetration depth of NO into the red cell would be less than for CO (Borland et al., 2017), because of large differences in Hb reaction rates (see previous section). A possible explanation for how geometric differences could account for ratios of DMNO/DMCO 4.0–4.4 (Coffman et al., 2017) is offered in a companion paper (Borland et al., 2017). Briefly the penetration depth $y = \log_e(CAi/CA/(\sqrt{(k_1/D)})$ where CA is the NO concentration at depth y, CAi is the surface concentration, k_1 is the pseudo first order rate constant for reaction of NO with oxyhaemoglobin and D_{NO} is the diffusion coefficient for NO within the red cell. Because k_{1NO} is two orders of magnitude greater for NO compared to CO (Meyer et al., 1990) whereas D_{NO} is similar to D_{CO} the penetration depth for NO is far shorter than for CO. Since from Fick's first law diffusion through a membrane is inversely proportional to membrane thickness (~penetration depth) then DMNO will be more than twice DMCO as predicted from the ratio of their water solubilities to molecular weight. (iii) Nitric oxide is certainly more reactive than CO, reacting with albumin. Aquaporin-1 has been claimed as an NO carrier at physiological concentrations (Herrera et al., 2006). However there is no evidence for facilitated diffusion for CO (Jones et al., 1982), nor for NO during single breath DL measurements (Borland, unpublished observations (see Appendix A)). In fact, facilitated diffusion across a biological membrane has never

been proven for any gas including CO (Jones et al., 1982) and would contravene the Meyer–Overton rule (Missner and Pohl, 2009).

5. Arguments proposed by advocates of infinite θ_{NO}

These workers (Coffman et al., 2017) find that single-breath DmCO calculated assuming finite θ_{NO} does not significantly increase during submaximal exercise in healthy volunteers. It would be expected from simple geometry that the proportional increase in DmCO with exercise would be less than Vc. If the capillary is thought of as a cylinder of diameter d which dilates on exercise then its volume will be proportional to $(d/2)^2$ whereas its area (and hence Dm) will be proportional to $d/2$. We also note from their data that the standard deviations are wide. This is to be expected from the way DmCO is calculated. It is calculated from a reciprocal which is approaching zero. It could be argued that the technique is not sensitive enough to detect a small increase among the scatter.

The second reason proposed for infinite θ_{NO} is that DM calculations using a finite θ_{NO} have not been tested against a "Gold Standard" method for DM and Vc that does not use NO; in other words the classical Roughton–Forster analysis measuring DLCO at different oxygen tensions and a $1/\theta_{CO}$–PO_2 equation. Unfortunately there is no accepted gold standard for the $1/\theta_{CO}$–PO_2 relationship. The pros and cons of various equations are discussed in the next section.

6. Choosing the best value for θ_{CO}

Using the 1957 value for θ_{CO} neatly divided the resistance to CO transfer (1/DLCO) into two equal resistances 1/DM representing the membrane and $1/\theta$Vc the blood (Roughton and Forster, 1957). However in 1987 Forster measured θ_{CO} again (Forster, 1987), this time at physiological pH 7.4 rather than pH 8 obtaining:

$$1/\theta_{CO} = (1.3 + 0.0041 \cdot P_AO_2) \quad (6)$$

Using this formula results in lower values for 1/DM, the intercept on the Roughton–Forster plot of 1/DL *versus* $1/\theta_{CO}$ and higher values for 1/Vc. Values for DM approaching infinity or even negative values (the extrapolated intercept approaching or going below zero) occur occasionally among trained volunteers. Forster stated: "Our present view is that the alveolar capillaries expose nearly naked red cells to alveolar gas and that the simultaneous diffusion and chemical reaction within the erythrocyte is a major rate limiting process even in normal subjects breathing air and the diffusing capacity of the membrane is so great as to be difficult to measure precisely" (Forster, 1983).

Forster's (1987) values for θ_{CO} yield larger, but believable (when not negative), values for DM. Indeed, the 2–3 time larger values for DMCO from the 1987 θ_{CO} relationship with the combined DLNO–DLCO technique approach, but do not equal, the morphometric measurements of DM of Weibel et al. (1993). Nevertheless, many researchers still use the 1957 θ_{CO} relationship with the multistep oxygen technique (Tamhane et al., 2001), and some advocate the θ_{CO} relationship of Reeves and Park's (Reeves and Park, 1992) with the DLNO–DLCO combination (Coffman et al., 2016), but both these approaches result in relatively low values for DMCO. Also, Table 8 in Hughes and Bates (2003) shows that the CO red cell resistance ($1/\theta_{CO}$Vc) as a fraction of the total resistance (1/DLCO) depends on the $1/\theta_{CO}$–PO_2 relationship chosen, *i.e.* 0.15 (Reeves and Park, 1992), 0.45 (Roughton and Forster, 1957) but 0.78 (Forster, 1987) and 0.82 (Borland and Cox, 1991) [NO–CO method]. 0.15 and 0.45 are too low when set against the value of 0.37 for the NO red cell resistance ($1/\theta_{NO}$Vc) (Borland et al., 2010).

7. Reeves and Park's value for $1/\theta_{CO}$

Reeves and Park's measurement of θ_{CO} was a new departure from the original continuous flow and later stopped flow estimates (see Hughes and Bates, 2003). They used a very thin layer of human blood (one–two red cells thick) sandwiched between two layers of Teflon mesh containing a CO oxygen mix. They made a step change in PCO and followed the reaction of CO with HbO_2 by spectroscopy. This technique had many potential advantages. It used human haemoglobin at physiological concentrations and included plasma CO and O_2 concentrations equivalent to those expected during a single breath DLCO manoeuvre. The pH used was physiological. Their account is very comprehensive. Their regression equation:

$$1/\theta_{CO} = 0.00211 + 0.00787 \cdot PO_2 \quad (7)$$

indicates negligible intracellular and extracellular diffusion resistance to blood CO transfer (from the low value, 0.00211, of the first, oxygen-independent, component). Indeed their regression was quantitatively identical to the Gibson–Roughton rate equation for a well mixed haemoglobin solution rather than for haemoglobin packaged within the red cell (Roughton and Forster, 1957).

Reeves and Park suggested that the larger diffusion resistance found by others was due to stagnant extracellular layers in both the continuous and stopped flow apparatus. They appear to dismiss any intracellular diffusion resistance. But several groups have directly measured the intracellular diffusion resistance for ligands. For example Ho and Hershey obtained a figure for the Fick's 1st law diffusion coefficient for oxygen (D_{O2}) in the red cell of 4.3×10^{-6} cm^2 s^{-1} (Ho and Hershey, 1974). The Cambridge group calculated intracellular D_{NO} at 37 °C for human red cells as 8.98×10^{-6} cm^2 s^{-1} and a figure for D_{CO} of half this value (Borland et al., 2014); in their oxygenator model D_{CO}, like D_{NO}, linearly declined with an increase in intraerythrocyte diffusion resistance. This was achieved by progressively adding sodium nitrite to the oxygenator producing an outer layer of methaemoglobin within the red cell of progressively increasing thickness. This layer presented an increasing barrier to intraerythrocyte diffusion. This procedure could not have changed stagnant layers. These values for the intracellular diffusion coefficient are threefold lower, *i.e.* resistance threefold higher, than plasma and physiological solutions (Borland et al., 2014). In addition, Reeves and Park's theoretical calculations can be challenged. The correct analysis for non-steady state combined diffusion and chemical reaction in a thin layer is given by

$$d(CO)/dt = D_{CO}d^2(CO)/dx^2 - l'_4(Hb_4(O_2)_3O_2)(CO) \quad (8)$$

which is a combination of Fick's second law and second order chemical kinetics. The left hand term represents changes in CO concentration with time, the middle with diffusion distance and the right hand term combination with oxyhaemoglobin. Solving this second order differential equation algebraically is complicated and a better approach would have been to calculate each change in CO over infinitesimal distances δx and time δt. This can be best done using a commercial software package. Furthermore we have shown the right hand term is a simple second order kinetic rate expression; this is true for $PO_2 > 200$ mmHg. In normoxia there would undoubtedly be unliganded Hb molecules. To complicate matters further, at the lowest CO concentrations used 2 mmHg (0.3% corresponding to a single breath DLCO), the tissue concentration of CO ($=2.16 \times 10^{-6}$ M) is substantially lower than Hb species (9×10^{-3} M) so that pseudo first order kinetics would then apply complicating any algorithm used for analysis.

There are a number of other concerns with their approach. At the end of the experiment they noted 22% of haemoglobin lay outside intact cells. Whilst they believe this occurred when sectioning

the cell after measurements were made we are not convinced by their argument and are concerned that free Hb could have artefactually reduced the diffusion resistance. Secondly, whilst stagnant layers are a feature of stopped-flow they are less likely with continuous flow. Thirdly, nobody has ever repeated the Buffalo group's work. Robert Forster writes (personal communication), "I doubted if a layer of blood between two flexible stretched teflon sheets an average1 micron apart would maintain a constant thickness in all areas and would be more than one cell thick". More philosophically, the impetus for their work was that stagnant extracellular layers impede CO diffusion in rapid reaction apparatus. In the rapid reaction apparatus each solution was accelerated down one limb of a Y shaped apparatus at 150 ml s^{-1} (*i.e.* a velocity of 2.8 m s^{-1}) whereas in the pulmonary capillary the blood velocity has been estimated as 1.78 mm s^{-1} (Horimoto et al., 1979). If stagnant layers occur in a rapid reaction apparatus then they must surely be present *in vivo*!

Reeve and Park's values for of θ_{CO} give the lowest value for Dm and for red cell resistance fraction ($[1/\theta_{CO}Vc]/[1/DLCO]$) and therefore the highest value for 1/Dm. Within and between studies, variance increases with Dm. The best statistical fit will be found using the value for $1/\theta$ that gives the highest value for 1/Dm (*i.e.* Reeves and Park), but a good fit is not necessarily the correct solution.

To conclude, we are wary of Reeves and Park's value for θ_{CO} on the following grounds: their value for a the intracellular CO diffusion resistance component is too small, and their calculations of the PO_2 term in Eq. (10) may be wrong. The good fit that the Mayo group (Coffman et al., 2016) obtained between DM using Reeve and Park's formula for θ_{CO} may just be statistical chance.

8. Guenard et al.'s value for $1/\theta_{CO}$

Given the dispersal of the *in vitro* estimates of the $1/\theta_{CO}$–$1/PO_2$ relationships in the literature Guénard et al. (2016) recently attempted to obtain in *vivo* values for θ_{CO} and θ_{NO} as follows. They measured DLNO and DLCO in ten non-smoking volunteers using mixtures containing 15% and 21% oxygen leading to alveolar PO2 of about 80 and 120 mmHg during the single breath manoeuvre, thus low and high normoxic conditions respectively. They used all published values for θ_{CO} to obtain Vc firstly from the traditional Roughton Forster two oxygen tension method then *via* DLNO/DLCO at the low and higher PO_2. This gave three estimates of Vc. They reasoned that if these Vcs are equal Vc can be substituted to obtain θ_{NO} from the two (high and low) values of θ_{CO}, DLCO and DLNO. Guénard et al. obtained values for θ_{NO} ranging from −2.8 to 4.8 min^{-1} $mmHg^{-1}$ and decided upon 4.5 (the literature value) as reasonable. They then tested a wide range values for a and b in $1/\theta_{CO} = aPO_2 + b$ using their high and low DLCO measurements. These yielded a range of pairs of Dm amd Vc. The values of a and b which minimised the difference between Dm and Vc in normoxia and hypoxia were chosen as the optimum values. In their study this was

$$1/\theta_{CO} = 0.0062PO_2 + 1.16 \quad (9)$$

This first "*in vivo*" measurement of the $1/\theta_{CO}$–PO_2 relationship is a statistical "best fit", and not an actual measurement of θ_{CO}. Guénard et al.'s data (Guénard et al., 2016) supports the θ_{CO} estimates made with the classical continuous flow rapid reaction apparatus (Hughes and Bates, 2003; Roughton and Forster, 1957) rather than the ingenious technique of Reeves and Park (Reeves and Park, 1992). DLCO was measured twice with the multi-step O_2 method, but over a narrow PO_2 range (80–120 mmHg). This was necessary to avoid haemodynamic changes which might have altered their prerequisite of maintaining a constant DMCO and Vc. Changes in DLCO from the lower to higher PO_2 were small but consistent (9% decrease at higher PO_2). There were no changes in the oxygen insensitive DLNO. We believe that Guénard et al.'s formula for the $1/\theta_{CO}$–PO_2 relationship is the best available at the present time.

9. Should we not perform DLNO/DLCO in hyperoxia?

What everybody is agreed upon is that in hyperoxia red cell resistance to CO transfer is almost entirely reaction limited and there is much closer agreement for the various values of $1/\theta_{CO}$. "At high pO_2 (>200 mmHg), $\theta_{CO} \propto \sim l'_4 PO_2$ and CO uptake by red cells is slowed to such an extent that diffusion of CO becomes relatively unimportant and θ_{CO} is approximately the same in intact red cells as in an equivalent concentration of haemolysate (Forster, 1987)." In hyperoxia DLCO would be even more dominated by CO-Hb kinetics and thus weighted heavily (over 95%) towards the pulmonary capillary bed.

A case could be made therefore for performing combined DLNO/DLCO in hyperoxia. Whilst NO oxidation would proceed faster:

$$1/NO_t = 1/NO_0 + 2kO_2t \quad (10)$$

where $k = 7.6 \times 10^{-10}$ ppm^{-2} min^{-1} loss of NO/generation of NO_2 would be negligible provided the mixture was inhaled within 5 min of preparation (Borland and Higenbottam, 1989).

While the idea of measuring DLNO–DLCO in hyperoxia is attractive, it would be difficult for practical and theoretical reasons. First, there is the time involved in pre-oxygenating the subjects (perhaps prolonged in patients), and secondly there is the uncertainty in calculating the PO_2 for the $1/\theta_{CO}$–PO_2 relationship. The "effective" PO_2 is the mean capillary PO_2 ($Pcap(mean)O_2$) and in hyperoxia with even minor VA/Q mismatch, it may differ substantially from the measured $PalvO_2$ (West, 1969).

10. Conclusions

Measurements of the blood conductance of NO and CO (θ_{NO} and θ_{CO}) are essential for the calculation of the membrane and red cell conductances for these gases from DLNO and DLCO measurements. But, controversy exists concerning the application of *in vitro* estimates of θ_{NO} and θ_{CO} to their values *in vivo*.

1. Does θ_{NO}, which is finite *in vitro* (about 8 times the value of θ_{CO} at 100 mmHg) behave "as if" infinite *in vivo*? There is no direct evidence for or against this proposition, but a companion paper (Borland et al., 2017) argues that an infinite value for θ_{NO} *in vivo* is feasible. Physiologically, an infinite θ_{NO} *in vivo* means a lower estimate of DMCO, (and higher 1/DMCO and so lower Rrc/RTot% for CO) and one which, even on exercise, is much lower than morphometric measurements of membrane diffusing capacity (Weibel et al., 1993). With a finite θ_{NO}, estimates of DMCO on exercise, using a combined NO–CO analysis, approach morphometric estimates.
2. Because of consistent differences between DMCO calculated with the classical multi-step O_2 technique and the newer combined DLNO–DLCO method, is it feasible that the DMNO/DMCO ratio is greater than the 1.97 predicted by the physics of tissue diffusion? We argue elsewhere (Borland et al., 2017) that it is possible.
3. What is the consensus about the $1/\theta_{CO}$–PO_2 relationship? There are several equations in the literature. The equation of Reeves and Park (1992) differs from the others, and has not been replicated by other researchers. Their approach is ingenious, but we are very critical of its method and theory. The recent "*in vivo*" estimate of Guénard et al. (2016) warrants further consideration.

Appendix A.

(1) Carlsen and Comroe *in vitro* reacted a 1:40 suspension of human red cells (2.25×10^{-4} M of Hb) with 1:40 NO (3×10^{-4} M) at high velocity in a continuous flow rapid reaction apparatus in the absence of oxygen and followed the reaction spectroscopically (Carlsen and Comroe, 1958). *In vivo*, in a combined DLNO/DLCO single breath test 40 ppm NO is inhaled with 0.27% CO. The tissue NO concentration will be $40 \times 10^{-6} \times 0.015 \times 10^{-3} \times 95 = 5.7 \times 10^{-8}$ M. (0.015 is water solubility of NO at 370°C in mmol L^{-1} kpa^{-1}, 10^{-3} converts mmol to M and 95 is barometric pressure less saturated vapour pressure of water at 37 °C). The molar concentration of Hb in a red cell at 14.6 g dL^{-1} is $9 \times 10^{-3}/0.45 = 2 \times 10^{-2}$ M. For CO with a single breath inhalation of 0.27% CO, the initial tissue concentration = $0.27 \times 10^{-2} \times 0.0081 \times 10^{-3} \times 95 = 2.08 \times 10^{-6}$ M where 0.0081 is water solubility of CO.

(2) Krogh modified Fick's and Graham's laws of diffusion to state that the rate of diffusion of a gas is inversely proportional to the square root of its molecular weight $\alpha/\sqrt{MW}$. This statement stands up to modern theory about the diffusion of gases in liquids.

Fick's first law states that the rate of diffusion per unit area (J) is:

$$J = -D\,dc/dy \tag{11}$$

J is rate of transfer per unit area in $length^3\ time^{-1}\ length^{-2}$, $\dot{M}$ is rate of transfer $length^3\ time^{-1}$, D is diffusion coefficient in $length^2\ time^{-1}$, A is surface area in $length^2$, dc concentration in $length^3\ length^{-3}$, and dy distance in units length.. Now $dc = \alpha dP$ where α is solubility and P partial pressure so rate of transfer through membrane

$$\dot{M} = -DA\alpha dP/dy \tag{12}$$

There are several formulae that are used to predict D from the physical properties of the solute (including a gas) and solvent. Wilke and Chang's formula is in widespread use (Wilke and Chang, 1955).

$$D = \text{Constant}(xMW)^{1/2}T/(\eta V^{0.6}) \tag{13}$$

x is association factor, a property of the solvent, 2.6 for water, MW is solvent molecular weight, T is absolute temperature, η is solvent viscosity and V solute molecular mass.

Comparing two different gases diffusing across a membrane with an identical partial pressure gradient and of a given area and thickness from Eq.(12) the rate of transfer will be proportional to the ratio of solubilities. Since $V^{0.6}$ will be similar to $\sqrt{MW}$ from Eq.(13) the rate will be approximately inversely proportional to the ratio of $\sqrt{MW}$.

(3) In a single individual DLNO at 0.7 ppm was 142 ml/min/mmHg whereas at 40 ppm it was 158 ml/min/mmHg (Borland, unpublished observations).

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