

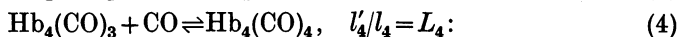
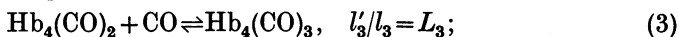
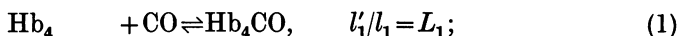
**THE DIRECT DETERMINATION OF THE VELOCITY  
CONSTANT OF THE REACTION  $\text{Hb}_4(\text{CO})_3 + \text{CO} \rightarrow \text{Hb}_4(\text{CO})_4$**

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According to the intermediate compound hypothesis of Adair (1925) combination between carbon monoxide and haemoglobin takes place in four stages:



where  $l'_1, l'_2, l'_3$  and  $l'_4$  are the combination velocity constants,  $l_1, l_2, l_3$  and  $l_4$  are the dissociation velocity constants and  $L_1, L_2, L_3$  and  $L_4$  the equilibrium constants for the intermediate stages. Adair's formulation became necessary following his demonstration that each molecule of haemoglobin contains four atoms of iron rather than a number varying between two and three as had earlier been supposed. For many years little practical use was made of equations (1) to (4), not because they were incompatible with the experimental results available, but because many different sets of values could be assigned to the constants, any one of which would give agreement within experimental error between theory and observation. This was true both of kinetic and equilibrium studies.

In recent years the precise work of Roughton and his school on the oxygen dissociation curve (Roughton, 1954; Roughton, Otis & Lyster, 1955) and on the upper end of the carbon monoxide dissociation curve has allowed close limits to be set to the values of certain of the equilibrium constants. This advance has led to renewed interest in the thoroughgoing application of equations (1) to (4) to the kinetics of these reactions, and Gibson & Roughton (1956) have given an account of kinetic work in which the over-all course of the reaction between reduced haemoglobin and carbon monoxide was followed in a stopped-flow apparatus. The resulting kinetic curves were then fitted with appropriate equations derived from (1) to (4), and the values of the combination velocity constants thus determined.

In spite of the greater precision of the new kinetic experiments (as compared with those previously available) such a procedure would have been impossible without an independent estimate of  $l'_4$ , which was obtained indirectly by combining Roughton's (1954) determinations of  $L_4$  with values for  $l_4$ .

The present paper describes a direct determination of  $l'_4$  which is of interest not only because of its bearing on the problem of fixing the values of the intermediate constants for carbon monoxide combination, but more generally because it offers a direct and dramatic confirmation of the differences between the properties of the intermediate  $\text{Hb}_4(\text{CO})_3$  and those of intermediate compounds containing fewer molecules of CO. These differences, which have now been observed directly, have been found to be at least at pH 9.1, of the order predicted by Gibson & Roughton (1956) as a result of their mathematical analysis.

#### METHODS

The flash photolysis apparatus used was that described by Gibson (1956) in a preceding paper. The preparation of the blood and buffers and the handling of the solutions were carried out as described by Gibson & Roughton (1956) except for the experiments using nitric oxide which were carried out as follows: nitric oxide haemoglobin was prepared by allowing the stock reduced haemoglobin solution to react with NO. Excess NO was then removed with a filter-pump and the solution repeatedly evacuated until the blood boiled, vigorous shaking being maintained for 5 min. The tonometer was finally filled with  $\text{O}_2$ -free  $\text{N}_2$ . Carboxyhaemoglobin was prepared similarly, except that the solution was evacuated only briefly (30 sec) before filling the tonometer with  $\text{N}_2$ . The mixtures of COHb and NOHb required were prepared by filling clean dry tonometers with  $\text{N}_2$  and transferring to them the required volumes of COHb and NOHb from burettes where the solutions were confined over mercury.

The mixtures were then heated to 40–45° C for an appropriate time (usually 15 min) to establish equilibrium between the intermediate compounds containing NO and CO, and finally diluted with 0.05 M-borate buffer pH 9.1 to a total pigment concentration of about 0.08 mM. Portions were transferred to the observation cell of the flash apparatus, equilibrated with the required gas mixture and examined as usual.

#### THEORY OF THE METHODS

##### *Method 1. Variation in flash intensity*

As is well known, carboxyhaemoglobin is sensitive to light, dissociating into reduced haemoglobin and carbon monoxide. According to the intermediate compound hypothesis this dissociation will take place in four steps corresponding to equations (4) to (1) with the formation, successively, of  $\text{Hb}_4(\text{CO})_3$ ,  $\text{Hb}_4(\text{CO})_2$ ,  $\text{Hb}_4(\text{CO})$  and  $\text{Hb}_4$ . In calculating the quantity of each intermediate formed when the amount of light applied is not enough to displace all the combined CO it has been assumed that the quantum yield is the same whichever intermediate compound is being irradiated. This assumption is supported by the work of Buecher & Negelein (1941–2) who found that the quantum efficiency of CO dissociation from COHb remained the same in mixtures of oxy- and carboxy-haemoglobin when the proportions of each were varied. Their solutions contained mixed molecules of the form  $\text{Hb}_4(\text{O}_2)_n(\text{CO})_{4-n}$  (see

Gibson & Roughton, 1955*a*) so that, in their case, variation in  $n$  did not influence quantum yield. Although conditions in the present experiments differ from those of Buecher & Negelein, the parallel is sufficiently close to substantiate the assumption of independence between individual haem groups.

With this assumption, if  $p$  and  $q$  are the amounts of CO remaining in combination and which have dissociated respectively, then the amounts of each intermediate are given by the successive terms in the expansion of  $(p+q)^4$ , the first term representing  $\text{Hb}_4(\text{CO})_4$  and so on (see Appendix). The proportions of the various intermediates present at different percentage dissociations of CO are shown graphically in Fig. 1.

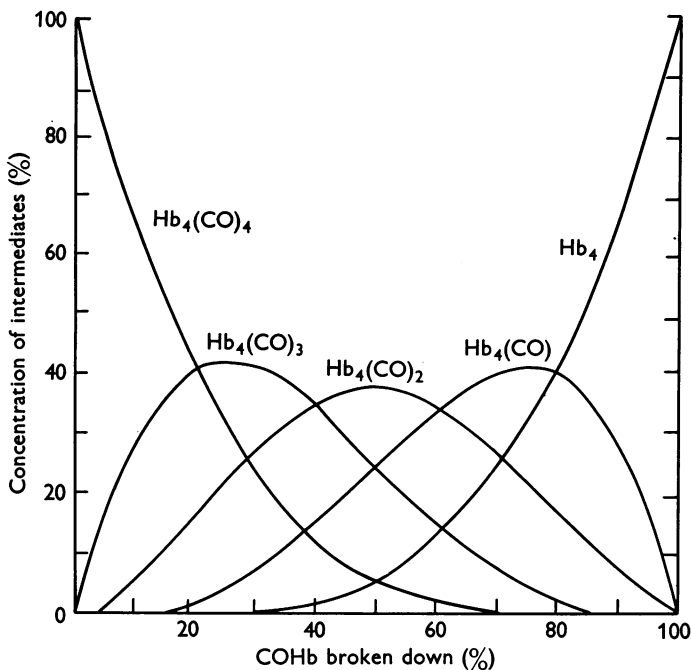


Fig. 1. The variation in the proportions of intermediate compounds present when carboxyhaemoglobin is caused to dissociate to varying degrees by a brief flash of light.

This calculation neglects the finite length of the flash, which lasts effectively for 0.2–0.3 msec. Such neglect is only justified if the half-time of the most rapid recombination reaction is considerably longer than 0.3 msec. In the present work this condition has been met by adjusting the concentrations of the reactants so that the shortest half-time measured has been of the order of 5–10 msec.

*Calculation of  $l'_4$ .* As Fig. 1 shows, the ratio of  $\text{Hb}_4(\text{CO})_3$  to lower intermediates (i.e. those with less combined CO) increases rapidly as the percentage of carboxyhaemoglobin broken down decreases. In measuring  $l'_4$  it is therefore

clearly desirable to work with the least practicable percentage breakdown of carboxyhaemoglobin. It has been possible to make measurements with as little as 5–10% dissociation. With 5% dissociation the solution contains 81.45%  $\text{Hb}_4(\text{CO})_4$ , 17.15%  $\text{Hb}_4(\text{CO})_3$ , 1.35%  $\text{Hb}_4(\text{CO})_2$  and only 0.05% of other intermediates. Similar calculations for 10% dissociation of COHb show that the solution then contains 65.61%  $\text{Hb}_4(\text{CO})_4$ , 29.16%  $\text{Hb}_4(\text{CO})_3$ , 4.86%  $\text{Hb}_4(\text{CO})_2$  and 0.37% of other intermediates. The initial rate of recombination of CO in each case may thus reasonably be taken as

$$-d[\text{CO}]/dt = l'_4[\text{Hb}_4(\text{CO})_3][\text{CO}] + l'_3[\text{Hb}_4(\text{CO})_2][\text{CO}] \quad (5)$$

if the lower intermediates, amounting to 0.05 and 0.37% respectively, be neglected.

It is possible to substitute numerical values in (5), obtaining  $-d[\text{CO}]/dt$  and the percentage of carboxyhaemoglobin broken down from the oscillograph traces, while the concentrations of the intermediates  $\text{Hb}_4(\text{CO})_3$  and  $\text{Hb}_4(\text{CO})_2$  are obtained by calculation from the proportion broken down, and the total concentration of carboxyhaemoglobin is determined spectrophotometrically on a portion of the solution. The concentration of CO in solution is initially equal to that originally present in physical solution plus that caused to dissociate by the light flash, leaving  $l'_3$  and  $l'_4$  as the only unknowns in (5). By substituting in (5) for two different proportions of carboxyhaemoglobin breakdown the two constants are, in principle, calculable. In practice, the initial value of  $-d[\text{CO}]/dt$  depends effectively only on the term  $l'_4[\text{Hb}_4(\text{CO})_3][\text{CO}]$  in the range of dissociation of COHb from 0 to 20%. This offers direct evidence that  $l'_4$  is much greater than  $l'_3$ , as has indeed already been suggested by Gibson & Roughton (1955*a*). Although this finding simplifies the calculation of  $l'_4$ , the corresponding disadvantage is that  $l'_3$  is not obtainable from simple measurements of initial values of  $-d[\text{CO}]/dt$ , but would require for its evaluation mathematical analysis of the time-course of the over-all reaction.

#### *Method 2. With nitric oxide haemoglobin*

Gibson & Roughton (1955*a*) have shown that when three of the four gas-binding groups of a mammalian haemoglobin molecule have already combined with ligand molecules, the rate of combination of ligand with the fourth gas-binding group is determined only by the nature of the fourth ligand molecule and is independent of the nature of the three molecules already combined. It should thus be possible to determine  $l'_4$  directly by exposing mixtures of NOHb and COHb to the flash and observing the rate of recombination of CO, provided that the proportion of NOHb to COHb is such that, on dissociation of the COHb, only small amounts of compounds with more than one free gas-binding group are formed. In practice this condition cannot be satisfied and it is necessary to allow for the presence of  $\text{Hb}_4(\text{NO})_2$  as well as

$\text{Hb}_4(\text{NO})_3$ . The calculation is formally exactly parallel to that in Method 1, since Gibson & Roughton (1955*a*) have shown that the distribution of a mixture of ligand molecules among the gas-binding groups of haemoglobin is random, provided that sufficient ligand is present to make the numbers of free gas-binding groups negligible. Thus if  $p$  is the proportion of  $\text{HbNO}$  and that of  $\text{HbCO}$ , then the amounts of  $\text{Hb}_4(\text{NO})_4$ ,  $\text{Hb}_4(\text{NO})_3\text{CO}$ ,  $\text{Hb}_4(\text{NO})_2(\text{CO})_2$ ,  $\text{Hb}_4(\text{NO})(\text{CO})_3$ , and  $\text{Hb}_4(\text{CO})_4$  will be given by successive terms in the expansion of  $(p+q)^4$ . The equivalent of equation (5) is

$$-d[\text{CO}]/dt = l'_4[\text{Hb}_4(\text{NO})_3][\text{CO}] + l'_2 n'_2 [\text{Hb}_4(\text{NO})_2][\text{CO}], \quad (6)$$

in which  $l'_2 n'_2$  is, however, not necessarily equal to  $l'_3$ . In equation (6)  $n'$  is used to denote the presence of  $\text{NO}$  molecules and to distinguish the combination constants of the mixed molecules from those employed in equations (1) to (4).

Although Method 2 is limited in principle as well as in practice to the evaluation of  $l'_4$ , the agreement with Method 1 adds greatly to the assurance with which the assumptions of Method 1 may be accepted, particularly with regard to the distribution of intermediate compounds.

## RESULTS

### *The determination of $l'_4$*

On cutting down the energy supplied to the flash tube the time-course of the recombination reaction changes markedly. The general nature of the effect is shown in Fig. 2 where the time-course of the reaction is plotted for two different degrees of dissociation of  $\text{COHb}$ . The figure, which represents directly the traces observed on the oscillograph screen, does not show the full effect of varying the proportion of  $\text{COHb}$  broken down by the flash as it does not take into account the smaller amount of  $\text{CO}$  present when the smaller amount of  $\text{COHb}$  is caused to dissociate. To take account of this the overall velocity constant  $l'$  in the familiar equation for a second-order reaction has been used

$$dz/dt = l'(\alpha - z)(\beta - z) \quad (7)$$

where  $z = [\text{COHb}]$ ,  $\alpha = [\text{CO}]$  at  $t_0$ ,  $\beta = [\text{Hb}]$  at  $t_0$  and  $l'$  is the second-order velocity constant. The change in value of  $l'$  with variation in the proportion of  $\text{COHb}$  broken down is represented in Fig. 3. The initial value of  $l'$  in (7) varies between the upper limit  $l'_4$  as the proportion of  $\text{COHb}$  broken down approaches zero, and the lower limit  $l'_1/4$  as it approaches 100%; values for these limits are suggested on the figure. The difference between these limits is a valid measure of the difference in behaviour between the first and further molecules of carbon monoxide to combine with mammalian haemoglobin.

A number of determinations of  $l'_4$  have been made on different blood samples at pH 9.1 and also at pH 7.1. The results of these experiments are given in

Table 1. The mean value of  $l'_4$  at pH 9.1 and 19° C of about 7000  $\text{mm}^{-1} \text{sec}^{-1}$  is substantially lower than the estimated value of 11,600  $\text{mm}^{-1} \text{sec}^{-1}$  obtained by Roughton (1954) as the product of  $L_4$  and  $l_4$ . It seems likely that the greater part of the difference is due to the value of 0.04  $\text{sec}^{-1}$  used by Roughton for  $l_4$ . Recent determinations on sheep blood from the Sheffield slaughterhouse (Gibson & Roughton, unpublished) suggest that a value of 0.026  $\text{sec}^{-1}$  may more nearly represent a fair average. With this new value for  $l_4$ , the

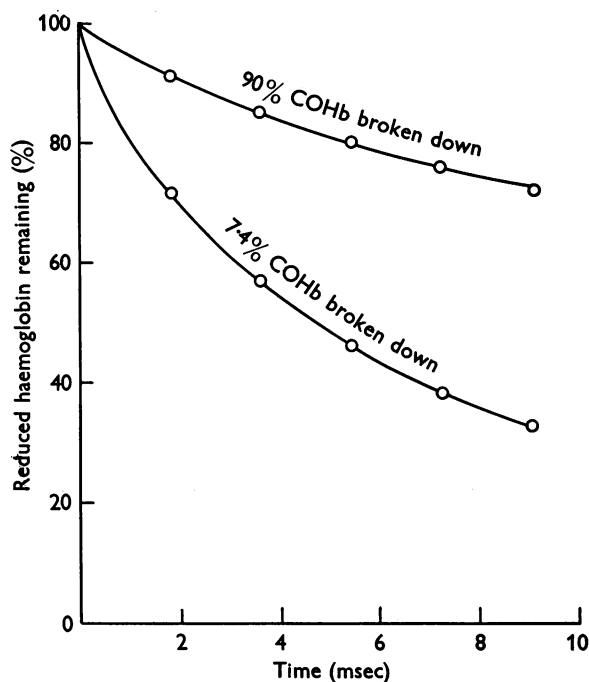


Fig. 2. The observed rate of recombination of CO with reduced haemoglobin when two different amounts of CO have been split off by exposure to light.

estimated value of  $l'_4$ , using Roughton's value of  $2.9 \times 10^5$  for  $L_4$ , is 7600  $\text{mm}^{-1} \text{sec}^{-1}$ , which agrees excellently with the value of 6900 obtained by direct determination; particularly when it is borne in mind that  $L_4$  has shown a large variation from one individual to another and that each of the three series of determinations (of  $L_4$ ,  $l_4$ , and  $l'_4$ ) was carried out at different times and on different samples of blood.

The relatively slight effect of pH does not agree, however, with Roughton's tentative conclusion that  $l'_4$  at pH 7.1 is only about one-third of the value at pH 9.1; if the new figure for  $l_4$  is accepted an upward revision of Gibson & Roughton's (1955*a*) estimate of the corresponding oxygen combination constant ( $k'_4$ ) is necessary.

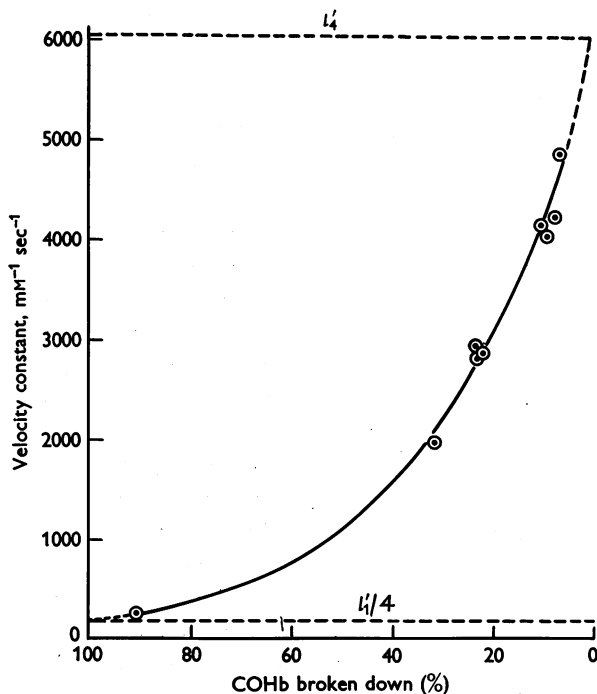


Fig. 3. Changes in the value of the second-order velocity constant for the combination of reduced haemoglobin with carbon monoxide when the proportion of COHb broken down by the photolysis flash is varied. The dotted lines indicate the limiting rates equal to  $l_4$  and  $l_4/4$ , which would be reached as the proportion of COHb broken down tended to 0% and 100% respectively. Borate buffer pH 9.1, 16.5° C.

TABLE 1. Values of the combination velocity constant for the reaction  
 $Hb_4(CO)_3 + CO \rightarrow Hb_4(CO)_4$

Temperature (°C)	COHb (mm)	$l_4$ observed (mm <sup>-1</sup> sec <sup>-1</sup> )		$l_4$ at 19°C (mm <sup>-1</sup> sec <sup>-1</sup> )	
		pH 7.1	pH 9.1	pH 7.1	pH 9.1
20.5	0.022	—	10,000	—	8800
23.5	0.020	7200	11,000	5000	7700
26.8	0.021	—	11,100	—	6000
24.0	0.023	—	9,000	—	6100
22.8	0.028	5300	7,100	4000	5300
24.5	0.033	7400	7,500	4800	4900
19.0	0.031	6800	9,400	6800	9400
		Average		5200	6900

The values of  $l_4$  at 19° C given in the last two columns of the table were calculated using the temperature coefficient given by Gibson & Roughton (1955). The values at pH 9.1 were obtained using 0.05 M-borate buffer, at pH 7.1 0.1 M-phosphate buffer. The concentrations of reduced haemoglobin and of carbon monoxide after exposure to the flash were the same. Light of mean wave-length 432 mμ, 0.14 cm cell.

Over the rather small number of samples examined,  $l'_4$  appears to be somewhat less variable than  $L_4$ , but, as it is not known if  $l'_4$  and  $l_4$  are correlated, this reduced variability need not necessarily reflect a real difference in the samples examined.

#### *Sources of error*

It is desirable to consider the sources of systematic error inherent in the methods used. One obvious danger is that of contamination of the solutions with oxygen, which on combining with haemoglobin will produce an observed optical density change about one-half as great as that corresponding to the combination of the same amount of carbon monoxide. The concentration of COHb used in many of the experiments of this paper (about 0.025 mm) corresponds to a partial pressure of CO of 20 mm Hg: with 5–10% photochemical dissociation of COHb, the partial pressure of CO in solution at the beginning of the recombination reaction is from 1 to 2 mm Hg. Since  $k'_4$  is three times greater than  $l'_4$  (Gibson & Roughton, 1955*a*), it is clear that a partial pressure of 0.1 mm Hg oxygen would increase the apparent initial rate of recombination of Hb with CO by about one-eighth. On several occasions experiments were carried out with and without small amounts of  $\text{Na}_2\text{S}_2\text{O}_4$ , and the same rate was observed in both trials, suggesting that the technique used for handling the solutions was adequate for its purpose.

A second danger is that the observation beam might break down a significant amount of COHb, producing an equilibrium concentration of CO in physical solution which would be available to increase the initial rate of recombination after exposure to the flash. It was found that if unfiltered light from the 24 W tungsten lamp passed through the solutions an equilibrium partial pressure of CO of 1–2 mm Hg was produced, so an additional filter of 2 mm Chance OB1 (deep blue) glass was placed before the observation cell, cutting down the photochemically active light to a safe level.

A third source of error arises from the risk that in attempts to remove  $\text{O}_2$  completely from the solutions CO may also be lost, thus reducing the initial COHb concentration below that estimated from the spectrophotometric determination of total pigment. The effect, to a first approximation, would be to reduce the value of  $l'_4$  in proportion to the loss of CO from the solutions.

The errors resulting from these three causes may be reduced by varying the conditions under which the determinations are made; in particular, by raising the concentration of CO in physical solution, the first and second difficulties are reduced and the third eliminated. A new difficulty appears, however, in that the observed rate is increased in proportion to the CO concentration and the calculation of concentrations of intermediates becomes less certain. A number of experiments have, however, been carried out with an increased CO concentration and have yielded results in agreement with those of Table 1 (see Table 2). The results of repeated determinations on the same solution are also



given in Table 2. In considering the size of this individual variation it should be pointed out that the total change in COHb concentration is perhaps 0.002 mm, and if the first tenth of the reaction record is measured to give an estimate of the initial rate, the measured change corresponds to 0.0002 mm: thus an error of 10% in the rate constant will correspond to an error of 0.00002 mm in the estimation of the COHb concentration. Expressed in another way, a 10% error corresponds to the change in light absorption when a film 0.062 in. thick of blood diluted 1:400,000 is converted from reduced haemoglobin to carboxyhaemoglobin. With this limitation in mind, it is considered that the individual values in Table 2 show satisfactory agreement with one another and with the values in Table 1.

TABLE 2. The determination of  $l'_4$  in the presence of 20 mm Hg partial pressure of CO

	COHb de- composed (%)	$l'_4$ observed ( $\text{mm}^{-1} \text{sec}^{-1}$ )	$l'_4$ at 19° C ( $\text{mm}^{-1} \text{sec}^{-1}$ )
A. Total pigment 0.104 mm, 17.3° C, pH 9.1 borate, 0.027 mm-CO in physical solution at equilibrium	8.3	5700	6500
	6.4	5600	6400
	4.7	4800	5500
	3.9	5300	6000
B. Total pigment 0.100 mm, 21° C, pH 9.1 borate, 0.026 mm-CO in physical solution at equilibrium	8.1	7300	6200
	6.2	7500	6400
	4.5	8700	7400
	4.2	7500	6400

*Comparison of Method 1 (COHb only) with Method 2 (COHb/NOHb mixtures)*

The practical application of the nitric oxide method for determining  $l'_4$  is difficult because of the slowness with which nitric oxide dissociates from combination with sheep haemoglobin, the half-life at 20° C and pH 9.1 being about 6 hr (Gibson & Roughton, 1955*b*). After mixing COHb and NOHb it is necessary to allow at least five half-lives of NOHb to pass before it can be assumed that the distribution of CO and NO molecules among the haem groups is random. In the first experiments mixtures of COHb and NOHb were kept for several days at room temperature, and under these conditions it was repeatedly found that  $l'_4$  by the NOHb method was 20–30% lower than the value obtained from the COHb method using freshly prepared solutions.

The temperature coefficient of the reaction  $\text{Hb}_4(\text{NO}) \rightarrow \text{Hb}_4(\text{NO})_3 + \text{NO}$  is unusually high, Gibson & Roughton (1955*b*) giving  $Q_{10} = 5.5$ , and by heating the COHb-NOHb mixtures to 40° C the half-time for the redistribution of CO and NO molecules can be reduced to a few minutes. It was found that determinations of  $l'_4$  by the NOHb method carried out by heating the mixtures for a relatively brief period to 40–45° C before examination in the flash apparatus agreed within experimental error with those by the COHb method. A full set of results for one experiment is given in Table 3.

TABLE 3. Determination of  $l'_4$  in mixtures of COHb and NOHb and in solutions of COHb only

	COHb de- composed (%)	$l'_4$ observed ( $\text{mm}^{-1} \text{sec}^{-1}$ )	$l'_4$ at 19° C ( $\text{mm}^{-1} \text{sec}^{-1}$ )
A. NOHb/COHb mixture. Total pigment 0.103 mm, 5% COHb, 95% NOHb, pH 9.1, 0.05 M-borate buffer, 21° C, 0.026 mm-CO.	93	8100	6900
	79	5500	4500
	70	8300	6800
	51	7700	6300
	20	7100	5800
	Average		6100
B. COHb alone. Total pigment 0.097 mm, 0.026 mm-CO in physical solution, pH 9.1, 0.05 M-borate buffer, 20° C. Determinations on the same sample of sheep blood as above.	8.0	8000	7400
	5.8	7100	6600
	3.9	8100	7500
	3.7	8400	7700
	Average		7300

## DISCUSSION

Although the intermediate compound hypothesis follows logically from the demonstration that the molecular weight of haemoglobin is 66,000, all previous attempts to show the existence of the intermediate compounds directly have been unsuccessful. There is, for example, apparently no spectrophotometric difference between the different intermediate compounds, and a mixture of oxy- and reduced haemoglobin behaves as though only two compounds were present, although Adair's (1925) hypothesis requires the presence of five.

The experiments reported here appear to offer the most striking and direct evidence of the existence of intermediate compounds so far obtained. They were designed, using the intermediate compound hypothesis, to observe the high rate of combination of  $\text{Hb}_4(\text{CO})_3$  with CO predicted from Roughton's (1954) equilibrium measurements and Gibson & Roughton's (1955*a*) kinetic observations on the over-all reaction of carbon monoxide with sheep haemoglobin.

The results have confirmed directly the earlier statements of Gibson & Roughton (1955*a*) that after three molecules of CO have combined there is a marked change in the behaviour of the molecule so that the last molecule to combine does so some seventy times faster than the first three.

In the present experiments, mixtures of intermediate compounds have been prepared photochemically and the initial rate of recombination used to yield values of  $l'_4$ . It would seem, at first sight, that similar information could be obtained by mixing solutions containing COHb and Hb with excess CO in a flow apparatus. Such solutions must contain partially saturated intermediates similar to those produced photochemically, and variations in the initial rate of combination of CO with the free gas-binding groups similar to those shown in Fig. 3 would be expected. Experiments of this kind have already been reported by Legge & Roughton (1950) who mixed 0.25 mm-CO: (a) with 0.25 mm-Hb, and (b) with 0.25 mm-Hb + 0.05 mm-COHb. They found

that the course of the reaction was the same in both cases. Similar experiments, covering a wider range of mixtures, were made in 1952 by Gibson & Roughton (unpublished) again with negative results.

The failure of these experiments was due to two unfavourable circumstances which were unknown at the time. First, Roughton (1954) has shown that  $L_4$  is very large indeed and probably much larger than the other intermediate compound equilibrium constants. This means that equilibrium mixtures of COHb and Hb contain only small concentrations of  $\text{Hb}_4(\text{CO})_3$ , the intermediate with kinetic properties conspicuously different from those of  $\text{Hb}_4$ .

Secondly, the value of  $l'_4$  is so great that, with the high concentrations of CO used by Legge & Roughton (1950) and Gibson & Roughton, such  $\text{Hb}_4(\text{CO})_3$  as was present would have disappeared before the flowing mixture had reached the point of observation. The success of the flash method is due to the possibility of preparing mixtures containing  $\text{Hb}_4(\text{CO})_3$  in which equilibrium has not been reached and in which the proportion of this intermediate is large, and to the shorter 'dead-time' of the apparatus.

It has long been recognized that the combination of haemoglobin with gases is readily affected by small changes in molecular structure and environment, and this lability of function has been ascribed to interaction between the four haem groups of the molecule. So far, this interaction has only been assessable in quantitative terms through the very precise measurements of the gas dissociation curves made by Roughton and his co-workers. These procedures are too exacting and time-consuming for wide use, so that determinations have not, as yet, been made under a wide range of conditions. The flash photolysis method is relatively quick and easy, and lends itself readily to wide variation in experimental conditions. It is hoped, accordingly, that it may make possible considerable extension of our quantitative information about haem-haem interaction.

#### SUMMARY

1. The theoretical basis for the determination of the rate of the reaction  $\text{Hb}_4(\text{CO})_3 + \text{CO} \rightarrow \text{Hb}_4(\text{CO})_4$  by a photochemical method is discussed.
2. Determinations of the velocity constant have been made using carboxyhaemoglobin alone and also in mixtures of carboxyhaemoglobin and nitric oxide haemoglobin.
3. At pH 9.1 and 19° C the value of  $l'_4$  was  $6.9 \times 10^3 \text{ mm}^{-1} \text{ sec}^{-1}$ , and at pH 7.1 and 19° C  $5.2 \times 10^3 \text{ mm}^{-1} \text{ sec}^{-1}$ . These values are some fifty times greater than those observed for the velocity of combination of the first molecule of CO taken up by haemoglobin.

I wish to thank the Medical Research Council and the Royal Society for grants which have helped this work.

## APPENDIX

*Calculation of the quantities of intermediates present after exposure to the flash.* The situation is the same as that which would arise if a population of coins showing the proportion  $p$  heads and  $q$  tails were to be distributed at random in groups of four. The proportion of groups with four heads will be  $p^4$ , while the probability that a group will arise with the first place filled by a tail and the other three places by heads is  $p^3q$ . If the order of heads and tails within the group is immaterial, any one of the four places may equally be occupied by a tail so the total proportion of groups containing three heads and a tail will be  $4p^3q$ . By extension of similar reasoning the frequencies of the other groups may be obtained and are found to be identical with the terms in the expansion of  $(p + q)^4$ .

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