The Photochemical Formation of a Quickly Reacting Form of Haemoglobin

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In describing the application of flash photolysis to some reactions of myoglobin and haemoglobin with carbon monoxide, Gibson (1956*a, b*) pointed out the possibilities of extending the use of the method to study their reactions with oxygen. It seemed that suitable photochemical procedures would offer important practical and theoretical advantages over flow methods. On carrying out experiments in systems containing oxygen, however, it was quickly found that the changes in extinction after flashing were much smaller than expected, that their size depended on the concentration of oxygen, and that both the extent of the changes in extinction and the rate of the reaction recorded were increased when the temperature was lowered.

Since the situation was clearly complex, no further work with oxygen was done until a new and more powerful flash apparatus had been completed. This used a monochromator instead of interference filters and gave much shorter and brighter flashes than the first model described by Gibson (1956*a*). The experiments described here show that after the photochemical decomposition of carboxyhaemoglobin the reduced haemoglobin formed has, for a brief period, a much increased rate of reaction with oxygen or carbon monoxide, though differing only slightly from ordinary reduced haemoglobin in its spectroscopic characters. The occurrence and some of the properties of this reactive haemoglobin have been examined and are discussed in relation to other observations on haemoglobin kinetics.

MATERIALS AND METHODS

Blood solutions. Sheep blood was used throughout, and was prepared by the method of Gibson & Roughton (1957). The blood was defibrinated by shaking with glass beads and the mixture strained through glass wool. To 1 vol. of blood 3 vol. of water was added and, after 10 min. at room temperature (about 20°), 5 g. of finely powdered solid borax/100 ml. After shaking for 2 min. the mixture was centrifuged for 20 min. at 3000 *g* and the clear supernatant decanted. It was transferred to a tonometer, reduced by evacuation and kept under O₂-free N₂ at 4° until required. The blood solution was discarded after 5 days and a fresh preparation made. This stock solution was diluted with buffers as required for use in the flash apparatus.

The choice of diluted blood rather than a purified haemoglobin preparation is arbitrary. The risk of altering

kinetic behaviour by denaturation in a purification procedure is balanced by the possibility that non-haemoglobin materials in the blood may influence the results. In the absence of definite knowledge which would dictate the use of any particular preparation, diluted blood was preferred because almost all kinetic work so far reported has been done with it.

Carbon monoxide. This was obtained from Imperial Chemical Industries Ltd.

Buffers. pH 5.0–6.3: 0.2*N*-citric acid with 0.2*N*-NaOH (Britton & Welford, 1937); pH 6.4–7.6: 0.2*M*-KH₂PO₄ and 0.2*M*-Na₂HPO₄ mixed in the proportions of Sørensen (1909); pH 7.8–9.1: 0.2*M*-H₃BO₃ in 0.2*M*-KCl with 0.2*N*-NaOH (Clark, 1928); pH 10–11: 0.1*M*-Na₂CO₃ with 0.1*N*-HCl (Kolthoff, 1925). Where exact pH values were important the figure was checked with a glass-electrode pH meter.

Spectrophotometry. Absorption curves in the visible and near ultraviolet were determined with a Unicam model 600 glass spectrophotometer with cells of 1 cm. path length for the visible and of 1 mm. path for the Soret regions of the spectrum. The concentration of haemoglobin solutions was determined by conversion into carboxyhaemoglobin (COHb) and the determination of the extinction at 540 mμ, taking the millimolar extinction coefficient on an iron basis as 14.5. Concentrations of all haemoglobin solutions are expressed as molarity of iron.

Flash-photolysis apparatus. The general lay-out and method of operation and calculation of results followed the procedure described by Gibson (1956*a*), with differences as follows: The commercial photographic flash tubes were replaced by U-shaped tubes of 12 mm. internal diameter with a discharge path of 20 cm. between tungsten electrodes 3.2 mm. in diameter. These tubes were made either from silica or from borosilicate glass. The ease of manipulation of the latter was offset by the short life of the tubes, whose walls crazed and released gases into the tube, making the initiation of the discharge difficult. The tubes were filled with argon (British Oxygen Co.) at 5–6 cm. pressure Hg. Two tubes were used and each was connected to a 4 μF condenser rated at 12.5 kv. The tubes were fired by an initiating pulse applied to one side of a spark gap included in the discharge circuit. The arrangement is shown in Fig. 1 and follows the practice of G. Porter (personal communication). With this circuit flashes of up to 600 J were obtained in an effective time of about 20 μsec. as measured photographically. The main discharge was followed by a much weaker light emission continuing for 300–400 μsec.

The interference filters were replaced by a Hilger D. 246 monochromator with glass prism, used with a Mazda 6v, 108 w ribbon-filament lamp. The wavelength setting was checked with a mercury lamp.

The reaction vessels were modified to allow accurate temperature control by circulating heating or cooling water through spaces on either side of the thin (1.6 or 3.2 mm.) layers of blood solution under examination. A drawing of one of these cells is given in Fig. 2. The cell was made of clear methacrylate resin and had an interchangeable joint which allowed it to be attached either to a tonometer or to leads which allowed solutions to be introduced without removing the cell from the flash apparatus.

Fig. 1. Circuit diagram of flash apparatus. C_1, C_2 , $4\mu\text{F}$ 12.5 kv working, C_3 , $0.25\mu\text{F}$ 2.5 kv working; R_1 15 k Ω , 2 w; T_1, T_2 , flash tubes; Co , spark coil; Sw_1 , contact on rotating shutter; Sw_2 , contacts on camera shutter; M , motor driving shutter; H.V., high-voltage supply; T.V., trigger-voltage supply.

Fig. 2. Temperature-controlled cell, part sectional. I , Water inlet; O , water outlet; S , solution space; W , water spaces. Cells of methacrylate resin; B14 standard ground-glass socket.

RESULTS

Preliminary observations. In the first experiments with the new flash apparatus the findings already mentioned in the introduction were confirmed. When a dilute solution of blood ($[\text{Hb}]$ 20 μM) in borate buffer, pH 9.1, is equilibrated with a moderate $p\text{O}_2$ of about 50 mm. Hg, giving $[\text{O}_2]$ 93 μM , and a low $p\text{CO}$ of about 5 mm., giving $[\text{CO}]$ 6.5 μM , about 95% COHb is formed in the dark. Such a solution was then exposed to a photolysis flash sufficiently powerful to break down about 99% of the COHb present and the subsequent changes in extinction followed at several wavelengths, the first observations being made 400 $\mu\text{sec.}$ after initiation of the photolysis flash. At 430 m μ , a wavelength at which oxyhaemoglobin (O_2Hb) and COHb have small and similar light-absorptions and haemoglobin has a large absorption, the change in extinction observed was only about 10–20% of that expected on the basis of the haemoglobin concentration and the extinction coefficients. This was not due to failure of the photolysis flash to bring about dissociation of COHb, for measurements at 421.5 m μ (where O_2Hb and haemoglobin have similar light-absorption and COHb has a greater absorption) showed that the COHb disappeared on flashing and was slowly re-formed in the dark. Of the various possible explanations the most probable seemed to be that when haemoglobin was formed photochemically from COHb the product was able to react very rapidly with O_2 to form O_2Hb . In systems containing haemoglobin, O_2Hb and COHb the number of possible intermediate compounds of the form $\text{Hb}_4(\text{O}_2)_n(\text{CO})_m$, where n and m may each vary between 0 and 4, and $n+m \leq 4$, is 15, so that the possibilities of kinetic analysis from optical measurements seemed distinctly limited. As similar effects could be obtained in systems containing CO only, almost all the experiments described in this paper have been carried out with the simpler system.

Reduced haemoglobin reacting with carbon monoxide. It was found that the change in extinction which could be recorded at 430 m μ was sometimes substantially smaller than would have been expected from the extinction coefficients of haemoglobin and COHb, and varied with the partial pressure of CO and with change of pH from 9.1 to 7.1. The results of one experiment are summarized in Table 1, which shows that the recorded change in extinction after flashing did not vary significantly with change in $[\text{CO}]$ between 100 and 1000 μM when the haemoglobin was diluted with phosphate buffer at pH 7.1. At pH 9.1 the change in extinction was smaller than at pH 7.1, and at the higher of the two concentrations of CO examined

Table 1. *Haemoglobin combining with carbon monoxide*

The combination of haemoglobin with CO at pH 7.1 and 9.1 as measured by the change in extinction at 430 m μ after flash photolysis. [Hb], 0.03 mM; 1.6 mm. cell; temp., 21°; flash, 400J. Change in extinction expected = 0.32.

	[CO] (μ M)	ΔE	$10^{-5} \times$ Velocity const. ($M^{-1} \text{ sec}^{-1}$)	Half-time of reaction (msec.)
pH 7.1	1030	0.27	1.2	6
	300	0.25	1.3	18
	100	0.27	1.3	56
pH 9.1	1030	0.12	1.7	4
	260	0.23	1.7	16

Fig. 3. Effect of pH on the reaction of CO with Hb. [COHb] 18 μ M; [CO] 220 μ M; pH values were as shown on curves; observations were made at 435 m μ ; flash, 200J; temp., 1.6°.

Fig. 4. Effect of varying [CO] on the division between 'fast' and 'ordinary' reactions. [COHb] 22 μ M; [CO] as shown on curves; observations were made at 435 m μ ; flash, 200J; temp., 1.0°; pH 9.1; k_1 220 sec^{-1} ; k^* $1.7 \times 10^6 M^{-1} \text{ sec}^{-1}$; for further explanation see text.

(1000 μ M) was only about half that found with a value of [CO] of 260 μ M. There are three explanations of these findings which require consideration:

(1) In describing the apparatus it was pointed out that the main flash was followed by a weaker 'tail' which prevented observation of changes in extinction until about 400 μ sec. after firing the flash. The reactions being observed, although rapid, have half-times of 4 and 6 msec. at pH 9.1 and 7.1 respectively, so that the differences in change in extinction given in Table 1 cannot be explained by the 'dead time' of the apparatus of 400 μ sec. In fact, a dead time of 13 msec. would be needed to allow the reaction records obtained with [CO] 1 mM to start from a common point both at pH 7.1 and at pH 9.1. The satisfactory working of the apparatus was confirmed by carrying out control experiments with myoglobin and CO, where, with widely varying [CO] and hence reaction rates, it appeared that observation could begin 370 μ sec.

after firing the flash. Further, when the rate was 2.5 times greater than the rate of the haemoglobin + CO reaction, 83 % of the expected reaction could be observed.

(2) There might be a rapid reaction taking place during the dead-time of the apparatus at pH 9.1 but not at all or to a less extent at pH 7.1.

(3) The absorption spectrum of the product of photodecomposition of COHb at pH 9.1 is different from that obtained at pH 7.1.

As will appear, both the second and third possibilities are realized, though the second is quantitatively more important than the third.

Experiments with carbon monoxide at low temperatures. When flash experiments were carried out at temperatures between 1° and 5° it was found that the recombination reaction was markedly biphasic, a rapid reaction during the first 5 msec. being

succeeded by a much slower one which yielded a second-order rate constant for the overall reaction haemoglobin + CO $\rightarrow$ COHb of $4 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$. This value is in agreement with the rates and temperature coefficient given by Gibson & Roughton (1957) in determinations made by the stopped-flow method, and it is suggested that the slow phase represents the combination of 'ordinary' reduced haemoglobin with CO.

The rapid phase of the reaction as observed at $435 \text{ m}\mu$ is shown in Figs. 3 and 4, which illustrate also its dependence on pH and concentration of CO.

Absorption spectrum of the rapidly reacting compound. To avoid repetition of phrases such as 'the rapidly reacting compound' the material giving the quick changes of extinction in Figs. 3 and 4 will be written Hb* and the material giving the slow changes referred to in the previous paragraph as 'ordinary' haemoglobin will be written Hb. Before attempting any analysis of the material in Figs. 3 and 4 the absorption spectrum of Hb* is needed. Two methods have been used:

(1) The variation with wavelength of the change in extinction due to flashing has been observed in conditions where the rapid reaction predominates (e.g. at pH 10.6; see Fig. 3) and also where the ordinary slow reaction is prominent (e.g. at pH 6.7; see Fig. 3). To work out the results in terms of absolute absorption spectra, solutions of Hb and COHb were introduced into the observation cell of

the flash apparatus and their light-absorption at different wavelengths was determined. Then, assuming that normal COHb is the final product in the absorption cell after flashing, the absorption spectrum immediately after the flash was obtained by addition of the changes due to flashing to the absorption of COHb at each wavelength. The results of an experiment of this kind are shown in Fig. 5, where the spectrum immediately after flashing at pH 10.6 is attributed to Hb* and that at pH 6.7 to Hb. It appears that Hb* has a slightly higher absorption than Hb at wavelengths longer than $435 \text{ m}\mu$, but shows a lower absorption maximum near $430 \text{ m}\mu$. The spectrum for Hb given in Fig. 5 agreed well with the direct static determinations made in the absorption cell of the flash apparatus.

(2) The conditions were arranged so that about half the observed change in extinction after flashing took place quickly (e.g. pH 9.1, [CO] $150 \mu\text{M}$, temp. 1°) when observed at $435 \text{ m}\mu$. The wavelength of the observation beam was then varied and the proportion of the change in extinction taking place quickly determined for each wavelength. The results showed that the rather small differences of Fig. 5 are real, the apparent proportion of rapid reaction increasing at wavelengths longer than $435 \text{ m}\mu$ and decreasing between 435 and $415 \text{ m}\mu$.

The close similarity between the spectra of Hb* and Hb suggests that the difference between them is unlikely to be due to a major change in the electronic configuration of the haem group (e.g. formation of a triplet), but is the reflexion in the spectrum of a change in the protein portion of the molecule.

Kinetic properties of Hb.* The curves of Fig. 4 show that both the rate of the initial change in extinction and the extent of the rapid reaction as a proportion of the whole change in extinction after flashing increase when the concentration of CO is raised. This result is most easily interpreted by supposing that Hb* not only combines with CO in a second-order process, giving COHb, but also reverts to Hb in a first-order reaction. This kinetic proposal is readily dealt with provided that the concentration of CO is large enough in comparison with the concentration of haemoglobin to be treated as approximately constant during the reaction.

Then, writing $[\text{COHb}] = y$, $[\text{Hb}^*] = x$, $[\text{Hb}] = z$, $[\text{CO}] = \alpha$, velocity constant for the reaction $\text{Hb}^* + \text{CO} \rightarrow \text{COHb} = l^*$, velocity constant for the reaction $\text{Hb} + \text{CO} \rightarrow \text{COHb} = l'$, velocity constant for the reaction $\text{Hb}^* \rightarrow \text{Hb} = k_1$, and taking the total pigment concentration as unity:

$$dy/dt = \alpha(l^*x + l'z), \quad (1)$$

$$x = e^{-(k_1 + \alpha l^*)t}, \quad (2)$$

$$x + y + z = 1, \quad (3)$$

Fig. 5. Absorption spectra of normal (●) and rapidly reacting (○) haemoglobin. Ordinate gives millimolar extinction coefficients. For methods used see text.

substituting from (2) and (3) in (1) gives, on solving by standard methods,

$$y = 1 - A e^{-(k_1 + \alpha l^*)t} + (A - 1) e^{-\alpha l^* t}, \quad (4)$$

where $A = \alpha(l^* - l') / (k_1 + \alpha l^* - \alpha l')$. The use of equation (4) is limited to circumstances: (1) where [CO] may be regarded as constant during the reaction, (2) where observations are made with light of a wavelength where Hb and Hb* are isosbestic, (3) where the sole immediate product of breakdown of COHb is Hb*, as stated in equation (2), and (4) where the length of the photolysis flash is short compared with the life of Hb*.

The chief difficulty in the use of equation (4) is to be sure that condition (3) is met, for any single reaction curve from Fig. 3 or Fig. 4 can be fitted equally well on the supposition that $k_1 = 0$ and that $A = \text{Hb}^* / (\text{Hb}^* + \text{Hb})$ at $t = 0$. It is, however, possible to show that condition (3) is met, or nearly so, at alkaline pH. Thus in Fig. 3 the curve for pH 10.6 was drawn from equation (4): the numerical values obtained show that 94% of the reaction taking place is represented by the process $\text{Hb}^* + \text{CO} \rightarrow \text{COHb}$, so that the products of COHb breakdown must have included at least 94% of Hb*. At pH 9.1, on the other hand, there is a quantitatively important contribution from the slow reaction even at the highest of the three values of [CO] used in obtaining the data of Fig. 4. In this case fulfilment of condition (3) can be inferred by considering the curves of the figure together. As [CO] is changed αl^* must change in direct proportion and hence both A and the first exponent ($k_1 + \alpha l^*$) of equation (4) must change predictably with [CO]. If, then, the reaction curves with several values of [CO] can be fitted with a single value for each of the constants k_1 , l^* and l' , it follows that Hb* must be the only important immediate product of COHb photolysis. The agreement between the observed points of Fig. 4 in which all three curves were calculated from equation (4) with

$$k_1 = 220 \text{ sec.}^{-1}, \quad l^* = 1.7 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1}$$

supports this assumption. Some values of k_1 and l^* have been worked out with equation (4) and are given in Table 2. The mean value of

$$l^* = 1.8 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1}$$

at pH 9.1 and 1° . It is about 50 times as great as the value for the overall reaction-velocity constant

for Hb combining with CO found by Gibson & Roughton (1957).

Effect of pH. Some reaction curves illustrating the effect of varying pH between 10.6 and 5.45 are given in Fig. 3. As the pH is reduced from 10.6, the rapid reaction becomes less obvious and is least at pH 6.7, increasing again in buffers more acid than this. The change with pH is not uniform over the range pH 9.1–6.1: experiments with buffers closer in pH than those shown in Fig. 3 give only a small change between pH 9.1 and 7.6, but a large change between pH 7.6 and 6.7.

It was hoped that the effects of pH could be expressed in terms of the constants k_1 and l^* , but this has proved impossible because at neutral pH Hb* is not the only product of the photodecomposition of COHb, large amounts of Hb being formed as well. This has been shown by experiments at pH 7.1 in which [CO] was varied. One such is illustrated in Fig. 6, where both curves are

Fig. 6. Comparison between observed and predicted effects of varying [CO] on the combination reaction with Hb at pH 7.1. Curves are drawn from equation (4) with $l^* = 5.1 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1}$, $k_1 = 450 \text{ sec.}^{-1}$, $\alpha = 3.55 \times 10^{-4} \text{ M}$ (lower curve), $\alpha = 1.08 \times 10^{-4} \text{ M}$ (upper curve). O, Experimentally observed [COHb] $20 \mu\text{M}$; [CO] $355 \mu\text{M}$; flash, 200 J; temp., 1.2° ; observations were made at $435 \text{ m}\mu$. ●, [CO] $108 \mu\text{M}$; other conditions were as given above.

Table 2. Values of k_1 and l^*

Dilute sheep blood was used; borate buffer, pH 9.1; 3.2 mm. cell; temp., 1° ; flash, 200 J; observation beam, $435 \text{ m}\mu$.

k_1 (sec. ⁻¹)	$10^{-6} l^*$ (M ⁻¹ sec. ⁻¹)	[CO] (μM)	[Hb] (μM)	No. of determinations
270	1.9	220	19	1
220	1.7	130, 260, 550	22	3
240	1.8	230	25	1
200	2.0	130	21	4

calculated from equation (4) with values of k_1 and l^* giving a reasonable fit to the experimental data obtained with the higher value of $[\text{CO}]$. The calculated curve for the lower value of $[\text{CO}]$ differs widely from the experimental points. In principle, the proportions of Hb and Hb* present immediately after the flash could be determined from pairs of curves similar to those in Fig. 6, but with three independent constants to dispose of, reliable results would be difficult to obtain. Although it has not been possible to give numerical expression to the results it is clear that change in pH influences not only the course of the recombination reaction but also the changes leading to the formation of Hb* in the photochemical reaction.

Effect of p-chloromercuribenzoate on Hb.* Gibson & Roughton (1957) have shown that when three molecules of CO have combined with a molecule of haemoglobin there is a sharp increase in the rate constant, the fourth molecule combining some 40 times as fast as the first. The high rate constants observed for the combination of Hb* with CO suggested that the two effects might be related. As Riggs & Wolbach (1956) have shown that haem-haem interaction (on which Gibson & Roughton's finding depends) is much influenced by blocking of sulphhydryl groups with mercurials, experiments were carried out to see if p-chloromercuribenzoate (PCMB) affected the formation and reactions of Hb*. In experiments at both pH 7.1 and pH 9.1 it was found that PCMB increased the proportion

of the reaction taking place quickly. The effects are most striking at pH 7.1, where little Hb* is formed in the absence of the mercurial. As shown in Fig. 7, after addition of PCMB, almost the whole reaction takes place rapidly. The rate was similar at both pH values with $l^* = 1.8 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 7.1 and 1.5° and $2.1 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 9.1. It appears that PCMB increases the yield of Hb* and also decreases its rate of reversion to Hb.

Effect of the length of photolysis flash. The most stable product of photolysis of COHb is Hb, and it would therefore be expected that on prolonged illumination Hb would become progressively more abundant, since Hb* would combine with CO, be broken down again in the light and have a further chance of yielding Hb by the first-order reaction already described. The length of flash is not easily varied without major change in the apparatus, and only two lengths of flash have been examined. One was the usual flash given by the apparatus described in the Materials and Methods section: the other was obtained by using a commercial photographic flash tube at a comparatively low voltage with high-capacity condensers, exactly as described by Gibson (1956a). The high-voltage apparatus yields flashes with a time constant of about $15 \mu\text{sec.}$, whereas the low-voltage system gives

Fig. 7. Effect of p-chloromercuribenzoate on the combination of CO with Hb. $[\text{COHb}]$ $25 \mu\text{M}$; $[\text{CO}]$ $100 \mu\text{M}$; $[\text{PCMB}]$ $50 \mu\text{M}$; flash, 200 J; temp., 1° ; pH 7.1; observations were made at 430 m μ .

Fig. 8. Effect of varying the length of photolysis flash on the recombination reaction between CO and Hb. Upper curve, 140 J dissipated in LSD3 flash tube from $60 \mu\text{F}$ condenser. Lower curve, 140 J in two quartz flash tubes from two $4 \mu\text{F}$ condensers. $[\text{COHb}]$ $26 \mu\text{M}$; $[\text{CO}]$ $280 \mu\text{M}$; temp., 1° ; pH 9.1.

flashes with a time constant of about $300\ \mu\text{sec.}$ (as determined by recording the light output with a photomultiplier and oscillograph). The voltages and capacities were adjusted to give the same energy dissipation and the course of the recombination reaction after flashing was recorded for the same solution. The results shown in Fig. 8 at first appeared to offer a gratifying confirmation of the theory put forward above.

To examine the matter quantitatively it was supposed: (1) that the reaction of Hb with CO could be neglected during the first few milliseconds after firing the photolysis flash, (2) that the light from the flash decayed exponentially, (3) that $[\text{CO}] \gg [\text{Hb}]$ and (4) that the flash, if applied under conditions where the back reaction was negligible, would produce 95% dissociation of COHb. Then, with the same notation as before and writing illumination intensity $I = k_2 e^{-k_3 t}$, where k_2 includes the effects of quantum efficiency, geometry of the apparatus, etc., and k_3 is the time constant of the flash:

$$dy/dt = \alpha I x - k_2 y e^{-k_3 t}, \quad (5)$$

$$dx/dt = k_2 y e^{-k_3 t} - (\alpha I + k_1) x. \quad (6)$$

These equations do not appear to be readily soluble and so have been integrated numerically with values corresponding to the conditions of Fig. 8, taking $I^* = 1.6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, $k_1 = 300 \text{ sec}^{-1}$, $\alpha = 2.8 \times 10^{-4} \text{ M}$. The resulting curves, plotted in Fig. 9, show that little difference due to the length of the flashes is to be expected, certainly much less than that shown in Fig. 8. Any explanation of this result is necessarily speculative, but it may be that

Fig. 9. Calculated course of COHb concentration during photolysis and recombination. Curve A, 'short' flash; curve B, 'long' flash. For further explanation see text.

formation of Hb* occurs only when the CO molecules in combination with all four haem groups are removed photochemically within a very short space of time.

Reactions of Hb* with oxygen. The effects observed with CO also occur with O_2 (Fig. 10). In this experiment a solution containing $20\ \mu\text{M}$ -Hb was equilibrated with a gas mixture containing 10 mm. $p\text{CO}$ and 20 mm. $p\text{O}_2$ and the changes in extinction after flashing were followed at $430\ \text{m}\mu$ (isosbestic for COHb and O_2Hb), at $421\ \text{m}\mu$ (isosbestic for Hb and O_2Hb) and $412\ \text{m}\mu$ (isosbestic for Hb and COHb). The records give the concentrations of Hb ($430\ \text{m}\mu$), COHb ($421\ \text{m}\mu$) and O_2Hb ($412\ \text{m}\mu$). As Fig. 10 shows, the disappearance of Hb in the first 2 msec. after flashing is almost wholly due to the formation of O_2Hb , the rate giving a velocity constant of $10^7 \text{ M}^{-1} \text{ sec}^{-1}$ at 1° . This is about 10 times as great as the value found for the combination of O_2 with Hb by the stopped-flow method. Thus the anomalous rate of combination found for CO is matched by a similar phenomenon with O_2 , though the quantitative difference between Hb* and Hb is smaller. It is interesting to note that the ratio between the velocity constant for O_2 and that for CO is 4:1, and to compare this figure with the value of 3.5 reported for the ratio k_4'/l_4' by Gibson & Roughton (1955). In their notation k_4' is the velocity constant for the reaction

and l_4' that for the reaction

Fig. 10. Changes in haemoglobin, oxyhaemoglobin and carboxyhaemoglobin after flashing in a solution with $[\text{Hb}] 20\ \mu\text{M}$; $[\text{O}_2] 37\ \mu\text{M}$; $[\text{CO}] 13\ \mu\text{M}$; flash, $200\ \text{J}$; temp., 1° ; pH 9.1. Two time scales, covering 0–2 msec. and 2 msec.–1.2 sec., are used.

Effect of temperature on the reaction of Hb with carbon monoxide.* The most satisfactory observations have been made at pH 10.6, where the reaction $\text{Hb}^* + \text{CO} \rightarrow \text{COHb}$ accounts for almost the whole observed change in extinction at 435 m μ . The temperature coefficient for the reaction is significantly smaller than that for the overall reaction $\text{Hb} + \text{CO} \rightarrow \text{COHb}$, where Gibson & Roughton (1957) found the energy of activation to be 10.5 kcal. The data shown in line A of Fig. 11 give a value of 5.6 kcal. The change in rate and the change in activation energy are not in the proportions required by classical theory, a change of 5 kcal. calling for 1000-fold increase in rate.

Experiments at pH 9.1 intended to give the temperature coefficient of k_1 yielded reaction records over the range 1.2–19.0° which differed little from one another, and which, on analysis with equation (4), gave a temperature coefficient for l^* of 1.2/10° temperature rise, appreciably less than that found at pH 10.6. The temperature coefficient for k_1 was 1.5/10°, corresponding to an energy of activation of about 6 kcal. In view of the discrepancy between this experiment and the one illustrated in Fig. 11 in the value for l^* this result must be regarded with some slight reserve.

Effect of urea and of 2M-sodium chloride. It is well established that the denaturation of haemoglobin by urea begins with the splitting of the molecule into two halves. This treatment might be expected to modify the accessibility of the haem groups to ligand molecules and the formation and properties of Hb*. The experiments were carried out at pH 7.1, where the formation of Hb* is normally slight. The effect of 5M-urea was to produce a dramatic increase in the rate of reaction, the haemoglobin now behaving very much as in a solution of pH 10.6, l^* at 1.2° being

$$1.9 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1}$$

The energy of activation was 5 kcal., as shown in Fig. 11, line B.

The effects of 2M-NaCl were less striking and also took some time to develop, a good effect being obtained after incubation of the dilute haemoglobin solutions for 3 hr. at room temperature. The effect was generally similar to that of urea, as would be expected from the work of Gutfreund (1949) on the osmotic pressure of haemoglobin in salt solutions. The greatest value of the recombination velocity constant was $8 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1}$ at 1° as compared with 1.9×10^6 with 5M-urea under similar conditions.

Effect of pigment concentration, and miscellaneous observations. The physical dimensions of the present flash apparatus largely dictate the pigment concentrations which can be most effectively and conveniently used, and all the experiments dealt with

so far have been carried out with blood diluted about 400 times, giving a haemoglobin concentration of about 30 μM . It is not possible to increase the pigment concentration much without producing grossly non-uniform illumination within the photolysis cell, but greater dilutions may be used. The results of some experiments at pH 9.1 and 7.1 are given in Table 3. At pH 9.1 they are expressed in terms of k_1 and l^* , which are little affected by dilution within the range studied. At pH 7.1 determinations with two values of $p\text{CO}$ gave results consistent with a value of k_1 much less than al^* . To allow the results to be worked out it has been supposed that $k_1 = 0$, and values of the percentage of Hb* formed and l^* given in Table 3 have

Fig. 11. Energy of activation of reaction of Hb* with CO. A, [Hb] 25 μM ; [CO] 240 μM ; 5M-urea in 0.2M-phosphate buffer, measured pH 7.1; flash, 200 J; observations made at 435 m μ . Interrupted line shows slope but not position of line relating $\ln l^*$ to $1/T$ for normal haemoglobin according to Gibson & Roughton (1957).

Table 3. *Effect of dilution on k_1 and l^**

Observations were made at 435 m μ ; 3.2 mm. cell; flash, 200 J; temp., 1.2°.

	[Hb] (μM)	k_1 (sec.^{-1})	$10^{-6} l^*$ ($\text{M}^{-1} \text{ sec.}^{-1}$)	Hb* (%)
pH 9.1	34	230	1.6	—
	19	180	1.3	—
	14	200	1.3	—
	8	170	1.2	—
pH 7.1	20	—	1.5	17
	4.6	—	2.2	45

been calculated on this basis. It appears that the proportion of Hb* formed on flashing is increased by dilution, and there may also be some rise in l^* . The effects of dilution at pH 7.1 are presented in a more striking form in Fig. 12, where the [CO] was held constant and the strength of the blood varied. The change in extent of the rapid reaction is obvious.

Although in ten experiments the effect of dilution at pH 7.1 was always in the direction shown in Table 3 and Fig. 12, the quantitative findings differed greatly from blood to blood, and from day to day with the same blood stored as described in the Materials and Methods section. The general conclusions reached were: that fresh blood shows the least effect on dilution; that storage as blood diluted with 3 vol. of borate (see Materials and Methods) at pH 9.1 and 4° gives a bigger change than storage at pH 7.1; and that dilution of materials present in the serum is not important, since similar results were obtained on dilution either with buffer or with buffer containing serum from the same blood sample.

Myoglobin. Several attempts have been made to look for anomalous effects with myoglobin, but even under the most favourable circumstances the reactions appeared to be accurately second-order both with O₂ and CO. These negative results are important because they suggest that the formation of Hb* is dependent on the protein part of the molecule and is not a general property of the haem group.

Fig. 12. Effect of dilution on the reaction of CO with Hb. pH 7.1, [CO] 130 μM; [COHb] as shown on curves; temp., 1°; flash, 200 J; observations were made at 435 mμ.

DISCUSSION

A qualitative explanation of the existence of a rapidly reacting form of haemoglobin can be offered in terms of Gibson & Roughton's (1957) finding that the fourth molecule of carbon monoxide to combine with sheep haemoglobin does so at about 40 times the speed of the first three. Their result can be correlated with Haurowitz's (1938) observation that the penetration of oxygen into a crystal of reduced haemoglobin is accompanied by a change in crystal form and double refraction, suggesting that a major structural reorganization accompanies binding of ligand. The rapidly reacting form of haemoglobin would then be pictured as a haemoglobin molecule which had lost its ligand molecules within a brief space of time by photochemical action, but which had not yet had an opportunity to undergo the structural alterations corresponding to the change from the saturated to the reduced state. The subsequent disappearance of the rapidly reacting form would then be regarded as a measure of the rate of this alteration.

It is not surprising that -SH groups are concerned, since there is already much evidence to implicate them in the phenomena of haem-haem interaction, and Riggs & Wollbach (1956) have given an excellent summary of the present position in describing their experiments on the effect of mercurials on the oxygen affinity of horse haemoglobin. The present results are fully consistent with the idea that the haem groups and -SH groups of sheep haemoglobin are in close proximity, and that the formation of linkages by them may be directly responsible for the alteration of protein configuration which accompanies haem-haem interaction.

At pH 7.1, where the formation of Hb* is not normally prominent, it is stimulated by mercurials, by urea, by strong salt solutions and by great dilution of the blood. Since three of these are known to favour splitting of the haemoglobin molecule it is natural to consider if the effect can be explained by a photochemically induced splitting followed by recombination of the fragments. If so, it would be expected that k_1 , which measures the re-formation of Hb from Hb*, would be inversely related to the dilution of the blood. This is not so, and a change of structure rather than an actual split appears more probable.

An opening up of the protein structure in terms of the 'crevice' hypothesis of St George & Pauling (1951) is not favoured by the data on the rate and energy of activation of the reaction between Hb* and carbon monoxide, since a change of this kind would be expected to affect the frequency factor rather than the activation energy. The experiments have shown that the activation energy is significantly reduced, and as there is not a corresponding

increase in the rate of reaction the frequency factor is also reduced.

It is interesting to speculate on the relation between the effects observed by Roughton (1934) and the present work. He found that when reduced haemoglobin was mixed with an amount of oxygen insufficient to saturate it a definite percentage saturation was not reached at a rate consonant with the velocity constants for the reaction of oxygen with haemoglobin as measured with larger amounts of oxygen. Instead, the combination of oxygen continued slowly for several seconds, giving a drift in percentage saturation. This was observed most strikingly with very dilute haemoglobin and at neutral pH. Although the time scale of this drift is of a different order from that of the effects discussed in this paper, being some hundreds of times longer, a reorganization of the protein structure may well be concerned in Roughton's effect also.

It is important to consider what relation, if any, the results given here have to the kinetics of haemoglobin reactions as studied by flow methods. There is no reason to suppose that in the spontaneous dissociation of ligand molecules from haemoglobin the dissociation can run ahead of the capacity of the protein to rearrange its structure since the value of k_1 is much greater than the fastest dissociation velocity constant for oxygen, so far measured, at the temperatures used in the present experiments. Further, the large effects due to variation in the length of the flash argue for a need to remove several ligand molecules within a very brief space of time if Hb* is to be formed in good yield. In fact, with the longer of the two flashes used, it can be calculated that half of the carbon monoxide had been removed in 60 μ sec., whereas the shorter flash would require only 4 μ sec. If the difference in yield of Hb* with the two flash lengths is due to this difference in time, the rate of removal of ligand in any spontaneous dissociation would be far too slow to give an appreciable yield of Hb*.

The method of flash photolysis has already been used by Gibson (1956b) and Ainsworth & Gibson (1957) in obtaining velocity constants for haemoglobin reactions under various conditions: it is important to consider how far the findings reported in this paper call for reinterpretation of the earlier work. In Gibson's determinations of l'_1 , the velocity constant of the reaction

the partial pressure of carbon monoxide was low, the temperature high and the flashes were of relatively long duration. These conditions all favour the completion of side reactions in a brief space of time, and it seems clear that Gibson's measure-

ments were carried out with normal haemoglobin. Some small uncertainty attaches, however, to the calculation of the amounts of $\text{Hb}_4(\text{CO})_3$ and $\text{Hb}_4(\text{CO})_2$ present immediately after flashing, since the side reactions might well alter their relative proportions.

In determining l'_1 Ainsworth & Gibson (1957) used a very low concentration of carbon monoxide and the recombination reactions were quite slow, with half-times of the order of tenths of a second: it is again likely that their measurements were correct. One observation in their work requires separate attention. They found that l'_1 as measured by the flash method was much increased (four- to eight-fold) by PCMB, whereas Gibson & Roughton (1955) have published results indicating that l'_1 was unlikely to be increased by more than 40% at the most. It now seems likely that the flash measurements were made on haemoglobin in an abnormally reactive state, k_1 being very small indeed in the presence of PCMB, and that each of the apparently discrepant findings was correct under its own experimental conditions.

A difficulty met by Ainsworth & Gibson in unpublished experiments on the temperature coefficient of l'_1 can also be explained by the results of this paper. They found that l'_1 decreased only by about 50% when the temperature of the solutions was lowered from 20° to 1°, whereas the calculations of Gibson & Roughton (1955) predicted a fourfold decrease. It is likely that at the lower temperature Hb* was being observed along with $\text{Hb}_4(\text{CO})_3$. It is clearly necessary to use the flash method with some care when studying haemoglobin kinetics.

It should perhaps be pointed out that although very many examples of photochemical effects on proteins have been reported they have usually concerned irreversible changes produced by ultraviolet light of wavelengths shorter than 300 m μ . In the present instance a quickly reversible change has been produced which can, apparently, be repeated many times without damage to the protein, since frequent repetition of the flash discharge does not alter the response of the haemoglobin. Indeed, were it not for the transient functional change observed, this effect of light might have gone for long undetected.

SUMMARY

1. When carboxyhaemoglobin is broken down by the action of light the newly formed reduced haemoglobin has, for a brief period, a higher rate of reaction with carbon monoxide or oxygen than reduced haemoglobin of greater age.

2. The observed reaction is influenced by pH and by -SH reagents. At neutral pH little quickly-

reacting haemoglobin (Hb*) is formed. If *p*-chloromercuribenzoate is added almost all the reduced haemoglobin formed is Hb*. At alkaline pH the chief product of photolysis is Hb* even in the absence of *p*-chloromercuribenzoate.

3. At alkaline pH Hb* reverts spontaneously to ordinary haemoglobin with a velocity constant of about 200 sec^{-1} at 1° .

4. The second-order velocity constant for the combination of Hb* with carbon monoxide is $1.8 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at 1° and the activation energy 5.6 kcal.

5. The bearing of these results on the use of flash photolysis in the study of haemoglobin kinetics is discussed.

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The Glutamic Acid and Creatine Content of Cock Seminal Plasma

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There is evidence that the semen of the domestic cock is wholly derived from the reproductive tract proximal to the protruding ejaculatory ducts, so that the possible sources of seminal plasma are the testes, vasa efferentia, epididymides and vasa deferentia (Lake, 1957a). The cock lacks the accessory reproductive organs, i.e. prostate and seminal vesicles, characteristic of mammals, and ampullae are absent or ill-defined (Lake, 1957b). These organs are the source of a large part of the seminal fluid in mammals. A study of the chemical composition of the seminal plasma of the cock is thus of physiological as well as of comparative interest, and should provide information essential to an understanding of the metabolism of the testis and spermatozoa, especially in the reproductive tract.

In the present investigation the free amino acid content of the seminal plasma has been examined. Gassner & Hopwood (1952) and Larson & Salisbury (1953) have studied this in bull seminal fluid. Since creatine is reported to occur in large amounts in the testes of some mammals (Hunter, 1928; Eggleton, Elsdon & Gough, 1943; Ennor & Stocken, 1948) and of certain invertebrates (Greenwald, 1946) it seemed profitable also to examine cock seminal plasma for guanidine bases. Only small amounts of creatine and creatinine have been reported to be present in mammalian semen (Ilyasov, 1933; McKenzie, Miller & Bauguess, 1938).

MATERIAL AND METHODS

Reproductively active Brown Leghorn cocks were maintained in battery cages and received the Poultry Research Centre diet (Bolton, 1958).

Seminal plasma. Samples of uncontaminated semen (0.12–0.6 ml./ejaculate) were collected according to Lake (1957a) and centrifuged at 1500 g for 15 min. at 2° within 5 min. of collection. The plasma was removed rapidly, before the spermatozoa began to move upwards, and added to 4 vol. of ethanol and heated in boiling water for 15 min. The precipitated protein was removed by centrifuging and 2 vol. of CHCl_3 was added to the supernatant to remove lipid. Before chromatography, the aqueous layer was taken to dryness at room temperature in a vacuum desiccator over P_2O_5 , and the residue was dissolved in 10% propanol. Appropriate dilutions of the aqueous layer were made for the estimation of non-protein nitrogen, ninhydrin-reacting substances, glutamic acid and creatine. Samples from individual cocks were used for these determinations and for chromatography when possible.

Chromatography. (a) Amino acids. One- and two-dimensional chromatography was carried out in phenol saturated with water (0.3% of NH_3) and butanol-acetic acid-water (4:1:5). *iso*Butanol-butan-2-one-water (70:50:30) (Kemble & MacPherson, 1954) was also used as a solvent for unidimensional chromatography. Dinitrophenyl (DNP) derivatives of amino acids were separated by one- and two-dimensional chromatography, with toluene-pyridine-2-chloroethanol-aq. 0.8N- NH_3 soln. (30:9:18:18) and 2M-phosphate buffer (pH 6) as solvents (Biserte & Osteux, 1951).