Biochem. J. (1971) **124**, 31–45
Printed in Great Britain

The Binding of Carbon Dioxide by Horse Haemoglobin

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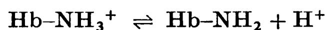
(*Received* 19 *March* 1971)

1. Three modified horse haemoglobins have been prepared: (i) $\alpha_2^c\beta_2^c$, in which both the α-amino groups of the α- and β-chains have reacted with cyanate, (ii) $\alpha_2^c\beta_2$, in which the α-amino groups of the α-chains have reacted with cyanate, and (iii) $\alpha_2\beta_2^c$, in which the two α-amino groups of the β-chain have reacted with cyanate. 2. The values of n (the Hill constant) for $\alpha_2^c\beta_2^c$, $\alpha_2\beta_2^c$ and $\alpha_2^c\beta_2$ were (respectively) 2.5, 2.0 and 2.6, indicating the presence of co-operative interactions between the haem groups for all derivatives. 3. In the alkaline pH range (about pH 8.0) all the derivatives show the same charge as normal haemoglobin whereas in the acid pH range (about pH6.0) $\alpha_2^c\beta_2^c$ differs by four protonic charges and $\alpha_2^c\beta_2$, $\alpha_2\beta_2^c$ by two protonic charges from normal haemoglobin, indicating that the expected number of ionizing groups have been removed. 4. $\alpha_2^c\beta_2$ and $\alpha_2^c\beta_2^c$ show a 25% decrease in the alkaline Bohr effect, in contrast with $\alpha_2\beta_2^c$, which has the same Bohr effect as normal haemoglobin. 5. The deoxy form of $\alpha_2^c\beta_2^c$ does not bind more CO_2 than the oxy form of $\alpha_2^c\beta_2^c$, whereas $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$ show intermediate binding. 6. The results reported confirm the hypothesis that, under physiological conditions, haemoglobin binds CO_2 through the four terminal α-amino groups and that the two terminal α-amino groups of α-chains are involved in the Bohr effect.

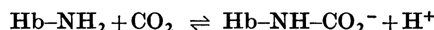
The direct combination between CO_2 and haemoglobin accounts for a significant fraction of the total CO_2 exchanged by the blood during respiration (Roughton, 1964). This combination is 'oxygen-linked', that is, at constant pCO$_2$ and pH, less CO_2 is bound to oxy- than to deoxy-haemoglobin. The mode of this combination is probably a direct reaction of CO_2 with an amino group to form a carbamino compound (–NH–CO_2H) rather than electrostatic binding of HCO_3^- (Kernohan, Kreuzer, Rossi-Bernardi & Roughton, 1966; Forster, Constantine, Craw, Rotman & Klocke, 1968). Since CO_2 will only react with uncharged amino groups (–NH_2) the most likely –NH_2 groups in the protein to combine with CO_2 would be the α-amino groups of the α- and β-chains; these would have a pK sufficiently low [in the range 7–8 (Tanford, 1961)] to be mainly uncharged at physiological pH. The α-amino groups of lysine residues would have pK values of about 10 (Tanford, 1961) and thus would be fully charged and unable to combine with CO_2 at physiological pH.

Thus the reaction of CO_2 with α-amino groups will depend on their pK:



$$\text{Hb–NH}_3^+ \rightleftharpoons \text{Hb–NH}_2 + \text{H}^+$$

and on the equilibrium constant (K_c) of the reaction:



$$\text{Hb–NH}_2 + \text{CO}_2 \rightleftharpoons \text{Hb–NH–CO}_2^- + \text{H}^+$$

where Hb represents haemoglobin. The pK of the carboxyl group of Hb–CO_2H would be about 6.0, as deduced from studies on model compounds (Roughton, 1943; Roughton & Rossi-Bernardi, 1970); thus it would be almost completely dissociated in the physiological pH range of 7.0–7.4.

Rossi-Bernardi & Roughton (1967*b*) calculated values of the pK of the CO_2 combining groups from the limited data obtained by other workers on carbamino CO_2 binding by haemoglobin (Ferguson & Roughton, 1934; Ferguson, 1936; Stadie & O'Brien, 1937). They obtained pK values between 7.2 and 7.7, well within the range of pK values for α-amino groups.

Chemical modification of the α-amino groups would provide another approach to the problem of identifying the CO_2 combining groups in haemoglobin, and also reveal the extent to which these groups are involved in the Bohr effect (Rossi-Bernardi & Roughton, 1967*a*).

To prepare a chemically modified haemoglobin suitable for this purpose, it is necessary to fulfil the

following conditions. (i) Total modification of the α-amino group and no other group. Thus an unambiguous interpretation of the result is possible. (ii) The functional properties of the modified protein not related to CO_2 binding, such as the sigmoid oxygen dissociation curve, should be unaltered. (iii) The structure of the modified protein must be as similar to the original protein as possible.

The only experimental technique which has so far supplied detailed information of the molecular structure of proteins is X-ray crystallography, and since horse haemoglobin has been studied in detail by Perutz and his collaborators, we decided to attempt to chemically modify the α-amino groups of horse haemoglobin.

A detailed investigation of the crystallographic structure of a chemically modified protein is necessary because if the particular effect is not present, then the possibility must be excluded that the chemical modification has caused a small structural change which has masked some other group on the protein really responsible for the original effect.

This work was presented at the 'Symposium on CO_2' held at Haverford, Pennsylvania, in August 1968 (Kilmartin & Rossi-Bernardi, 1970) and a part of it has been published in preliminary form (Kilmartin & Rossi-Bernardi, 1969).

MATERIALS

Reagents. Potassium [^{14}C]cyanate (15.8mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. It was diluted to an approximate specific radioactivity of 0.079mCi/mmol with potassium cyanate (recrystallized from a mixture of equal volumes of ethanol and water). The exact specific radioactivity was measured by the method of Rimon & Perlmann (1968). Iodo[1-^{14}C]-acetamide (26.8mCi/mmol) was also obtained from The Radiochemical Centre and was diluted to an approximate specific radioactivity of 0.27mCi/mmol with iodoacetamide (recrystallized from hot water). Its specific radioactivity was measured after reaction with glutathione (Benesch & Benesch, 1957), and isolation of the carboxymethylated glutathione by paper chromatography.

[U-^{35}S]Cystamine was obtained from The Radiochemical Centre, its specific radioactivity was not measured accurately.

Cystamine dihydrochloride and *p*-hydroxymercuribenzoate (sodium salt) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Dithiothreitol was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Resins. Amberlite CG-50 (200–400 mesh), Amberlite IR-120 (analytical grade) and Amberlite IRA-400 (analytical grade) were obtained from BDH (Chemicals) Ltd., Poole, Dorset, U.K. Dowex AG 50W-X2 (200–400mesh) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Sephadex was obtained from Pharmacia, Uppsala, Sweden. CM-cellulose (Whatman CM-52) was obtained from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Glass beads (Ballotini no. 11) were obtained from Jencons, Hemel Hempstead, Herts., U.K.

Enzymes. Trypsin (twice recrystallized) was obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. Papain was obtained from Sigma Chemical Co. Leucine aminopeptidase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Prolidase was purified to Step 2 as described by Davis & Smith (1957).

Gases. Nitrogen (>99.999%), carbon monoxide (>99.5%), oxygen (>99.95%) and carbon dioxide (>99.95%) were obtained from S10, Milan, Italy.

Horse blood. Horse blood was obtained through the kindness of the Equine Research Station, Newmarket, Suffolk, U.K. Blood from the same animal was used throughout this work; it contained the normal fast and slow components of horse haemoglobin (Bangham & Lehmann, 1958) but had only phenylalanine at position 24 of the α-chains of both the fast and slow components (Kilmartin & Clegg, 1967).

Scintillation chemicals. The 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen (BBOT) was obtained from CIBA Ltd., Basle, Switzerland, and Thicxin was obtained from Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, U.K.

METHODS

All manipulations of haemoglobin were carried out at 4°C unless otherwise stated.

Preparation of horse haemoglobin. Horse blood was collected in a flask containing 1.7g of sodium oxalate for each litre of blood. The cells were washed four times with 4vol. of 0.9% (w/v) NaCl. The cells were lysed after addition of an equal volume of deionized water, left for 15min, and then NaCl was added to give a final concentration of 3% (w/v). The solution was then immediately spun for 1h in the GSA rotor of the Sorvall centrifuge at 14600*g*. After careful decantation the supernatant was spun again for 30min. A 500ml sample of this haemoglobin solution was then passed through a column ($50cm^2 \times 40cm$) of Sephadex G-25 (fine) equilibrated with 25mM-Na_2HPO_4. The solution was saturated with CO and used within a month.

The carbonmonoxyhaemoglobin concentration was measured in CO-saturated 0.05M-sodium phosphate buffer, pH6.5, after the addition of a few crumbs of sodium dithionite to reduce any methaemoglobin to carbonmonoxyhaemoglobin. The values of the extinction of this solution at 540 and 569nm (which should be equal) were divided by 8.03 to give the concentration of carbonmonoxyhaemoglobin in g/100ml (Perutz, 1968).

Preparation of the fast component. The fast component of horse haemoglobin was purified by a modification of the method of Clegg & Schroeder (1959). Amberlite CG-50 (200–400 mesh) was treated as described by Hirs, Moore & Stein (1953). After the final water wash, the wet resin was passed through a 200-mesh sieve and that retained by a 300-mesh sieve was collected. The resin was equilibrated with sodium phosphate buffer (2.52g of Na_2HPO_4/l and 2.262g of $NaH_2PO_4,2H_2O$/l, pH7.03 at 25°C) at 4°C. A 20g sample of horse haemolysate was passed through a column ($50cm^2 \times 40cm$) of Sephadex G-25 (fine) equilibrated with the Amberlite column buffer; after saturation with CO the solution (400ml) was loaded on to a column ($38cm^2 \times 50cm$) of Amberlite CG-50 as described by Clegg & Schroeder (1959). If there was a small amount

of precipitate in the haemoglobin solution then a layer of glass beads was placed on top of the resin before loading the haemoglobin solution on to the column. The sodium phosphate buffer to be used for eluting the haemoglobin off the Amberlite column was saturated with CO by using the system in Fig. 1. The bottle containing the buffer had an inlet connected to a scooter inner tube, so that when buffer was removed from the bottle it would be replaced by CO from the inner tube, hence atmospheric pressure would be maintained inside the bottle as the buffer was removed. The buffer was saturated with CO by first shaking in a vacuum to remove dissolved air and then in an atmosphere of CO. After the haemoglobin had been loaded on to the Amberlite column, all the air between the top of the resin and the rubber bung was displaced by the CO-saturated buffer and elution was begun at 10ml/min. When the main band containing the fast component had reached the bottom, this part of the column was immersed in water at 25°C for 3h. The fast component was then eluted by restarting the flow at room temperature into a cooled flask. The slow component can be removed from the resin by stirring it in the sodium phosphate buffer heated to 50°C. The solution of the fast component was adjusted to pH8.5 by addition of a solution containing 5M-KOH and 4M-KH_2PO_4. It was concentrated by addition of dry Sephadex G-25 (coarse) to give a moist paste, which was placed on a sintered-glass-disc funnel, then addition of phosphate buffer at pH8.5 eluted the haemoglobin. Each of these Sephadex treatments decreased the volume by about half. The pH was made alkaline to prevent precipitation of the haemoglobin in the Sephadex at high concentrations. When the volume of haemoglobin solution was down to about 100ml [with a concentration of about 10% (w/v)], it was resaturated with CO and stored in a bottle under slightly positive (10cmHg) CO pressure. Such solutions of fast component were stable (in the sense of not oxidizing to methaemoglobin) for several months. The Sephadex was regenerated by washing with water and drying in a rotary evaporator. If the recommended method was used, i.e. drying with ethanol, then considerable quantities of ethanol remained in the Sephadex; when this Sephadex was used to concentrate haemoglobin then the adsorbed ethanol was eluted with the haemoglobin.

Measurement of radioactivity. The counting efficiency for the first three methods described below was measured by using horse haemoglobin that had reacted with iodo[1-^{14}C]acetamide (Guidotti & Konigsberg, 1964). Method (i) was used for measuring the specific radioactivity of $K^{14}CNO$ and for counting the radioactivity of valine hydantoin.

(i) Discs of Whatman no. 1 filter paper (2.5cm diam.) were stuck on to 3cm-diam. aluminium planchets. A 0.05ml sample of 2.5% (w/v) haemoglobin [salt-free, prepared by passing haemoglobin through a column of Sephadex G-25 (fine) equilibrated with 10μM-NaOH] was pipetted on to these discs and left for 1h at room temperature before drying in an oven.

(ii) Salt-free haemoglobin (2.5%, w/v) (0.025ml) was precipitated by addition of 5% (w/v) trichloroacetic acid. This was collected on a Millipore filter, dried and stuck to an aluminium planchet.

(iii) Salt-free haemoglobin solution (2.5%, w/v) (0.1ml) was pipetted into a 20ml scintillation vial and freeze-dried. A scintillation mix of 1g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen, 5g of Thicxin and 125ml of toluene was mixed in a Waring Blendor and 10ml was pipetted into the vial. The vial was shaken vigorously to disperse the haemoglobin in the gel. Longer periods of agitation did not change the counting efficiency. In contrast with the results of Schwartz & Nathan (1967) there was heavy quenching with increasing amounts of haemoglobin. There was no difference in counting efficiency between carbonmonoxy- and met-haemoglobin.

(iv) The radioactivity of the effluent from the CM-cellulose (CM-52) columns run in 8M-urea was counted by pipetting 0.2ml samples into 2in×0.5in glass tubes. A 4ml portion of a scintillation mix containing 1500ml of toluene, 1000ml of methylCellosolve, 20g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen and 6.25ml of 10M-NaOH was added to the 0.2ml of column effluent. The NaOH should be added to the scintillation mix immediately before use. After vigorous shaking, this vial was placed inside a 20ml scintillation vial and the radioactivity counted. The counting system showed a linear response for up to 1mg of radioactive α- and β-chains/vial.

Separation of the α- and β-chains. The method of Clegg, Naughton & Weatherall (1966) was used with several modifications. The 0.05M-mercaptoethanol was replaced by 50mg of dithiothreitol/l. This abolished the large changes in E_{280} (probably due to oxidation of the mercaptoethanol) which occurred after the fractions had been left for several hours at room temperature. The globin was prepared by the method of Hill, Konigsberg, Guidotti &

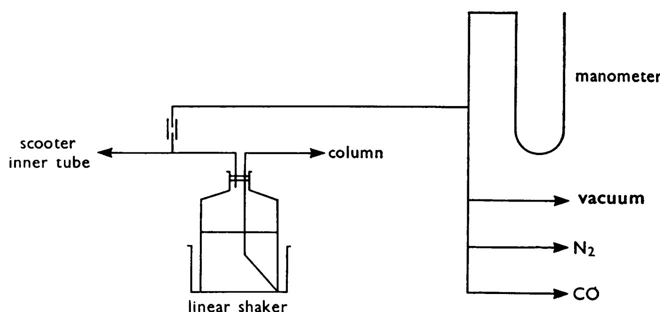


Fig. 1. Apparatus for deoxygenation of buffers and haemoglobin and their saturation with CO.

Craig (1962) since the yield of globin prepared from small amounts of haemoglobin was much higher than with the acid–acetone method used by Clegg *et al.* (1966). The compositions of the three buffers used were (g/l): buffer 1, Na_2HPO_4 (0.129), $NaH_2PO_4,2H_2O$ (1.279); buffer 2, Na_2HPO_4 (0.236), $NaH_2PO_4,2H_2O$ (1.022); buffer 3, Na_2HPO_4 (0.328), $NaH_2PO_4,2H_2O$ (0.840). Each of these buffers contained 8M-urea and 50mg of dithiothreitol/l.

After removal of the haem, the sample of globin (20–100mg) was passed through a column ($3cm^2 \times 15cm$) of Sephadex G-25 (fine) equilibrated with buffer 1, after which it was loaded on to a column ($0.8cm^2 \times 10cm$) of CM-cellulose (CM-52) also equilibrated with buffer 1. The acidic pH of buffer 1 ensured that the protein stuck firmly to the column. If a more alkaline buffer was used then there was often a splitting of the most acidic component; part of it came straight through the column and part remained to be eluted by the gradient. If the sample was thought to contain an acidic β-chain, then, after it had been loaded in buffer 1, the column was re-equilibrated with buffer 2, then a linear gradient of 150ml of buffer 2 (which was 0.01M with respect to Na^+) and 150ml of buffer 2 (0.05M with respect to Na^+, i.e. five times as concentrated). If the sample did not contain an acidic β-chain, then after re-equilibration with buffer 3 the gradient was 150ml of buffer 3 (which was 0.01M with respect to Na^+) and 150ml of buffer 3 (0.05M with respect to Na^+). These narrow-range gradients could easily separate a chain modified at its α-amino group from the modified chain.

The pooled fractions containing the peaks were aminoethylated as described by Raftery & Cole (1966) for the aminoethyl-insulin β-chain. This method of aminoethylation was used in preference to that of Clegg *et al.* (1966) since it prevented aggregation of the horse α-chains during tryptic digestion. After aminoethylation, the solution was adjusted to pH5.0, passed through a column ($12cm^2 \times 40cm$) of Sephadex G-25 (fine) equilibrated with 0.5% (v/v) formic acid, and freeze-dried.

Tryptic digestion and 'fingerprinting'. The aminoethylated α- or β-chain (10mg/ml) in 1% (w/v) $(NH_4)_2CO_3$ was digested for 3h at 37°C by trypsin in an enzyme/substrate ratio of 1:50. The digest was freeze-dried three times to remove the $(NH_4)_2CO_3$ and applied to Whatman no. 3 chromatography paper (1mg of digest/cm) and electrophoresis was carried out in pH6.5 buffer [pyridine–acetic acid–water (25:2:223, by vol.)] for 50min at 3kV in the apparatus described by Ryle, Sanger, Smith & Kitai (1955). The strip containing the separated peptides was stitched on to a second sheet of Whatman no. 3 paper and chromatographed overnight in solvent BAWP [butanol–acetic acid–pyridine–water (15:3:10:12, by vol.)]. Occasionally peptides were cut out from the run in solvent BAWP and separated by electrophoresis for 50min at 3kV in pH3.5 buffer [pyridine–acetic acid–water (1:10:189, by vol.). Radioautographs were obtained by exposing Kodak Blue Brand film to the 'fingerprint' for 2–3 days.

Elution of peptides and amino acid analysis. After staining guide strips with 0.25% (w/v) ninhydrin in acetone, peptides were eluted with 0.1M-NH_3; or ninhydrin spots (located by staining with 0.025% ninhydrin) were eluted with 6M-HCl directly into capillary glass tubes (Sanger & Tuppy, 1951). After elution with 0.1M-NH_3 the peptide was dried under vacuum, 0.5ml of 6M-HCl added and the tube was evacuated and sealed. Hydrolysis was allowed to continue for 18–72h at 105°C and the products were dried under vacuum with NaOH pellets. Amino acid analysis of the hydrolysed peptide was carried out with a Beckman 120B amino acid analyser (Spackman, Stein & Moore, 1958).

Removal of carbon monoxide. Chromatographically purified oxyhaemoglobin oxidizes to methaemoglobin much faster than oxyhaemoglobin in a haemolysate. This is probably because the reducing systems present in the haemolysate (Hegesh & Avron, 1967) are removed during the chromatographic purification. All the chromatographically purified haemoglobins described in this work were prepared and stored in an atmosphere of CO. When functional studies on a particular haemoglobin involving removal of ligand were required then the CO was removed immediately beforehand. This was easily accomplished by replacing with oxygen. The sample of carbonmonoxyhaemoglobin, concentration less than 5% (w/v) in phosphate buffer, pH8.5, was placed in a tonometer (a strong glass bottle with parallel sides) at least 200 times the volume of the sample. The tonometer was flushed by evacuation and filled with oxygen several times, finally being filled with oxygen at a pressure of 1200mmHg. The tonometer was then rotated horizontally at 100rev./min at 4°C under strong light (200W bulb), with a fan to prevent the sides of the bottle being heated by the light. After 5min a sample of haemoglobin was removed for the determination of carbonmonoxyhaemoglobin by the van Slyke procedure (Peters & van Slyke, 1932), the tonometer was flushed, refilled with oxygen and rotated for two more 5min periods. The results of the carbonmonoxyhaemoglobin analyses (Fig. 2) show that the CO exchanges rapidly with oxygen. For complete removal of CO, the

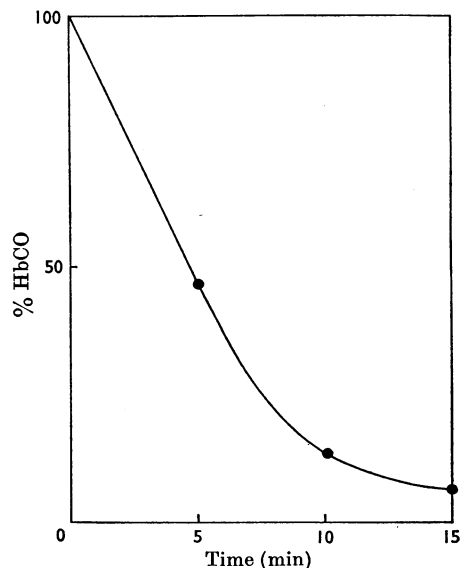


Fig. 2. Removal of CO from carbonmonoxyhaemoglobin (HbCO) after rotation in an atmosphere of oxygen.

sample was rotated and flushed alternately for periods of 3, 3, 4, 7 and 8 min. At the end of this procedure the residual carbonmonoxyhaemoglobin was less than 5%. Thus by this method all the carbon monoxide can be removed from several grams of carbonmonoxyhaemoglobin in less than 30 min. No oxidation of the reactive thiol group (cysteine-93β) could be detected after titration with p-mercuribenzoate (Benesch & Benesch, 1962). The methaemoglobin concentration was less than 5%. This was measured by recording the extinction (E) at 540, 560 and 580 nm of a solution of the haemoglobin in CO-saturated buffer. Addition of a few crumbs of dithionite converts this completely into carbonmonoxyhaemoglobin (HbCO) and extinction values are read again. The extinction of the same concentration of methaemoglobin (met-Hb) in the same buffer was calculated for these wavelengths, then:

$$\%\ \text{methaemoglobin} = \frac{\sum E\,(100\%\text{HbCO} - \text{mixture CO and met-Hb})}{\sum E\,(100\%\ \text{HbCO} - \text{met-Hb})}$$

The oxygen was removed from the haemoglobin by carrying out the same flushing and rotation procedure in the presence of nitrogen. Three 10 min rotations were usually enough to remove all the oxygen.

Titration curves. Titration curves of the oxy- and deoxyhaemoglobins were used to compare the total charge the derivatives carried at various pH values and also to measure the Bohr effect. The CO was removed from the haemoglobin by the methods described above. The oxyhaemoglobin was then passed through a Dintzis deionizing column (Nozaki & Tanford, 1967). This consists of a top layer of a mixture of Amberlite IR-120 (NH_4^+ form) and Amberlite IRA-400 (acetate) and a bottom layer of a mixture of Amberlite IR-120 (H^+ form) and Amberlite IRA-400 (OH^- form). A conductivity meter, previously calibrated with solutions of KCl (Robinson & Stokes, 1959), was used to measure the conductivity of the effluent. If this was less than 7×10^{-6} mho/cm at 4°C (a value in agreement with that of Wallach, Gail & Janeway, 1966), 2 M-KCl was added to give a final concentration of 0.2 M and the solution was deoxygenated. If the conductivity was any greater than the above value the solution was again passed through the deionizing column, otherwise an anomalously high pH was recorded at the isoionic point. Deoxyhaemoglobin was transferred from the tonometer under pressure of nitrogen directly into a 5 ml burette. In this way, only the haemoglobin at the very top of the burette became oxygenated. An accurately measured sample of this solution was transferred to a glass vessel, through which nitrogen (saturated with water at 25°C) was flushed to provide a barrier to oxygen and CO_2. An appropriate amount of deoxygenated 10 mM-KOH or 10 mM-HCl in 0.19 M-KCl was added, together with enough deoxygenated 0.2 M-KCl to keep the concentration of haemoglobin and the ionic strength the same for each point on the titration curve. The pH of the deoxyhaemoglobin solution was measured by using the system described by Rossi-Bernardi & Roughton (1967*a*); the pH of the haemoglobin in the oxy form was measured by passing O_2 instead of N_2 over the solution.

The accurate measurement of haemoglobin concentrations was very important for comparison of the titration curves of different haemoglobin derivatives. The methaemoglobin cyanide method was used as described by Van Kampen & Zijlstra (1961). Oxyhaemoglobin was added to Drabkin's reagent [200 mg of $K_3Fe(CN)_6$, 50 mg of KCN and 140 mg of KH_2PO_4/l], and after 30 min the extinction at 540 nm was read and the methaemoglobin cyanide concentration obtained by using $E_{1\,\text{M}}^{1\,\text{cm}}$ 11000. The conversion of oxyhaemoglobin into methaemoglobin cyanide is pH-dependent; thus the slightly acid pH of the Drabkin's reagent used here ensured a rapid conversion. It was essential to use oxyhaemoglobin since carbonmonoxyhaemoglobin was converted into methaemoglobin cyanide very slowly in this system.

Oxygen dissociation curves. The method of Riggs (1951) was used with several modifications. A 1 cm cuvette was welded via a glass stalk on to the bottom of a 250 ml tonometer. The top of the tonometer contained an inlet, for evacuation and flushing with gas, and a Quickfit plastic screw cap (ST 52/18) with a silicone pad for injection of air. The major error in this procedure was found to be the position of the cuvette in the spectrophotometer, in agreement with the results of Keyes, Mizukami & Lumry (1967). This error may be detected by gently rocking the tonometer (covered by a black cloth) while it is in the light-beam of the spectrophotometer. There should be no changes in the extinction.

The tonometer containing 5 ml of oxyhaemoglobin (0.05%, 0.2 M-KCl, 0.015 M-potassium phosphate buffer, pH 7.25 or 7.4) was evacuated and flushed with N_2 several times, finally being filled with a slight positive pressure of N_2 to keep air out. The solution was gently rocked for 3–4 min in a linear shaker. This procedure was repeated several times until the colour of the haemoglobin was purple, and its spectrum was then checked to ensure that the solution was deoxygenated. Air was added by injection through the silicone cap with a Hamilton gas-tight syringe. The solution in the tonometer was equilibrated for 10 min by gentle rocking inside a glass water jacket maintained at 25°C. The E_{558} was then determined. Usually five points in the range 30–80% saturation of haemoglobin were measured, sufficient to give a value of the oxygen affinity at 50% saturation (p_{50}), and the Hill constant n, which was calculated from the slope of the line obtained when $\log\left(\frac{y}{100-y}\right)$ is plotted against $\log pO_2$, where y is the percentage saturation and pO_2 the partial pressure of oxygen. The methaemoglobin at the end of this procedure was always less than 10% of the total haemoglobin. When the curves were determined in the presence of CO_2, then 46 mmHg of CO_2 was added by observing the change in pressure on a mercury manometer as the gas was added. The buffer was now 0.05 M-$KHCO_3$–0.15 M-KCl–0.015 M-potassium phosphate, pH 7.4 at 25°C. The pH was measured at the end of the curve by using the positive pressure of gas in the tonometer to transfer the solution into a burette. Then the thin-polythene-tubing end of the microelectrode (Radiometer E.5021) was inserted into the end of the burette and the solution drawn anaerobically into the electrode. Thus at no time during

the pH measurement was the CO_2 in the solution exposed to the atmosphere.

Measurement of CO_2 *binding.* The indirect method of Rossi-Bernardi & Roughton (1967*b*) was used to measure CO_2 binding to haemoglobin. This simple method measures the total CO_2 by the gasometric van Slyke method when the partial pressure of CO_2 (pCO_2) is constant and the pH is varied within the range 7.0–7.5 by addition of appropriate amounts of HCO_3^-. The total CO_2 in a solution of haemoglobin consists of dissolved CO_2, HCO_3^-, CO_3^{2-} and CO_2 combined with haemoglobin as carbamino CO_2; thus the total CO_2 in deoxyhaemoglobin solutions will be greater than in oxyhaemoglobin solutions at the same pCO_2 and pH because although the dissolved CO_2, HCO_3^- and CO_3^{2-} are the same in each case there is more CO_2 combined with deoxyhaemoglobin. Thus the difference in total CO_2 between solutions of the same concentration of deoxy- and oxy-haemoglobin equilibrated with the same pCO_2 and at the same pH corresponds to the difference in carbamino CO_2 bound by deoxyhaemoglobin compared with oxyhaemoglobin.

Since the carbamino CO_2 combined with haemoglobin is only a small fraction of the total CO_2 in solution, it was essential to use highly concentrated haemoglobin solutions (at least 13%, w/v) to make this fraction as large as possible. Unfortunately, it was impossible to use the Sephadex method to prepare the haemoglobin because at very high concentrations it precipitated in the gel and could not be eluted. The modified vacuum dialysis (Fig. 3) system was used to concentrate the haemoglobin instead. After the CO had been removed (the concentration of haemoglobin had to be less than 5%, w/v, for this procedure) the oxyhaemoglobin was passed through a column of Sephadex G-25 (fine) equilibrated with 0.2M-KCl and then concentrated once with Sephadex to about 7% (w/v). The correct amount of acid to bring the pH of the solution to 7.0 was calculated from the titration curve and added to the haemoglobin solution. This lowered the oxygen affinity sufficiently to allow deoxygenation with N_2. The deoxygenated haemoglobin was then transferred to a burette whose tip was inserted inside the glass tube at the top while N_2 was passed through the apparatus (Fig. 3) via the three-way tap. The deoxyhaemoglobin solution (under positive pressure of N_2) was concentrated to 13% (w/v) within 3–4h. The concentrated deoxyhaemoglobin was removed by suction into a burette to which a long piece of glass tubing was attached to reach down into the dialysis bag. N_2 was again passed through the three-way tap during this process. The concentration process was carried out on deoxyhaemoglobin because it was more stable than oxyhaemoglobin. After concentration, 1.4ml of deoxyhaemoglobin was transferred anaerobically to a jacketed tonometer (250ml) maintained at 37°C, previously flushed with a gas mixture of CO_2+N_2 (6:94). An appropriate amount of 1M-$KHCO_3$ was added to give a final pH of about 7.4 after equilibration with the CO_2. The solution was equilibrated by gentle rocking for 15min with the gas mixture at atmospheric pressure. This was repeated three times, the gas mixture being changed each time. It was then transferred (methaemoglobin about 13%) by a burette, 0.5ml to the van Slyke apparatus for total CO_2 measurement, and the rest to a small chamber (Siggaard-Anderson, 1963) maintained at 37°C and flushed with humidified CO_2+N_2 (6:94) also at 37°C.

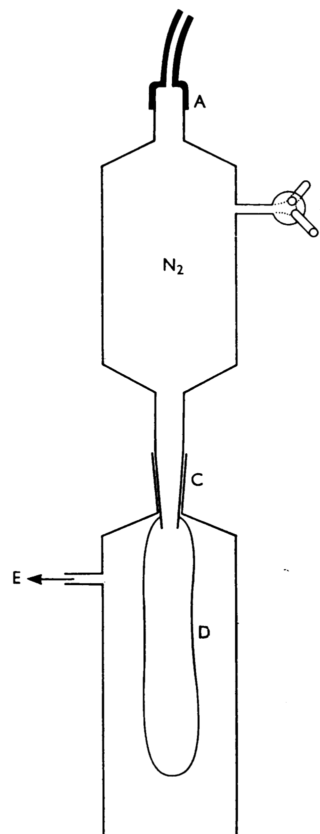


Fig. 3. Apparatus for concentrating deoxygenated haemoglobin by vacuum dialysis. The glass tube at the top (A) was sealed with plastic tubing and a clamp after the sample had been added. B is a three-way tap for evacuation and flushing with N_2, C a ground-glass joint, D the dialysis bag containing deoxyhaemoglobin and E an outlet for connexion to a vacuum.

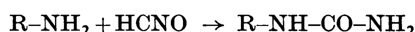
The pH of the solution was then measured with the microelectrode. Next, the total CO_2 in solutions of carbonmonoxyhaemoglobin was measured (Peters & van Slyke, 1932). Carbonmonoxyhaemoglobin was used instead of oxyhaemoglobin because it is more stable at the high temperature (37°C) used in these measurements. This temperature was used because there is less CO_2 in solution, making the difference in total CO_2 between oxy- and deoxy-haemoglobin a higher fraction of the total CO_2 and thus easier to measure accurately. After the deoxyhaemoglobin was put inside the tonometer, it was filled with CO to saturate it. Then the tonometer was filled with CO_2+N_2 (6:94) at atmospheric pressure and about 1cmHg pressure of CO was added to keep the haemoglobin in the carbonmonoxy form. After equilibration, the CO_2 content and pH were measured as before.

RESULTS

Preparation of the carbamoylated haemoglobins

Stark (1965*b*) and Smyth (1967) showed that

cyanate reacts with amino groups in the following way:



$$R\text{–}NH_2 + HCNO \rightarrow R\text{–}NH\text{–}CO\text{–}NH_2$$

thus the positive charge carried by the amino group at acid pH values is abolished, which makes it readily separable from unchanged protein by ion-exchange methods. The reaction of cyanate with carboxyl groups is not reversible (Stark, 1965*b*,*c*). However, the reaction with the phenolic hydroxyl group of tyrosine, the imidazole group of histidine and the thiol group of cysteine is reversible at alkaline pH (Stark, 1964, 1965*a*; Smyth, 1967).

Protection of the reactive thiol group (*cysteine* β93). During preliminary work on the reaction of cyanate with haemoglobin it was found that after alkaline treatment (pH8.5 at 4°C for 12h) a small proportion of the reactive thiol group was not available to titration with *p*-mercuribenzoate. Presumably not all the cyanate that had reacted with this group was readily removable. Thus it was decided to protect the reactive thiol group. Cystamine was used since it reacts readily with this group (Taylor, Antonini, Brunori & Wyman, 1966; Smithies, 1965) to form a disulphide bond which would be readily reversible with thiol reagents. The reversibility of this reaction was checked by using [^{35}S]cystamine. Carbonmonoxyhaemoglobin, 6% (w/v) in 0.05M-sodium phosphate buffer, pH8.5, was treated with 15mol of [^{35}S]cystamine dihydrochloride/mol of αβ-dimer for 1h at 4°C. The excess of cystamine was removed by passage through a column of Sephadex G-25 (fine), the radioactivity incorporated into the protein was 47770c.p.m./mg, and the reactive thiol group was completely blocked as shown by titration with *p*-mercuribenzoate. The blocked carbonmonoxyhaemoglobin (2%, w/v) was then treated with 30mol of dithiothreitol/mol of αβ-dimer in 0.05M-sodium phosphate buffer, pH8.5, for at least 12h at 4°C. After passage through another column of Sephadex G-25 (fine) to remove the dithiothreitol all of the reactive thiol group was available for titration with *p*-mercuribenzoate and the radioactivity left on the protein was 938c.p.m./mg. Thus about 2% of the cystamine that had reacted with the protein remained.

Reaction with cyanate. A 100ml sample of 8% (w/v) cystamine-treated horse carbonmonoxyhaemoglobin (fast component) was passed through a column ($50cm^2 \times 40cm$) of Sephadex G-25 (fine) equilibrated with 0.2M-sodium phosphate buffer, pH6.0 (21.2g of $NaH_2PO_4,2H_2O$ and 4.56g of Na_2HPO_4/l). After dilution to 2% (w/v) with the pH6.0 buffer, 50mol of recrystallized potassium cyanate/mol of αβ-dimer was added and the reaction allowed to continue for 1h at 25°C under CO. Then the reaction mixture was passed through a column ($50cm^2 \times 40cm$) of Sephadex G-25 (fine) equilibrated with 0.025M-disodium hydrogen phosphate to remove the potassium cyanate, and dithiothreitol added as described above (the pH was adjusted to 8.5 if necessary). The next day the haemoglobin was concentrated twofold with Sephadex and passed through a column ($50cm^2 \times 40cm$) of Sephadex G-25 (fine) equilibrated with the Amberlite column buffer. It was then loaded on to the Amberlite column ($28cm^2 \times 50cm$) as described earlier for the preparation of the fast component. Fig. 4 shows the elution pattern obtained on a smaller-scale experiment when cystamine-treated carbonmonoxyhaemoglobin was treated with potassium [^{14}C]cyanate. An identical elution pattern was obtained for the larger-scale reactions. After elution the peaks were concentrated with Sephadex and stored under CO.

Chemical characterization of the carbamoylated haemoglobins. The radioactivities of samples from each of the peaks were counted and the results (±S.D.) are shown in Table 1. All of the counting techniques described in the Methods section were used, and the results for each method were very similar. The α- and β-chains of each of the peaks were separated as described in the Methods section. The elution patterns are shown in Fig. 5. Buffers 1 and 3 were used in these chromatograms. When an acidic β-chain was present, as in peaks 1 and 2, the peak marked β′ was eluted ahead of the main β-chain peak. Peak β′ was a chromatographic artifact and was identical with peak β since it had the same 'fingerprint' and the same radioactive peptide. In fact, when the α- and β-chains of peak 1 were chromatographed with buffers 1 and 2, peak β′ did not appear. Thus, as discussed in the Methods section, peak β′ was a consequence of using too high a pH for the chromatogram and was absent when a lower pH (buffer 2) was used. The pooled fractions (the total recovery of radioactivity from the columns ranged between 83 and 88%) were aminoethylated and digested with trypsin. After digestion the pH was lowered to 6.5, the precipitate was spun off, washed once and the distribution of counts in the soluble and insoluble portions determined by using the same counting system as was used for counting the radioactivity of the CM-cellulose column effluent. The percentage of counts soluble in the tryptic digests of the various peaks ranged from 91 to 96%. Electrophoresis at pH6.5 was used to separate the soluble peptides and the resulting strips of paper were scanned for radioactivity by using a Packard radiochromatograph scanner. The peptides were then chromatographed in solvent BAWP to give a 'fingerprint' which was radioautographed (Plate 1). The radiochromatograph scans showed that the spots on the radioautograph were the only radioactive peptides present. These peptides were eluted, hydrolysed with 6M-hydrochloric

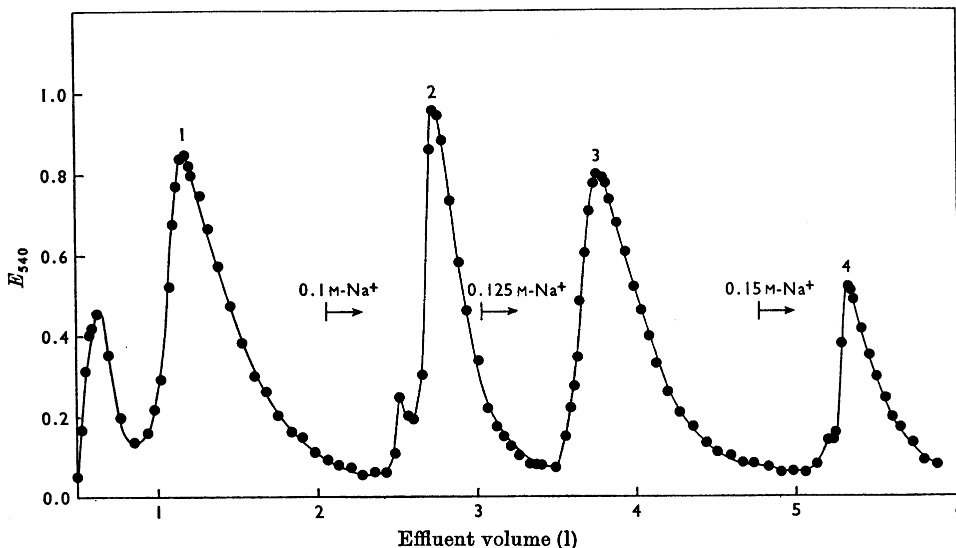


Fig. 4. Elution pattern of 2.0g of fast component after reaction with cyanate after passing through a column ($12cm^2 \times 50cm$) of Amberlite CG-50, which was equilibrated with 0.05M-sodium phosphate buffer, pH6.5, at 4°C (1.802g of Na_2HPO_4 and 3.85g of $NaH_2PO_4,2H_2O$/l). The column was developed with a discontinuous salt gradient at the points indicated.

Table 1. *Incorporation of potassium* [^{14}C]*cyanate into cystamine-treated* CO-*haemoglobin*

For experimental details see the text. The peaks are those eluted from Amberlite CG-50 as shown in Fig. 4.

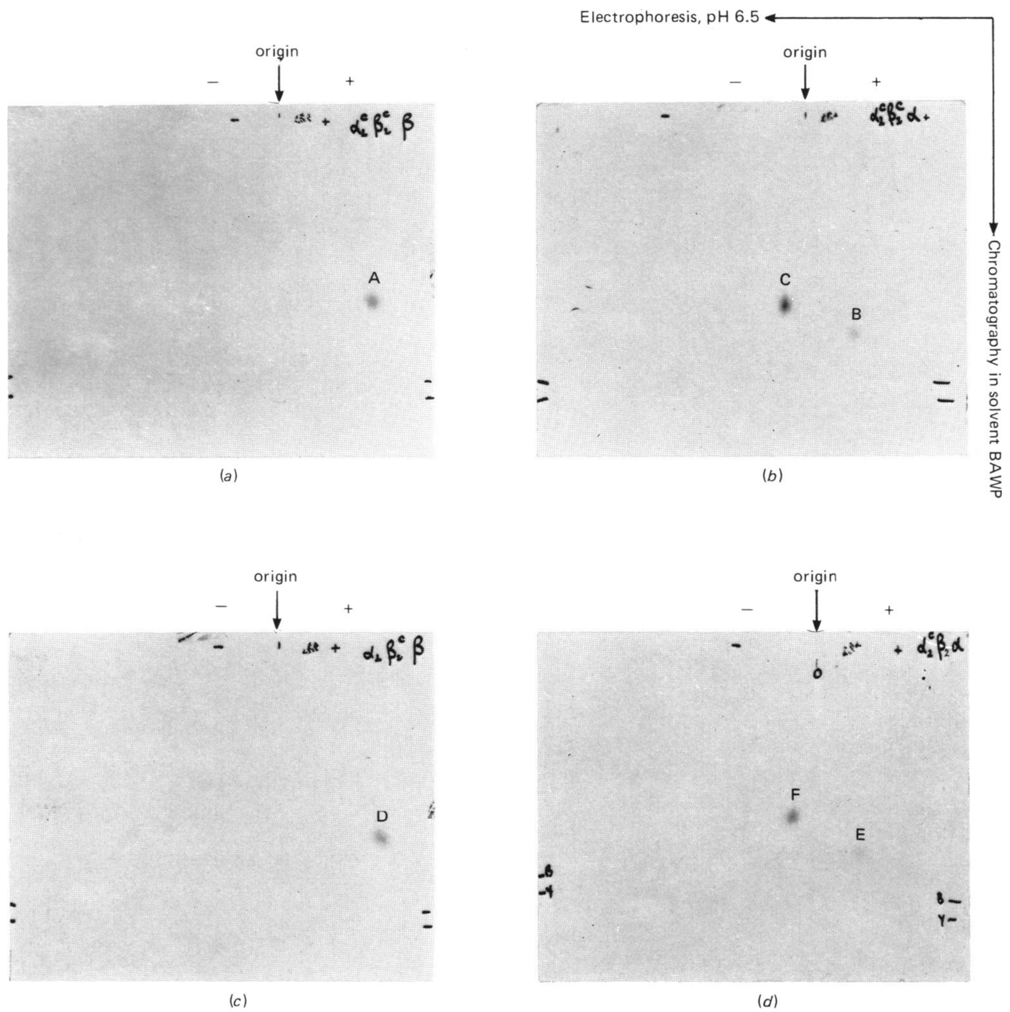
	Mol of [^{14}C]CNO^-/mol of $\alpha\beta$-dimer
Peak 1	1.86 ± 0.04
Peak 2	1.03 ± 0.02
Peak 3	0.96 ± 0.03
Peak 4	0.09 ± 0.005

acid for 24h and their amino acid compositions determined (shown in Table 2). The amino acid compositions of peptides A and D correspond to residues 1–8 of the β-chain (Smith, 1968), and similarly B, E and C, F correspond to residues 1–7 and 1–11 respectively of the α-chain (Braunitzer & Matsuda, 1963). The low value of valine in each peptide would indicate that the cyanate had reacted with the α-amino group. Stark & Smyth (1963) showed that complete regeneration of valine from carbamoylvaline in 6M-hydrochloric acid required 96h hydrolysis at 110°C. The 'fingerprints' were stained with ninhydrin and found to be identical with those published by Kilmartin & Clegg (1967) except for the following. In peak 1β peptide β1 is missing from the β-chain 'fingerprint'. In peak 1α peptide α1,2 is missing from the α-chain 'fingerprint'. It could not be seen whether peptide α1 was missing. The radioactive peptide α1,2 was superimposed. Peak 2β was identical with peak 1β. Peak 2α was a normal α-chain 'fingerprint'. Peak 3β was a normal β-chain 'fingerprint', as was peak 3β'', which may be the same as peak X in Kilmartin & Clegg (1967). Peak 3α was identical with peak 1α. Peak 4β and 4α had normal β-chain and α-chain 'fingerprints'.

These results would indicate that peak 1 had the α-amino groups of both chains modified with cyanate, peak 2 the α-amino group of only the β-chain modified, peak 3 the α-amino group of only the α-chain modified and peak 4 was unchanged material. These derivatives are given the notation: peak 1, $\alpha_2^c\beta_2^c$, peak 2, $\alpha_2\beta_2^c$ and peak 3, $\alpha_2^c\beta_2$, where the superscript c denotes reaction at the α-amino group with cyanate.

If cyanate had reacted with the N-terminal α-amino groups only in $\alpha_2^c\beta_2^c$, $\alpha_2\beta_2^c$ and $\alpha_2^c\beta_2$, then all the radioactivity should be recovered as valine hydantoin if the protein were treated as described by Stark & Smyth (1963). They showed that if the α-amino groups of a protein have been carbamoylated then hydrolysis with 6M-hydrochloric acid at 100°C for 1h would quantitatively release the N-terminal acid as the hydantoin, which can be separated from all the other amino acids and peptides produced by the acid hydrolysis on a column of Dowex 50W-X2.

The application of this procedure should lead to the recovery of all the radioactivity [measured by



EXPLANATION OF PLATE I

Radioautographs of 'fingerprints' of the radioactive (*a*) β- and (*b*) α-chains from peak 1, (*c*) the radioactive β-chain from peak 2 and (*d*) the radioactive α-chain from peak 3.

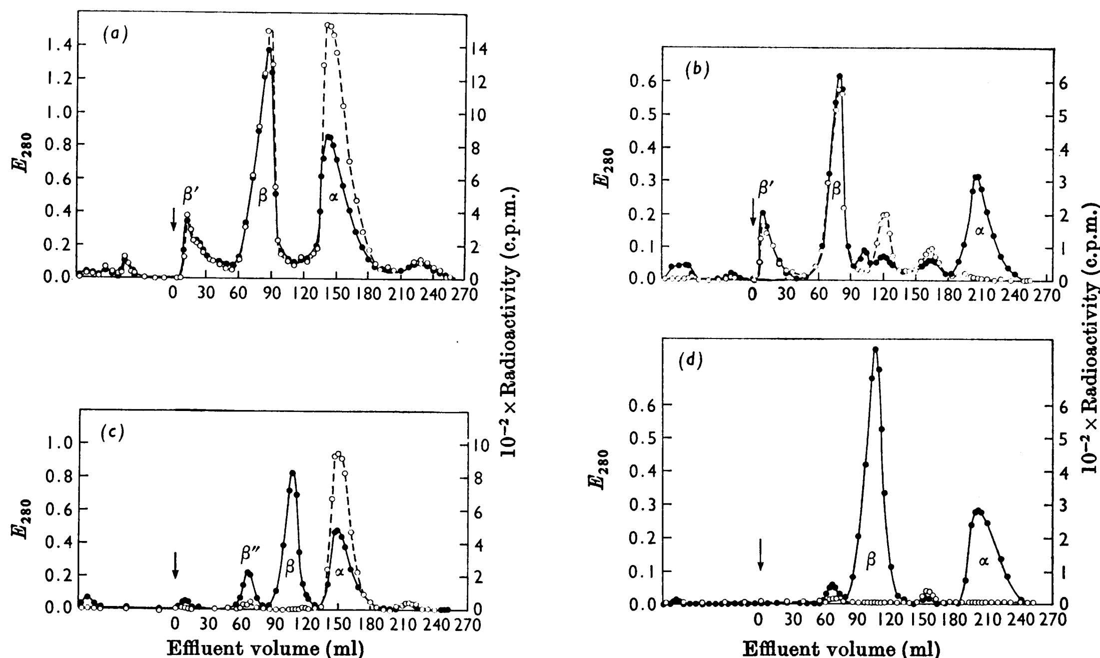


Fig. 5. Separation of the α- and β-chains of the peaks from the Amberlite column. The arrow marks the beginning of a linear salt gradient. Globin (50–100mg) was loaded on to a column (0.8cm^2 × 10cm) of CM-cellulose at 25°C. ●, E_{280}; ○, radioactivity. (*a*) Peak 1, (*b*) peak 2, (*c*) peak 3, (*d*) peak 4.

Table 2. *Amino acid compositions of carbamoylated haemoglobin peptides after tryptic digestion*

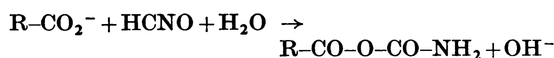
For experimental details see the text. n.d., not determined

	Residues/molecule of peptide								
	Peak 1			Peak 2	Peak 3		*N*-Terminal tryptic peptides from normal horse haemoglobin		
Peptide ...	A	B	C	D	E	F	α1	α1,2	β1
Amino acid									
Lys	0.9	n.d.	1.9	1.0	n.d.	2.1	1.0	2.0	1.0
Asp		0.9	2.3		1.0	2.0	1.0	2.0	
Thr			1.1			1.0		1.0	
Ser	0.9	0.9	1.1	0.9	0.9	1.1	1.0	1.0	1.0
Glu	3.2			3.2					3.0
Gly	1.0			1.1					1.0
Ala		2.2	1.9		2.1	2.1	2.0	2.0	
Val	0.6	0.6	1.7	0.9	0.7	1.9	1.0	2.0	1.0
Leu	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Yield ...	40%	5%	33%	37%	5%	10%			

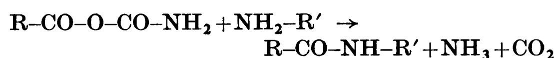
method (i) as described in the Methods section] from these haemoglobin derivatives as valine hydantoin. This is converted into valine by alkaline hydrolysis and thus can also be measured by amino acid analysis. The percentages of counts released as valine hydantoin were for $\alpha_2^c\beta_2^c$, 80%, $\alpha_2\beta_2^c$, 78%, and for $\alpha_2^c\beta_2$, 83%. In the case of $\alpha_2^c\beta_2^c$, the amount of valine released was measured by amino acid analysis and was 84%. These results therefore show that practically all the radioactivity is located at the α-amino groups of the haemoglobin derivatives. The yield of valine hydantoin is a little low. Stark

& Smyth (1963) give values of 85–95% recovery of the *N*-terminal amino acid of various proteins; however, Bradshaw, Rogers, Hill & Buettner-Janusch (1965) obtained only a 70% yield of valine hydantoin after applying the cyanate procedure to the α-chains of lemur haemoglobin, which have the same *N*-terminal sequence, Val-Leu-Ser, as the α-chain of horse haemoglobin.

Under the slightly acid reaction conditions (pH 6.0), the reaction of cyanate with carboxyl groups is possible (Stark, 1965*b*,*c*). However, since all the radioactivity incorporated into the protein can be accounted for by the reaction of cyanate with the α-amino groups, then reaction with carboxyl groups would be without incorporation of radioactivity into the protein. Stark (1965*b*,*c*) has demonstrated that such reactions can occur:



$$R\text{–}CO_2^- + HCNO + H_2O \rightarrow R\text{–}CO\text{–}O\text{–}CO\text{–}NH_2 + OH^-$$



$$R\text{–}CO\text{–}O\text{–}CO\text{–}NH_2 + NH_2\text{–}R' \rightarrow R\text{–}CO\text{–}NH\text{–}R' + NH_3 + CO_2$$

Thus a cross-link between an aspartic acid or glutamic acid and lysine could occur without any label from [^{14}C]cyanate transferred to the protein.

No evidence for such reactions was found in the 'fingerprints' of the tryptic digests of the α- and β-chains of the derivatives. A more quantitative method of detecting these reactions was to totally digest the α- and β-chains from $\alpha_2^c\beta_2^c$ with enzymes and measure the amount of lysine, aspartic acid and glutamic acid by amino acid analysis. Any decrease from the expected values of these residues in either the α- or β-chains would indicate that some cross-linking reactions had occurred. The enzymic hydrolysis was carried out on chains in which the thiol groups had reacted with iodoacetic acid (Crestfield, Moore & Stein, 1963). The method of Hill & Schmidt (1962) was used, and this involved hydrolysis first with papain and then with a mixture of leucine aminopeptidase and prolidase (the latter completely hydrolysed peptides containing proline). The amino acid compositions (averaged from duplicate determinations) measured from these total enzymic hydrolyses are shown in Table 3. In the acid hydrolysate, for comparative purposes, the number of nmol of each amino acid was assumed to correspond exactly to the known number of residues/molecule (Smith, 1968; Braunitzer & Matsuda, 1963). Then the number of nmol for the amino acids in the enzymic hydrolysate (after slight adjustment so that the same total amount of protein was used) expressed as residues/molecule were compared with the values obtained from the acid hydrolysate. Any discrepancy would indicate incomplete enzymic hydrolysis. However, the values are in good agreement, indicating total hydrolysis. The high value of serine in the enzymic hydrolysate was due to the presence of asparagine, which chromatographs in the same position as serine. The low value of serine and threonine in the acid hydrolysate of the normal β-chain was due to the prolonged hydrolysis (80h) necessary to release the valine and leucine. The low

Table 3. *Amino acid compositions of α- and β- chains after total enzymic hydrolysis*

For experimental details see the text. CMCys, carboxymethylcysteine.

	Residues/molecule of chain					
Chain ...	24h acid hydrolysate α	Enzymic hydrolysate α	Enzymic hydrolysate α^c	80h acid hydrolysate β	Enzymic hydrolysate β	Enzymic hydrolysate β^c
Amino acid						
Lys	10.0	9.8	9.7	11.0	11.0	10.9
His	10.0	10.2	10.2	9.0	8.8	8.9
Arg	3.0	3.0	3.1	4.0	4.1	4.2
CMCys	1.0	1.2	1.1	1.0	1.1	1.1
Asp	13.0	9.1	9.0	14.0	7.8	7.9
Thr	9.0	8.6	8.5	(3.0)	4.0	4.1
Ser	13.0	17.0	17.3	(6.0)	17.7	17.7
Glu	5.0	3.2	3.3	12.0	8.8	8.7
Pro	6.0	5.9	5.7	5.0	4.5	4.8
Gly	10.0	9.7	9.9	14.0	14.0	14.0
Ala	16.0	16.1	16.2	15.0	15.0	15.2
Val	12.0	11.5	10.6	17.0	17.0	16.0
Met	1.0	0.6	0.8	1.0	0.8	0.9
Leu	21.0	20.8	20.2	19.0	19.0	18.9
Tyr	2.0	0.9	1.4	3.0	1.4	1.7
Phe	8.0	7.9	7.9	8.0	7.9	8.0

value of tyrosine in the enzymic hydrolysates, observed also by Hill & Schmidt (1962), was probably due to the presence of Mn^{2+} in the digestion mixture. This ion was necessary for the activity of both leucine aminopeptidase and prolidase. A comparison of the amino acid compositions (from the enzymic hydrolysates) of the normal and chemically modified α-chains shows practically no differences except that 1.0 residue of valine and 0.6 residue of leucine are missing. Since leucine is the residue next to the N-terminal valine, this is not surprising. Similarly the only difference between the amino acid compositions of the β-chains is one residue of valine. Since the number of residues/molecule of lysine, aspartic acid and glutamic acid is the same in the chemically modified chains as in the normal chains, then reaction of cyanate with carboxyl groups has not occurred.

Sedimentation coefficients of the carbamoylated haemoglobins. Sedimentation coefficients were measured as described by Benesch, Benesch & Williamson (1962). The values of $s_{20,w}$ for carbonmonoxy and deoxy fast component were 4.3 and 4.2 respectively and for carbonmonoxy and deoxy $\alpha_2^c\beta_2^c$ were 4.2 and 4.2 respectively. Thus under these conditions (0.4% haemoglobin, 15mM-phosphate buffer, pH 7.4, 0.2M-KCl, 20°C) no change in molecular weight after carbamoylation could be detected.

Determination of the structure of $\alpha_2^c\beta_2^c$ by crystallographic methods. Crystals of both the met and deoxy forms of $\alpha_2^c\beta_2^c$ were obtained. As described before, no conformation changes in either of the two forms could be detected (Kilmartin & Rossi-Bernardi, 1969).

Oxygen dissociation curves. The value of the Hill constant n ($\pm$S.D.) and the oxygen affinity at 50% saturation are shown in Table 4. The value of the Hill constant n for $\alpha_2^c\beta_2^c$ and $\alpha_2^c\beta_2$ is practically the same as that for the fast component, showing that these derivatives retain full haem–haem interaction. Control experiments showed that the treatment with cystamine and its removal made no difference to the value of n or the oxygen affinity of the fast component.

Table 4 also shows the lowering of the oxygen affinity by CO_2 at constant pH (Margaria & Green, 1933); this is due to the formation of oxygen-linked carbamino CO_2 (Kernohan *et al.* 1966) and is completely abolished when formation of carbamino CO_2 is prevented by carbamoylation of the α-amino groups in $\alpha_2^c\beta_2^c$. Intermediate effects are seen in the case of $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$, indicating that all four α-amino groups participate in carbamino CO_2 binding.

Effect of carbon monoxide on CO_2 binding. Just as CO_2 causes a lowering of oxygen affinity, i.e. causes oxyhaemoglobin to unload oxygen, O_2 or CO causes deoxyhaemoglobin to unload CO_2. This effect is most conveniently measured from the values of total CO_2 present in solutions of concentrated carbonmonoxy- and deoxy-haemoglobin at constant pCO_2 and pH, as explained in the Methods section. The results are shown in Fig. 6. The fast component ($\alpha_2\beta_2$) exhibits the usual difference in total CO_2 between carbonmonoxy- and deoxy-haemoglobin of 0.2mol of CO_2/mol of haem. This value corresponds to the extra carbamino CO_2 carried by deoxyhaemoglobin compared with carbonmonoxyhaemoglobin. $\alpha_2^c\beta_2^c$ shows no difference in total CO_2 between carbonmonoxy- and deoxy-haemoglobin, in agreement with the lack of effect of CO_2 on the oxygen affinity of this derivative.

Measurement of the alkaline Bohr effect. Titration curves of each of the derivatives were measured; the results are shown in Table 5. The Bohr effect is defined as the difference in negative charge per haem at constant pH between oxy- and deoxy-haemoglobin. It is due to the uptake of protons by haemoglobin on deoxygenation above pH 6.0. This part of the Bohr effect is usually called the alkaline Bohr effect; below pH 6.0 there is a release of protons by haemoglobin on deoxygenation, the acid Bohr effect. The negative charge per haem group (between pH 5.0 and 9.0) is the amount of potassium hydroxide added (in mequiv.) divided by the haemoglobin concentration (in mequiv. of haem groups). For each addition of potassium hydroxide or hydrochloric acid, the pH of deoxyhaemoglobin was measured (pH_{Hb}), then after addition of oxygen

Table 4. *Hill constants* ($\pm$S.D.) *and oxygen affinity at* 50% *saturation of various haemoglobin components*

For experimental details see the text.

	Hill constant n	Oxygen affinity at 50% saturation at pH 7.4 and 25°C (mmHg)	Oxygen affinity at 50% saturation in the presence of CO_2 at pH 7.4 and 25°C (mmHg)	Bohr effect at pH 7.4 and 25°C (difference in negative charge/haem group)
$\alpha_2^c\beta_2^c$	2.5 ± 0.2	4.2	4.2	0.48
$\alpha_2\beta_2^c$	2.0	5.9	7.4	0.62
$\alpha_2^c\beta_2$	2.6 ± 0.1	3.9	4.9	0.48
Fast component	2.7 ± 0.2	6.6	9.3	0.62

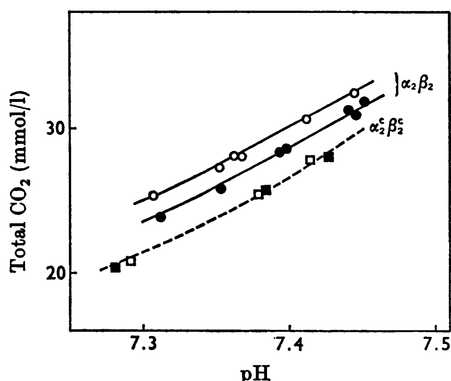


Fig. 6. Total CO_2 content of solutions of horse carbonmonoxy- (○ and □) and deoxy- (● and ■) $\alpha_2\beta_2$ (○, ●) and $\alpha_2^c\beta_2^c$ (□, ■) in 0.2M-KCl. The haemoglobin concentration was 8.0mequiv./l, the temperature 37°C and the partial pressure of CO_2 48mmHg.

the pH of oxyhaemoglobin (pH_{HbO_2}). The titration curve represents a plot of negative charge per haem (B/haem in Table 5) against pH_{Hb} and pH_{HbO_2} assuming no bound Cl^- ion. The Bohr effect is obtained from the vertical difference between the titration curves of oxy- and deoxy-haemoglobin, which is the difference in negative charge per haem group at constant pH. A plot of the Bohr effect at different pH values for these derivatives has already been published (Kilmartin & Rossi-Bernardi, 1969). The values of the Bohr effect at pH 7.4 for each of the derivatives is shown in Table 4. It can be seen that there was a 25% decrease in the alkaline Bohr effect when the α-amino group of the α-chain was carbamoylated. Since no conformational change could be detected from examination of crystals of $\alpha_2^c\beta_2^c$ by X-ray methods, the α-amino group of the α-chain should be directly responsible for 25% of the alkaline Bohr effect.

If the chemical modification had not affected the ionization of any other groups, modified and unmodified haemoglobins should carry the same total charge at pH 8.5, where α-amino groups that had not reacted would be uncharged; however, at pH 6.0 they will be almost fully charged, whereas the α-amino groups that had reacted (R–NH–CO–NH_2) will still be uncharged. Thus at pH 6.0 there should be a difference of 1.0 protonic charge for each amino group modified. The differences in charge at various pH values of the derivatives in the deoxy and oxy state relative to the normal deoxy and oxy fast component are shown in Fig. 7. The expected difference of 1 charge/haem for $\alpha_2^c\beta_2^c$ and about 0.5 charge/haem group for $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$ is seen. The results obtained at the two ends of the pH range shown here were less accurate than those obtained in the intermediate range. Below pH 6.0 there was occasional denaturation of the haemoglobin due to the large amount of hydrochloric acid added and above pH 8.0 the glass electrode was less accurate owing to the lower buffer power of the solution. Within these limitations, it can be seen that the difference curve $\alpha_2\beta_2^c$–$\alpha_2\beta_2$ represents the titration curve of the α-amino group of the β-chain; similarly the difference curve $\alpha_2^c\beta_2$–$\alpha_2\beta_2$ represents the titration curve of the α-amino group of the α-chain. The pH at which this difference is exactly half the total charge carried by the group gives a value for the pK of the group. For the α-chain α-amino group, these values are about pK_{oxy} 7.3 and pK_{deoxy} 7.7, and for the β-chain α-amino group the pK is about 7.2, there being no change in pK value between oxy- and deoxy-haemoglobin. It must be emphasized that these are very approximate values owing to the limitations already discussed.

DISCUSSION

Cyanate would appear to be one of the most suitable reagents for the modification of an amino group in a protein when the requirement is the abolition of a positive charge without conformational change. Its specificity of reaction has been carefully investigated by Stark (1964, 1965*a*,*b*,*c*) and Smyth (1967), who have shown that the only stable bond cyanate forms after reaction with a protein is with an amino group. There is also a possibility of irreversible reaction with a carboxyl group at acidic pH values (Stark, 1965*b*,*c*). Other reagents used, in particular fluorodinitrobenzene, are most likely to cause extensive conformational changes, due to the placing of a large hydrophobic group on the hydrophilic surface of a protein. Thus Neer & Konigsberg (1968) prepared human haemoglobin where the α-amino group of the α-chain had specifically reacted with fluorodinitrobenzene. This haemoglobin had lost all haem–haem interactions and had no Bohr effect.

Our finding that the α-chain α-amino group is responsible for about one-quarter of the alkaline Bohr effect found a structural interpretation after a careful examination of the low-resolution (5Å) Fourier map of human deoxyhaemoglobin (Muirhead & Perutz, 1963). It was found that the *C*-terminus of one α-chain was linked to the *N*-terminus of the other α-chain, and Perutz *et al.* (1969) proposed the existence of a salt bridge between the carboxyl group of the *C*-terminal arginine (141α) and the positively charged α-amino group (valine-1α) of the opposite α-chain. The proximity of the negatively charged carboxyl group to the α-amino group would stabilize the positive charge and thereby raise its pK value. These residues cannot be seen

Table 5. *Titration curves of haemoglobin derivatives*

For experimental details see the text. B/haem is mequiv. of added base per mequiv. of haem group.

Fast component			$\alpha_2\beta_2^c$			$\alpha_2^c\beta_2^c$			$\alpha_2^c\beta_2$		
B/haem	pH_{Hb}	pH_{HbO_2}	B/haem	pH_{Hb}	pH_{HbO_2}	B/haem	pH_{Hb}	pH_{HbO_2}	B/haem	pH_{Hb}	pH_{HbO_2}
−3.360	5.632	5.752	−3.260	5.470	5.612	−2.951	5.405	5.538	−3.000	5.79	5.875
−2.939	5.958	5.982	−2.538	5.825	5.895	−1.599	6.200	6.170	−2.400	6.08	6.073
−2.773	6.078	6.066	−1.980	6.216	6.190	0	7.024	6.870	−1.600	6.485	6.408
−1.680	6.610	6.488	−1.447	6.498	6.402	1.737	7.771	7.600	−0.800	6.824	6.704
−0.853	6.934	6.782	−0.725	6.852	6.700	3.474	8.810	8.690	0	7.138	6.983
0	7.258	7.072	0	7.150	6.950	4.135	9.320	9.265	0.760	7.442	7.295
0.802	7.552	7.378	0.687	7.436	7.238				1.520	7.712	7.582
1.596	7.828	7.650	1.375	7.710	7.507				3.800	9.038	9.014
2.407	8.210	8.044	2.410	8.143	7.950						
3.192	8.620	8.489	3.600	8.910	8.825						
3.925	9.190	9.153									
4.005	9.337	9.310									

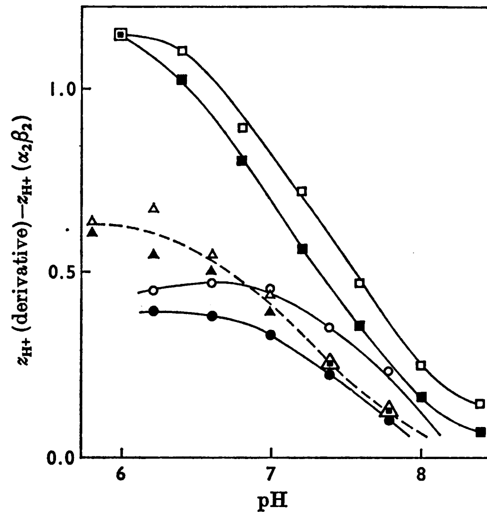


Fig. 7. Difference between the total charge (z_{H^+}) carried by the derivatives and the total charge carried by normal haemoglobin ($\alpha_2\beta_2$) is plotted against pH. Open symbols are the differences between deoxy titration curves, and filled symbols the differences between oxy titration curves. □ and ■, differences between $\alpha_2^c\beta_2^c$ and $\alpha_2\beta_2$; △ and ▲, differences between $\alpha_2\beta_2^c$ and $\alpha_2\beta_2$ (----); ○ and ●, differences between $\alpha_2^c\beta_2$ and $\alpha_2\beta_2$.

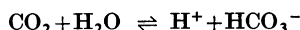
in the high-resolution Fourier map of horse oxyhaemoglobin (Perutz, Muirhead, Cox & Goaman, 1968), and are probably floating freely in solution. Therefore the salt bridge cannot occur and the p*K* of the α-chain α-amino group would revert to a lower value.

The existence of the salt bridge between the α-chain *C*-terminal carboxyl group and the α-amino group of the opposite α-chain has been confirmed on examination of the high-resolution Fourier maps of deoxyhaemoglobin (Muirhead & Greer, 1970; Bolton & Perutz, 1970). No salt bridges involving the β-chain α-amino group have been found in the structures of oxy- and deoxy-haemoglobin. Thus its p*K* should have the same value in oxy- and deoxy-haemoglobin.

Perutz *et al.* (1969) proposed that most of the rest of the Bohr effect comes from histidine-146β, which is floating free in solution in oxyhaemoglobin but forms a salt bridge with aspartic acid-94β (of the same β-chain) in deoxyhaemoglobin. This was confirmed by the preparation of des-(His-146β)-human haemoglobin, which had co-operative interactions but had lost half of its alkaline Bohr effect (Kilmartin & Wootton, 1970).

The lack of any effect of CO_2 on the oxygen affinity of $\alpha_2^c\beta_2^c$ coupled with the lack of any conformational change in this derivative indicates that

the α-amino groups are the sole source of oxygen-linked CO_2 effects. Since HCO_3^- ion has only a small effect on oxygen affinity (Kernohan *et al.* 1966) and since our approximate values of the pK of the α-amino groups lie within the physiological pH range, the oxygen-linked CO_2 effects must be mediated via carbamino CO_2. These results are in agreement with the measurements of total CO_2 in solutions of carbonmonoxy- and deoxy-haemoglobin at constant pCO_2, as shown in Fig. 6. In the case of $\alpha_2\beta_2$, the total CO_2 in the solution corresponds to the dissolved CO_2, HCO_3^-, CO_3^{2-} and the carbamino CO_2. Thus, at the same pH, the difference between the two curves corresponds to the extra carbamino CO_2 carried by deoxyhaemoglobin. In the case of $\alpha_2^c\beta_2^c$ there is no extra carbamino CO_2 carried by the deoxy form, and if there is no CO_2 bound to $\alpha_2^c\beta_2^c$ in either the oxy or deoxy form then the total CO_2 in solutions of $\alpha_2^c\beta_2^c$ should correspond to the dissolved CO_2, HCO_3^- and CO_3^{2-}. Since the CO_3^{2-} in solution at these pH values is very small the concentration of dissolved CO_2 and HCO_3^- can be calculated from the value of the equilibrium constant of the reaction:



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

assuming that haemoglobin has no effect on the activity coefficient of HCO_3^-. The method of Stadie & Hawes (1928) was used to calculate the amount of dissolved CO_2 and HCO_3^- in solution at several of the pH values in Fig. 6. The values obtained (after making allowance for the effect of haemoglobin on the volume of water) are shown by the broken line in Fig. 6. This corresponds exactly to the white squares, which represent the data for $\alpha_2^c\beta_2^c$. Thus, taking into account the assumptions involved in the calculation, there was practically no CO_2 or HCO_3^- bound to $\alpha_2^c\beta_2^c$.

The binding of carbamino CO_2 to haemoglobin causes a large decrease in the Bohr effect (Rossi-Bernardi & Roughton, 1967*b*). As expected, this decrease was abolished when the Bohr effect of $\alpha_2^c\beta_2^c$ was measured in the presence of CO_2 (Kilmartin & Rossi-Bernardi, 1969; Roughton, 1970).

The combination of CO_2 with the α-chain α-amino group of deoxyhaemoglobin must be accompanied by a small conformational change. This is because, at physiological pH 7.4 in the absence of CO_2, a positive charge is stabilized on the group due to the proximity of the α-carboxyl of arginine-141α (Perutz, 1970). On the other hand, in the presence of CO_2, a negative charge ($-NH-CO_2^-$) is now present on this group. This negative charge must have a greater stabilizing effect on the deoxyhaemoglobin structure than the positively charged amino group since deoxyhaemoglobin has a high affinity for carbamino CO_2. Since the extra salt bridges present in deoxyhaemoglobin stabilize its structure compared with oxyhaemoglobin (Perutz, 1970), the carbamino group ($-NH-CO_2^-$) must form a strong salt bridge. Examination of a deoxyhaemoglobin model (Muirhead & Greer, 1970; Bolton & Perutz, 1970) shows that the α-amino groups of lysine-7α and -139α are in the near vicinity of the α-chain α-amino group and could possibly form a salt bridge with the carbamino group. However, the exact structure of deoxyhaemoglobin combined with CO_2 will remain unknown until the complex is crystallized and examined.

Benesch & Benesch (1969) have shown that organic phosphate molecules such as D-2,3-diphosphoglycerate and ATP are bound more to deoxyhaemoglobin than to oxyhaemoglobin. Rental, Benesch, Benesch & Bray (1970) and Bunn & Briehl (1969) have shown that the major site of 2,3-diphosphoglycerate conformation with deoxyhaemoglobin is in the central cavity running through the molecule along the dyad axis of symmetry. The groups within bonding distance of 2,3-diphosphoglycerate would be the two N-terminal amino groups of the β-chains, the imidazoles of the histidine-143β and the ϵ-amino groups of lysines-82β. We have shown that CO_2 can also bind at the two N-terminal amino groups of the β-chains, and a competition between 2,3-diphosphoglycerate and CO_2 is to be expected. Such an interaction was first demonstrated by Bauer (1969) and later quantitatively investigated by Pace, Rossi-Bernardi & Roughton (1970). By determining the total CO_2 in solutions of human oxy- and deoxy-haemoglobin at constant pCO_2 at 37°C in the presence of a ratio of up to 2mol of ATP or 2,3-diphosphoglycerate/mol of haemoglobin tetramer, Pace *et al.* (1970) showed that organic phosphate molecules decreased the total amount of CO_2 bound by deoxyhaemoglobin by 25% whereas the total CO_2 bound by oxyhaemoglobin was not affected. Thus extrapolation of our physicochemical results and those of Rossi-Bernardi & Roughton (1967*b*) to physiological conditions requires consideration of at least one more ligand, ATP or 2,3-diphosphoglycerate, besides the three ligands (O_2, protons and CO_2) already considered in detail.

J.V.K. thanks the Medical Research Council and L.R.-B. the Consiglio Nazionale delle Ricerche, Rome, for support. J.V.K. thanks Professor E. Trabucchi for his generous hospitality in Milan and Dr M. F. Perutz for suggesting this project and for his interest throughout its course.

REFERENCES

Bangham, A. D. & Lehmann, H. (1958). *Nature, Lond.*, **181**, 267.

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

Benesch, R. & Benesch, R. E. (1957). *Biochim. biophys. Acta*, **23**, 643.

Benesch, R. & Benesch, R. E. (1962). In *Methods of Biochemical Analysis*, vol. 10, p. 56. Ed. by Glick, D. New York: Interscience Publishers Inc.

Benesch, R. & Benesch, R. E. (1969). *Nature, Lond.*, **221**, 618.

Benesch, R. E., Benesch, R. & Williamson, M. E. (1962). *Proc. natn. Acad. Sci. U.S.A.* **48**, 2071.

Bolton, W. & Perutz, M. F. (1970). *Nature, Lond.*, **228**, 551.

Bradshaw, R. A., Rogers, L. A., Hill, R. L. & Buettner-Janusch, J. (1965). *Archs Biochem. Biophys.* **109**, 571.

Braunitzer, G. & Matsuda, G. (1963). *J. Biochem., Tokyo*, **53**, 262.

Bunn, H. F. & Briehl, R. W. (1970). *J. clin. Invest.* **49**, 1088.

Clegg, J. B., Naughton, M. A. & Weatherall, D. J. (1966). *J. molec. Biol.* **19**, 91.

Clegg, M. D. & Schroeder, W. A. (1959). *J. Am. chem. Soc.* **81**, 6065.

Crestfield, A. M., Moore, S. & Stein, W. H. (1963). *J. biol. Chem.* **238**, 622.

Davis, N. C. & Smith, E. L. (1957). *J. biol. Chem.* **224**, 261.

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

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

Forster, R. E., Constantine, H. P., Craw, M. R., Rotman, H. H. & Klocke, R. A. (1968). *J. biol. Chem.* **243**, 3317.

Guidotti, G. & Konigsberg, W. (1964). *J. biol. Chem.* **239**, 1474.

Hegesh, E. & Avron, M. (1967). *Biochim. biophys. Acta*, **146**, 397.

Hill, R. J., Konigsberg, W., Guidotti, G. & Craig, L. C. (1962). *J. biol. Chem.* **237**, 1549.

Hill, R. L. & Schmidt, W. R. (1962). *J. biol. Chem.* **237**, 389.

Hirs, C. H. W., Moore, S. & Stein, W. H. (1953). *J. biol. Chem.* **200**, 493.

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

Keyes, M., Mizukami, H. & Lumry, R. (1967). *Analyt. Biochem.* **18**, 126.

Kilmartin, J. V. & Clegg, J. B. (1967). *Nature, Lond.*, **213**, 269.

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

Kilmartin, J. V. & Rossi-Bernardi, L. (1970). In *Carbon Dioxide: Chemical, Biochemical and Physiological Aspects*, p. 73. Washington: U.S. Government Printing Office (N.A.S.A. no. SP-188).

Kilmartin, J. V. & Wootton, J. F. (1970). *Nature, Lond.*, **228**, 766.

Margaria, R. & Green, A. A. (1933). *J. biol. Chem.* **102**, 611.

Muirhead, H. & Greer, J. (1970). *Nature, Lond.*, **228**, 516.

Muirhead, H. & Perutz, M. F. (1963). *Nature, Lond.*, **199**, 633.

Neer, E. J. & Konigsberg, W. (1968). *J. biol. Chem.* **243**, 1966.

Nozaki, C. & Tanford, C. (1967). In *Methods in Enzymology*, vol. 11, p. 733. Ed. by Hirs, C. H. W. New York and London: Academic Press.

Pace, M., Rossi-Bernardi, L. & Roughton, F. J. W. (1970). *Biochem. J.* **119**, 67 P.

Perutz, M. F. (1968). *J. Cryst. Growth*, **2**, 54.

Perutz, M. F. (1970). *Nature, Lond.*, **228**, 726.

Perutz, M. F., Muirhead, H., Cox, J. M. & Goaman, L. C. G. (1968). *Nature, Lond.*, **219**, 131.

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

Peters, J. P. & van Slyke, D. D. (1932). In *Quantitative Clinical Chemistry*, vol. 2, p. 261. London: Baillière, Tindall and Cox.

Raftery, M. A. & Cole, R. D. (1966). *J. biol. Chem.* **241**, 3457.

Rental, R., Benesch, R. E., Benesch, R. & Bray, B. A. (1970). *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 732.

Riggs, A. F. (1951). *J. gen. Physiol.* **35**, 23.

Rimon, S. & Perlmann, G. F. (1968). *J. biol. Chem.* **243**, 3566.

Robinson, R. A. & Stokes, R. H. (1959). *Electrolyte Solutions*, 2nd ed., p. 462. London: Butterworths.

Rossi-Bernardi, L. & Roughton, F. J. W. (1967*a*). *J. biol. Chem.* **242**, 784.

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

Roughton, F. J. W. (1943). *Harvey Lect.* **39**, 96.

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. (1970). *Biochem. J.* **117**, 801.

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

Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.

Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.

Schwartz, E. & Nathan, D. G. (1967). *J. Lab. clin. Med.* **70**, 841.

Siggaard-Anderson, O. (1963). *The Acid–Base Status of the Blood*, 3rd ed., p. 102. Copenhagen: Munksgaard.

Smith, D. B. (1968). *Can. J. Biochem.* **46**, 825,

Smithies, O. (1965). *Science, N.Y.*, **150**, 1595.

Smyth, D. G. (1967). *J. biol. Chem.* **242**, 1579.

Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.

Stadie, W. C. & Hawes, E. R. (1928). *J. biol. Chem.* **77**, 265.

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

Stark, G. R. (1964). *J. biol. Chem.* **239**, 1411.

Stark, G. R. (1965*a*). *Biochemistry, Easton*, **4**, 588.

Stark, G. R. (1965*b*). *Biochemistry, Easton*, **4**, 1030.

Stark, G. R. (1965*c*). *Biochemistry, Easton*, **4**, 2363.

Stark, G. R. & Smyth, D. G. (1963). *J. biol. Chem.* **238**, 214.

Tanford, C. (1961). *Physical Chemistry of Macromolecules*, p. 556. New York: John Wiley and Sons Inc.

Taylor, J. F., Antonini, E., Brunori, M. & Wyman, J. (1966). *J. biol. Chem.* **241**, 241.

Van Kampen, E. J. & Zijlstra, W. G. (1961). *Clinica chim. Acta*, **6**, 538.

Wallach, D. F. H., Gail, M. H. & Janeway, C. A. (1966). *Analyt. Biochem.* **15**, 300.