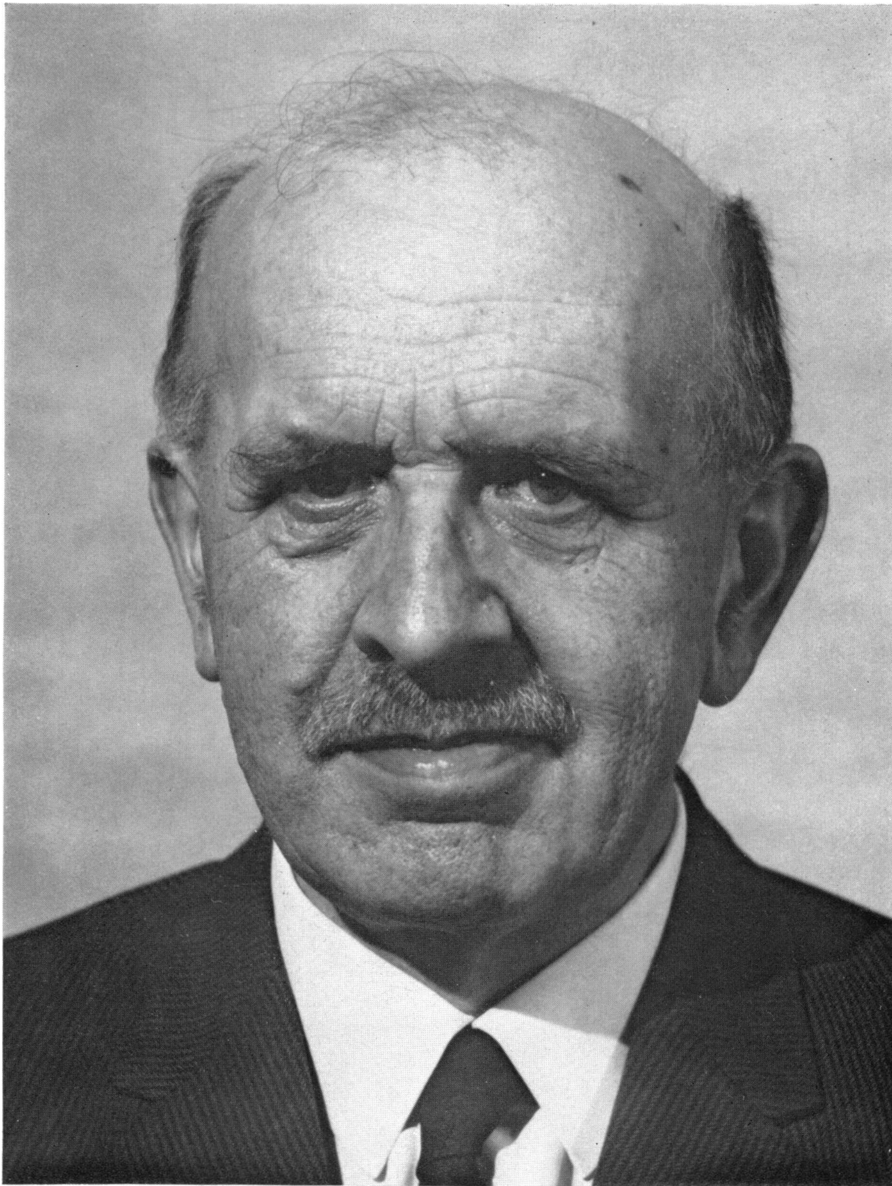


PROFESSOR F. J. W. ROUGHTON, F.R.S.

Biochem. J. (1970) **117**, 801–812
Printed in Great Britain

Some Recent Work on the Interactions of Oxygen, Carbon Dioxide and Haemoglobin

THE SEVENTH HOPKINS MEMORIAL LECTURE

By F. J. W. ROUGHTON*

Delivered at a Meeting of the Biochemical Society on 17 *April* 1969 *at the Sciences Lecture Theatre Building, University of Liverpool, Liverpool, U.K.*

An early personal contact with Frederick Gowland Hopkins

The Hopkins Memorial Medal and Lectureship are two of the greatest honours in the gift of our Biochemical Society or indeed of biochemists anywhere in the world. No scientist chosen to receive them should, or indeed could, fail to feel deeply grateful and proud but at the same time humble—all the more so if he had had, as I had in my earlier youth, the privilege of knowing Professor Hopkins intimately and of being greatly inspired by this unforgettable man.

It was said of Robert Boyle in the Seventeenth Century that he was 'the Father of Chemistry and Uncle of the Earl of Cork'. Sir Frederick Gowland Hopkins, or 'Hoppy' as he was so widely and affectionately known, was certainly the Father of British Biochemistry, but not, to my knowledge the uncle of any earl or other aristocrat. Nevertheless he had a much more important avuncular function than that of Robert Boyle. He was in fact for at least half a century a kind of Universal Uncle, and indeed Fairy Godfather, to a host of younger scientists, not only biochemical, from all parts of the world. It is now some 22 years since his death, just before the first International Biochemical Congress at Cambridge in 1948.

Some of his friends and pupils prepared a special book for that first Congress and named it *Hopkins and Biochemistry*. Read it if you have not already done so and read it again if you have, for you will then recapture the thrills of Hoppy's time as readily as I did when I reread it last year. Particularly I would urge on you Hoppy's own romantic biography at the beginning of the book. At the end is a roster of some 400 people who worked with him or came closely in other ways under his influence at some periods of their lives. I do not doubt that every one of these fortunate men and women have found that they have owed something or many things of great value alike in their scientific work and their personal outlooks. I myself was no exception—I was indeed uniquely lucky in getting to know him well even in my freshman year at Cambridge, when I was only 19 and he 57, just three times my age. Before I come to my chosen scientific theme I would like to recall some of this early personal contact, for it was typical of the catalytic influence which Hoppy so often exerted on very much younger disciples.

I had won an entrance scholarship in mathematics, physics and chemistry at Trinity College, Cambridge, in December 1917, just 11 months before the Armistice that ended World War I. Owing to a heart anomaly I had been rejected for all military service except office work, and my advisers therefore thought it best for me to go straight up to Cambridge in January 1918 and start on my intended career in medicine—the profession of my Roughton ancestors for the five preceding generations, father to son. Academic life at Cambridge at that time was much more severely affected than in World War II. Undergraduate numbers were down to 5% of their pre-War figures and most of the dons under 50 were away on war service. I was, indeed, in 1918 one out of only three medical students at Trinity, and it was an extraordinary piece of luck that Hoppy, who as Professor of Biochemistry held a professorial fellowship at Trinity, was called out to direct our studies. I thus was given the earliest possible opportunity to get to know this remarkable man and his wonderful new subject. I hope I did not bore him too much by the frequent use I made of this golden opportunity—if so, he dissembled with his usual charm.

Of the contacts with him in 1918 the one I remember most vividly occurred on a November morning, when he was walking round our diminutive class in Practical Biochemistry. He stopped and asked me what I was reading in physiology. I replied with the name of a standard textbook of that time for medical students. 'Oh,' he said, 'that is all very well as a cram book for the M.B., but a young man like you ought to aim much higher. Why don't you try Bayliss's *Principles of General Physiology*, that is really fascinating?'. So I

* Address: Laboratory of Biophysical Chemistry and Colloid Science, University of Cambridge, Cambridge CB2 3RJ, U.K.

scrambled as fast as I could through the rest of the morning's practical work and then bolted round to my usual bookshop, where they by good luck did have one copy of Bayliss in stock. For this I disgorged the sum of 24 shillings, big money in those times and for me! I then hurried with the precious volume to my rooms in Trinity Great Court and was soon so immersed in it that I completely forgot my twelve o'clock lecture and almost forgot my lunch an hour later—which would have been quite a thing in those tightly rationed days. 'Bayliss' with its extraordinary medley of physical chemistry, physiology, biochemistry with touches of many other branches of science and culture was—in its way—as stimulating as a book as Hopkins was as a man. Incidentally Bayliss's chapter on haemoglobin was a particularly inspiring one, even though its point of view did not survive for long. Here then were two of the first academic catalysts for my path in biophysical chemistry 50 years ago—a trail which has, I hope, mostly wound upwards and has now brought me in my seventieth year to the tops of the twin peaks of the Hopkins Memorial and Lectureship.

The end of World War I brought home a spate of other catalysts to Cambridge. Hoppy was succeeded as director of medical studies by E. D. Adrian (later Lord Adrian) and to this mentor I owed much. My own budding research interests, however, turned rather to Kings College, where Joseph Barcroft, A. V. Hill (previously at Trinity) and Hamilton Hartridge were by the summer of 1919 back again in full fettle. These three Fellows of Kings, but especially Barcroft, had before the War done outstanding work on haemoglobin, and all of them were after World War I to have a great influence on my subsequent work on the physical chemistry of the reactions of haemoglobin with gases, which has been ever since one of my major research interests. By an amusing coincidence the names of all these men, Hopkins, Bayliss, Hill, Barcroft and Hartridge began either with capital H or capital B, whilst the customary abbreviation for haemoglobin is of course Hb. Barcroft used indeed to recall that a student once insisted to him that haemoglobin is one of the simplest of all chemical substances for its formula is just Hb!

Some structural features of the haemoglobin molecule in relation to its reactions with oxygen and carbon dioxide

Actually the brilliant amino acid sequence and X-ray crystallographic work of the last two decades has shown that myoglobin and haemoglobin are much more complicated than Barcroft's somewhat naive student imagined. The three-dimensional structure of myoglobin, as Kendrew first showed, resembles a bunched necklace or coiled serpent, formed of a single chain of 153 amino acids joined to one another by peptide links, so that at one end of the chain there is a free α-amino group and at the other end a free carboxyl group. Attached to certain histidine residues in the chain are the haem prosthetic groups, with which the oxygen molecule reacts reversibly.

The normal haemoglobin molecule, with four times the molecular weight of myoglobin, is formed of four amino acid chains, similar in character though shorter than the single chain of myoglobin. Two of the chains are named 'alpha', i.e. α_1 and α_2, and the other two 'beta', i.e. β_1 and β_2. Each haem group is linked to the imidazole groups of two histidine residues (at positions 93 and 64 in the case of the β chains, each amino acid residue being numbered by its distance from the N-terminus of the chain). It is with these terminal α-amino groups of the four chains of the haemoglobin molecule that CO_2 has been postulated, in the last 35 years, to combine reversibly at physiological pH and temperature. The suggested reaction belongs to the general carbamate type:

$$RNH_2 + CO_2 \rightleftharpoons RNH{\cdot}CO_2^- + H^+ \quad (1)$$

which has, since about 1917, been studied continuously and exhaustively over a very wide range of amines by Faurholt and his school at Copenhagen. During the past 2 years the truth of this carbamate hypothesis as regards haemoglobin seems to have been placed beyond reasonable doubt by the dramatic work of Kilmartin & Rossi-Bernardi (1969), which will be described below.

O_2 and CO_2 are thus held at quite different sites in the haemoglobin molecule, but their respective reactions, which are thus of such a different type, nevertheless influence each other in a marked way, which is both of great physicochemical interest and physiological importance. These mutual interactions do indeed form an excellent example, and perhaps one of the earliest ones, of what are now known as allosteric effects, by virtue of which a reaction at one site of a large molecule affects greatly some other reaction at quite another site in the same molecule. Such allosteric phenomena are of wide and deep interest in current biochemistry and molecular biology, and it is largely for this reason that the present lecture is mainly devoted to a detailed review of this particular example, with which my colleagues and I have been intimately concerned for upwards of 40 years.

Some notes on the background of the subject (1837–1937)

The origins of the subject of this lecture go back over a century and are well treated in the historical accounts by Forbes (1931) and Perkins (1964).

Magnus (1837) suggested that CO_2 might assist in the liberation of O_2 from blood: this was convincingly shown some 70 years later by the classical work of Bohr, Hasselbalch & Krogh (1904) at Copenhagen. Their demonstration that increase of PCO_2 lowers the amount of O_2 combined with haemoglobin at a given PO_2 was referred to by Haldane (1922, p. 114) as the 'Bohr effect', by which it and allied phenomena became generally known. It is probable, however, that most of the credit should have been given to the junior author, A. S. Krogh, who was at that time assistant to Bohr, even whom he was later to outshine.

Just 2 years after Hoppy's birth in 1861 Holmgren (1863), working in the laboratory of Ludwig, who has been described as 'the Father of Modern Physiology', brought to light the reciprocal phenomenon, namely that uptake of O_2 helps in the discharge of CO_2 from the blood. That such an interrelationship should exist was realized by Bohr, who in his last years made several unsuccessful attempts to measure quantitatively the effect of O_2 on the affinity of blood for CO_2, but consistently failed to reveal any physiologically significant effects of this kind. The matter was not in fact clinched until 2 years after Bohr's untimely death in 1911, when Christiansen, Douglas & Haldane (1914) showed that at a given PCO_2 reduced blood does indeed take up significantly more CO_2 than does oxygenated blood. It is interesting to recall that at the time of this equally classical work Miss Christiansen was a young visitor from Copenhagen to Haldane's laboratory in Oxford and that Haldane (1922, p. 224) himself gratefully acknowledged the great debt he—and indirectly other British physiologists—owed to Christian Bohr for indoctrinating him in blood-gas methods and theory during a visit to Copenhagen about 1892.

Christiansen *et al.* (1914) correctly pointed out that the influence of CO_2 on the unloading of O_2 from the blood (the Bohr effect) is in the living body much slighter than the influence of O_2 in the dissociation of CO_2 from the blood (later known as the 'Haldane effect'), *pace* Bohr, whose experiments, as just mentioned, had failed to reveal it. During exercise the rate of exchange of gases between the blood and the muscles may be increased tenfold or more, chiefly due—as Krogh later showed—to a great opening up of blood capillaries in the muscles. This anatomical factor is ten or more times more important than the Haldane effect, and still more so than the Bohr effect.

The exact thermodynamic relation between the Bohr effect and the Haldane effect was deduced a few years later by Adair (1923) in an all too rarely quoted communication. Fig. 1 shows a graphic check of his equation in which PO_2 for 80% oxygenation of the blood at various values of PCO_2 (as

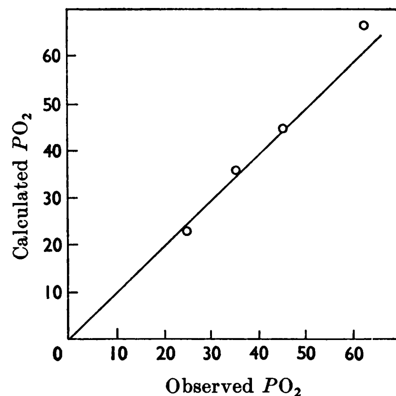


Fig. 1. Test of Adair's equation. The abscissa gives the measured values of PO_2 for 80% O_2Hb at various values of PCO_2 (data of Barcroft); the ordinate gives the values of PO_2 for 80% O_2Hb calculated by Adair's equation from data of Christiansen *et al.* (1914) on the CO_2 dissociation curves of blood.

measured by Barcroft) is plotted as abscissa against the PO_2 calculated by Adair's equation from the CO_2 dissociation curves of oxygenated blood and reduced blood reported by Christiansen *et al.* (1914). If the experimental and theoretical data tally the points should fall on a 45° straight line through the origin, as they are indeed seen to do. Later the matter was taken up by L. J. Henderson and extensively since World War II by Wyman.

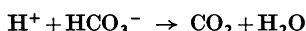
Bohr *et al.* (1904) did not put forward any detailed physicochemical explanation as to the mechanism of the Bohr effect, but Christiansen *et al.* (1914) made several suggestions as to the mechanism of the Haldane effect, one of which—namely that oxygenated haemoglobin (O_2Hb) is a stronger acid than reduced haemoglobin (Hb)—soon turned out a 'winner', both as to the interpretation of the Haldane effect and in many allied blood-gas phenomena. According to the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K + \log[\mathrm{HCO_3}^-] - \log[\mathrm{CO_2}] \qquad (2)$$

At a PCO_2 of 40 Torr (1 Torr ≡ 1 mmHg pressure) normal reduced blood at 37°C was found to contain about 24.3 mM of chemically bound CO_2, whereas for oxygenated blood under the same conditions the figure was 21.8 mM. If the bound CO_2 is all in the form of bicarbonate, then by eqn. (2) the pH of the oxygenated blood should have been lower than that of the reduced blood by log (24.3/21.8), i.e. by about 0.06 unit. The existence of such a difference could not have been tested in 1914, since there were no accurate methods available then for measuring the pH of whole blood. Careful determinations with

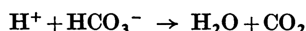
modern glass electrodes have, however, shown more recently (see e.g. Rossi & Roughton, 1962) that the actual difference of pH is only half or less of that calculated on the basis of eqn. (2). The discrepancy is largely, if not entirely, explained by the extra amount of CO_2 bound to haemoglobin in the carbamate form when the haemoglobin is freed of O_2.

The idea of a direct compound of CO_2 with haemoglobin was long ago suggested by Bohr, who coined for it the name 'carbhaemoglobin'. No unequivocal evidence for such a compound, however, came to hand until the pioneer kinetic work of Henriques (1928)—also at Copenhagen but some 16 years after Bohr's death. Henriques found that when serum or plasma containing CO_2 and bicarbonate at physiological pH is violently shaken *in vacuo* the CO_2 is liberated at a slow steady rate governed by the velocity of the uncatalysed reaction:



$$H^+ + HCO_3^- \rightarrow CO_2 + H_2O$$

With haemoglobin solutions on the other hand there was a strikingly different effect. Part of the CO_2 was evolved very rapidly, whereas the remainder escaped into the gas phase at a rate similar to that found with plasma. Henriques attributed the first rapid phase to the presence and fast dissociation of a reversible carbamate type compound of CO_2 with haemoglobin, and believed that there was no catalyst in the blood for the slow reaction:



$$H^+ + HCO_3^- \rightarrow H_2O + CO_2$$

since there was no reason why such a catalyst should not also be active during the second slow phase. Nevertheless there is such a catalyst in the blood, as was shown soon afterwards by Hawkins & Van Slyke (1930) and by Brinkman & Margaria (1931): finally the catalyst was isolated in the same year by Meldrum & Roughton (1932*a*,*b*) and given the name carbonic anhydrase. Next year the same enzyme was independently discovered by Stadie & O'Brien (1933), who had apparently missed the earlier announcements by Meldrum & Roughton (1932*a*,*b*). Carbonic anhydrase was found to be sensitive to heavy-metal poisons, e.g. cyanides and sulphides; Keilin & Mann (1940) showed that it contains zinc at or near its active centre and that is markedly inhibited by sulphanilamide and even more so by derivatives of the latter, e.g. acetazolamide (Diamox). It was a curious mischance that Henriques failed to discover the enzyme: haemoglobin prepared by the method he used is usually richly contaminated by carbonic anhydrase, which must have got accidentally inhibited during his laboratory procedure. 'Interdum dormitat bonus Homerus' were Henriques's words to us in 1932, in the course of a generous letter of appreciation on the discovery of carbonic anhydrase.

Meldrum & Roughton (1933) were able to reproduce the biphasic curves of Henriques, both as regards CO_2 output and CO_2 uptake, if, but only if, sufficient cyanide was added to eliminate the catalysis by carbonic anhydrase, cyanide being the most suitable inhibitor at that time. Many manometric experiments under a wide range of conditions gave strong support to Henriques's suggestions that the rapid reversible compound of CO_2 with haemoglobin is indeed of a carbamate type and is more markedly formed with Hb than with O_2Hb. These ideas were soon after clinched by direct chemical estimations of the CO_2 bound to Hb and O_2Hb under physiological conditions (Ferguson & Roughton, 1934; Ferguson, 1936; Stadie & O'Brien, 1937). The method for estimating the amount of haemoglobin carbamate ($HbCO_2$ for short) was a modification of the classical $BaCO_3$ precipitation method used by Faurholt for the carbamate compounds of simple amines, e.g. ammonia, glycine etc. With its aid it was found that a significant proportion (one-third to three-quarters according to conditions) of the extra CO_2 found by Christiansen *et al.* (1914) in reduced blood as compared with oxygenated blood is accounted for by carbamate compounds. Recently Rossi-Bernardi, Pace, Roughton & Van Kempen (1970) have modified the method, by separating the $HbCO_2$ by means of gel filtration rather than by $BaCO_3$ precipitation. It is hoped that this new technique will prove both more accurate and of greater applicability to other proteins, e.g. plasma proteins and myoglobin.

Relative effects of carbamate and bicarbonate on the equilibrium between oxygen and haemoglobin

If oxygenation decreases the amount of CO_2 bound to haemoglobin in the carbamate form, then reciprocally the combination of CO_2 in blood should lessen the affinity of haemoglobin for oxygen, over and above the similar effect produced by lowering the pH. The first clear-cut experiments on this issue, which had been debated for many years after the original paper by Bohr *et al.* (1904), were those reported by Margaria & Green (1933). These authors showed that P_y, the oxygen pressure for $y\%$ saturation of horse Hb with O_2, is about 1.4 times greater at pH 7.37 in $NaHCO_3$–CO_2 solutions than in NaCl solutions of the same ionic strength (0.1 M). Their results were confirmed and extended in a comprehensive paper by Hermann, Hudoffsky, Netter & Travia (1939), who also gave a valuable review of previous work on the subject. Since 1950 the problem has been further investigated

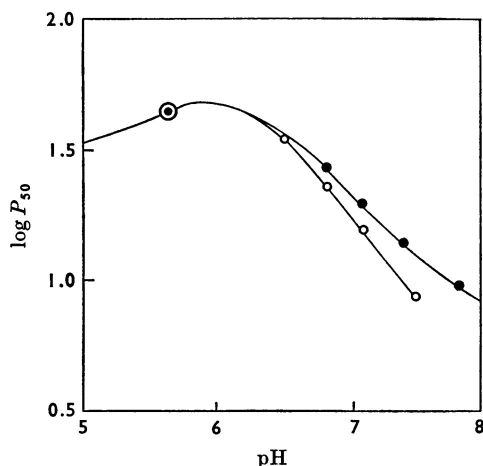


Fig. 2. Relation between $\log P_{50}$ (O_2 pressure for 50% O_2Hb) and pH for horse Hb solution at 37°C, ionic strength 0.15M. ○, PCO_2 0; ●, PCO_2 30 Torr.

in several laboratories: almost all recent workers have agreed that CO_2 does have a specific influence, both in: (i) haemoglobin solutions—the work of the Milan School since World War II has been summarized by Margaria (1957) in a useful review, which, however, contains some mistakes that have since been rectified; (ii) whole human blood under physiological conditions (see especially Naeraa, Petersen & Boye, 1963; Naeraa, Petersen, Boye & Severinghaus, 1966).

The specific effect of CO_2 in haemoglobin solutions (the whole blood work will be referred to later) is well brought out in an unpublished graph by Dr Marchi & Dr Rossi, who have kindly allowed me to include it here. Fig. 2 shows the relation they found between $\log P_{50}$ and pH, with PCO_2 equal to 0 and to 30 Torr at 37°C and total ionic strength 0.15M.

The value of $d(\log P_{50})/d(pH)$, which is taken as a quantitative measure of the Bohr effect, is seen to be practically constant over the range pH 6.8–7.4, the values for PCO_2 equal to 0 and to 30 Torr being 0.63 and 0.44 respectively. CO_2 is thus seen to have an antagonistic influence on the Bohr effect, as had also been shown by Rossi-Bernardi & Roughton (1967) in their studies of the differential titration curves of Hb and O_2Hb in the presence and in the absence of CO_2.

It is clear from Fig. 2 that the specific effect of CO_2 steadily increases as the pH is made more alkaline, as would be expected from the increased carbamate formation at constant PCO_2 when the pH is raised (see Fig. 3 below). There is also, however, a concurrent rise in bicarbonate concentration under these circumstances (see eqn. 2), and it is only recently that Kernohan, Kreuzer, Rossi-Bernardi & Roughton (1966) have tried to discriminate between these two factors, i.e. carbamate and bicarbonate. Fuller sets of experiments have since been carried out and will, it is hoped, be published before long.

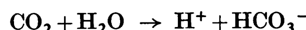
The procedure used by Kernohan *et al.* (1966) was as follows.

A CO_2-free solution of bovine or human haemoglobin in 0.2M-KCl and saturated with O_2 to, say, 50% at 37°C was mixed in a rapid-reaction apparatus of the Hartridge–Roughton type with (*a*) a solution of CO_2 in 0.2M-KCl or (*b*) a dilute solution of HCl in 0.2M-KCl, and the PO_2 and pH of the continuously flowing mixture were recorded electrometrically after a lapsed time of 0.2s from mixture. No appreciable bicarbonate can be formed from the dissolved CO_2 in 0.2s provided 1mM-acetazolamide is added to the Hb solution so as to inhibit any accompanying traces of carbonic anhydrase. On the other hand 0.2s is long enough at 37°C for the dissolved CO_2 to reach carbamate equilibrium with the Hb and for any accompanying changes in the O_2–Hb equilibrium to occur.

In a typical experiment (on 13 March 1969) a solution of CO_2-free human Hb, containing 6.1mequiv. of haem/l, 60% saturated with O_2, PO_2 17 Torr and pH 7.33, was mixed with: (*a*) 4.3mM-CO_2 in 0.2M-KCl: after 0.2s PO_2 = 20.5 Torr, pH = 7.19: (*b*) 0.8mM-HCl in 0.2M-KCl: after 0.2s PO_2 = 19.6 Torr, pH = 7.10. Ideally the HCl concentration in (*b*) should have been such as to give, on mixing with the Hb solution, exactly the same pH as in case (*a*), namely 7.19. A small correction, based on the known effect of pH on the O_2–Hb equilibrium curve in the absence of CO_2, decreased the figure of 19.6 Torr at pH 7.10 to 18 Torr at pH 7.19.

Thus the specific effect is equal to $100\times(20.5-18)/18 = 14\%$. This figure tallies satisfactorily with the mean of similar experiments on Hb solutions prepared from the blood of 17 other individuals, namely 15.8 (±1.0S.D.)% at the same PCO_2, pH 7.0–7.2.

The solution which had passed through the apparatus was collected anaerobically and after 2min or more (which is enough for the reaction:



$$CO_2+H_2O \rightarrow H^+ + HCO_3^-$$

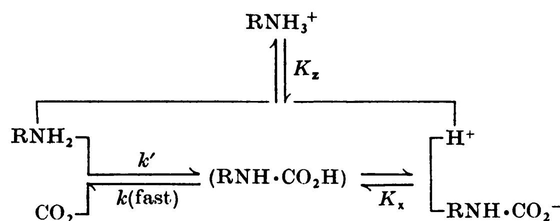
to reach equilibrium at 37°C even if the carbonic anhydrase is completely inhibited) showed a very small rise in PO_2, namely from 20.5 to 20.9 Torr, but a much more appreciable fall in pH, namely from 7.1 to 7.03. A similar calculation now showed a negligible difference from the control, namely an effect of $100\times(20.9-21)/21$, i.e. of only −0.5% (21 Torr is the calculated equilibrium PO_2 of the Hb–O_2Hb mixture at pH 7.03).

This comparatively feeble effect of bicarbonate was confirmed by converse experiments in which the same CO_2-free Hb–O_2Hb solution was mixed with (*c*) 0.05M-$KHCO_3$+0.15M-KCl and (*d*) 0.2M-KCl containing sufficient KOH to produce on mixture as nearly as possible the same pH as in case (*c*). In a typical such experiment (on 8 May 1969): Hb+(*c*): after 0.2s $PO_2 = 21.3$ Torr, pH = 7.35; on standing, $PO_2 = 24.3$ Torr, pH = 7.50; Hb+(*d*): after 0.2s $PO_2 = 20.7$ Torr, pH = 7.33. On standing there was no change either in PO_2 or pH. The specific effect of bicarbonate after 0.2s thus equals $100\times(21.3-20.2)/20.2 = 5.4\%$, 20.2 being the corrected value for the control at pH 7.35. In four of the most recent and best controlled experiments on human Hb+0.05M-$KHCO_3$ in the pH range 7.25–7.40 the percentage effect ranged from 3 to 8, with a mean of 5.1%.

It is thus clear that the effect due to CO_2+carbamate formation is qualitatively much greater than that due to HCO_3^-, though the latter seems not quite negligible. This conclusion is strikingly supported by the marked changes in the Hb+(c) mixture on standing. The pH rose from 7.35 to 7.50, but the PO_2 instead of falling with increase of alkalinity (the usual Bohr effect) rose appreciably from 21.3 to 24.3 Torr. This paradoxical behaviour is readily explained by the depressing effect on the O_2–Hb affinity produced by the $HbCO_2$, formed after the dehydration of HCO_3^- to CO_2. The lowering of the O_2 affinity in the present case is thus $100\times(24.3-17.6)/17.6 = 38\%$, i.e. an unmistakable effect, some 7 times that attributed to that of HCO_3^- *per se*. The effect of CO_2 (as distinct from that of pH) on the O_2 affinity of haemoglobin at constant pH being in the main mediated by carbamate can thus—in reverse—be used as another pointer for the existence of carbamate compounds with Hb and O_2Hb: an important use of this principle will be given later in discussing the very recent work of Kilmartin & Rossi-Bernardi (1969).

Physical chemistry of carbamate formation of simpler amines and of haemoglobin

Scheme 1 shows the general reaction scheme for reaction of CO_2, as established by the pioneer work of Faurholt (1925; see also earlier references given therein) on ammonia, methylamine, dimethylamine and glycine, with subsequent extension to a very wide range of amines, including especially glycylglycine (Roughton & Rossi-Bernardi, 1966; Jensen, 1970). This dipeptide was chosen for intensive study, as being a model for the analogous, but more complex, kinetics and equilibria of CO_2 with the terminal α-amino groups of haemoglobin. The glycylglycine equilibria were accordingly measured, not only by the classical $BaCO_3$ precipitation



$$K_z = [H^+][RNH_2]/[RNH_3^+]$$

$$K_x = [H^+][RNH\cdot CO_2^-]/[RNH\cdot CO_2H] \qquad (pK_x \sim 5.2)$$

$$K_c = \frac{[H^+][RNH\cdot CO_2^-]}{[CO_2][RNH_2]} = K_x\frac{k'}{k}$$

$$\frac{[\text{Carbamate}]}{[\text{Total N}]} = \frac{K_cK_z[CO_2]}{K_cK_z[CO_2]+K_z[H^+]+[H^+]^2}$$

Scheme 1. General scheme for reactions of CO_2 with amines.

method of Faurholt, but also by the CO_2 method of Forster, Constantine, Craw, Rotman & Klocke (1968) and by the 'total-equilibrium' method of Stadie & O'Brien (1936, 1937). Reasonably concordant results by these three independent methods were obtained for K_c, the fundamental equilibrium constant for the carbamate reaction, the average value at 20°C being $10^{-4.85}$, i.e. pK_c 4.85, when corrections had been inserted for the activity coefficients of the ions.

It will be noted that CO_2 does not react with the cationic form of the amine (RNH_3^+); with RNH_2, however, the reaction is very rapid, without the aid of any added catalyst. For an extensive range of amino acids Chipperfield (1966) found that the velocity constant of carbamino combination, k', is approximately related to the ionization constant of the amine, K_z, by the empirical equation (at 25°C):

$$\log k' = 0.262\,pK_z + 1.197 \qquad (3)$$

where $pK_z = -\log K_z$. The relation was tested over the range of pK_z 7–11. For glycylglycine at 25°C $pK_z = 8.25$ and hence k' by eqn. (3) is 2280 as compared with the observed value of 2030. The same equation probably applies roughly to the kinetics of combination of CO_2 with Hb and O_2Hb (Forster *et al.* 1968; Kernohan & Roughton, 1968), though here there are not yet any independent accurate values for the pK_z of the groups with which CO_2 combines.

Preliminary experiments have shown (Roughton & Rossi-Bernardi, 1970) that k, the velocity constant for the dissociation of glycylglycine carbamic acid ($HO_2C\cdot CH_2\cdot NH\cdot CO\cdot CH_2\cdot NH\cdot CO_2H$) into the free dipeptide plus CO_2 is about $200\,s^{-1}$ at 5°C,

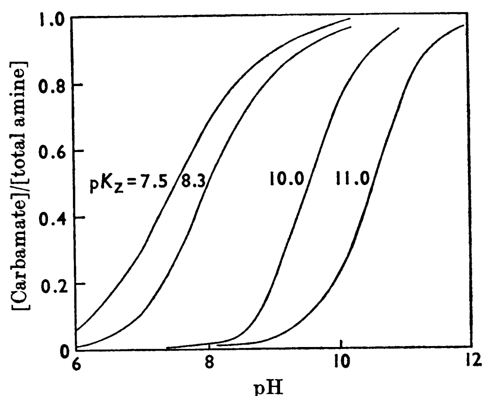


Fig. 3. Relation between pH and proportion of carbamate formed at constant PCO_2 (25 Torr) and constant pK_c (4.5) but with various values of pK_z.

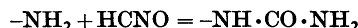
and that K_x, the ionization constant of the unstable carbamic acid, is about $10^{-5.2}$, i.e. pK_x 5.2. If the haemoglobin carbamic acids have pK_x of the same order they would not have any significant buffer power, *per se*, at physiological pH (7.0–7.4).

The proportion of carbamate formed at constant PCO_2 at pH values in the physiological range is very sensitive to the values of the pH and pK_z, as is shown in Fig. 3, in which [carbamate]/[total N of amine] at 1 mM-CO_2 is plotted against pH: (i) for pK_z 7.5–8.3, corresponding to the range for the terminal α-amino groups of peptides, e.g. glycylglycine, glycylglycylglycine etc., with pK_c 4.5; (ii) for pK_z 10–11, corresponding to the ε-amino group of lysine, with pK_c 4.5. Actually pK_c for amino acids and peptides varies very much less than pK_z: thus at 18°C, for glycine pK_z is 9.88 and pK_c 4.90; for glycylglycine pK_z is 8.46 and pK_c 4.90; for ε-aminocaproic acid (6-aminohexanoic acid) pK_z is 11.06 and pK_c 4.60. Probably the values of pK_z and pK_c for the ε-amino group of lysine are about the same as those for ε-aminocaproic acid.

It is clear from Fig. 3 that the proportion of carbamate at physiological pH (7.0–7.4) is not appreciable except in the case of peptides such as glycylglycine, wherein the peptide link causes a lowering of the pK_z of the adjacent α-amino group of about 1.4 units as compared with glycine. If, then, haemoglobin behaves in a similar way to simple amino acids and peptides, it would be only the terminal α-amino groups of the four amino acid chains that would be capable of combining appreciably with CO_2 at pH 7.0–7.4. For the pK_z of the ε-amino group of the lysine side chains, numerous as they are (i.e. five or more may be free for each haem group in the haemoglobin molecule), is too high for them to combine significantly with CO_2 until the pH is raised at least to 8.0. The same is even more true of the guanidine groups of the arginine residues, with their pK_z in the range 12–13. The pK_z of the imidazole ring of histidine, namely 6.0–8.0, though highly favourable for buffering in the physiological range, is not suited for carbamate formation, since nitrogen in closed rings appears to have no significant power of combining with CO_2 (Booth & Roughton, 1938). Nitrogen in the form of amides ($RCO{\cdot}NH_2$) or in the peptide link likewise lacks power to form carbamate at physiological pH. Thus by elimination the only likely nitrogen groups in the haemoglobin molecule to form carbamate at pH 7.0–7.4 are the terminal α-amino groups of the two α and β chains. Serum albumin has nearly the same molecular weight as haemoglobin and possesses actually more lysine residues per molecule; it has, however, only one terminal α-amino group, which may not in fact be free to react, and thus its relatively feeble power of forming carbamate at pH 7.0–7.4 (as compared with that of haemoglobin) is just what would be expected on the α-amino group hypothesis.

Sole responsibility of the four terminal α-amino groups of haemoglobin for carbamate formation at pH 7–7.4

Our working hypothesis would be greatly strengthened if the terminal α-amino groups could be modified to a form incapable of carbamate formation, without at the same time affecting the structure, conformation and reactivity of the rest of the haemoglobin molecule significantly. This idea had been in mind for some time—I remember, for example, it being mooted by Dr Reinhold Benesch in 1962 in the discussion of a paper on the reactions of CO_2 with haemoglobin which I gave in New York that year. Attempts have been made since then with various reagents, but no success was obtained until the triumph by Kilmartin (1969) at Cambridge very recently. The reaction he used was the carbamoylation (synonymous with the older term carbamylation) of the α-amino groups by cyanate, i.e.:



$$-NH_2 + HCNO = -NH{\cdot}CO{\cdot}NH_2$$

This reaction is, in a sense, the primeval reaction of organic chemistry, since it was by its means that Wohler in 1828 obtained urea, until then regarded as a substance of typically biological origin, from an inorganic, non-biological, precursor, i.e. ammonium cyanate.

Working with the electrolytically fast component of normal horse haemoglobin, Kilmartin (1969) succeeded in preparing and separating by chromatographic techniques compounds in which (i) the

α-amino groups of the α chains alone (ii) the α-amino groups of the β chains alone or (iii) the α-amino groups of both the α and β chains had been thus modified. The corresponding carbamoylated compounds are written as: (i) $\alpha_2^c\beta_2$, (ii) $\alpha_2\beta_2^c$ and (iii) $\alpha_2^c\beta_2^c$.

The X-ray crystallographic structure of the several carbamoylated compounds, together with a study of their equilibria in solution with oxygen, failed to indicate significant changes in the conformation and reactivity of other parts of their molecules.

If these carbamoylated compounds, i.e. $HbNH_2\cdot CO\cdot NH_2$, behave like typical amides such as acetamide ($CH_3\cdot CO\cdot NH_2$) and urea ($NH_2\cdot CO\cdot NH_2$), they should act as very weak bases with pK values in the range 0–1 instead of 7–8 and should fail to show any carbamate reactions at physiological pH. These deductions have been brilliantly confirmed by Kilmartin & Rossi-Bernardi (1969).

Fig. 4 shows the titration curves of the CO compounds of the normal horse Hb, $\alpha_2\beta_2$, and of the modified compounds, $\alpha_2^c\beta_2$, $\alpha_2\beta_2^c$ and $\alpha_2^c\beta_2^c$, over the range pH 6.0–9.0 at 25°C in 0.2M-KCl. The diagram may be most easily understood if we start with all the compounds at pH 9.0 and then consider the course of their back-titrations with HCl back to pH 6.0. It will be noted that $\alpha_2^c\beta_2^c$ needs the least acid to change it to pH 6.0 because all its terminal α-amino groups have had their pK values shifted to a very acid pH range and hence have lost their buffering power, or power of combining with acid, in the range pH 6.0–9.0. The compounds $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$ have each lost only two effective α-amino groups per tetramer haemoglobin molecule, so that their buffer power over the range pH 6.0–9.0 is only about half as much affected as that of $\alpha_2^c\beta_2^c$. Their titration curves do in fact coincide or practically so, thus showing that the pK values of the respective α-amino groups of the α and β chains must be about equal and do lie in the range pH 7–8, as must be so if they are to form carbamates under physiological conditions.

Three clear-cut lines of evidence were obtained with regard to the CO_2 reactions of these compounds in the unliganded form (Hb) and in the liganded form (O_2Hb or COHb, the latter being often preferred in view of its relative insensitivity to methaemoglobin formation, which is greatly exaggerated in these highly purified haemoglobin compounds). Fig. 5 shows measurements of the total CO_2 (by Van Slyke analysis) in four different haemoglobin solutions (concentration 8 mequiv. of Fe/l) each equilibrated at 37°C at PCO$_2$ 48 Torr at different pH values over the range 7.3–7.5. The top (continuous) line through the white circles is for the unliganded Hb ($\alpha_2\beta_2$), whereas the continuous line below it, through the black circles, is for the same haemoglobin combined with CO. The difference in total CO_2 in the two cases corresponds with that found by Rossi-Bernardi & Roughton (1967) for normal human and bovine haemoglobin and is attributed in the main, if not entirely, to the extra carbamate at a given PCO$_2$ and pH in Hb as compared with O_2Hb or COHb solution. Similar estimations on Hb ($\alpha_2^c\beta_2^c$) and on COHb ($\alpha_2^c\beta_2^c$) are

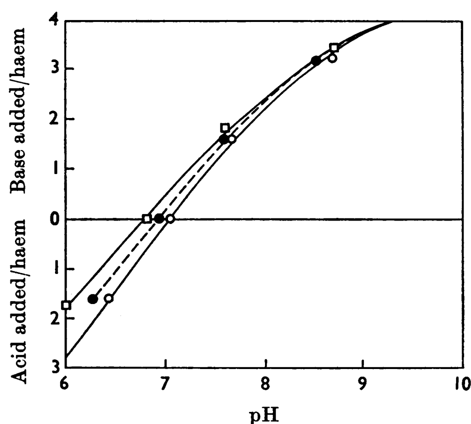


Fig. 4. Titration curves at 25°C of the CO compounds of normal Hb ($\alpha_2\beta_2$) (○), the carbamoylated derivatives $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$ (indistinguishable) (●) and the derivative $\alpha_2^c\beta_2^c$ (□). This figure and also Figs. 5, 6 and 7 have been redrawn from figures published by Kilmartin & Rossi-Bernardi (1969), through the courtesy of the authors and the publishers of *Nature*.

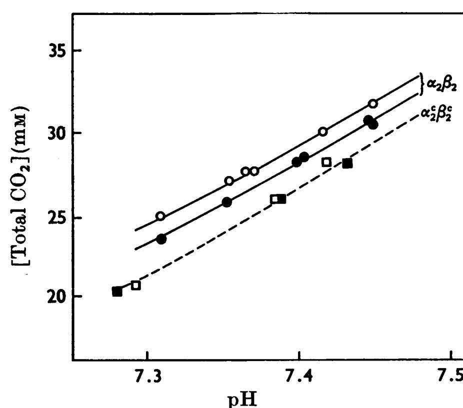


Fig. 5. Relation between pH and [total CO_2] at 37°C and constant PCO$_2$ 48 Torr for 8 mequiv. of COHb/l and 8 mequiv. of Hb/l. ○, Hb ($\alpha_2\beta_2$); ●, COH b($\alpha_2\beta_2$); □, Hb ($\alpha_2^c\beta_2$); ■, COHb ($\alpha_2^c\beta_2^c$).

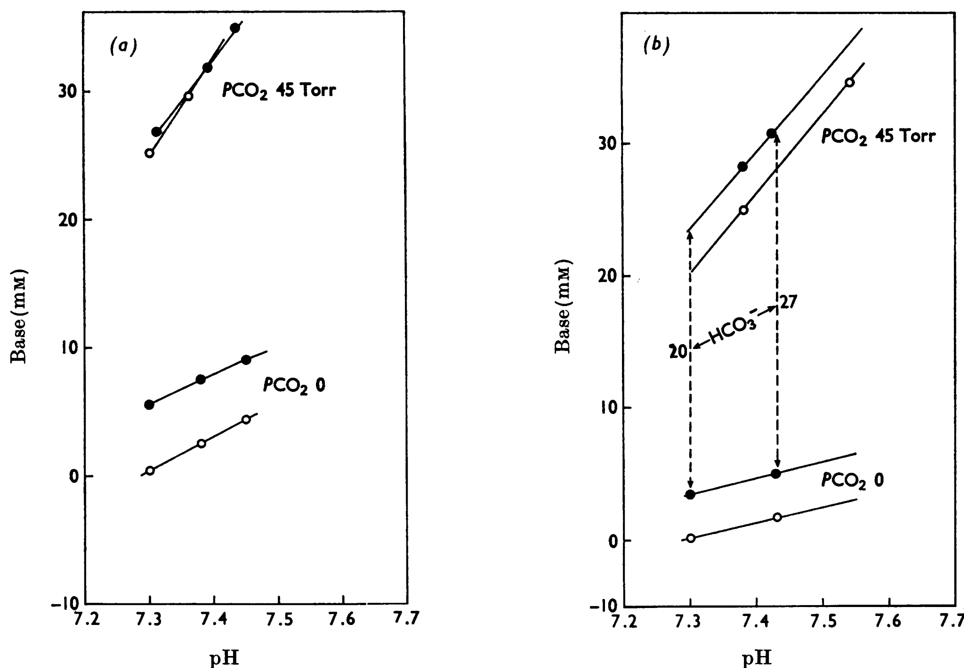


Fig. 6. (a) Titration curves at 37°C of 8.2 mequiv. of COHb/l (●) and 8.2 mequiv of Hb/l (○) for normal haemoglobin ($\alpha_2\beta_2$) in the presence of CO_2 (45 Torr) and in the absence of CO_2. (b) Titration curves at 37°C of 8.2 mequiv. of COHb ($\alpha_2^c\beta_2^c$)/l (●) and of 8.2 mequiv. of Hb ($\alpha_2^c\beta_2^c$)/l (○) in the presence of CO_2 (45 Torr) and in the absence of CO_2.

plotted as white squares and black squares respectively: these fall, within experimental error, on a single line (shown broken), as should indeed be the case if the carbamoylation of all the terminal α-amino groups has abolished their carbamate-forming power in the pH range 7.3–7.5.

Application of another of the tests used by Rossi-Bernardi & Roughton (1967) was equally conclusive. Fig. 6(a) shows the titration curves at 37°C, over the pH range 7.3–7.5, of solutions (8.2 mequiv./l) of Hb and COHb for the uncarbamoylated compounds $\alpha_2\beta_2$, in the absence and in the presence of CO_2 (PCO$_2$ 45 Torr). The Bohr effect is eliminated in the latter case, just as Rossi-Bernardi & Roughton (1967) found for normal human and bovine haemoglobin. This they explained by their theory as being due to the differing effect of carbamate compounds on the buffering powers of Hb and O_2Hb. Fig. 6(b) shows with the $\alpha_2^c\beta_2^c$ compounds that the Bohr effect, as measured by the vertical distance between the titration curves for Hb and COHb, is unaffected in the presence of CO_2, just as would be expected if no carbamate was formed either by Hb ($\alpha_2^c\beta_2^c$) or by COHb ($\alpha_2^c\beta_2^c$). The carbamoylated compounds $\alpha_2^c\beta_2^c$ do in fact behave in their acid–base and titration-curve relationships in just the way in which normal haemoglobin had generally been supposed to behave prior to the work of Rossi-Bernardi & Roughton (1967).

Direct carbamate estimations on the carbamoylated compounds have not yet been possible, owing to the difficulty of preparing sufficient quantities of the various derivatives, especially of $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$. Their carbamate reactions have, however, been investigated by the indirect method of Margaria & Green (1933) (see above). Fig. 7 shows that the $\alpha_2\beta_2$ compound gives the normal specific effect of displacing the O_2 equilibrium curve to the right, whereas for $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$ the effect is diminished by about a half, and for $\alpha_2^c\beta_2^c$ the CO_2 effect vanishes entirely.

All these tests of Kilmartin & Rossi-Bernardi (1969) thus seem to leave no reasonable doubt that the terminal α-amino groups of the α and β chains of purified haemoglobin are equally—and solely—responsible for the carbamate reactions at physiological pH.

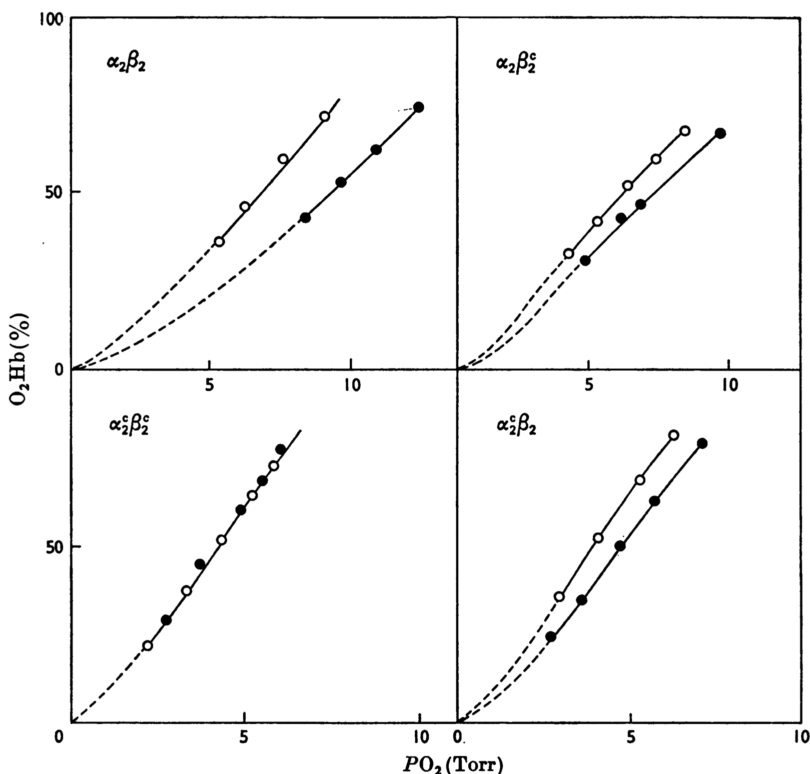


Fig. 7. Specific effect of CO_2 on the O_2 dissociation curves of the haemoglobin derivatives $\alpha_2\beta_2$, $\alpha_2\beta_2^c$, $\alpha_2^c\beta_2$ and $\alpha_2^c\beta_2^c$ as a test of their respective carbamate-forming power at pH 7.4 at 25°C. The haemoglobin concentration was 0.05g/100ml of solution: ○, 15mM-potassium phosphate in 0.2M-KCl (PCO_2 0); ●, 15mM-potassium phosphate in 0.15M-KCl–0.05M-$KHCO_3$ (PCO_2 38 Torr).

Note on some very recent work by Dr M. F. Perutz and his colleagues

Since the delivery of the Hopkins Memorial Lecture in April 1969 a highly important and relevant paper has been published by Perutz *et al.* (1969) on the 'Identification of Residues responsible for the Alkaline Bohr Effect in Haemoglobin'. I am greatly indebted to Dr M. Perutz for discussing and explaining this work to me. He and his colleagues believe that they have identified 'two ionizable groups with a pK in the neutral region which change their interactions with the solvent in going from oxyhaemoglobin to deoxyhaemoglobin. In oxyhaemoglobin the imidazole of the terminal histidine of the β-chain, i.e. HC3(146)β, would have a normal pK, while in deoxyhaemoglobin its pK would be raised due to its specific interaction with the aspartate at position 94 in the same β-chain. The terminal valine of the α-chain, i.e. NA1(1)α, would have a normal pK for its α-NH_2 group in the case of deoxyhaemoglobin, where it is free, and an abnormally low pK in oxyhaemoglobin due to its proximity to the positively charged guanidinium group of the C-terminal arginine of the other chain'. This last suggestion is still tentative.

The statement in inverted commas has been very kindly given to me by Dr M. Perutz, and I have only added a few explanatory words to it. Further work on these lines is in progress by him and his colleagues. It has obvious repercussions on the carbamate question, particularly the section of it referring to the α-amino group of the terminal valine of the α chain. Future developments will be awaited with great interest.

Physiological role of haemoglobin carbamic acid compounds in carbon dioxide transport by the circulating blood

Assuming explicitly that haemoglobin in the erythrocytes behaves like haemoglobin in dialysed solution as regards the O_2-linked carbamate

reactions with CO_2, it has been calculated that in normal resting men the carbamate mechanism should be responsible for 25–30% of the physiological transport of CO_2 in the circulating blood (see Rossi-Bernardi & Roughton, 1967; Roughton, 1964, and earlier references given therein). It is possible, however, that the reactivity towards CO_2 of the α-amino groups of the haemoglobin may be different in the highly concentrated and diverse milieu of the erythrocyte, and in fact lower than their reactivity in purified solutions of haemoglobin, in which moreover the protein molecules may well have become stripped of their organic phosphate (especially 2,3-diphosphoglycerate). Such stripped Hb molecules have, as Benesch, Benesch & Yu (1969, and earlier references given therein) have brilliantly shown, an unnaturally high affinity for O_2: according to present views 2,3-diphosphoglycerate combines at or close to the α-amino groups of the β chains of the haemoglobin and may thus lower the reactivity of that part of the molecule towards CO_2. In favour of such an effect as regards CO_2 is the recent finding by Bauer (1969) that the antagonistic effect of CO_2 on the Bohr effect in dialysed solutions, which has been attributed (see above) to the carbamate combination of CO_2, is itself countered by adding 2,3-diphosphoglycerate. Unfortunately, however, all the experiments seem to have been done in fairly concentrated phosphate buffer solutions (0.15M), which may—and according to unpublished evidence do—affect the reactions of Hb with CO_2. Direct comparisons are accordingly needed of the carbamate affinity of O_2Hb and Hb for CO_2, in the presence and in the absence of 2,3-diphosphoglycerate. Nevertheless it will be useful now to discuss the existing evidence as to the carbamate reactivity of haemoglobin in its natural habitat, the erythrocyte.

Kinetic studies of the carbamate reactions inside the intact erythrocyte

Early manometric experiments of the Henriques type on the rate of CO_2 uptake by erythrocyte suspensions, in which the carbonic anhydrase was inhibited by sulphanilimide or its derivatives, gave diphasic uptake effects like those seen in Hb solutions. The size of the rapid phase was greater if the cells were deoxygenated, thus showing that haemoglobin in the erythrocyte does retain a significant amount of O_2-linked carbamate reactivity. This finding has been confirmed by recent experiments by Constantine, Craw & Forster (1965), in which the rate of CO_2 uptake was measured in a Hartridge–Roughton rapid-reaction apparatus and the change in dissolved CO_2 recorded by CO_2 electrodes sited at various distances from the mixing chamber (i.e. at various lapsed times from the start of the reaction).

Perhaps the most convincing quantitative evidence has been that by Kernohan & Roughton (1968), who measured by rapid thermal methods the rates of CO_2 uptake at 25°C at physiological pH by (*a*) suspensions of oxygenated and deoxygenated bovine erythrocyte suspensions, with and without added Diamox; (*b*) haemolysed solutions of the same oxygenated and deoxygenated blood, also with and without Diamox (to inhibit the carbonic anhydrase); the Hb in these solutions was not further purified and may still have been combined with 2,3-diphosphoglycerate. The observed rates for the carbamate reaction were the same to within 20%, whether the Hb was in solution or in the erythrocyte. Furthermore, the rates of the reactions were, at constant pH and PCO_2, twice as high for the deoxygenated as for the oxygenated Hb (whether in solution or in the erythrocytes), thus confirming that the Hb in the erythrocyte maintains its physiologically important O_2-linked character as regards its carbamate reactions. Incidentally, though less germane to the present purpose, it was noted that the carbonic anhydrase activity of the intact erythrocytes also tallied to within 20% with that of the haemolysed solutions.

Effect of oxygenation on the amount of carbon dioxide bound to haemoglobin at equilibrium in the erythrocytes

Ferguson (1936) found that the decrease in carbamate when dialysed Hb solutions at 37°C are oxygenated at pH 7.2–7.4 and PCO_2 40 Torr is directly proportional to the percentage of O_2Hb. His carbamate estimations showed that $\Delta[HbCO_2]/\Delta[O_2Hb]$ for human haemoglobin is −0.25, but the pH of his Hb solutions was about 0.05 unit more alkaline than that of his O_2Hb solutions. Approximate correction for this change of pH alters his value of $\Delta[HbCO_2]/\Delta[O_2Hb]$ to −0.21 at constant PCO_2 (40 Torr) and constant pH (7.3).

No direct estimations are available as to the value of $\Delta[HbCO_2]/\Delta[O_2Hb]$, for the Hb in the interior of the erythrocyte, but indirect calculations can be made from the experimental data on the effect of PCO_2 on the O_2 equilibrium curve of blood at constant pH (Naeraa *et al.* 1966).

According to eqn. (11) of Rossi-Bernardi & Roughton (1967):

$$\left(\frac{\partial(a\text{-}CO_2)}{\partial(\ln PO_2)}\right)_{\mathrm{pH},PCO_2} = \left(\frac{\partial(a\text{-}O_2)}{\partial(\ln PCO_2)}\right)_{\mathrm{pH},PO_2} \quad (4)$$

where (a-CO_2) is the total bound CO_2 and (a-O_2) the total bound O_2, i.e. [O_2Hb]. Now, if pH and PCO_2

are both constant the bicarbonate concentration must also be constant and equation simplifies to:

$$\left(\frac{\Delta[HbCO_2]}{\Delta(\ln PO_2)}\right)_{pH,PCO_2} = \left(\frac{\Delta[O_2Hb]}{\Delta(\ln PCO_2)}\right)_{pH,PO_2} \quad (5)$$

where the Δ symbols refer to small finite changes in the corresponding quantities.

The following results were found by Naeraa *et al.* (1966) for the effect of PCO_2 on the O_2–Hb equilibrium of human blood at 37°C at a constant PO_2 32 Torr and a constant plasma pH 7.4 (the corresponding pH of the interior of the erythrocyte would be approx. 7.2):

PCO_2 (Torr)	10.6	21.4	39.6	83.9
O_2Hb (%)	66.4	65.3	62.7	57.1

A graphic plot of these data shows that there is, quite closely, a straight-line relation between PCO_2 and $[O_2Hb]$. This empirical finding eases the application of eqn. (5) considerably.

Preliminary calculations, which it is hoped to give in detail in a later paper, suggest a value for $\Delta[HbCO_2]/\Delta[O_2Hb]$ in the erythrocytes, at PCO_2 40 and pH 7.4, of only about half that obtained by Ferguson (1936) for dialysed human Hb solutions. Further work on whole blood is thus very much needed and is at present being planned: if this confirms these preliminary indications it would entail a significant downward revision of the role hitherto assigned to the carbamate mechanism in the physiological transport of CO_2.

REFERENCES

Adair, G. S. (1923). *J. Physiol., Lond.*, **58**, ix.

Bauer, C. (1969). *Life Sci.* **8**, 1041.

Benesch, R. E., Benesch, R. & Yu, C. G. (1969). *Biochemistry, Easton*, **8**, 2567.

Bohr, C., Hasselbalch, K. A. & Krogh, A. S. (1904). *Skand. Arch. Physiol.* **16**, 402.

Booth, V. H. & Roughton, F. J. W. (1938). *Biochem. J.* **32**, 2049.

Brinkman, R. & Margaria, R. (1931). *J. Physiol., Lond.*, **72**, 26*P*.

Chipperfield, J. R. (1966). *Proc. R. Soc.* B, **164**, 401.

Christiansen, J., Douglas, C. G. & Haldane, J. S. (1914). *J. Physiol., Lond.*, **48**, 244.

Constantine, H. P., Craw, M. R. & Forster, R. E. (1965). *Am. J. Physiol.* **208**, 801.

Faurholt, C. (1925). *J. Chim. phys.* **22**, 1.

Ferguson, J. K. W. (1936). *J. Physiol., Lond.*, **88**, 40.

Ferguson, J. K. W. & Roughton, F. J. W. (1934). *J. Physiol., Lond.*, **83**, 87.

Forbes, W. H. (1931). Ph.D. Dissertation: University of Cambridge.

Forster, R. E., Constantine, H. P., Craw, M. R., Rotman, H. H. & Klocke, R. A. (1968). *J. biol. Chem.* **243**, 317.

Haldane, J. S. (1922). *Respiration.* New Haven: Yale University Press.

Hawkins, J. A. & Van Slyke, D. D. (1930). *J. biol. Chem.* **87**, 265.

Henriques, O. M. (1928). *Biochem. Z.* **200**, 1.

Hermann, H., Hudoffsky, B., Netter, H. & Travia, L. (1939). *Pflügers Arch. ges. Physiol.* **242**, 311.

Holmgren, F. (1863). *Sber. Akad. Wiss. Wien Abt. II*, **48**, 614.

Jensen, M. B. (1970). In *Carbon Dioxide: Chemical, Biochemical and Physiological Aspects*, p. 33. Washington: U.S. Government Printing Office (NASA no. SP-188).

Keilin, D. & Mann, T. (1940). *Biochem. J.* **34**, 1163.

Kernohan, J. C., Kreuzer, F., Rossi-Bernardi, L. & Roughton, F. J. W. (1966). *Biochem. J.* **100**, 49P.

Kernohan, J. C. & Roughton, F. J. W. (1968). *J. Physiol., Lond.*, **197**, 345.

Kilmartin, J. V. (1969). Ph.D. Dissertation: University of Cambridge.

Kilmartin, J. V. & Rossi-Bernardi, L. (1969). *Nature, Lond.*, **222**, 1243.

Magnus, H. G. (1837). *Ann. Phys. Chem.* **40**, 583.

Margaria, R. (1957). *Clin. Chem.* **3**, 306.

Margaria, R. & Green, A. A. (1933). *J. biol. Chem.* **73**, 311.

Meldrum, N. U. & Roughton, F. J. W. (1932*a*). *J. Physiol., Lond.*, **75**, 3*P*.

Meldrum, N. U. & Roughton, F. J. W. (1932*b*). *J. Physiol., Lond.*, **75**, 15*P*.

Meldrum, N. U. & Roughton, F. J. W. (1933). *J. Physiol., Lond.*, **80**, 143.

Naeraa, N., Petersen, E. S. & Boye, E. (1963). *Scand. J. clin. Lab. Invest.* **15**, 141.

Naeraa, N., Petersen, E. S., Boye, E. & Severinghaus, J. W. (1966). *Scand. J. clin. Lab. Invest.* **18**, 96.

Perkins, J. F. (1964). In *Handbook of Physiology, Section 3: Respiration*, vol. 1, chapter 1. Ed. by Fenn, W. O. & Rahn, H. Washington: American Physiological Society.

Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J. & Kilmartin, J. V. (1969). *Nature, Lond.*, **222**, 1240.

Rossi, L. & Roughton, F. J. W. (1962). *J. Physiol., Lond.*, **162**, 17*P*.

Rossi-Bernardi, L., Pace, M., Roughton, F. J. W. & Van Kempen, L. (1970). In *Carbon Dioxide: Chemical, Biochemical and Physiological Aspects*, p. 61. Washington: U.S. Government Printing Office (NASA no. SP-188).

Rossi-Bernardi, L. & Roughton, F. J. W. (1967). *J. Physiol., Lond.*, **189**, 1.

Roughton, F. J. W. (1964). In *Handbook of Physiology, Section 3: Respiration*, vol. 1, p. 767. Ed. by Fenn, W. O. & Rahn, H. Washington: American Physiological Society.

Roughton, F. J. W. & Rossi-Bernardi, L. (1966). *Proc. R. Soc.* B, **164**, 381.

Roughton, F. J. W. & Rossi-Bernardi, L. (1970). *In Carbon Dioxide: Chemical, Biochemical and Physiological Aspects*, p. 37. Washington: U. S. Government Printing Office (NASA no. SP-188).

Stadie, W. C. & O'Brien, H. (1933). *J. biol. Chem.* **103**, 521.

Stadie, W. C. & O'Brien, H. (1936). *J. biol. Chem.* **112**, 723.

Stadie, W. C. & O'Brien, H. (1937). *J. biol. Chem.* **117**, 439.