Some Observations on the Kinetics of the Reactions with Gases of Natural and Reconstituted Haemoglobins

By E. ANTONINI AND Q. H. GIBSON

Istituto di Chimica Biologica, Rome, and Department of Biochemistry, University of Sheffield

(Received 12 February 1960)

The first experiments in which protohaem was removed from haemoglobin and the native globin used to prepare haemoglobins containing protohaem, mesohaem and deuthaem were reported by Hill & Holden (1926). The only reports of work on the oxygen equilibria of reconstituted protohaemoglobin which we have been able to trace appear to be those of Hill, quoted by Lemberg & Legg (1949), and of Davies, quoted by Wyman (1948). Hill found that the oxygen-dissociation curve was hyperbolic, a finding confirmed by Davies, who also found that the oxygen pressure for half-saturation was decreased to about one-quarter of that required for natural haemoglobin, and that the Bohr effect was absent.

More recently Rossi-Fanelli, Antonini & Caputo (1958) have prepared globin from human haemoglobin which could be coupled with protohaematin to give a product having the same absorption spectrum, sedimentation constant and electrophoretic behaviour as natural human methaemoglobin. After reduction by means of an enzyme system the oxygen-dissociation curve was the same as that for natural haemoglobin; the oxygen concentration for half-saturation, the degree of inflexion of the curve and the Bohr effect all agreed closely (Rossi-Fanelli & Antonini, 1959*a*).

Although the evidence presented by Rossi-Fanelli & Antonini (1959*a*) suggests strongly that haem-haem interaction had survived unchanged in the reconstituted protein, the form of the dissociation curve over the middle range of saturations is not, by itself, fully conclusive. Thus Roughton (1949) has pointed out that on the Adair (1925) hypothesis many sets of equilibrium constants describing the consecutive reactions of oxygen molecules with reduced haemoglobin can be found which yield dissociation curves scarcely differing from each other or from a given model over the range 10–90% saturation. Values of the individual equilibrium constants can, so far, only be obtained by exacting gasometric procedures which require prolonged training and experience of the operator (see, for example, Roughton, Otis & Lyster, 1955). Procedures have, however, been devised which will allow the determination of several kinetic constants corresponding to individual partial reactions

of haemoglobin with gases. Such determinations offer not only information about haem-haem interaction similar to that embodied in the values of the individual equilibrium constants, but in addition give a further insight into the mechanism of the reactions.

In this paper it is shown that reconstituted human protohaemoglobin prepared by the methods of Rossi-Fanelli *et al.* (1958) gives the same values for the individual kinetic constants as the natural protein in every case which we have been able to examine. Comparative measurements have also been made on haemoglobins in which mesohaem and deuthaem have replaced protohaem, thus extending the work already initiated by Rossi-Fanelli & Antonini (1959*b*), and Rossi-Fanelli, Antonini & Caputo (1959) in the kinetic field.

EXPERIMENTAL

Notation. According to the intermediate-compound hypothesis of Adair (1925) the reaction between haemoglobin (Hb) and oxygen (or any other ligand) takes place in four stages: the kinetic and equilibrium constants may be written as shown.

The reactions with carbon monoxide are dealt with in the same way except the letters *L* and *l* are used instead of *K* and *k* to distinguish them from the oxygen reactions.

Materials. Protohaemin was obtained from British Drug Houses Ltd.; deuthaemin was prepared by the method of Fischer & Orth (1940*a*); mesohaemin was prepared by the method of Erdmann & Corwin (1947); mesoporphyrin was prepared from protohaemin by the method of Fischer & Orth (1940*b*); human globin was prepared by the method of Rossi-Fanelli *et al.* (1958); carbon monoxide was supplied by Imperial Chemical Industries Ltd.

Buffers. All reactions were carried out in 0.1 M-phosphate buffer, pH 7.1, made by mixing 5 vol. of 0.1 M- Na_2HPO_4 with 2 vol. of 0.1 M- KH_2PO_4 .

Reconstituted haemoglobins. These were prepared and reduced with the enzyme system of Rossi-Fanelli & Antonini (1959*a*).

Concentrations of haem pigments. These were determined as the CO compounds with a Unicam SP. 600 spectrophotometer.

Table 1. *Reactions of haemoglobin with oxygen and carbon monoxide*

All determinations were carried out at 20.5° in 0.1 M-phosphate buffer, pH 7.1, with total pigment concentrations of: 3 μ M for k_{43} and l_{43} ; 40 μ M for k_{34} and l_{34} ; 10 μ M for spectrophotometric determination of M . Values of K_4 and L_4 were calculated from the kinetic constants and M from K_4 and L_4 . Units: combination velocity constants, $\text{M}^{-1} \text{sec}^{-1}$, based on haemin iron; dissociation velocity constants, sec^{-1} ; equilibrium constants, M^{-1} .

	Natural protohaemoglobin	Reconstituted protohaemoglobin	Mesohaemoglobin	Deuterohaemoglobin
k_{34}	3.2×10^7	3.0×10^7	1.4×10^7	5.7×10^7
k_{43}	34	34	8	60
l_{34}	7.0×10^8	7.0×10^8	7.0×10^8	8.8×10^8
l_{43}	0.031	0.031	0.022	0.038
K_4	9.4×10^8	8.8×10^8	3.0×10^8	9.5×10^8
L_4	2.2×10^8	2.2×10^8	3.2×10^8	2.3×10^8
M	230	250	110	240
M by static spectrophotometry	330	350	130	240

meter, with the wavelengths and extinction coefficients of Rossi-Fanelli *et al.* (1959).

Kinetic experiments. These were carried out with the stopped-flow apparatus of Gibson (1954), to give the values of k_{43} by the method of Gibson & Roughton (1955), and of l_{01} by the method of Gibson & Roughton (1957*a*). Values of k_{34} and l_{34} were obtained by the method of flash photolysis with the apparatus of Gibson (1959). The procedure for determining l_{34} was that described by Gibson (1956); for k_{34} the method was modified by increasing the flash energy to correspond to the lower photosensitivity of oxyhaemoglobin (Gibson & Ainsworth, 1957), and omitting the step of converting into carboxyhaemoglobin; otherwise the determinations were carried out as for l_{34} . The value of l_{43} was obtained by replacement of CO by NO with the spectrophotometric method of Gibson & Roughton (1957*b*).

Partition of haemoglobin between oxygen and carbon monoxide

When haemoglobin is equilibrated with a mixture of O_2 and CO such that a negligible proportion of gas-binding groups remains free, the partition coefficient

$$M = L_4/K_4 = ([\text{O}_2] \cdot [\text{COHb}]) / ([\text{CO}] \cdot [\text{O}_2\text{Hb}]).$$

This coefficient forms a useful check on the kinetic work in which k_{34} , k_{43} , l_{34} and l_{43} were determined, since $K_4 = k_{34}/k_{43}$ and $L_4 = l_{34}/l_{43}$. A value of M is thus obtained from kinetic measurements which can be compared with the value determined directly. The method used was to place a dilute (10 μ M) solution of oxyhaemoglobin in a tonometer attached to a spectrophotometer cell and record the absorption spectrum between 380 and 450 $\text{m}\mu$ (with an Optica recording spectrophotometer, Optica Ltd., Newcastle upon Tyne). The haemoglobin was then equilibrated in the dark with a gas mixture containing $\text{CO} + \text{O}_2$ (1:250, v/v), and the spectrum again recorded. The tonometer was finally filled with pure CO and the spectrum of the CO-compound recorded. The three records were made on the same piece of chart, and the extinction was measured for each record at the wavelength of maximum absorption of the CO-compound. The proportions of oxy- and carboxyhaemoglobin in the mixture were calculated assuming Beer's law to apply.

Table 2. *Combination of the first molecule of carbon monoxide with fully reduced haemoglobin*

Reaction velocities were measured by the stopped-flow method. Total pigment concentration after mixing was 5 μ M (as haematin iron); concentration of CO, 0.11 mm; phosphate buffer, 0.1 M, pH 7.1; temperature, 20°. Wavelengths: proto-haemoglobin 430 $\text{m}\mu$, mesohaemoglobin and deuterohaemoglobin 421 $\text{m}\mu$. Values of l_{34} are taken from Table 1.

Haemoglobin	$10^{-5} \times l_{01}$ ($\text{M}^{-1} \text{sec}^{-1}$)	Ratio l_{34}/l_{01}
Natural protohaemoglobin	4.4	16
Reconstituted protohaemoglobin	4.8	15
Mesohaemoglobin	11.6	6
Deuterohaemoglobin	7.6	12

RESULTS

The results of experiments on the rate of combination and dissociation of the fourth molecule of oxygen and carbon monoxide are summarized in Table 1. The values are all averages of satisfactory duplicate experiments. It should be noted that in every case the natural protohaemoglobin gave results agreeing within experimental error with those obtained for reconstituted protohaemoglobin. The values agree well with those reported by Gibson, Kreuzer, Meda & Roughton (1955) for human haemoglobin at pH 7.1, although their experiments did not cover so wide a range of velocity constants and were usually obtained with much higher concentrations of haemoglobin. Although the values of M calculated from the kinetic experiments differ appreciably from those directly determined, the possibilities of cumulative error in five independent determinations are such that the agreement is considered satisfactory. Inspection of Table 1 shows that the largest differences between protohaemoglobin and mesohaemoglobin and deuterohaemoglobin are found in the values for k_{43} . The other three kinetic constants determined show much smaller differences.

Table 3. *Comparison of observed and calculated results for the combination of carbon monoxide with reconstituted protohaemoglobin, mesohaemoglobin and deuterohaemoglobin*

In each case l_{34} was taken to be infinitely large. The experimental conditions were those of Table 2.

Time (msec.)	Protohaemoglobin		Mesohaemoglobin		Deuterohaemoglobin	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
5	—	—	85.5	86.9	—	—
10	87.7	87.2	74.2	74.3	81.0	80.7
15	—	—	63.3	62.5	—	—
20	75.8	75.8	53.8	52.9	61.3	62.0
30	64.5	64.6	38.1	37.8	44.8	45.1
40	54.6	54.5	26.9	27.1	31.3	31.5
50	45.7	46.0	19.2	19.0	21.3	21.2
60	37.4	37.5	—	—	14.2	13.8
70	30.7	31.0	—	—	—	—

Ratios used in calculating figures for the Table.

	l_{01}	l_{12}	l_{23}
Protohaemoglobin	4	3	2
Mesohaemoglobin	4	10	1.5
Deuterohaemoglobin	4	3.2	3

Values for the rate of combination of the first molecule of carbon monoxide are given in Table 2. They were obtained by the extrapolation procedure described by Gibson & Roughton (1957*a*). There is no significant difference between natural and reconstituted protohaemoglobins, but both mesohaemoglobin and deuterohaemoglobin show a substantially higher rate of reaction. Comparison of the form of the progress curve of the reaction of carbon monoxide with the three haemoglobins showed striking differences: protohaemoglobin showed a gentle increase in rate as the reaction progressed, which was closely similar to that observed by Gibson & Roughton (1957*a*) with sheep haemoglobin. With deuterohaemoglobin an increase was again observed, which was, however, appreciably sharper than that seen with protohaemoglobin. With mesohaemoglobin little or no increase in rate occurred during the reaction. Gibson & Roughton (1957*a*) have dealt with the problem of evaluating all four combination velocity constants from data of this type. Their full procedure requires extensive experimental information and expert statistical analysis which were not available to us. We have, however, made a rough estimate of the relative sizes of the four constants, by somewhat simpler methods. Thus Table 2 shows that the ratio l_{34}/l_{01} is in every case much greater than the statistical value of 0.25. Gibson & Roughton (1957*a*) have shown that any value of this ratio greater than about 6 may be treated as being infinitely large. Taking the values of l_{01} from Table 2, and l_{34} as ∞ , attempts to fit the experimental curves by Desk Method II of Gibson & Roughton (1957*a*) have been made on a trial-and-error basis. The fit achieved is illustrated in Table 3, which gives also the values of the ratios $l_{01}:l_{12}:l_{23}$ used in

working out the calculated values. The general result emerges that the ratios for protohaemoglobin and deuterohaemoglobin do not differ widely from each other or from the statistical set of ratios, 4:3:2. Mesohaemoglobin does not fit into this pattern, and reasonable agreement between the observed and calculated values requires that l_{12} shall be considerably above the statistical value and l_{23} below it. The precision with which l_{12} can be estimated is, however, low: it can only be said with any assurance that it must be large by comparison with l_{01} and l_{23} .

DISCUSSION

Natural and reconstituted protohaemoglobins

Rossi-Fanelli & Antonini (1959*a*) have shown that the oxygen-dissociation curves of natural and reconstituted protohaemoglobins are similar, and the kinetic work reported here now extends their findings to include individual kinetic and equilibrium constants. The results suggest strongly that the natural and reconstituted proteins are, within the limits of experimental error, exactly equivalent in their functional properties, and that this equivalence extends even to such an easily disturbed character as haem-haem interaction, which can be profoundly modified by relatively slight changes in the protein molecule. It is difficult to believe that such close similarity of behaviour between the natural and reconstituted haemoglobins could be observed unless the haem groups return, on reconstitution, to positions exactly analogous to those which they occupied in the natural protein. This view is exactly contrary to the conclusion reached by Wyman (1948), on the experimental evidence of Davies. The reasons for

the discrepancy are not clear, but no doubt arise from the three main differences in the experimental procedure: (i) Davies used horse haemoglobin whereas the present work has been carried out with human haemoglobin; (ii) the procedure of Rossi-Fanelli *et al.* (1958) for preparing the apo-protein is carried out under mild conditions; (iii) methaemoglobin has been reduced enzymically instead of with dithionite. It should also be pointed out that the preparation of human haemoglobin used by Rossi-Fanelli *et al.* (1958) has been very fully studied not only in regard to its reversible reactions with ligands, but also in its ultracentrifugal and electrophoretic behaviour, and in its resistance to heat and alkaline denaturation. The close agreement found in every property studied justifies, in our opinion, the belief that the natural and reconstituted haemoglobins are, in fact, identical.

The finding (Table 3) that the first three molecules of carbon monoxide to combine with protohaemoglobin do so at rates in the ratios 4:3:2 is unexpected, since the indications from equilibrium work suggested that a sequence such as 4:10:2 was more likely. The determinations of Lyster (1957) showed that with horse and with human haemoglobins K_2 was considerably greater than K_1 , whereas Roughton *et al.* (1955) found with sheep haemoglobin that K_1 , K_2 and K_3 were all of the same order. There is no information available about the values of L_1 and L_2 , however, for any species of haemoglobin, and very little weight can be attached to speculations grounded on comparisons of kinetic work with carbon monoxide and equilibrium work with oxygen.

Mesohaemoglobin and deuterohaemoglobin

The kinetic results summarized in Tables 1-3 show that the combination of the first molecule of carbon monoxide with fully reduced mesohaemoglobin and deuterohaemoglobin takes place two or three times as fast as with protohaemoglobin. This result is similar to the findings of Smith & Gibson (1959) and Smith (1959) that carbon monoxide combined more rapidly with several derivatives of mesohaem and deuterohaem than with the corresponding protohaem compounds. In their work, however, mesomyoglobin formed an exception, combining with carbon monoxide at the same rate as did protomyoglobin.

The differences between mesohaemoglobin and deuterohaemoglobin and protohaemoglobin do not extend to the combination of the fourth molecule of carbon monoxide, where the rate is substantially similar for all three proteins. The effect of this is that haem-haem interaction, as measured by the ratio l_{34}/l_{01} , is smaller for mesohaemoglobin and deuterohaemoglobin than for protohaemoglobin.

In the properties discussed so far, mesohaemo-

globin and deuterohaemoglobin have shown similar behaviour. The analysis of the course of the reaction with carbon monoxide (Table 3), however, indicates considerable differences between them. Thus deuterohaemoglobin shows a pattern of kinetic constants similar to that for protohaemoglobin, where the marked increase in rate of combination is restricted to the reaction $\text{Hb}_4(\text{CO})_3 + \text{CO} \rightleftharpoons \text{Hb}_4(\text{CO})_4$. Mesohaemoglobin, on the other hand, behaves more as if the haem-haem interaction took place between pairs of haems, the second and fourth velocity constants both being relatively large. This finding depends on the analysis of the time course of the combination reaction; although the differences between mesohaemoglobin and deuterohaemoglobin are well outside the limits of experimental error, their sense is such that it would be possible for heterogeneity in the kinetic properties of the mesohaemoglobin preparation to produce the observed result. There is, however, no reason to suppose that the mesohaemoglobin preparation was heterogeneous.

The dissociation velocity constant for carbon monoxide, l_{43} , does not show outstandingly great differences between the three haemoglobins. The corresponding oxygen constant, k_{43} , however, shows a large difference between mesohaemoglobin and deuterohaemoglobin, which differ by seven to eight times in their rate of reaction. This is much the largest difference found and is chiefly responsible for the differences in K_4 and M which distinguish these haemoproteins. There is a lack of correlation between k_{43} and l_{43} , another example of the breakdown of Haldane's second principle [see Wyman's (1948) discussion].

Rossi-Fanelli & Antonini (1959*b*) and Rossi-Fanelli *et al.* (1959) have examined the oxygen-dissociation curves of mesohaemoglobin and deuterohaemoglobin, which showed an increased affinity for oxygen together with a decreased inflexion, n in Hill's (1910) equation being 1.7 as against 2.7 for protohaemoglobin. Unfortunately our data are not sufficient to allow us to discuss the kinetic origins of these equilibrium effects fully. The differences in affinity between mesohaemoglobin and deuterohaemoglobin are, however, in the direction to be expected from the values of k_{43} and k_{34} . Preliminary experiments on the overall oxygen-combination reaction suggest that mesohaemoglobin may be particularly suitable for more detailed study, especially if its reactions can be accounted for in terms of interaction between pairs of haems, rather than between groups of four haems.

The roles of the haem and of the protein in producing the differences between protohaemoglobin, mesohaemoglobin and deuterohaemoglobin cannot, at present, be precisely defined. The results

of Smith (1959) suggest, however, that the combination velocity rates may be determined chiefly by the chemical configuration of the haems, which apparently all combine with the same site on the protein surface (Rossi-Fanelli & Antonini, 1959*b*), though not necessarily forming identical linkages. Determinations have shown that the ultracentrifugal molecular weight is the same in all three cases, so that the lower degree of haem-haem interaction shown by mesohaemoglobin and deuterohaemoglobin cannot be attributed to splitting to half-molecules. The results reported here suggest that experiments with more widely different haems may help in understanding the mechanism of haemoglobin function.

SUMMARY

1. The rates of combination of natural and reconstituted protohaemoglobin with carbon monoxide have been compared both for the first and fourth molecules of carbon monoxide to combine. The natural and reconstituted proteins give rates agreeing with one another.

2. The rates of dissociation of the first molecule of oxygen and of carbon monoxide from fully saturated natural and reconstituted protohaemoglobin have been similarly compared and found to agree.

3. The corresponding reactions of mesohaemoglobin and deuterohaemoglobin have been examined. The rate of combination of the first molecule of carbon monoxide is greater than for protohaemoglobin. The rate of dissociation of the first molecule of oxygen from saturated haemoglobin is slow for mesohaemoglobin (k_{43} 8 sec.⁻¹) and rapid for deuterohaemoglobin (k_{43} 60 sec.⁻¹).

We are indebted to the Consiglio Nazionale delle Ricerche, to the Research Fund of the University of Sheffield and to the Rockefeller Foundation for grants in aid of expenses and equipment.

REFERENCES

- Adair, G. S. (1925). *J. biol. Chem.* **63**, 529.
 Erdmann, J. G. & Corwin, A. H. (1947). *J. Amer. chem. Soc.* **69**, 750.
 Fischer, H. F. & Orth, H. O. (1940*a*). *Die Chemie des Pyrrols*, vol. 2, part 1, p. 414. Leipzig: Akademische Verlagsgesellschaft.
 Fischer, H. F. & Orth, H. O. (1940*b*). *Die Chemie des Pyrrols*, vol. 2, part 1, p. 445. Leipzig: Akademische Verlagsgesellschaft.
 Gibson, Q. H. (1954). *Disc. Faraday Soc.* **17**, 137.
 Gibson, Q. H. (1956). *J. Physiol.* **134**, 123.
 Gibson, Q. H. (1959). *Biochem. J.* **71**, 293.
 Gibson, Q. H. & Ainsworth, S. (1957). *Nature, Lond.*, **180**, 1416.
 Gibson, Q. H., Kreuzer, F., Meda, E. & Roughton, F. J. W. (1955). *J. Physiol.* **129**, 65.
 Gibson, Q. H. & Roughton, F. J. W. (1955). *Proc. Roy. Soc. B*, **143**, 310.
 Gibson, Q. H. & Roughton, F. J. W. (1957*a*). *Proc. Roy. Soc. B*, **146**, 206.
 Gibson, Q. H. & Roughton, F. J. W. (1957*b*). *Proc. Roy. Soc. B*, **147**, 44.
 Hill, A. V. (1910). *J. Physiol.* **40**, iv.
 Hill, R. & Holden, H. F. (1926). *Biochem. J.* **20**, 1326.
 Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience Publishers Inc.
 Lyster, R. L. J. (1957). Ph.D. Thesis: University of Cambridge.
 Rossi-Fanelli, A. & Antonini, E. (1959*a*). *Arch. Biochem. Biophys.* **80**, 299.
 Rossi-Fanelli, A. & Antonini, E. (1959*b*). *Arch. Biochem. Biophys.* **80**, 308.
 Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1958). *Biochim. biophys. Acta*, **30**, 608.
 Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1959). *Arch. Biochem. Biophys.* (in the Press).
 Roughton, F. J. W. (1949). *Haemoglobin*, p. 83. London: Butterworths Scientific Publications.
 Roughton, F. J. W., Otis, A. B. & Lyster, R. L. J. (1955). *Proc. Roy. Soc. B*, **144**, 29.
 Smith, M. H. (1959). *Biochem. J.* **73**, 90.
 Smith, M. H. & Gibson, Q. H. (1959). *Biochem. J.* **73**, 101.
 Wyman, J. (1948). *Advanc. Protein Chem.* **4**, 408.

Biochem. J. (1960) **76**, 538

Study of a Proteolytic Enzyme from Rabbit Spleen

BY C. LAPRESLE AND T. WEBB

Service de Chimie microbienne, Institut Pasteur, Paris

(Received 12 February 1960)

It has long been known that aqueous extracts of spleen exhibit proteinase activity (Hedin, 1903). The intracellular enzymes responsible for this activity and a similar activity found in other tissues have been named cathepsins (Willstätter &

Bamann, 1929). The cathepsins of ox spleen have been studied in some detail and classified according to their specificity toward synthetic substrates (Tallan, Jones & Fruton, 1952).

In order to study the catabolism of a protein