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Chemical Studies on Haemoglobins A_1 and A_0

BY M. Z. ATASSI*
Division of Laboratories and Research, New York State Department of Health, Albany, N.Y., U.S.A.

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The heterogeneity of adult human haemoglobin is well established on the basis of alkali denaturation studies (Brinkman & Jonxis, 1935), starch-block electrophoresis (Kunkel & Wallenius, 1955), and solubility techniques with variable solvents (Roche, Derrien & Roques, 1952) and variable solute tests (Allison & Tombs, 1957). Chromatography on ion exchangers (Morrison & Cook, 1955; Huisman & Prins, 1955) confirmed these observations. Schneck & Schroeder (1961) were the first to investigate the minor components with great care and to work out a correlation between the fractions isolated by chromatography and by starch-block electrophoresis. The haemoglobin of a normal adult individual was separated into four major fractions (Huisman & Meyering, 1960) by chromatography on CM-cellulose. These fractions were termed, in the order of their appearance from the column, HbA_1, HbA_0, HbF and HbA_2. In addition, small amounts of methaemoglobin reductase and unidentified proteins were present. A_1 is the most rapidly moving electrophoretic component and can be resolved on CM-cellulose into three components, namely A_1^A, A_1^B and A_1^C. This component was termed A_3 by Schneck & Schroeder (1961). A_0 is the major component (about 80 % of total), which was termed A_1 by Schneck & Schroeder (1961). On electrophoresis, it moves at a position intermediate between the most-slowly-moving component, A_2 (by both systems of nomenclature), and the most-rapidly-moving component. Foetal haemoglobin F can be resolved into two components, F_1 and F_0 (Huisman & Meyering, 1960). F_1 contains an acetyl group at the *N*-terminal end of one of its polypeptide chains (Schroeder, Cua & Fenninger, 1962). No differences in oxygen affinities were observed with HbA_1,

* Present address: Biochemistry Department, School of Medicine and Dentistry, State University of New York at Buffalo, N.Y., U.S.A.

HbA_0, HbF_1 and HbF_0 (Meyering, Israels, Sebens & Huisman, 1960). The main haemoglobin component, HbA_0, could be partially converted into the corresponding fast-moving haemoglobin, HbA_1, by the addition of sodium chromate to haemoglobin solutions (Meyering *et al.* 1960). Kunkel, Ceppellini, Muller-Eberhard & Wolf (1957) had observed previously that, after salt precipitation, exposure to acid and alkaline pH, treatment with urea, or prolonged dialysis and concentration, an increase in fast-migrating material was revealed by starch-block electrophoresis, when it formed a streak from the main peak. Hill, Swenson & Schwartz (1962) have obtained similar results by chromatography on Amberlite IRC-50 after treatment with acid, alkali, ethanol, urea, or freezing and thawing.

Reported below is the interconversion of HbA_1 or HbA_0 (either in their cyanmet or in their oxy forms)* by simply allowing what was originally an electrophoretically and chromatographically homogeneous fraction to stand in a buffered solution at 0–2°. The effects of different conditions on this transformation have been investigated, and studies on the nature of the physicochemical differences between $MetA_1$ and $MetA_0$ are reported.

EXPERIMENTAL

All experiments were carried out at 0–2°, unless otherwise stated.

Preparation of haemoglobin. Citrate-treated blood, freshly drawn from a normal human male adult, was centrifuged, the red cells were washed four times each with 0·15 M- and 0·21 M-NaCl and lysed with toluene (Clegg & Schroeder, 1959), and the haemoglobin was crystallized twice by the method of Drabkin (1946). The solutions were converted into MetHb with a threefold molar excess of 0·02 M-$K_3Fe(CN)_6$ in 0·01 M-potassium phosphate buffer, pH 7·00, for 1 hr., and completion of the conversion was confirmed spectrally in the range 700–330 mμ. Excess of ferricyanide was removed by dialysis against distilled water. The concentrations of haemoglobin solutions were determined from their extinctions, in the CN-MetHb form, at 541 mμ. The molar extinction coefficient (identical for both CN-$MetA_1$ and CN-$MetA_0$) at 541 mμ is $4·59 \times 10^4$.

Chromatography of haemoglobin. Solutions were chromatographed in the CN-MetHb form. This procedure prevented the appearance of extraneous zones since CN-MetHb and oxyhaemoglobin have identical chromatographic behaviour (Clegg & Schroeder, 1959). For preparative work haemoglobin solutions (approx. 1 g. in 25 ml.) were chromatographed on columns (2·5 cm. × 60 cm.) of CM-cellulose (Sigma Chemical Co.), with gradient elution (Huisman & Meyering, 1960) from pH 6·8 to 7·7 in 0·01 M-phosphate buffers containing KCN (0·01%). The extinctions of the fractions (15 ml. each) were read at 280 and 541 mμ in a Beckman spectrophotometer. For analytical work, stepwise elution of small columns (0·9 cm. × 60 cm.) was employed. CN-MetHb samples (2 mg. in 1–2 ml.) were applied at pH 7·30 in 0·01 M-phosphate buffer containing KCN (0·01%). Under these conditions CN-$MetA_1$ was unabsorbed, after which the pH of the eluting buffer was raised to 7·58, when CN-$MetA_0$ was recovered. Small fractions (1–2 ml.) were collected and extinctions were read at 412 mμ. The relative amounts of CN-$MetA_1$ and CN-$MetA_0$ in a mixture were determined after chromatography from the total extinctions of each component at 541 mμ in concentrated solutions or at 412 mμ in dilute solutions. Dilute haemoglobin solutions were also concentrated on CM-cellulose. The pH of the fractions was lowered to 7·00 only, to avoid denaturation of CN-$MetA_1$, by the addition of 0·01 M-NaH_2PO_4 containing KCN (0·01%). The fraction was then applied on a short CM-cellulose column (3 cm. × 20 cm.) equilibrated at that pH and eluted with 0·01 M-phosphate buffer, pH 7·30, containing KCN (0·01%), whereby CN-$MetA_1$ was collected. At this stage dilute solutions of CN-$MetA_0$, which had been adjusted to pH 7·30, could be applied on the same column, which was washed again with pH 7·3 buffer to elute any CN-$MetA_1$ that had formed from CN-$MetA_0$ on standing. CN-$MetA_0$ could then be eluted by raising the pH of the eluting buffer to 7·58. By this method 20–40-fold concentration of purified fractions was obtained.

For quick changing of solvent, when dialysis was considered undesirable, the solutions were applied on short columns (2·2 cm. × 20 cm.) of Sephadex G-25 that had been equilibrated with the desired buffer solution. About 80% of the haemoglobin sample could be eluted in 1·5–2 times its original volume.

Analytical methods. The identity of each sample was confirmed by starch-gel electrophoresis, at room temperature, by the discontinuous buffer system of Poulik (1957) for their electrophoresis in the met or oxy forms. For electrophoresis of CN-MetHb fractions the gels and buffer contained KCN (0·02%) to avoid complications that might arise on partial saturation of methaemoglobin with cyanide (Itano, 1958). The gels were stained with Amido Black (Smithies, 1959). Spectral investigations in the range 230–700 mμ were carried out in a Cary model 14 spectrophotometer. Ultracentrifugal studies were made at 5–7° on 0·3–0·8% solutions of the proteins in 0·01 M-phosphate buffer, pH 7·2, containing KCN (0·01%), at full speed (59 780 rev./min.). For amino acid analysis haemoglobin samples were dialysed against distilled water and freeze-dried. Portions (2–4 mg.) were then dissolved in 1·0 ml. each of constant-boiling HCl (triple-distilled), flushed with nitrogen, evacuated, sealed and heated at 110° for 22 or 72 hr. The hydrolysates were freed of excess of HCl on a rotary evaporator and assayed with an amino acid analyser similar to that described by Spackman, Stein & Moore (1958).

Titration of the thiol groups. (*a*) Reaction with *p*-hydroxymercuribenzoate. The method of Boyer (1954) was employed. Because of the high extinction of the sample (1–2 mg./ml.) at 250 mμ, a pair of cells, each containing 3 ml. of solution, was placed in each light-path: (i) Blank: in cell 1, MetHb solution in 0·05 M-phosphate buffer, pH 7·21; in cell 2, 0·05 M-phosphate buffer, pH 7·21; (ii) sample: in cell 3, 0·05 M-phosphate buffer, pH 7·21; in cell 4, MetHb

* Abbreviations: CN-MetHb, cyanmethaemoglobin; CN-$MetA_1$, cyanmethaemoglobin A_1; CN-$MetA_0$, cyanmethaemoglobin A_0; the corresponding methaemoglobins (ferrihaemoglobins) are MetHb, $MetA_1$ and $MetA_0$ respectively.

solution in 0·05 M-phosphate buffer, pH 7·21. Cells 1 and 3 were left undisturbed throughout the determination. Portions of 1 mM-*p*-hydroxymercuribenzoate were added only to cells 2 and 4 and the extinction at 250 mμ was read in a Zeiss model PMQ II spectrophotometer after the solutions had stood for 30 min. after each addition.

For determining total SH groups, MetHb solutions were in 5 M-urea in 0·05 M-phosphate buffer, pH 5·2.

(*b*) Reaction with iodoacetamide. MetHb solutions (1·5–2·0 mg./ml.) were treated with a tenfold molar excess of iodoacetamide and the pH was kept constant at 7·20 by titration with 0·01 N-NaOH (Benesch & Benesch, 1962*a*). After dialysis, freeze-drying and hydrolysis of the samples, their contents of *S*-carboxymethylcysteine and amino acids were determined.

(*c*) Reaction with *N*-ethylmaleimide. Reaction was carried out at room temperature by adding a fivefold molar excess of *N*-ethylmaleimide to MetHb solution in 0·1 M-phosphate buffer, pH 7·30. The *N*-ethylmaleimide uptake was determined at 5 min. intervals by a method utilizing perchloric acid precipitation (Benesch & Benesch, 1961).

Reconstitution of haemoglobin. Soluble globin was prepared by the method of Anson & Mirsky (1930) from unchromatographed haemoglobin. Recombination was carried out in phosphate buffer according to the method of Rossi-Fanelli, Antonini & Caputo (1959).

Labelling of methaemoglobins A_1 and A_0 with chromium. Samples of fresh CN-MetHb solutions, prepared by column chromatography, were dialysed against distilled water to remove all buffer. The solutions were then adjusted to the same concentration (10–20 mg. in 20 ml.), on the basis of the extinction at 541 mμ, and a tenfold molar excess of 1·25 mM-$K_2{}^{51}CrO_4$ (2·228 μc/ml.) was added. After standing for 6 hr. the solutions were dialysed against eight changes of distilled water to remove unbound chromate, and the counts/min. were determined on 20 ml. samples in a 25 ml. conical flask in a multichannel analyser γ-scintillation counter with a 4 × 4 thallium-activated sodium iodide crystal. Suitable standards were also counted.

RESULTS

Characterization of fractions A_1 and A_0

The present study deals with the equilibrium between two species of methaemoglobin. It was therefore essential to ensure that a meaningful comparison could be made between the methaemoglobin components studied in the present work and the haemoglobins described by other investigators. Such a comparison is possible only if the two components described in the present work are well characterized.

Chromatographic and electrophoretic behaviour. A sample of oxyhaemoglobin (900 mg. in 30 ml.) was chromatographed on CM-cellulose by gradient elution from pH 6·8 to 7·7 in 0·01 M-phosphate containing potassium cyanide (100 mg./l.). The haemoglobin components obtained in this experiment had chromatographic behaviour identical with those obtained from chromatography of CN-MetHb described below. Electrophoretic mobilities, on starch gel, of the present fractions were also identical with those of the corresponding CN-MetHb fractions. The oxyhaemoglobin A_1^C component had a mobility of 1·14 (relative to that of oxyhaemoglobin A_0).

Amino acid analysis. Analyses of $MetA_1$, $MetA_0$ and their globins, obtained after different times of hydrolysis, are shown on Table 1 as g. of amino

Table 1. *Amino acid composition of hydrolysates of methaemoglobins A_1 and A_0 and their globins*

Experimental details are given in the text. The values are expressed in g. of amino acid/100 g. of protein (on ash-free and moisture-free basis).

	$MetA_1$		$MetA_0$		Globin A_1	Globin A_0
Duration of hydrolysis ... / Amino acid	22 hr.*	72 hr.	22 hr.*	72 hr.	22 hr.	22 hr.
Aspartic acid	9·94	9·80	9·64	9·82	10·18	9·66
Threonine†	5·15	4·28	5·18	4·62	5·27	5·09
Serine†	4·09	3·01	3·83	3·18	4·12	4·17
Glutamic acid	6·65	7·17	6·89	7·04	6·78	7·06
Proline	4·95	5·25	4·67	5·10	5·00	5·17
Glycine	4·43	4·35	4·25	4·37	4·49	4·28
Alanine	9·45	9·20	9·14	9·28	9·40	9·24
Half-cystine‡	1·06 (5·56)	0·892 (4·92)	0·874 (4·82)	0·823 (4·53)	0·652 (3·59)	0·646 (3·56)
Valine	10·35	10·44	10·13	10·00	10·22	9·88
Methionine	1·27	1·21	1·34	1·23	1·35	1·24
Isoleucine	0·05	< 0·02	0·03	< 0·02	< 0·02	< 0·02
Leucine	13·26	13·42	13·29	13·44	13·50	13·26
Tyrosine	2·78	2·47	2·76	2·75	2·97	2·82
Phenylalanine	6·64	6·80	6·92	6·94	6·87	6·56
Lysine	8·98	9·24	9·13	9·18	9·00	9·20
Histidine	8·30	8·14	8·44	8·03	7·94	8·07
Arginine	2·76	2·99	2·94	2·80	2·92	2·54

* Values represent the average of two determinations.

† Not corrected for decomposition on hydrolysis.

‡ Values in parentheses indicate numbers of residues/molecule of mol.wt. 66 000. The corresponding values obtained by titration in 5 M-urea at pH 5·2 were: $MetA_1$, 4·85; $MetA_0$, 4·47; globin A_1, 4·65.

acid/100 g. of protein on an ash-free and moisture-free basis.

These two fractions and their globins had identical amino acid compositions, which were in turn identical with that of adult haemoglobin A reported by Stein (1958).

Determination of total thiol groups. When haemoglobin is oxidized to MetHb, SH groups can be oxidized, under certain conditions (Taylor, 1958), to the level of disulphides and even to the level of sulphinates or sulphonates. It was therefore necessary to make certain that the equilibria described between the two species of MetHb were not related to the above processes. Titrations in 5 M-urea with *p*-hydroxymercuribenzoate showed an uptake of 4·85 moles/mole of $MetA_1$ of mol.wt. 66 000. The corresponding value for $MetA_0$ was 4·47 moles/mole.

Factors affecting the $A_1 \rightleftharpoons A_0$ transformation

The purified haemoglobin forms were prepared by column chromatography (Fig. 1) and rechromatography on CM-cellulose. The total recovery, based on the extinction at 541 mμ, was 98·2 %, and comprised: A_1^A, 2·45 %; A_1^B, 1·65 %; A_1^C, 11·8 %; A_0, 77·8 %; F_1, 1·47 %; F_0, 0·45 %; A_2, 2·95 %. Non-haemoglobin fractions accounted for 3·9 %. After rechromatography on CM-cellulose, CN-$MetA_1$ and CN-$MetA_0$ were homogeneous by electrophoresis on starch gel containing potassium cyanide (0·02 %) and by immediate rechromatography on CM-cellulose. CN-$MetA_1$ was left in 0·01 M-phosphate buffer, pH 7·3, containing potassium cyanide (0·01 %) (30 ml. containing 2·660 mg./ml.). The solution of CN-$MetA_0$ was adjusted to pH 7·3, by the addition of 0·01 M-sodium dihydrogen phosphate containing potassium cyanide (0·01 %), giving a final solution (30 ml.) of 2·620 mg./ml. The two samples were allowed to stand and 1 ml. portions were withdrawn at intervals and analysed by chromatography on CM-cellulose. The conversion of CN-$MetA_1$ into CN-$MetA_0$ was rapid during the first few days and then became slower. The conversion of CN-$MetA_0$ into CN-$MetA_1$ showed a different behaviour. No CN-$MetA_1$ could be detected in the first 2 weeks, but thereafter it accumulated as shown in Fig. 2. After 200 days the mixture contained about equal amounts of the two forms. The attainment of a mixture containing about equal amounts of the two forms was, however, facilitated by freezing and more so by freeze-drying or treatment with toluene. The effects of freezing, freeze-drying, toluene and cyanide on a solution of $MetA_0$ in water are shown in Table 2. In Table 3 the effects of these treatments on the conversion of $MetA_1$ into $MetA_0$ are shown. Cyanide seemed to facilitate the shift CN-$MetA_1$ → CN-$MetA_0$ only. The converted products appeared in each case to be identical with the original products both electrophoretically and spectrally in the range 230–700 mμ as well as in their chromatographic behaviour. On freezing and thawing, $MetA_1$ was continuously and irreversibly precipitated in appreciable quantities. $MetA_1$ also suffered irreversible precipitation on dialysis, vacuum-evaporation in the cold, or in the presence of toluene. The met form was more susceptible to

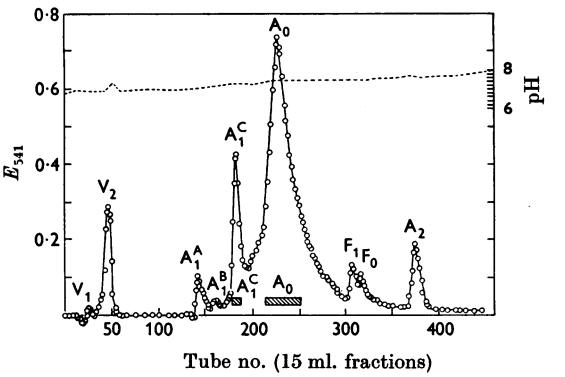

Fig. 1. Chromatogram of CN-MetHb on CM-cellulose. A 1 g. sample of CN-MetHb was chromatographed on a column (2·5 cm. × 60 cm.) by gradient elution from pH 6·8 to 7·7 in 0·01 M-phosphate buffers containing KCN (0·01 %). Experimental details are given in the text.

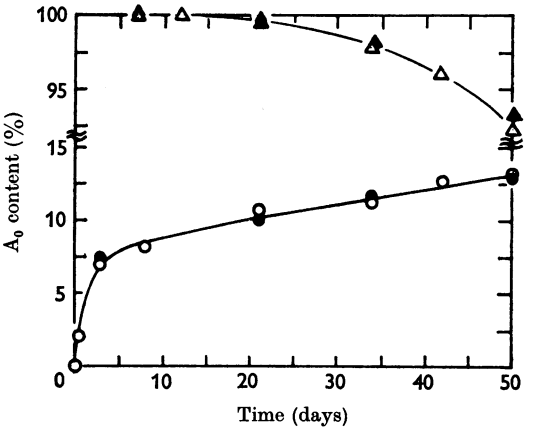

Fig. 2. Transformation of CN-$MetA_1$ and CN-$MetA_0$ at 0–2° in 0·01 M-phosphate buffer, pH 7·32, containing KCN (0·01 %). Samples were analysed by column chromatography (△, ○) and occasionally by starch-gel electrophoresis (▲, ●) for their CN-$MetA_0$ and CN-$MetA_1$ contents, which are expressed as percentages of CN-$MetA_0$ from the total; △, ▲, CN-$MetA_0$ (2·66 mg./ml.) conversion into CN-$MetA_1$ analysed by chromatography or as the average of two independent electrophoretic separations; ○, ●, CN-$MetA_1$ (2·62 mg./ml.) conversion into CN-$MetA_0$ analysed by chromatography or as the average of two electrophoretic separations. Experimental details are given in the text.

this denaturation than the cyanmet form, and when freeze-dried was about 70–80 % insoluble. Under all these conditions, however, $MetA_0$ showed complete solubility. More CN-$MetA_0$ was formed from CN-$MetA_1$ as the concentration of the latter in the starting solution increased (Table 3).

Studies on the nature of the transformation

The possibility of the presence of a monomer $\rightleftharpoons$ dimer/polymer (or sub-units) equilibria was investigated in the ultracentrifuge. With an equilibrium mixture a single sharp peak was obtained which sedimented at the same rate as the single peaks obtained with the chromatographically and electrophoretically homogeneous CN-$MetA_1$ and CN-$MetA_0$ solutions of the same concentration.

$MetA_1$ and $MetA_0$ had identical spectra typical of methaemoglobin in the range 700–230 mμ. However, in 0·01 % cyanide solution (about 700-fold molar excess of cyanide) in phosphate buffer, pH 7·3, both proteins exhibited the typical CN-MetHb spectra, except that fresh CN-$MetA_0$ had a maximum at 420 mμ in the Soret region, whereas CN-$MetA_1$ had a maximum at 415 mμ (Fig. 3). The three chromatographic components of CN-$MetA_1$, namely A_1^A, A_1^B and A_1^C, all gave peaks at 415–416 mμ. The possibility of acid and alkaline methaemoglobin forms (existing despite the presence of cyanide) at equilibrium with each other, like the one reported for myoglobin by Perkoff, Hill, Brown & Tyler (1962), was also ruled out as being the cause of the spectral shift. This was investigated spectrally on solutions of $MetA_1$ and $MetA_0$, with appropriate controls at acid and alkaline pH, in water, before and after the addition of cyanide. To investigate the role of phosphate and chloride in the spectral shift, analysis of solutions in 0·01 M-phosphate at acid and alkaline pH before and after the addition of cyanide and similarly of another set of solutions in 0·15 M-sodium chloride, all with appropriate controls, were carried out. Before addition to haemoglobin samples, cyanide solutions were either at pH 7·0 in 0·01 M-phosphate buffer or adjusted to that pH with dilute hydrochloric acid. Spectra of CN-$MetA_0$ solutions were all identical, having a peak at 420 mμ. On the other hand, all spectra of $MetA_1$ (except for those at acid pH which were denatured) showed a peak at 415 mμ. This ruled out the possibility that binding of phosphate or chloride caused this spectral shift. It was, however, possible to effect a shift from 415 to 420 mμ by the addition of a large excess of cyanide. Fig. 4 shows the change in position of the Soret peak of $MetA_1$ and $MetA_0$, both in 0·01 M-phosphate buffer, pH 7·3, on the addition of buffered cyanide. $MetA_0$ ($\lambda_{max.}$ 409 mμ) was already completely converted into the cyanmet derivative ($\lambda_{max.}$ 420 mμ) in the presence of 100-fold molar excess of potassium cyanide. At the same

Table 2. *Conversion of methaemoglobin A_0 into methaemoglobin A_1*

The MetHb concentration was 2·60 mg./ml. Experimental details are given in the text. Treatment was carried out on the met form, but after chromatography or electrophoresis the fractions were in their cyanmet forms. The values represent the average of two duplicate chromatographic separations and two independent starch-gel-electrophoretic determinations.

Treatment	Time (days)	CN-MetA formed (%) Treated	Untreated control
Freezing (10 ×)	3	39·5	2
Freeze-drying	—	52·9	—
Toluene*	2	49·1	0
0·08 M-KCN	1	0	0

* After the removal of the toluene layer, solutions were dialysed against 12 changes of buffer before chromatography or electrophoresis.

Table 3. *Conversion of methaemoglobin A_1 into methaemoglobin A_0*

The MetHb concentration was 2·66 mg./ml. Conditions were as given in Table 2.

Treatment	Time (days)	CN-$MetA_0$ formed (%) Treated	Untreated control
Freezing (1 ×)	200	4	42·1
Freezing (10 ×)	3	36·1	7·4
Freeze-drying	—	35·5	—
Toluene	2	52·0	6·3
0·08 M-KCN	1	30	4·9
Effect of concentration of CN-$MetA_1$			
190 μg./ml.	112	—	4·1
2660 μg./ml.	112	—	25·0

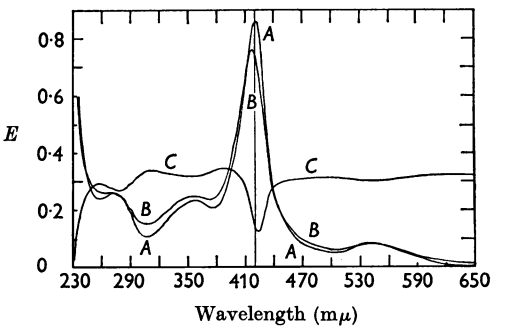

Fig. 3. Spectra of freshly prepared CN-$MetA_0$ (*A*), CN-$MetA_1$ (*B*) and their difference spectrum (*C*). Solutions were in 0·01 M-phosphate buffer, pH 7·3, containing KCN (0·01 %), and contained 120 μg./ml. Spectra were read in a Cary model 14 spectrophotometer. CN-$MetA_1$ has a Soret band at 415 mμ and CN-$MetA_0$ one at 420·5 mμ.

cyanide concentration, $MetA_1$ showed a shift from 409 to only 415 mμ, and gave spectra with $\lambda_{max.}$ at 420 mμ only at cyanide concentrations greater than 3100 moles of potassium cyanide/mole of $MetA_1$. As mentioned above, the addition of this excess of cyanide caused a faster formation of CN-$MetA_0$ (Table 3).

Figs. 5 and 6 show the effect of pH change on the spectra of CN-$MetA_1$ and CN-$MetA_0$ in 0·01 M-phosphate containing potassium cyanide (0·01 %; 700-fold molar excess). CN-$MetA_0$ could be brought to any pH between 10·5 and 3·0 without suffering any detectable irreversible damage. At pH values below 4·12 a Soret peak shift towards the ultraviolet was observed (e.g. $\lambda_{max.}$ at pH 3·4 was at 368 mμ; Fig. 6). This shift, which was completely reversible on readjustment of the pH to neutrality, might indicate haem dissociation taking place at the acid pH. On the other hand CN-$MetA_1$, at pH values lower than 6·7, lost all spectral characteristics typical of cyanmethaemoproteins, giving a non-specific steady increase in extinction below 380 mμ (Fig. 5). This spectral change was not reversible on readjustment of the pH to 7·0 or higher, or on the addition of more cyanide. CN-$MetA_1$ also suffered excessive precipitation under these low pH conditions and also after readjustment of the pH to 7·0 or higher. These irreversible changes at pH values below neutrality again point to the instability of CN-$MetA_1$ and its ease of denaturation. The Soret peak of both $MetA_1$ and $MetA_0$ could be shifted to a lower wavelength by the addition of salts (409 mμ in 0·01 M-phosphate to 408 mμ in 0·05 M-phosphate, or to 406 mμ in 0·15 M-sodium chloride at the same pH).

It is significant that freshly reconstituted haemoglobin from haem and soluble globin was completely in the A_0 form as indicated by starch-gel electro-

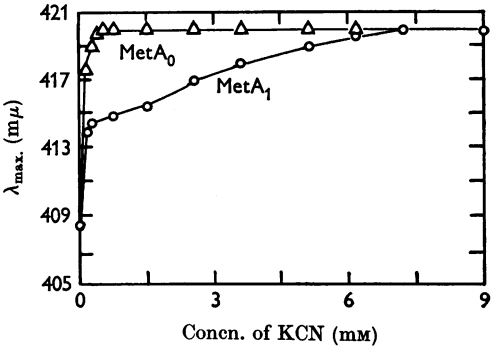

Fig. 4. Change in position of the Soret peak on the addition of buffered cyanide. Experiments were carried out on 430–450 μg. of MetHb in 0·01 M-phosphate buffer, pH 7·30. The position of the Soret peak is plotted against cyanide concentration. △, $MetA_0$; ○, $MetA_1$.

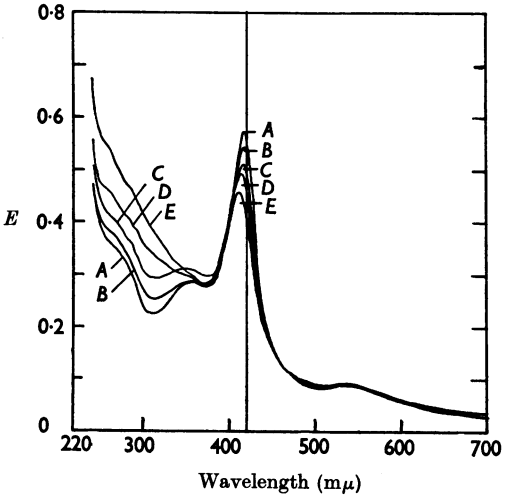

Fig. 5. Effect of change in pH on the spectrum of CN-$MetA_1$ in 0·01 M-phosphate buffer, containing KCN (0·01 %), at: pH 7·5 (*A*); pH 8·05 (*B*); pH 10·0 (*C*); pH 6·73 (*D*); pH 6·08 (*E*). The spectra *D* and *E* did not change (i.e. still lacked any definition below 380 mμ) on readjustment of the pH to 7·0 or higher, or on the addition of more cyanide to bring it to a concentration of 0·5 % KCN.

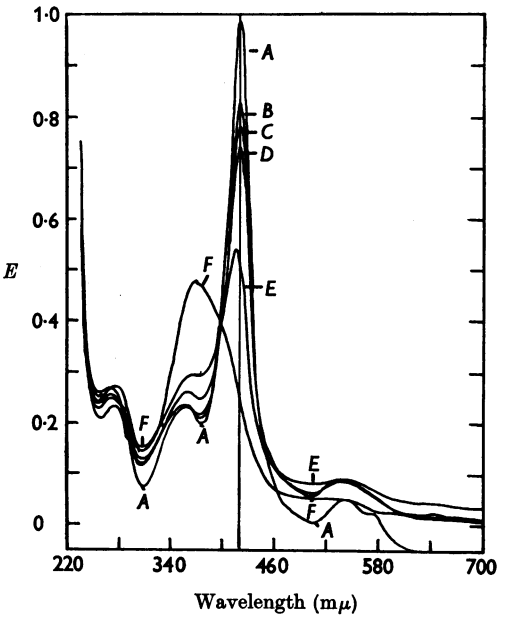

Fig. 6. Effect of the change of pH on the spectrum of CN-$MetA_0$ in 0·01 M-phosphate buffer, containing KCN (0·01 %), at: pH 9·63 (*A*); pH 8·50 (*B*); pH 6·35 (*C*); pH 5·20 (*D*); pH 4·12 (*E*); pH 3·40 (*F*). On readjustment of the pH of *F* to 5·2 or higher, the spectrum became similar to *A*–*D*.

phoresis and by chromatography. However, on standing in the CN-MetHb form at pH 7·0 in 0·05 M-phosphate containing potassium cyanide (0·01 %), at a concentration of 2·2 mg./ml., it suffered the usual transformation into CN-$MetA_1$, reaching a 1:1 mixture in 6–7 months.

Titration of the available thiol groups

Reaction of p-*hydroxymercuribenzoate with methaemoglobins A_1 and A_0.* The titration of pure $MetA_0$ was not difficult since its conversion into $MetA_1$ was not rapid. On the other hand, the titration of pure $MetA_1$ proved difficult owing to its fast conversion into $MetA_0$ when it is taken through the necessary steps of concentration and removal of cyanide. Nevertheless, by taking portions of the samples to be titrated and subjecting them to chromatography on the analytical CM-cellulose column and/or four simultaneous electrophoretic runs on starch gel and by scanning the latter after staining, the relative proportions of the two components in the mixture could be calculated. Table 4 shows the results of the titration of five mixtures of different composition.

When the values in Table 4 were plotted and the straight line was extrapolated to 100 % of $MetA_1$ and 100 % of $MetA_0$, the values for *p*-hydroxymercuribenzoate uptake were 0·20 and 1·96 moles/mole of $MetA_1$ and $MetA_0$ respectively.

Reaction with N-*ethylmaleimide.* The *N*-ethylmaleimide uptake, determined by the method of Benesch & Benesch (1961), was 1·88 moles/mole for a mixture containing 90·5 % of $MetA_0$. In another experiment a solution of MetHb mixture in 0·01 M-phosphate buffer, pH 7·19 (1·0 g. in 39 ml.), was mixed with a tenfold molar excess of *N*-ethylmaleimide (1·5 ml. of 0·1 M-*N*-ethylmaleimide) and the solution allowed to stand for 2 hr., after which it was equilibrated with 0·01 M-phosphate buffer, pH 6·8, containing potassium cyanide (0·01 %), on Sephadex G-25, and immediately subjected to chromatography on CM-cellulose. Two peaks were obtained. The first peak (30·0 % of the total) was eluted completely at an effluent pH value of 7·38, and was identical with CN-$MetA_1$ spectrally and electrophoretically. After concentration on CM-cellulose and conversion into its met form in 0·05 M-phosphate, it suffered 27·9 % transformation into $MetA_0$ and had 0·88 titrable SH group/molecule of haemoglobin. The second peak (68·2 % of total recovery) was eluted at an effluent pH value of 7·50, at its apex. It had an electrophoretic mobility of 0·96 (relative to that of CN-$MetA_0$) at pH 8·8 and gave a peak in the Soret region at 420·5 mμ. When concentrated and titrated with *p*-hydroxymercuribenzoate in 0·05 M-phosphate buffer, pH 7·20, no titratable SH groups were detected. On titration with *p*-hydroxymercuribenzoate in 5 M-urea at pH 5·2, 1·56 moles/mole of $MetA_0$–*N*-ethylmaleimide were consumed.

Reaction with iodoacetamide. Titration of MetHb, to which a tenfold molar excess of iodoacetamide had been added, with 0·01 N-sodium hydroxide to keep the pH constant at 7·00, gave unsatisfactory results. These were thought to be due to the interference of other groups like the sulphur of methionine residues, which might be expected to react at neutral pH (Gundlach, Moore & Stein, 1959). It is well known also that, when proteins are treated with iodoacetate to block the thiol groups, quantitative analyses for methionine give results that are occasionally slightly low (Gundlach *et al.* 1959). This is due to the formation of a methionine sulphonium salt that will decompose on acid hydrolysis. The products of decomposition of the sulphonium salt of free methionine have been identified by Gundlach

Table 4. *Titration of equilibrium mixtures of methaemoglobins A_1 and A_0 of different compositions with* p-*hydroxymercuribenzoate*

Experimental details are given in the text.

$MetA_0$ in mixture (%)	Uptake of *p*-hydroxymercuribenzoate (moles/mole of MetHb)
90·8*†	1·74
	1·74
80·0*	1·56
68·8*†	1·42
41·1*†	0·97
38·2*	0·88

* Values represent the average of four independent electrophoretic determinations.

† Values represent the results of one chromatographic determination.

Table 5. *Labelling of haemoglobins A_1 and A_0 with chromium*

A 20 ml. sample of haemoglobin (0·31 μM) was mixed with a tenfold molar excess of 1·25 mM-$K_2{}^{51}CrO_4$ (2·228 μC/ml.) and dialysed before counting. Experimental details are given in the text.

	Haemoglobin	Net radioactivity (counts/min.)	Total radioactivity (μC)	Chromium bound (atoms of Cr/mole)	HbA_0/HbA_1 molar binding ratio
Expt. 1	HbA_0 (11·7 mg.)	8806	0·683	2·17	1·23
	HbA_1 (11·2 mg.)	6892	0·540	1·79	
Expt. 2	HbA_0 (17·35 mg.)	4307	0·976	2·09	1·19
	HbA_1 (17·35 mg.)	3454	0·820	1·76	

et al. (1959) as *S*-carboxymethylhomocysteine and homoserine, together with some regenerated methionine. However, as pointed out by these authors, a sulphonium salt bound in peptide linkage may decompose differently from the salt of free methionine.

Amino acid analyses of a MetHb mixture, containing 39·5 % of $MetA_1$, which had been treated with iodoacetamide showed that the half-cystine value decreased from 1·06 g./100 g. of protein (equivalent to 5·84 residues/molecule of MetHb) to 0·636 g./100 g. (3·50 residues/molecule), i.e. a loss of 2·34 SH groups/molecule. This decrease was accompanied by the appearance of *S*-carboxymethylcysteine equivalent to 2·3 SH groups/molecule of MetHb. On the other hand, methionine decreased from 1·34 g./100 g. of protein (5·93 residues/molecule) to 0·968 g./100 g. of protein (4·28 residues/molecule), i.e. a decrease of 1·65 residues/molecule. An approximately equivalent amount of methionine sulphone appeared. These values represent the average of two independent amino acid determinations and two independent reactions with iodoacetamide. It is not possible, from these experiments, to determine if one or both of the haemoglobin species reacted with iodoacetamide.

Labelling of haemoglobin with ^{51}Cr

To ensure that haemoglobin was completely saturated with chromium, ^{51}Cr was diluted with ^{52}Cr. The final solution contained 1·25 mM-potassium chromate. Table 5 shows that 2·17–2·09 Cr atoms were bound/molecule of $MetA_0$ and 1·79–1·76 Cr atoms/molecule of $MetA_1$. Similar results were obtained when oxyhaemoglobins A_0 and A_1 were labelled with ^{51}Cr.

DISCUSSION

The $A_1 \rightleftharpoons A_0$ transformation suggests that there is an equilibrium in solution between the two forms of adult human haemoglobin and it throws considerable light on some hitherto unexplainable phenomena. Because this conversion is highly dependent on the concentration of haemoglobin and is influenced by organic solvents, the interpretation of some previous reports, where conditions were not standardized, on contents of haemoglobins A_1 and A_0 in various pathological blood specimens becomes difficult. The contents of these haemoglobins will probably depend on, among other factors, the amount of water used for lysing blood cells, whether or not toluene was employed, the concentration of buffers, salts and their ionic species, and possibly anticoagulants. These contents will also depend on the presence and concentration of cyanide in solution, and on the time elapsed during the preparation. This two-way transformation also poses the question as to which form is actually made in the body and which one is present in the blood cell. Experiments with [^{14}C]valine suggest that haemoglobin A_0 is made first and that it undergoes modification on aging of the cell whereby it is changed to a more negatively charged species (Rosa, Dreyfus & Schapira, 1960). Recent studies on the denaturation of haemoglobin in the single erythrocyte (Matioli & Thorell, 1963) seem to indicate that three differently reacting types of haemoglobin coexist in the single cell from human foetal or adult chicken blood. This $A_1 \rightleftharpoons A_0$ transformation might be one explanation for the well-known antigenic heterogeneity of chromatographically purified adult haemoglobin.

Benesch & Benesch (1962*b*) observed that fresh adult human deoxyhaemoglobin shows an increase in the number of its SH groups reactive with *N*-ethylmaleimide on standing for 5 days. This might be due to some form of transformation. Their observation of the dependence of the number of the reactive SH groups on the anticoagulant used might be due to the effect of the anticoagulant on the $A_1 \rightleftharpoons A_0$ transformation. Haemoglobin A_0 showed more stability and resistance to denaturation. As was to be expected, freshly reconstituted haemoglobin was completely in the A_0 form. This was most likely due to the irreversible denaturation of any haemoglobin A_1 during treatment with acid acetone.

The results of cyanide titration could be due to the different nature of the environment of approximately half the haem groups in the two forms. In the present work, $MetA_1$ (or CN-$MetA_1$) had an electrophoretic mobility of 1·14 (relative to that of $MetA_0$) towards the anode at pH 8·6. Assuming equal interactions with starch for the two haemoglobin forms and also that haemoglobin A_0 carries a net of 14 negative charges (Scheinberg, 1958), it was deduced that haemoglobin A_1 had two more negative charges than haemoglobin A_0. It is therefore likely that the configurational changes responsible for the appearance of two negative charges on the molecule are also responsible for the unavailability of certain haem groups to cyanide. The possibility should not be excluded, however, that the larger excess of cyanide required by $MetA_1$ to shift the Soret peak from 409 to 420 mμ might be due to the reaction of cyanide with other available groups and that the peaks at 415 and 420 mμ may represent, in fact, complete haem saturation with cyanide for both CN-$MetA_1$ and CN-$MetA_0$. If the two additional negative charges in A_1 are presumed to arise from two S^- groups, the unavailability of the latter to thiol reagents is understandable since these reagents (except for iodoacetamide) react with SH rather than S^- groups. A strong positive environment

near the SH groups of A_1 could account for their existence as S^- groups considerably below their pK.

Reports that A_1 is responsible for the ^{51}Cr-binding capacity of haemoglobin do not take into account the possibility that Cr-labelled A_0 is more negatively charged than A_0 and migrates with A_1 on electrophoresis. It was difficult, therefore, to determine the amount of A_1 that was converted into A_0 after chromatography and during the labelling procedure. However, the results point strongly to the possibility that A_1 does not bind chromium, contrary to other reports (Meyering *et al.* 1960; Malcolm, Ranney & Jacobs, 1963). Pearson, Van Metre & Vertrees (1963) found no significant difference in binding capacity of haemoglobins A_0 and A_1 isolated before labelling. Their results did not take into account the then unknown $A_1 \rightleftharpoons A_0$ transformation. The labelling has been identified in the β-chains (Heisterkamp & Ebaugh, 1962). It is not yet clear whether the mechanism responsible for unmasking the SH groups is also responsible for the affinity for chromium.

SUMMARY

1. The two forms of adult human haemoglobin, A_1 and A_0, have identical amino acid composition, and (in their met, cyanmet or oxy forms) are interconvertible, the conversion being influenced by the concentration of haemoglobin, chloride and cyanide. The attainment, from one component, of a mixture containing equal amounts of the two forms is facilitated by toluene, freezing and freeze-drying.

2. Methaemoglobin A_1 requires a large excess of cyanide, as compared with methaemoglobin A_0, to reach complete cyanide saturation as indicated by a shift of the Soret peak from 409 to 420 mμ. Chloride, phosphate and pH play no part in this spectral shift, although they have some influence on the spectra of methaemoglobins A_1 and A_0, and in the case of pH on cyanmethaemoglobins A_1 and A_0. Aggregation or dissociation to sub-units was not responsible for the $A_1 \rightleftharpoons A_0$ transformation.

3. The met, cyanmet or oxy forms of haemoglobin A_1 have two more negative charges than those of haemoglobin A_0 and are more easily denatured. Methaemoglobin A_0 carries two titratable SH groups that react with *N*-ethylmaleimide, *p*-hydroxymercuribenzoate and iodoacetamide, whereas methaemoglobin A_1 has no titratable SH groups. Methaemoglobin A_0 or oxyhaemoglobin A_0 has a higher binding capacity for chromium than have the corresponding forms of haemoglobin A_1.

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