J. Physiol. (1957) 136, 507–526

THE KINETICS AND EQUILIBRIA OF THE REACTIONS OF NITRIC OXIDE WITH SHEEP HAEMOGLOBIN

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(*Received* 31 *December* 1956)

During the past 50 years an enormous amount of work has been done on the equilibria and the kinetics of the reversible reaction of mammalian haemoglobin with oxygen. Such work has not only been of obvious importance in respiratory physiology, but has also helped greatly towards the elucidation of the detailed physico-chemical mechanism of this vitally important reaction. The kinetics and equilibria of the reactions of haemoglobin with carbon monoxide have likewise been studied in much detail, not only because their close physico-chemical analogies with the oxygen-haemoglobin reactions might well throw further light on the latter, but also because of their importance in the understanding of (*a*) the poisonous action of carbon monoxide, and (*b*) the use of carbon monoxide as a physiological tool, especially in the determination of the diffusing capacity of the human lung and of the average time spent by the blood in the lung capillaries (see, for example, Roughton, 1945).

There are a few other substances which combine reversibly with reduced haemoglobin in a similar way to oxygen and carbon monoxide. Of these the best-known are nitric oxide and the isocyanides. St George & Pauling (1951) have studied the effect on the affinity for haemoglobin of varying the size of the group to which the isocyanide radical is attached, and from their results have inferred that the iron centres in haemoglobin are buried in crevices, a view which has been strongly controverted by Keilin (1953). Little or no quantitative work seems, however, to have been done on the physical chemistry of the reaction of haemoglobin with nitric oxide since Hermann's discovery of nitric oxide haemoglobin almost a century ago. Hermann (1865) found, *inter alia*, that nitric oxide has at least five times greater affinity than carbon monoxide for haemoglobin, and accordingly is able to displace CO readily from combination with haemoglobin. His careful gasometric experiments showed that the volume of carbon monoxide displaceable from fully saturated carboxyhaemoglobin is equal to the volume of nitric oxide absorbed by the haemoglobin, and

therefore that the gas-combining capacity of haemoglobin is the same for nitric oxide as for carbon monoxide. This conclusion is confirmed in a more direct way in the present paper. Adair's (1925) intermediate compound hypothesis should therefore apply to nitric oxide haemoglobin, in the same manner as it is now generally believed to apply to oxyhaemoglobin and carboxyhaemoglobin. The kinetics and equilibria of the reactions of haemoglobin with nitric oxide should, accordingly, conform to the four-stage scheme:

$$\mathrm{Hb_4} + \mathrm{NO} \underset{j_1}{\overset{j_1'}{\rightleftharpoons}} \mathrm{Hb_4(NO)}, \quad \frac{j_1'}{j_1} = J_1; \tag{1}$$

$$\mathrm{Hb_4(NO)} + \mathrm{NO} \underset{j_2}{\overset{j_2'}{\rightleftharpoons}} \mathrm{Hb_4(NO)_2}, \quad \frac{j_2'}{j_2} = J_2; \tag{2}$$

$$\mathrm{Hb_4(NO)_2} + \mathrm{NO} \underset{j_3}{\overset{j_3'}{\rightleftharpoons}} \mathrm{Hb_4(NO)_3}, \quad \frac{j_3'}{j_3} = J_3; \tag{3}$$

$$\mathrm{Hb_4(NO)_3} + \mathrm{NO} \underset{j_4}{\overset{j_4'}{\rightleftharpoons}} \mathrm{Hb_4(NO)_4}, \quad \frac{j_4'}{j_4} = J_4. \tag{4}$$

In these equations j_1', j_2', j_3' and j_4' are the combination velocity constants, j_1, j_2, j_3, and j_4 the dissociation velocity constants and J_1, J_2, J_3 and J_4 the equilibrium constants of the corresponding intermediate reactions.

During the early stages of combination of nitric oxide with haemoglobin, the speed of the over-all process, which has proved to be faster than for any previously measured haemoglobin-ligand combination, should be given approximately by the equation

$$\frac{\mathrm{d[NOHb]}}{\mathrm{d}t} = j' [\mathrm{NO}] [\mathrm{Hb}], \tag{5}$$

where

[NOHb] = total molar concentration of NO combined in all forms

$$= [\mathrm{Hb_4(NO)}] + 2[\mathrm{Hb_4(NO)_2}] + 3[\mathrm{Hb_4(NO)_3}] + 4[\mathrm{Hb_4(NO)_4}], \tag{6}$$

$$[\mathrm{Hb}] = 4[\mathrm{Hb_4}] + 3[\mathrm{Hb_4(NO)}] + 2[\mathrm{Hb_4(NO)_2}] + [\mathrm{Hb_4(NO)_3}], \tag{7}$$

and

$$j' = \tfrac{1}{4} j_1'. \tag{8}$$

Analogous equations to (5), (6), (7) and (8) have previously been derived and used for the early stages of combination of haemoglobin with oxygen and with carbon monoxide (see, for example, Gibson & Roughton (1955*a*) and references therein). The first experimental subsection contains approximate measure-

ments of j' and j'_1 under various conditions by means of Gibson's stopped flow method.

Of the dissociation velocity constants only j_4 is so far readily measurable. The rate of the reaction $Hb_4(NO)_4 \rightarrow Hb_4(NO)_3 + NO$ has turned out to be so slow that it can be followed by rotating a dilute solution of NOHb in an all-glass tonometer containing carbon monoxide at 1 atm pressure for periods of several hours and determining, by means of the Hartridge reversion spectroscope, the percentage of residual NOHb after varying intervals.

Direct determinations of the equilibrium between nitric oxide and haemoglobin are rendered extremely difficult by the remarkably high affinity of haemoglobin for nitric oxide, which, as will be shown later, is of the order of 3000 times greater than for carbon monoxide. Indirect values of J_4 can, however, be derived from the equation

$$J_4 = NL_4, \tag{9}$$

where L_4 = the equilibrium constant of the reaction $Hb_4(CO)_3 + CO \rightleftharpoons Hb_4(CO)_4$, which can be estimated by the method described by Roughton (1954), and N is the partition equilibrium constant of haemoblogin between nitric oxide and carbon monoxide when shaken with mixtures of these gases sufficient to saturate the haemoglobin. Equation (9) is analogous to equation (16) of the appendix to Roughton's (1954) paper, and is deducible in exactly the same manner, provided that similar theoretical assumptions are applicable to fully saturated haemoglobin in equilibrium with CO-NO gas mixtures as with $CO–O_2$ gas mixtures. Actually

$$N = [\text{NOHb}]\,[\text{CO}]/[\text{COHb}]\,[\text{NO}], \tag{10}$$

or

$$\frac{[\text{NOHb}]}{[\text{NOHb}]+[\text{COHb}]} = N\frac{[\text{NO}]}{[\text{NO}]+[\text{CO}]}. \tag{10a}$$

Values of N are easily obtainable by rotating dilute solutions of NOHb or COHb for several hours in tonometers containing appropriate proportions of NO and CO and then measuring at equilibrium the ratio of [NOHb] to [COHb] by means of the reversion spectroscope.

Preliminary accounts of the present work have already been given elsewhere (Gibson & Roughton, 1955*b*, *c*). It should be noted that the symbols in the present paper for the velocity constants and equilibrium constants of the nitric oxide reactions differ from those used in our two previously published notes. The symbols now chosen are more convenient and fall better into line with those already used for the reactions of haemoglobin with oxygen and with carbon monoxide.

METHODS

Sheep blood solutions were prepared and handled as described by Gibson & Roughton (1955*a*), using tonometers adapted to fit the syringes of the stopped-flow apparatus. The concentrations used ranged from $1{\cdot}6 \times 10^{-5}$ M to 8×10^{-5} M corresponding to blood dilutions of 1 in 500 to 1 in 100.

Nitric oxide was prepared by the reaction of sodium nitrite and potassium iodide in acid solution (Farkas & Melville, 1939), traces of iodine and other impurities being removed by washing with alkali. It was stored under positive pressure in all-glass tonometers of 1·5–3 l. capacity, such volumes being sufficient for a large number of experiments. The gas was analysed by absorption with 20% ferrous sulphate in about 2% sulphuric acid, and was usually found to be about 99% nitric oxide.

Solutions of nitric oxide of concentrations ranging from $1{\cdot}6 \times 10^{-5}$ M to $6{\cdot}4 \times 10^{-5}$ M were prepared by equilibrating the appropriate buffer solutions with gas mixtures containing partial pressures of nitric oxide ranging from 6 to 24 mm Hg. The concentrations of the solutions were calculated from the partial pressures and solubility coefficients of nitric oxide. Meticulous care was necessary to exclude all traces of oxygen, in view of the property of nitric oxide of combining spontaneously with oxygen to form nitrogen peroxide.

Combination of NO with Hb. This reaction was measured with the stopped-flow apparatus described by Gibson & Roughton (1955*a*), all records being made at least in duplicate. In all cases observations were made with light of mean wave-length 432 mμ, as given by an interference filter. The results are expressed in terms of the over-all velocity constant j' (see equations (5) and (8)).

Measurement of NOHb:COHb by the reversion spectroscope

Nitric oxide haemoglobin, like oxyhaemoglobin and carboxyhaemoglobin, has two absorption bands in the visible part of the spectrum. Of these the α-band is about 75 Å to the red side of the corresponding α-band of carboxyhaemoglobin and is appreciably less sharp than the latter. The reversion spectroscope can, however, be used for estimating the ratio of NOHb to COHb in mixtures of the two pigments, but is rather less accurate than for the familiar estimation, with its aid, of the ratio of COHb to O_2Hb.

Fig. 1 shows a calibration curve, obtained by mixing various proportions of 1 in 150 blood solution saturated with NO and 1 in 150 blood solution saturated with CO and reading the position of the α-bands of the resulting mixtures with the reversion spectroscope. The NOHb and COHb solutions were prepared by first reducing 100 ml. of 1 in 150 blood solution thoroughly by shaking several times with oxygen-free N_2. 50 ml. of the stock solution of reduced haemoglobin was then transferred to a 200 ml. tonometer containing about 0·5% NO in O_2-free N_2 and shaken so as to saturate the reduced Hb with NO. The NO-N_2 gas mixture was removed from the tonometer by evacuation, the tonometer was then washed twice with O_2-free N_2 and the blood solution finally shaken with O_2-free N_2 so as to reduce the amount of dissolved NO in the blood solution to a negligible figure. A second 50 ml. portion of the reduced blood solution was similarly treated with CO. The NOHb and COHb solutions were then transferred from their respective tonometers to separate burettes, from which suitable volumes were measured out into stoppered tubes of about 1·5 cm diameter, with minimal air contact throughout.

The rate of dissociation of NO from NOHb

In these measurements it is absolutely essential to avoid contamination with appreciable amounts of oxygen. The following, somewhat elaborate, procedure was therefore adopted:

(*a*) A stock of haemoglobin solution (usually laked blood diluted with 150 parts of buffer solution) was thoroughly shaken in a tonometer, *A*, with excess of O_2-free carbon monoxide at a slight positive pressure so as to saturate the haemoglobin with carbon monoxide and to remove all oxygen (physically dissolved as well as chemically combined).

(*b*) A second—all glass—tonometer, *B*, of about 200 ml. capacity and with a closed cylindrical end of about 1·5 cm diameter was filled to a slight positive pressure with O_2-free nitrogen.

(*c*) A rubber tip was fitted to the stem of tonometer *A*, the latter placed in the cup of a Van Slyke gasometric chamber, with graduations at 10 ml. as well as at 0·5 and 2·0 ml. (see Fig. 1 A of Roughton (1954)), and the top of the tonometer cautiously opened so as to wash out the stem thoroughly with haemoglobin solution, the latter overflowing gently into the Van Slyke cup. When 3 or 4 ml. of haemoglobin solution had thus run out, the rubber tip of the stem on the tonometer was firmly seated into the bottom of the Van Slyke cup (as in Fig. 1 B of Roughton's (1954) paper) and 10 ml. of the Hb solution drawn into the Van Slyke chamber, using the 10 ml. graduation mark on the latter for the measurement.

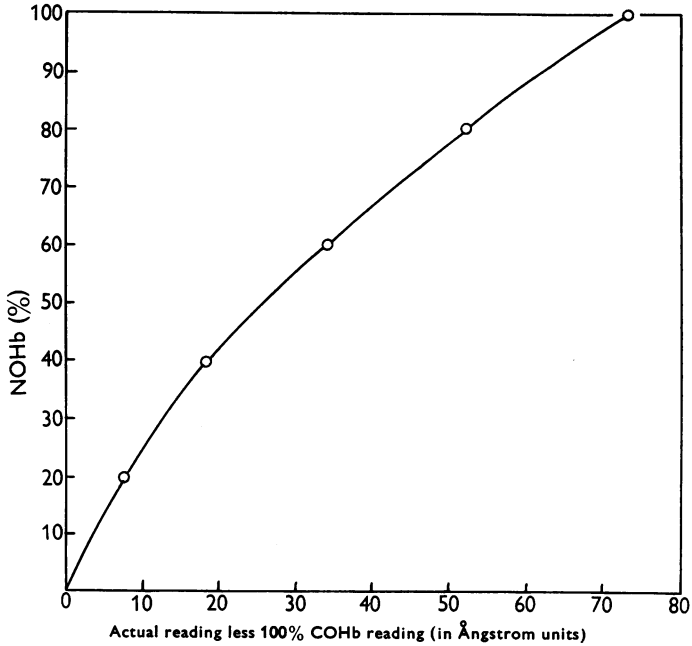

Fig. 1. Calibration curve for determination of mixtures of NOHb and COHb by means o the reversion spectroscope.

(*d*) The stem of tonometer *B* was also fitted with a rubber tip, and the excess pressure of nitrogen blown off under a layer of mercury in the cup of the Van Slyke chamber, which had just been charged with the 10 ml. of COHb solution. The latter was then transferred directly into tonometer *B* by raising the mercury reservoir of the Van Slyke apparatus and by suitably manipulating the various taps.

(*e*) The physically dissolved carbon monoxide in the COHb solution in tonometer *B* was then removed by shaking with three washings of oxygen-free nitrogen: the tonometer being finally left with a slight positive pressure of the latter.

(*f*) About 2 ml. of nitric oxide gas were transferred into the chamber of the Van Slyke apparatus with similar precautions to avoid all contact with atmospheric air.

(*g*) The stem of tonometer *B* was flushed with a small amount of Hb solution (by cautiously opening the tonometer tap), and then seated into the bottom of the Van Slyke cup, so that the 2 ml. of NO could next be transferred from the Van Slyke chamber to the tonometer, without any contact with the outside air.

(*h*) Tonometer *B* was then rotated in a water bath at 19° C for at least 15 min, so as to permit the nitric oxide in the gas phase to displace the carbon monoxide practically completely from combination with the haemoglobin.

(*i*) Tonometer *B* was then removed from the water-bath, evacuated and shaken with three washings of oxygen-free nitrogen so as to remove completely all the nitric oxide from the gas phase and from physical solution. The NOHb, however, is so stable that no appreciable dissociation of the latter can occur during this process. Finally the tonometer was evacuated, filled with oxygen-free carbon monoxide to an appropriate pressure (usually 1 atm) and then rotated for a series of periods in a constant temperature water-bath.

(*j*) At the end of each rotation period the tonometer was removed from the water-bath and held vertically so that all the haemoglobin solution drained into the cylindrical tube at the end of the tonometer. The percentage NOHb in the cylindrical tube was then estimated by means of the reversion spectroscope. If the tonometer had been rotated at other than room temperature, the cylindrical tube at the end of the tonometer was immersed for a few minutes in a beaker of water at room temperature before the spectroscopic determination was made. The reason for the latter precaution was that the positions of the spectral absorption bands of haemoglobin compounds are sensitive to temperature.

The partition equilibrium of haemoglobin between carbon monoxide and nitric oxide

A similar combination of gasometric and spectrometric procedure was used for this purpose. Steps (*a*), (*b*), (*c*) and (*d*) were the same as in the determination of the rate of dissociation of nitric oxide from NOHb as just described. The oxygen-free nitrogen in tonometer *B* was then replaced by oxygen-free carbon monoxide and an accurately measured volume of nitric oxide, ranging from 0·02 to 0·10 ml., was then transferred into the tonometer from the Van Slyke chamber. This was done by taking into the Van Slyke chamber a suitable volume of nitric oxide, measuring its pressure p_1 at the 0·5 ml. mark, forcing the whole of the nitric oxide into tonometer *B* and then measuring again the pressure p_2. The volume of nitric oxide, in ml. (s.t.p.), $=(p_1-p_2)\times 0{\cdot}5\times 273\times$ percentage purity of nitric oxide $\div 760\times(273+$temperature (° C) of Van Slyke chamber). The tonometer was then rotated in the constant temperature bath for periods ranging from 2 hr at 38° C to 12 hr at 19° C. These periods of rotation were sufficient to secure equilibrium in the case of the dilute haemoglobin solutions used, as was shown by control experiments in which (*a*) the rotation was continued for several hours longer and (*b*) NOHb instead of COHb was equilibrated with the same gas mixture. At the end of the equilibration the tonometer was removed from the bath and the percentage NOHb determined by the reversion spectroscope. The partition equilibrium constant was then calculated, as in the following worked example:

Volume of tonometer = 183 ml.

Volume of 1 in 150 blood solution (pH 9·1) introduced into tonometer = 10 ml.

Gas combining capacity of the 10 ml. blood solution = 0·01 ml. (s.t.p.).

Volume of nitric oxide introduced into tonometer = 0·0883 ml. (s.t.p.).

Percentage NOHb after 11 hr rotation at 19° C = 65.

Volume of nitric oxide combined with haemoglobin

$$=0{\cdot}01\times 65/100=0{\cdot}0065 \text{ ml. (s.t.p.)}$$

Residual nitric oxide in gas phase of tonometer

$$=0{\cdot}0883-0{\cdot}0065=0{\cdot}0818 \text{ ml. (s.t.p.).}$$

Carbon monoxide in gas phase of tonometer

$$=\frac{(183-10)\times 273\times 723}{(273+19)\times 760}=153{\cdot}9 \text{ ml. (s.t.p.).}$$

The figure 723 = (barometric pressure − water vapour pressure) × percentage purity of carbon monoxide/100.

Therefore
$$N=\frac{65}{35}\times\frac{153{\cdot}9}{0{\cdot}0818}\times\frac{\text{solubility of CO}}{\text{solubility of NO}}=1745.$$

RESULTS

The combination of nitric oxide with reduced haemoglobin

Fig. 2*A* gives the results of an experiment in which 1 in 300 reduced blood solution was mixed with NO solution of equivalent strength at 7·5° C and pH 9·1 (0·05 M borate solution). The concentrations of the reagents just after mixture were $1{\cdot}3 \times 10^{-5}$ M [Hb] and $1{\cdot}3 \times 10^{-5}$ M [NO]. Even at these low concentrations and low temperature the reaction is half complete in only

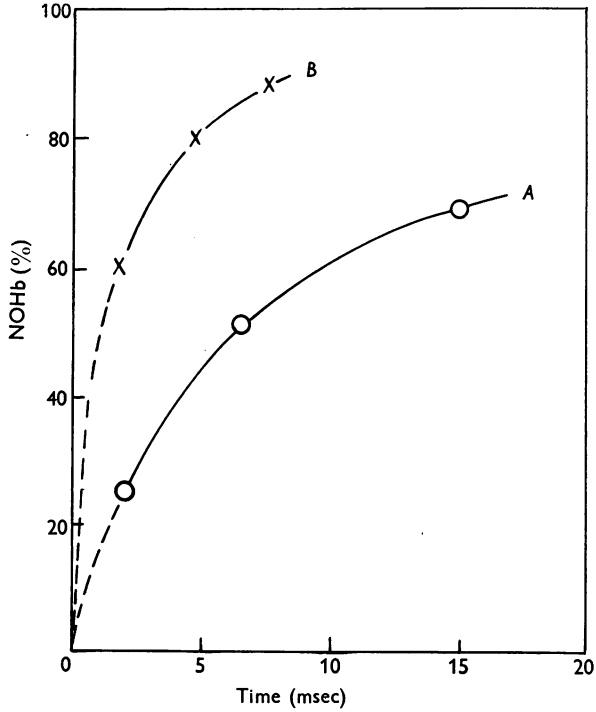

Fig. 2. Rate of combination of nitric oxide with haemoglobin in 0·05 M borate buffer, pH 9·1. (*A*) Temperature 7·5° C. [NO]=[Hb]=$1{\cdot}3 \times 10^{-5}$ M, just after mixture. (*B*) Temperature 24·5° C. [NO]=[Hb]=$2{\cdot}1 \times 10^{-5}$ M, just after mixture.

about 6 msec, the value of j' (the over-all velocity constant), viz. $1{\cdot}3 \times 10^{7}$ (M^{-1} sec^{-1}), being about four times greater than that of k', the over-all velocity constant for the rate of combination of oxygen with reduced haemoglobin under similar conditions. Unlike the latter reaction, the rate of combination of NO with Hb is sensitive to temperature, having a Q_{10} (temperature coefficient/10° C) of about 1·5. Fig. 2*B* shows an experiment at 24·5° C and pH 9·1 on the Hb solution prepared from the same blood as in Fig. 2*A*, but with higher concentrations, i.e. [Hb]=[NO]=$2{\cdot}1 \times 10^{-5}$ M. In this case, a large fraction of the total reaction fell within the 'dead time' of the apparatus, so

that the best value of j' that can be deduced from the experiment, viz. $2{\cdot}6 \times 10^7$ (M^{-1} sec^{-1}), is open to some doubt. Though the same reservation also applies to many of the individual data assembled in Table 1, it is clear that the over-all rate of the NO + Hb reaction is far greater than that of any other known ligand + Hb reaction, being in fact of the same order of speed as the fastest enzyme-substrate combinations so far recorded. Most of the experiments in Table 1 were performed at pH 9·1; Expts. 4*a* and 4*b*, however, show the effect of change of pH from 9·1 to 7·1. The threefold decrease in j' needs

TABLE 1. Values of j', the over-all velocity constant of combination of NO with Hb

Expt.	[NO] ($M \times 10^5$)	[Hb] ($M \times 10^5$)	Temp. (° C)	pH	j' (M^{-1} $sec^{-1} \times 10^{-7}$)
1	2·2	2·2	21	9·1	2·4
2	1·5	1·1	20	9·1	3·5
3*a*	1·7	1·7	14	9·1	2·6
3*b*	1·7	1·7	24·5	9·1	3·7
4*a*	2·1	2·1	21	9·1	4·5
4*b*	2·1	2·1	21	7·1	1·5

confirmation by further experiments, preferably with methods in which the earliest time of observation can be reduced to 0·5 msec, as in the most rapid of existing 'moving-flow' methods (as distinguished from the 'stopped-flow' method) or by a further development of the flash photolysis method (Gibson, 1956). Expts. 3*a* and 3*b* provide a further instance of the effect of temperature; the value of Q_{10} derived therefrom, viz. 1·4, agrees satisfactorily with that already found in the experiments plotted in Fig. 2.

The average value of j' at pH 9·1, 20° C, from the experiments so far carried out is about $3{\cdot}5 \times 10^7$ M^{-1} sec^{-1}: the corresponding value of j'_1, which is four times greater, is thus $1{\cdot}4 \times 10^8$ M^{-1} sec^{-1}.

The velocity constant, j_4, of the reaction $Hb_4(NO)_4 \rightarrow Hb_4(NO)_3 + NO$

The theoretical basis of the method, of which the experimental details have already been fully given, is the same as that previously adopted by Roughton (1934) for measuring l_4, the velocity constant of the reaction $Hb_4(CO)_4 \rightarrow Hb_4(CO)_3 + CO$. If a small volume of dilute solution of $Hb_4(NO)_4$ is rotated or shaken with a large volume of carbon monoxide gas, the following pair of reactions takes place at the start:

$$Hb_4(NO)_4 \rightharpoonup Hb_4(NO)_3 + NO, \qquad (11a)$$

$$Hb_4(NO)_3 + CO \rightharpoonup Hb_4(NO)_3CO. \qquad (11b)$$

Next a molecule of NO dissociates from $Hb_4(NO)_3CO$ and is replaced by a molecule of CO to give $Hb_4(NO)_2(CO)_2$ and so on until finally $Hb_4(CO)_4$ is formed. At pressures of CO of the order of 1 atm it is found that reactions of the type of (11*b*) take place so much more rapidly from left to right than reactions of the type (11*a*) that in the earliest stages when no intermediates con-

taining less than 3 molecules of combined NO are present the rate of NO dissociation conforms to the equation

$$-\frac{d[Hb_4(NO)_4]}{dt}=j_4[Hb_4(NO)_4], \tag{12}$$

or

$$-\frac{d[NOHb]}{dt}=j[NOHb], \tag{13}$$

where

$$j=\tfrac{1}{4}j_4.$$

Equation (13) will apply throughout the *whole* of the replacement process as well as to the earliest stages, provided that the velocity constant for dissociation of NO from a *fully saturated* mixed intermediate, $Hb_4(NO)_a(CO)_{4-a}$, is equal to a $\frac{1}{4}aj_4$, i.e. is proportional to the actual number, a, of combined NO molecules in the intermediate compound. This principle has been fully discussed by Gibson & Roughton (1955*a*) for the analogous case of oxygen replacement by carbon monoxide (and vice versa), and has been substantiated by them in numerous and varied experimental tests, for a detailed discussion of which reference should be made to their paper. Similar tests have shown that the same statistical kind of principle holds good in the case of CO-NO replacement and vice versa.

If equation (13) is valid then a plot of $\log_{10}$ (% NOHb) against time should give a straight line. Fig 3 shows a typical example, in which 10 ml. of 1 in 150 blood solution 93% saturated with NO (pH 9·1, 0·2 M borate buffer) were rotated at 16·5° C for periods up to 10 hr in a 189 ml. tonometer containing carbon monoxide at 1 atm pressure. The values of $\log_{10}$ (% NOHb) plotted in Fig. 3 are those which were actually determined in each experimental observation, but the values of the corresponding times have in every case been corrected for the effect of the back reaction, as described in the Appendix. The size of the correction increases, of course, with the extent of the replacement, but even in the case of the observation at 9·2 hr only amounts to about 7% of the latter. The actual value of j_4 for the experiment of Fig. 3 is 0·000092 sec^{-1}, and is thus far smaller than any previously recorded dissociation velocity constant of a haemoglobin-ligand reaction, being in fact only of the order of one two-hundredth of l_4, the velocity constant of the $Hb_4(CO)_4 \rightarrow Hb_4(CO)_3 + CO$ reaction.

Effect of variations of pCO, [Hb] and individual sheep blood samples

If the theory underlying the method is correct then the measured value of j_4 should be unaffected by a change in the pressure of carbon monoxide with which the NOHb solution is rotated. Table 2 shows that a threefold variation from 0·5 to 1·5 atmCO has in fact no significant effect on j_4.

If the reaction is truly unimolecular then the measured value of j_4 should be independent of haemoglobin concentration. Table 2 shows that increase of

[Hb] to three times its normal value only affects j_4 by 5% at most, both at pH 9·1 and at pH 5·8. Such an effect is well within the limits of experimental error.

Table 2 shows that the value of j_4 under standard conditions of pH (i.e. 9·1) and temperature (i.e. 19° C), varies appreciably from sheep to sheep, presumably owing to individual differences in the globin portions of the haemo-

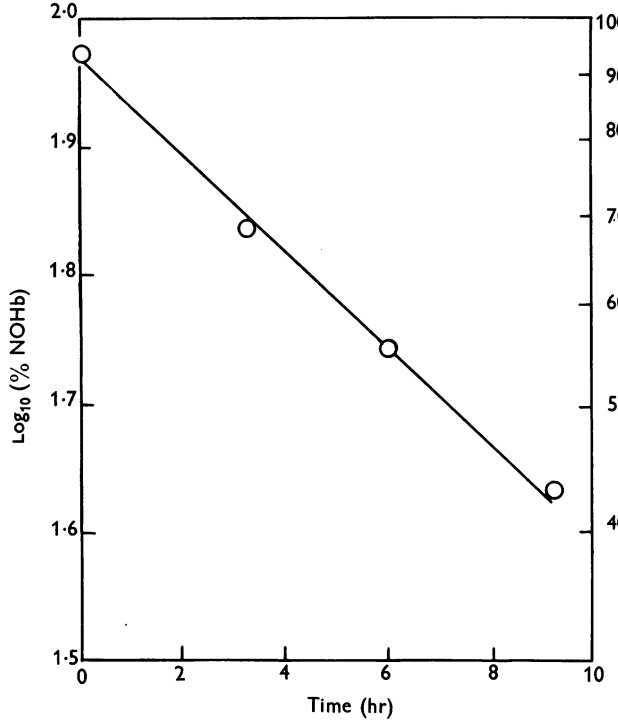

Fig. 3. Rate of dissociation of NOHb at 16·5° C, pH 9·1 (0·2 M borate), 1 in 150 blood solution.

TABLE 2. Effects of [CO], [Hb] and individual variations within the species on j_4

	pH	Temp. (° C)	[Hb] (M × 10^5)	pCO (atm)	j_4 (sec^{-1})
A. [CO] variation	9·1	19	5	1·5	0·000111
	9·1	19	5	0·5	0·000114
B. [Hb] variation	9·1	17	15*	1·0	0·000077
	9·1	17	5	1·0	0·000081
	6·8	17	15*	1·0	0·000174
	6·8	17	5	1·0	0·000182
C. Individual blood variations	9·1	19	5	1·0	0·000119
	9·1	19	5	1·0	0·000101
	9·1	19	5	1·0	0·000160
	9·1	19	5	1·0	0·000092

* In the case of the higher haemoglobin concentration, it was necessary to run the blood solution out of the tonometer into a 5 mm parallel-sided glass trough before the absorption bands were measured with the reversion spectroscope.

globin molecule. The magnitude of the variations in j_4 is of the same order as has been previously seen in the case of other haemoglobin velocity constants. It should be noted that the comparative tests in Table 2, parts *A* and *B*, were all made on blood from the same sheep.

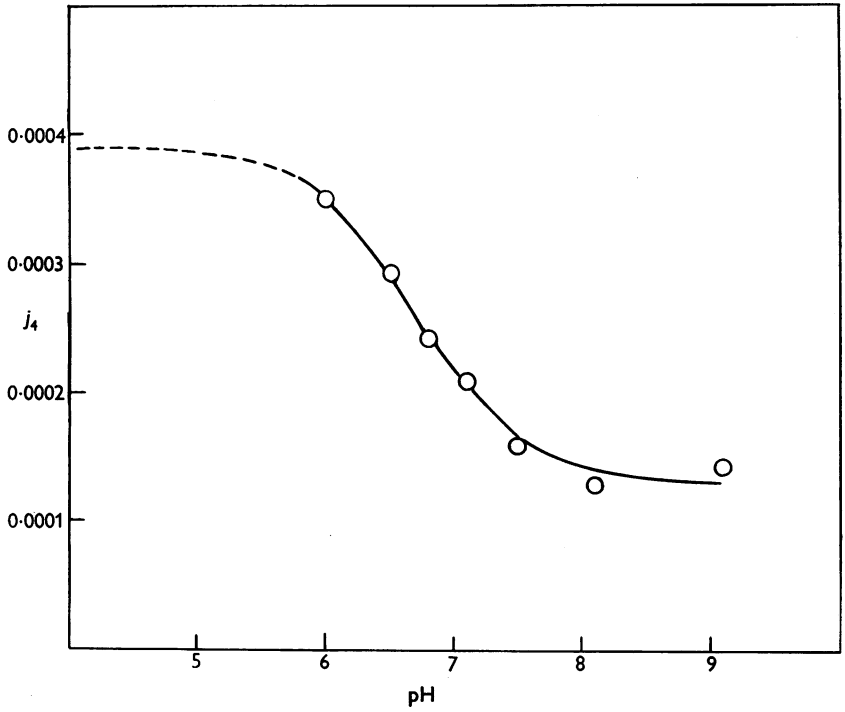

Fig. 4. Effect of pH on j_4.

Effect of pH

Experiments over the range pH 6·0–9·1 were carried out on laked blood diluted to 1 in 150 with 0·05 M phosphate or 0·2 M borate buffer. The results were essentially the same with blood from three different sheep. Fig. 4 shows a plot of the data obtained at 20·3° C in the most complete case studied. The circles represent the actual values of j_4 at each pH whilst the full curve, which is similar to the titration curve of a weak acid, is a theoretical one calculated on the assumptions that

(i) sheep haemoglobin contains a NO-labile acid group with a pK = 6·7;
(ii) the value of j_4 for the un-ionized form = 0·00039, whereas the value of j_4 for the ionized form = 0·00013;
(iii) at any intermediate hydrogen-ion concentration, h,

$$j_4 = \frac{0{\cdot}00039h + 0{\cdot}00013 \times 10^{-6{\cdot}7}}{h + 10^{-6{\cdot}7}}, \qquad (14)$$

since on assumption (i) the proportions of nitric oxide haemoglobin in the unionized and ionized forms are $h/(h+10^{-6\cdot7})$ and $10^{-6\cdot7}/(h+10^{-6\cdot7})$ respectively. The observed results in Fig. 4 agree very satisfactorily with the gas-labile ionizing group theory, which has for many years been generally accepted for oxyhaemoglobin, carboxyhaemoglobin and reduced haemoglobin. It is furthermore of interest to note that the numerical value for the pK of the gas-labile group in nitric oxide haemoglobin agrees closely with the values for this group in oxyhaemoglobin and carboxyhaemoglobin. A more complete test of equation (14) would have been obtained by extending the observations down to pH 5·0. Owing, however, to the instability of haemoglobin below pH 6·0, such an extension was not practicable.

Effect of temperature

Table 3 shows the effect of temperature over the range 20–38° C, on the value of j_4 at pH 6·8 and at pH 9·0, together with the value of the energy of activation, A, as calculated from the equation

$$\ln\{(j_4 \text{ at } T_2)/(j_4 \text{ at } T_1)\} = \frac{A}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right), \tag{15}$$

where T_1, T_2 are temperatures in degrees absolute and R is the usual gas constant (1·98 cal). The corresponding values of Q_{10}, the temperature coefficient per 10° C, are 5·3 at pH 6·8 and 5·6 at pH 9·0. These energies of activation and temperature coefficients are much higher than have been observed in the case

TABLE 3. Effect of temperature on j_4.

	Temp. (° C)	j_4 (sec^{-1})	A (cal)
pH 6·8	20·2	0·000256	29,500
	38·2	0·004850	
pH 6·8	19·0	0·000134	28,900
	38·0	0·002840	
		Mean	29,200
pH 9·0	20·2	0·000144	31,500
	38·2	0·003440	
pH 9·0	19·0	0·000101	29,100
	38·0	0·002190	
		Mean	30,300

of any previous haemoglobin reaction velocity constant, but are in quantitative accord, on classical physico-chemical theory, with the extremely low value of j_4. This matter is further considered in a contribution to the recent Faraday Society Discussion on 'The Physical Chemistry of Enzymes' (Gibson & Roughton, 1955*c*).

Effect of species variation

No detailed studies have yet been made, but preliminary comparisons have shown that the value of j_4 at pH 9·1 for human haemoglobin is 3–5 times smaller than for sheep haemoglobin. Measurements of j_4, and the effect of temperature thereon, should obviously be extended to a wide range of species.

Comparison of the rate of the reaction in solution and in the red cell

Roughton (1932) has shown that the faster the haemoglobin reaction, the greater the discrepancy is between the observed rate in solution and in the red cell, owing to the limiting effect of diffusion in the membrane and interior of the red cell. Thus for the combination of oxygen there is about a tenfold discrepancy, whereas for the slower combination of carbon monoxide the discrepancy falls to threefold. In the case of the very much slower dissociation of carboxyhaemoglobin the observed rate is the same in solution and in the red cell, the limiting effect of diffusion in this case being negligible. Since nitric oxide haemoglobin has been shown to dissociate about a hundred times more slowly than carboxyhaemoglobin it should be expected that this reaction also would take place at the same rate in solution and in the red cell.

The partition equilibrium constant of haemoglobin between NO and CO

Verification of equations (10) *and* (10*a*). Fig. 5 shows the results obtained in a typical experiment at pH 9·1 at 19° C, in which sheep haemoglobin solution (0·1 g/100 ml.) was rotated for periods of 18 hr with gas mixtures containing CO at 740 mm Hg and four different partial pressures of NO ranging from 0·14 to 0·84 mm Hg. The circles represent actual observed results, whereas the full line curve is a rectangular hyperbola corresponding to the theoretical equation (cf. equation (10*a*)),

$$\% \text{ NOHb}/100 = 3700\, p\text{NO}/(p\text{NO} + p\text{CO}). \tag{16}$$

The points clearly fall on the curve within the limits of experimental error, and the corresponding value of N as defined by equation (10)

$$= \frac{3700 \times \text{solubility coefficient of CO}}{\text{solubility coefficient of NO}} = 1810.$$

A similar satisfactory concordance between experiment and theory has been found in all the determinations of N (upwards of 20 in number) so far carried out on sheep haemoglobin solution.

Table 4 summarizes values of N obtained at pH 9·1, 19° C on haemoglobin solutions prepared from five different samples of sheep blood, together with values of N at pH 6·8, 19° C in the case of the last two blood samples. There is a slight individual variation, of the order of 10% from the mean, and there is also a slight effect of pH, i.e. about 1·2 times, over the range pH 6·8–9·1.

A similar though larger effect of pH on M, the partition equilibrium constant of haemoglobin between CO and O_2, has been reported by Roughton (1954).

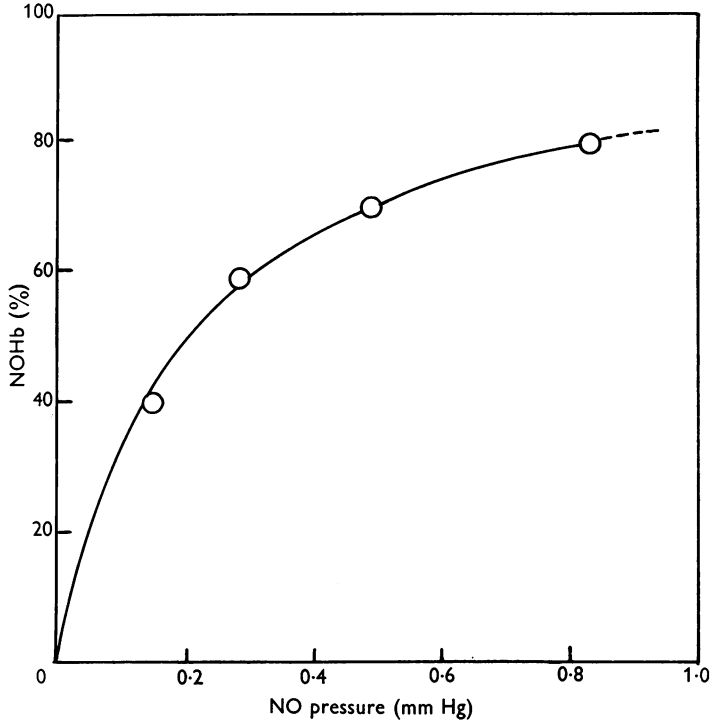

Fig. 5. Partition equilibrium of haemoglobin between NO and CO when shaken with gas mixtures containing small partial pressures of NO in gas phases containing CO at a partial pressure of 740 mm Hg (pH 9·1, 19° C). Abscissa = partial pressure of NO. Ordinate = % NOHb at equilibrium.

TABLE 4. Values of N at pH 9·1 and pH 6·8, 19° C

Blood sample	N at pH 9·1	N at pH 6·8
1	1545	—
2	1545	—
3	1810	—
4	1540	1350
5	1630	1360
Mean	1614	1355

Effect of temperature. Table 5 shows the effect of temperature, over the range 19–38° C on the value of N at pH 6·8 and at pH 9·0, together with the values of ΔH, the heat of the reaction NO + COHb → CO + NOHb, as calculated from the Van't Hoff isochore

$$\ln\left(\frac{N \text{ at } T_2}{N \text{ at } T_1}\right) = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right). \qquad (17)$$

The negative value of ΔH means that the reaction $NO + COHb \rightarrow CO + NOHb$ is exothermic, as is the case also for the reaction $CO + O_2Hb \rightarrow O_2 + COHb$, the heat of which (in the case of sheep haemoglobin) is, however, distinctly larger over the range 0–19° C (Roughton, 1954).

Owing to lack of time no study has yet been made of the effect of species variation on N. It would also be desirable to determine the effect, if any, of varying haemoglobin concentration.

TABLE 5. Effect of temperature on N

	Temp. (° C)	N	ΔH (cal)
pH 6·8	19	1360	−2570
	37·5	1050	
pH 9·0	19	1630	−2230
	37·5	1310	

Possibility of side reactions of nitric oxide: equivalence of NO- and CO-capacities of haemoglobin

Control experiments, already mentioned in the Methods section, have shown that once equilibrium is reached further rotation, for several hours, of the blood solution with the NO-CO gas mixture in the tonometer causes no change in the equilibrium percentage NOHb. This result, *per se*, is good evidence against progressive disappearance of nitric oxide by side reactions. Nitric oxide is, however, usually stated to be unstable in contact with water and it was therefore decided to determine directly the amount of nitric oxide absorbed by oxygen-free buffer solution over a period of 24 hr.

2·5 ml. of 3% borax solution was thoroughly de-aerated in a Van Slyke gasometric chamber. About 0·5 ml. of nitric oxide was then introduced into the chamber, the mercury lowered to the 50 ml. mark and the solution equilibrated with the nitric oxide by shaking for 3 min at 17° C. The pressure of nitric oxide was read at the 2 ml. mark and the mercury lowered again to the 50 ml. mark. The pressure at the 2 ml. mark was read at varying intervals during the following day. At the end of 24 hr the pressure of nitric oxide had only dropped by about 1% of its original value, thus showing that hydrolysis or other slow reactions with water could not have been of significance during 24-hour rotation periods at room temperature.

Nitric oxide is a relatively reactive gas and might, of course, react with other groups in the haemoglobin molecule besides the iron atoms. This possibility was examined in the following way:

(*a*) 5 ml. of water containing 4 drops of octyl alcohol was thoroughly evacuated in the Van Slyke chamber, all extracted gas completely expelled and the pressure reading, p_1, at the 2 ml. mark taken with only aqueous vapour and solution present in the chamber.

(*b*) About 0·5 ml. of nitric oxide, of known purity, was then introduced anaerobically into the Van Slyke chamber and the pressure, p_2, again read at the 2 ml. mark. From the difference, $p_1 - p_2$, and the percentage purity, the volume of nitric oxide (s.t.p.) is calculated in the usual way.

(*c*) 40 ml. of 1 in 4 laked sheep blood, containing 1% boric acid as preservative, was completely deoxygenated by repeated shaking with oxygen-free nitrogen in a 100 ml. tonometer fused to a 30 ml. burette graduated in 0·1 ml. divisions.

(*d*) Exactly 10 ml. of the deoxygenated blood solution was transferred from the tonometer to the Van Slyke chamber, the mercury lowered to the 50 ml. mark and the blood solution shaken with

the nitric oxide gas phase for successive periods of 10 min, the pressure at the 2 ml. mark being read at the end of each such period. It was found that at 14° C the nitric oxide absorption was 99 % complete in 10 min, and that after 20 min no further detectable absorption occurred. During the shaking the pressure of nitric oxide, i.e. about 1·5 % atm, was about 10 times higher than in the determinations of N, whereas the concentration of haemoglobin was nearly 40 times greater. It therefore seems most improbable that any significant amount of nitric oxide could be slowly absorbed by the dilute haemoglobin solutions, used in the N determinations, even over periods as long as 24 hr.

(*e*) To complete the determination of the NO-capacity of the blood solution, any CO_2 evolved into the gas phase of the chamber was then absorbed by running in 1 ml. of de-aerated N-NaOH, at the end of which the pressure at the 2 ml. mark was again read $=p_3$.

(*f*) The percentage nitric oxide in the chamber gas was determined by expelling a bubble of the gas into a Scholander-Roughton syringe and analysing for NO by absorption with acid $FeSO_4$, by the same technique as Roughton & Root (1945) used for analysis of carbon monoxide bubbles, save that in the latter case Winkler's solution was used as absorbent.

(*g*) The remaining gas in the chamber was then completely expelled, and a final reading taken at the 2 ml. mark (p_4). From $p_3 - p_4$ and the percentage NO at stage (*f*) the residual unabsorbed nitric oxide is calculated. Subtraction of the latter from the total nitric oxide originally introduced gives the volume of nitric oxide absorbed whence, after allowance for physically dissolved nitric oxide, the NO-capacity of the 1 in 4 blood solution can be calculated as in the following example:

$p_1 = 354{\cdot}5$, $p_2 = 151$, $p_1 - p_2 = 203{\cdot}5$ mm Hg at 14·1° C.

Purity of initial nitric oxide = 98 %.

Volume of nitric oxide introduced initially = 0·502 ml. (s.t.p.).

$p_3 = 174$, $p_4 = 99{\cdot}2$, $p_3 - p_4 = 74{\cdot}8$ mm Hg at 13·9° C.

Purity of final nitric oxide in chamber = 81·2 % (remainder = N_2).

Volume of nitric oxide in physical solution = 15 (total volume of fluid) × 0·048 (solubility coefficient of NO) $\times \frac{2}{35} \times \frac{74{\cdot}8}{760} \times \frac{81{\cdot}2}{100}$ (pressure of NO in atm when mercury is at 50 ml. mark) = 0·003 ml. (s.t.p.).

Volume of nitric oxide in chemical combination = 0·350 − 0·003 = 0·347 ml. (s.t.p.).

Since 10 ml. of blood diluted 1 in 4 were used, NO capacity of original blood = 13·88 vol. %.

An exactly similar experiment on a second 10 ml. sample from the same stock of reduced haemoglobin solution, but with carbon monoxide in place of nitric oxide, gave a CO-capacity = 13·60 vol. %, in satisfactory agreement with the above figure for the NO-capacity of the same blood. Hermann's (1865) original demonstration of the equivalence of these two capacities is thus confirmed in an entirely independent manner.

It was noted that the rate of carbon monoxide absorption during the shaking process was about half the rate of nitric oxide absorption at the same gas pressures. Just such a difference is to be expected since the solubility coefficient of carbon monoxide is about half that of nitric oxide.

DISCUSSION

Absence of interaction effects at high percentage saturations of haemoglobin

The previous work of Roughton (1934) and of Gibson & Roughton 1955*a*) has provided strong and varied evidence for their tenets that:

(I) If all four iron atoms in the haemoglobin molecule are combined with oxygen, i.e. Hb_4O_8, or with carbon monoxide, i.e. $Hb_4(CO)_4$, or partly with oxygen and partly with carbon monoxide i.e. $Hb_4(O_2)_a(CO)_{4-a}$ (where

$a=3$, 2, or 1), then the velocity constant for dissociation of an oxygen or carbon monoxide molecule is simply proportional to the total number of combined oxygen or carbon monoxide molecules in the dissociating complex. In these circumstances there are thus no interactive effects from neighbouring iron atoms.

(II) If three out of the four iron atoms are combined with oxygen, i.e. Hb_4O_6, or with carbon monoxide $Hb_4(CO)_3$, or partly with oxygen and partly with carbon monoxide, i.e. $Hb_4(O_2)_b(CO)_{3-b}$ (where $b=2$ or 1), then the velocity constant for combination of oxygen with the last free iron atom on the haemoglobin is the same in all four cases: similarly for the velocity constant of combination of carbon monoxide with any such molecule, though the numerical values of the combination velocity constants are of course different in the case of oxygen and carbon monoxide.

The work in the present paper on the rate of dissociation of NO-haemoglobin in presence of high concentrations of carbon monoxide indicates that tenet I can now be extended to nitric oxide. The validity of tenet II, as regards nitric oxide, has also been confirmed by us, *inter alia*, by measurements of the rate at which nitric oxide replaces carbon monoxide from combination with haemoglobin, when solutions of carboxyhaemoglobin are rotated with gas mixtures of carbon monoxide plus very small percentages of nitric oxide (as in the determination of N). The experimental details and results of this work are described in another paper (Gibson & Roughton, 1957), which also contains a varied range of further evidence, all of which goes to support the validity of tenets I and II not only as regards nitric oxide but also as regards carbon monoxide.

Calculation of J_4. From equation (9), it follows that at pH 9·1, 19° C

J_4 = average value of N (see Table 4) × average value of L_4 (as given by Roughton, 1954)

$= 1600 \times 2{\cdot}9 \times 10^8$

$= 4{\cdot}6 \times 10^{11}$ M^{-1}.

From equation (9) it also follows that the heat of the reaction $Hb_4(NO)_3 + NO$ (solution) $\rightarrow Hb_4(NO)_4$ = the heat of the reaction $NO + COHb \rightarrow CO + NOHb$ plus the heat of the reaction $Hb_4(CO)_3 + CO$ (solution) $\rightarrow Hb_4(CO)_4 = -2200$ (see Table 7) -10500 (Roughton, 1954) $= -12{,}700$ cal at pH 9·1. The corresponding value of Q_{10}, the temperature coefficient per 10° C rise, is 0·49. (The values given in Table 4 of Roughton's (1954) paper are for the heat of the reaction $Hb_4(CO)_3 + CO$ (gas) $\rightarrow Hb_4(CO)_4$ and for our present purpose need to be corrected for the heat of solution of carbon monoxide.)

No sufficiently accurate values of L_4 at pH 6·8 are yet available to permit calculation of J_4 at that pH.

Calculation of j_4'. From equation (4)

$$j_4' \text{ at pH } 9{\cdot}1,\ 19^\circ \text{ C} = J_4 \times \text{average value of } j_4 \text{ (see Table 2)}$$
$$= 4{\cdot}6 \times 10^{11} \times 1{\cdot}07 \times 10^{-3}$$
$$= 4{\cdot}9 \times 10^{8} \text{ M}^{-1} \text{ sec}^{-1}.$$

The numerical value of j_4' is over three times greater than j_1', thus showing that interaction effects as regards combination velocity constants still persist in the nitric oxide-haemoglobin reactions, which are by far the fastest ligand-haemoglobin combinations yet investigated, but in less degree than in the slower combinations of haemoglobin with oxygen or with carbon monoxide. The interaction effect, if the ratio of the fourth combination velocity constant to the first combination velocity be taken as an index, does indeed appear to decrease progressively as the absolute value of the velocity of combination of ligand with haemoglobin increases. It would be of interest to find out whether this relation also applies to combinations of haemoglobin with other ligands, e.g. the isocyanides, the kinetics of which have not yet been investigated.

Calculation of Q_{10}, the temperature coefficient per 10° C, of j_4' at pH 9·1. From equation (4) it also follows that

$$Q_{10} \text{ of } j_4' = Q_{10} \text{ of } J_4 \times Q_{10} \text{ of } j_4$$
$$= 0{\cdot}49 \times 5{\cdot}6$$
$$= 2{\cdot}75.$$

The corresponding energy of activation = 17,600 cal. These values for the Q_{10} and energy of activation are both remarkably high for such a fast reaction.

The partition equilibrium constant of the hypothetical reaction $NO + O_2Hb \rightleftharpoons O_2 + NOHb$. This reaction cannot be isolated owing to the well-known spontaneous combination of nitric oxide with oxygen to form nitrogen peroxide ($2NO + O_2 \rightarrow 2NO_2$). It is indeed this fact which prevents nitric oxide from being, in practice, an excessively poisonous gas. The numerical value of the partition equilibrium constant of this hypothetical reaction must, however, according to the Law of Mass Action

$= N \times M$ (the partition equilibrium constant of the reaction $CO + O_2Hb \rightleftharpoons O_2 + COHb$)

$= 1600 \times 240 = 384{,}000$ at pH 9·1, 19° C

$= 1350 \times 180 = 243{,}000$ at pH 6·8, 19° C.

SUMMARY

1. Hermann's (1865) finding that nitric oxide combines with haemoglobin in the same definite proportions as do oxygen and carbon monoxide has been confirmed by more precise methods. It has furthermore been shown that nitric oxide reacts only with the haem groups, even on prolonged exposure.

2. Nitric oxide has about 1500 times greater affinity for haemoglobin than has carbon monoxide. This result was obtained by equilibrating haemoglobin with gas mixtures containing 99–100% CO and 0–1% NO, and determining the final proportions of COHb and NOHb with the reversion spectroscope.

3. This remarkable affinity of NO for Hb is chiefly due to the slow rate of breakdown of NOHb. The half-time for dissociation of the first NO molecule from $Hb_4(NO)_4$ at 19° is about 8 hr at pH 9 and about 3 hr at pH 6·0. The Q_{10} is about 5·5.

4. The combination of nitric oxide with reduced haemoglobin takes place 5–20 times faster than the reaction with oxygen and is, indeed, the fastest direct reaction so far measured of an oxygen-carrying pigment with any ligand.

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APPENDIX

Allowance for the back reaction in the rate of dissociation of NOHb

According to the present paper the velocity of the forward reaction

$$=j_4[Hb_4(NO)_4]=n[\text{NOHb}], \qquad (1\,\text{A})$$

where $n=j_4/4$. Experiments described elsewhere (Gibson & Roughton, 1957) show that the velocity of the back reaction

$$=n'[\mathrm{NO}][\mathrm{COHb}]/[\mathrm{CO}], \tag{2A}$$

where $n'=n\times N$. The complete expression for the rate of dissociation of NOHb is therefore

$$\frac{dw}{dt}=n(a-w)-n'\frac{[\mathrm{NO}]}{[\mathrm{CO}]}w, \tag{3A}$$

where a=[NOHb] at zero time, $a-w$=[NOHb] at time t. Since the carbon monoxide is in great excess it may be taken as constant, and since furthermore [NO] must be proportional to w, equation (3A) may be replaced by the simpler form

$$\frac{dw}{dt}=n(a-w)-n''w^2, \tag{4A}$$

where $n''=\theta n'$

$$\theta=\alpha_{\mathrm{NO}}V_{\mathrm{L}}/(\alpha_{\mathrm{CO}}V_{\mathrm{G}}\times \mathrm{CO\ pressure\ in\ atm}),$$

α_{NO} = solubility coefficient of NO,

α_{CO} = solubility coefficient of CO,

V_{L} = volume of liquid phase in tonometer,

V_{G} = volume of gas phase in tonometer.

The solution of equation (4A) by standard methods, is

$$w=\frac{a}{\frac{1}{2}+\sqrt{\left(\frac{1}{4}+\frac{an''}{n}\right)}\coth\left[nt\sqrt{\left(\frac{1}{4}+\frac{an''}{n}\right)}\right]}$$

$$=\frac{a}{\frac{1}{2}+\sqrt{(\frac{1}{4}+a\theta N)}\coth\left[nt\sqrt{(\frac{1}{4}+a\theta N)}\right]} \tag{5A}$$

Table 1A shows the calculated effect of the back reaction in a typical example at pH 9·1 at 19° C in which $n=3\times10^{-5}$ (i.e. $j_4=4n=1{\cdot}2\times10^{-4}$), $N=1500$; $a=4\times10^{-5}$ M NOHb, CO $=9\times10^{-4}$ M, $V_{\mathrm{L}}=10$ ml., $V_{\mathrm{G}}=175$ ml.

Up to 50% dissociation the error in the calculation of n, if the back reaction is neglected, is seen to be less than 5·0% but thereafter mounts rapidly.

TABLE 1A. Effect of back reaction in typical example in which $n=3\times10^{-5}$ sec^{-1}, $N=1500$

Time (min)	% NOHb if back reaction neglected	$\log_{10}\left(\frac{a}{a-w}\right)$ if back reaction neglected	% NOHb if back reaction included	$\log_{10}\left(\frac{a}{a-w}\right)$ if back reaction included	% correction to value of n due to back reaction
0	100	0	100	0	0
163	74·5	0·1275	74·6	0·1270	0·4
326	55·6	0·2550	56·4	0·2489	2·4
489	41·4	0·3825	43·2	0·3640	4·8
652	30·9	0·5100	34·5	0·4629	9·2