The Haem-Globin Linkage

1. ARTIFICIAL HAEMOGLOBINS FROM HAEMATINS LACKING FREE CARBOXYL GROUPS

By J. E. O'HAGAN

Biochemistry Department, Red Cross Blood Transfusion Service, Brisbane, Queensland, Australia

(Received 20 May 1959)

Differing opinions have been expressed as to which groups of the haems are essential for coupling to globin. Thus Haurowitz & Waelsch (1929) claimed to have prepared from native globin and dimethyl-mesohaemin (mesohaemin with esterified propionate groups) a haemoglobin capable of reversible combination with molecular oxygen, and Haurowitz (1949) described use of the same compound to demonstrate that ferrohaemoglobin contained a water molecule co-ordinated with the iron. On the other hand, Granick & Gilder (1946) considered that 'the co-ordination of the central iron atom is not sufficiently strong by itself to bind the haem', and Blyumenfeldt (1952) believed that the main linkages were between carboxyl groups of the haems and basic groups in the globin.

In an attempt to reconcile the conflicting concepts: (1) that an iron-protein link was adequate, and (2) that haem propionate-protein links were necessary, Lemberg & Legge (1949) suggested that the ester might have been hydrolysed in the process of combination. To settle in an unequivocal manner whether haematins lacking free carboxyl groups could combine with native globin, aetiohaemin (with inert methyl and ethyl side chains, see Fig. 1) was employed, some preliminary results with which have already been reported (O'Hagan, 1950, 1951, 1955). Haurowitz's finding that artificial haemoglobins can be prepared from methylated haemins has been confirmed and the studies have been extended to experiments which substantiate the hypothesis that whereas the essential linkage for oxygenation is through the iron atom, linkages through the haem propionate side chains may be necessary to impart stability.

METHODS AND MATERIALS

Hydrogen-ion concentration. This was measured with a Jones electronic pH meter (N. L. Jones, Melbourne) calibrated with the phthalate, phosphate and borate buffers described in British Standard 1647 (1950).

Paper chromatography. The technique of Nicholas & Rimington (1949) was used chiefly to ensure that no pigments with free carboxyl groups contaminated the specimens of aetio and methylated porphyrins and haemins. That the spots ran with the solvent front (R_F 1) was interpreted

as meaning that no free carboxyl groups were present, but, of course, did not, without reference to other evidence, prove that the specimens were essentially pure compounds. For the coproporphyrin preparation, the chromatography of the esters as described by Chu, Green & Chu (1951) was performed as a check on purity as the extraction proceeded. The technique was performed at 21° and it was found best to view the spots under u.v. light before the solvent dried.

Measurement of spectra. A Hilger Uvispek spectrophotometer was employed; the wavelength scales were calibrated against a mercury arc, the extinction scales against the copper sulphate and cobalt sulphate solutions of Gibson (1950). The glass prism was used for all experiments reported here; the wavelength error over the range 390–1100 m μ was $\pm 1\text{\AA}$ and, after realignment of the attenuator network, the extinction scales were within the manufacturer's tolerance of ± 0.0016 unit.

A Beck Hartridge reversion spectroscopy was checked for wavelength accuracy with a mercury-arc lamp; the scale was true to $\pm 1\text{\AA}$ over the range 450–650 m μ .

Chemicals. Methanol A.R. grade was used for the combination experiments. Sodium dithionite (British Drug Houses Ltd.) was dispensed, on opening a 500 g. bottle, into a number of $\frac{1}{2}$ oz. MacCartney bottles. A fresh one of these bottles was used as soon as the granules showed any tendency to stick together, which was taken as an indication of deterioration.

Aetiohaemin III. The method of Schumm (1928, 1929) was used to prepare several batches of the aetioporphyrin from protohaemin, with a yield of 4.6%. The heating in liquid paraffin B.P. (1958) was performed in 250 ml. batches in a 500 ml. round-bottom Pyrex flask fitted with a short wide air condenser, heating with a Tower's Isomantle being controlled by a Simmerstat to keep the temperature between 344 and 352° for 80 min. The flask was occasionally shaken during the period. The porphyrin was twice recrystallized from CHCl_3 -methanol and gave the following absorption bands in solution (Hartridge reversion spectroscopy). In 10% HCl: I, 590.5; II, 547.4 m μ (order of intensity: II > I). In CHCl_3 : I, 620.0; II, 570.1; III, 532.7; IV, 497.6 m μ (decreasing order of intensity: IV, III, II, I). In ether: I, 622.3; II, 611.8; III, 597.8; IV, 577.9; V, 569.8; VI, 559.0; VII, 527.3; VIII, 496.2 m μ (decreasing order of intensity: VIII, VII, V, I, IV, III, II, VI). Bands III, II and VI were of very low intensity and were just detectable in saturated solutions. They probably originate from a trace of another porphyrin as impurity. In conc. H_2SO_4 : I, 592; II, 549 m μ (order of intensity: II > I). These measurements compared well with those given by Schumm (1929) and by Fischer & Treibs (1930).

On paper chromatography with lutidine-water-NH₃ by the method of Nicholas & Rimington (1949) the porphyrin repeatedly gave a single spot with R_F 1.0 at 21°. Kehl & Stich (1952), using 2:4-lutidine-water at 15°, found R_F 0 for aetioporphyrin (isomer unspecified), with slight movement from the point of application at 25°, but they found that copro- and uro-porphyrin esters moved with R_F 1.0. The difference could be due to the isomer used, or result from adsorption on application to the paper. It may perhaps be explained by the finding of R_F 0 when CHCl₃ was used as a solvent for the aetioporphyrin, for when pyridine was employed the porphyrin ran with the solvent front; the presence or absence of an NH₃ atmosphere made no difference with this porphyrin. By the method of Chu *et al.* (1951) the porphyrin had R_F 1.0 at 21°. The aetioporphyrin

was completely insoluble in dilute aqueous alkali, further indicating no trace of pigments with carboxylic acid groups.

The haemin was prepared from the porphyrin by introducing iron by the method of Zaleski (1904). The absorption spectra then obtained were as follows. In CHCl₃: I, 641.0; II, 543.0; III, 501 m μ onward (decreasing order of intensity: II, I, III). In pyridine: I, 632.0; II, 556.5; III, 521.5 m μ measured immediately (decreasing order of intensity: III, II, I). In pyridine + hydrazine hydrate: I, 546.3; II, 515.3 m μ (order of intensity: II > I).

The spectrum in CHCl₃ was close to that given by Fischer & Orth (1937) but the spectra of the pyridine solutions did not agree well. This may have been due to change of position of these bands with time or concentration or both.

Protohaemin IX

Mesohaemin IX

Aetiohaemin III

Coprohaemin III

Fig. 1. Structures of haemins mentioned in text.

Chromatography with lutidine-water-NH₃ gave R_F 1.0 at 21°, and no traces of haemins soluble in aqueous alkali were present.

Dimethylprotohaemin IX. Two methods of preparation were employed: A, esterification of haemin with methanolic HCl by the method of Küster & Schlayer (1927); B, by insertion of iron into the dimethylprotoporphyrin prepared by the method of Grinstein (1947). The porphyrin gave absorption maxima agreeing with those of Grinstein, namely: I, 631.1; II, 575.0; III, 541.3; IV, 507.4; V, 407.3 m μ (decreasing order of intensity: V, IV, III, I, II). Lutidine-water-NH₃ chromatography gave a single spot, R_F 1.0 at 21°. Iron was introduced by solution of the porphyrin in boiling CHCl₃ and addition of a freshly prepared boiling ferrous acetate solution in acetic acid saturated with NaCl. After cooling, the dimethylprotohaemin was taken into ether, the acid removed with dilute sodium acetate solution and then water, and the ether was evaporated.

Both preparations gave the following absorption spectra. In CHCl₃: I, 635.8; II, 547.5; III, 505 m μ (decreasing order of intensity: II, I, III). In pyridine + hydrazine hydrate: I, 556.8; II, 527.0 m μ onward (order of intensity: II > I). The second spectrum compared fairly well with that given by Fischer & Orth (1937), namely: I, 560; II, 527.5 m μ , but the spectrum in CHCl₃ (as with that obtained above for actiohaemin in pyridine) was considerably different from that listed by Fischer & Orth, namely: I, 625; II, 565; III, 515 m μ . Both preparations A and B gave the same spectral peaks, so it is most likely that the Fischer & Orth figures are in error. Chromatography of preparation A at 21° with lutidine-water-NH₃ gave one major component with R_F 1.0 and a trace of a component with R_F 0.7, probably some free protohaemin. Preparation A was used for the combination experiments with globin.

Dimethylmesohaemin IX. Mesoporphyrin IX was prepared from dimethylprotoporphyrin IX by the method of Grinstein & Watson (1943). It had, on chromatography with lutidine-water-NH₃, R_F 0.8 at 21°. It was converted into the dimethyl ester by treatment with methanolic HCl overnight, the mixture diluted with water, the ester taken into CHCl₃, the solution dried with anhydrous Na₂SO₄ and crystallized after addition of methanol and concentration on the hot plate. The ester gave a single spot with R_F 1.0 on chromatography with lutidine-water-NH₃ at 21°. Addition of iron, as described for dimethylprotohaemin, did not alter the R_F of the single spot obtained, and it was completely insoluble in dilute aqueous alkali.

Tetramethylcoprohaemin III. The porphyrin was prepared from the Mg(OH)₂ adsorbate of the porphyrins obtained during the preparation of *Corynebacterium diphtheriae* toxoid as described by Gray & Holt (1948). The adsorbate was supplied through the courtesy of Mr R. W. Patterson of the Commonwealth Serum Laboratories, Melbourne. During the extraction it was found that some of the red pigment present was not behaving as a normal porphyrin and could not be removed with acid, but was soluble in 2% (w/v) NaOH. After passage through ether and removal of all fluorescent porphyrin with 2–10% (w/v) HCl, the pigment could be eluted from the ether with 2% NaOH and precipitated from this by acidification with HCl. In the 2% NaOH the spectrum had peaks at 564.0 and 529.2 m μ . After being washed and dried, the precipitated pigment dissolved in dioxan had peaks at 562.3

and 526.3 m μ , which suggested that the pigment was essentially copper coproporphyrin, since a solution of this complex porphyrin prepared by the method of Fischer & Orth (1937) from some of the free coproporphyrin from the main batch had peaks in the same position. Enquiry from Mr Patterson revealed that the method used by Gray & Holt had been modified to increase the toxoid yield by the addition of 10 ml. of 1% (w/v) CuSO₄/l. of medium, as recommended by Mueller (1939). The free porphyrin could be obtained from the copper complex by solution in conc. H₂SO₄, immediate dilution with water and precipitation at pH 3.4. The porphyrin was esterified with methanolic HCl, chromatographed on CaCO₃ from benzene and extracted with CHCl₃-benzene (1:10, v/v). This chromatographed material from the copper-complex fraction still contained a white impurity of waxy nature and was not used for later work. Paper chromatography of the porphyrin by the method of Chu, *et al.* (1951) revealed two spots, a major one with R_F 0.63 and a minor one with R_F 0.37 (probably from tetramethylcoproporphyrin and hexamethylcarboxylic acid porphyrin). Spots with these values and one of 0.21 (octamethyluroporphyrin I) were obtained from the crude porphyrin of the main batch before chromatography on CaCO₃.

The main batch of the porphyrin ester (the first from the CaCO₃ column) was rechromatographed on CaCO₃ and recrystallized from methanol-CHCl₃. After drying in a vacuum desiccator over silica gel, the solid had m.p. 147°, with remelt at 179.5° (uncorr.), which compared well with the values of Jope & O'Brien (1945).

To prepare the haemin, 10 mg. of the ester was dissolved in acetic acid saturated with NaCl, and freshly prepared ferrous acetate in acetic acid was added at the boiling point. No crystals appeared after 2 days, so CHCl₃ was added, the mixture washed with water and the CHCl₃ evaporated. The tetramethylcoprohaemin III was then used without further purification. It was completely insoluble in aqueous alkali.

Coprohaemin III. A portion (5 mg.) of tetramethylcoprohaemin III was heated with 10% (w/v) NaOH for several hours (Fischer & Orth, 1937), the hydrolysate of the ester precipitated with dilute HCl and the free coprohaemin taken into pyridine. Crystallization was attempted from acetic acid saturated with NaCl. After 2 weeks, with gradual evaporation of the acetic acid, no crystals had appeared, so ether and water were added; the coprohaemin precipitated in the aqueous phase and was collected, dried over NaOH under vacuum and used without further purification.

The peaks in the absorption spectra from the free coprohaemin were: in pyridine: I, 557; II, 524.5 m μ (order of intensity II > I); in pyridine + hydrazine hydrate: I, 547; II, 518 m μ onward (order of intensity II > I). These compare well with the values of Schumm (1927) for the pyridine haemochrome.

Native globin. Considerable difficulty was experienced with this preparation since the methanol used to effect attachment of the haematin, the sodium dithionite used for reduction and changes in H⁺ ion concentration often precipitated traces of denatured globin that were present in solution, and made exact spectrophotometric studies impracticable. The addition of buffers in the presence of methanol, although permissible with apomyoglobin (O'Hagan & George, 1960), produced clouding, so buffered

solutions were not used, the ionic strength being kept as low as possible and the pH measured after haematin combination had occurred. Several methods of globin preparation were tried, that of Joep, Joep & O'Brien (1949) proving most suitable. However, after adjustment of the native globin solution to the isoelectric point of the denatured material (pH 7.8), addition of $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation and precipitation of denatured globin, a perfectly clear solution was obtained, but, after dialysis at 1° to remove $(\text{NH}_4)_2\text{SO}_4$ [in the equipment described by O'Hagan (1959*a*)], adjustment to the isoelectric point at room temperature (21°) brought down more precipitate]. Rossi-Fanelli, Antonini & Caputo (1958*a*), preparing globin from crystalline human haemoglobin, also found this precipitation when the solutions were brought to room temperature. The concentration of the globin was measured by the micro-Kjeldahl technique. All the work reported here was performed with human globin. The reputedly more stable horse and ox globin were tried but gave similar trouble.

EXPERIMENTAL AND RESULTS

Aetiohaemoglobin, mesohaemoglobin and derivatives

For some experiments, a solution of 5 mg. of aetiohaemin in 3.5 ml. of warm methanol was first made, chilled to 0° and added slowly to 50 ml. of 0.34% globin in water in a 100 ml. conical flask that was swirled in ice-water. The solution was allowed to come to room temperature, filtered and spun at 18 000 *g* for 10 min. to remove uncombined aetiohaemin. Since a variable fraction of the aetiohaemin precipitated, and since from the absorption curves it appeared that another fraction did not combine (but did not precipitate), it was considered unjustifiable to attempt to obtain quantitative absorption curves or determine extinction coefficients. Therefore most curves were obtained by adding a saturated solution of the haemin in methanol to a slight excess of native globin solution and centrifuging. The best absorption curves obtained for the oxyaetiohaemoglobin and the ferroaetiohaemoglobin are shown in Fig. 2.

The spectra closely resembled those obtained from mesohaemoglobin derivatives in the position of the absorption peaks but were not so sharp, as can be seen from Fig. 3.

Difficulty was experienced with the dithionite, as has been noted by Dalziel & O'Brien (1957), who found that even native haemoglobin was irreversibly affected. Numerous attempts failed to obtain the α - and β -oxyhaemoglobin peaks at the same relative height as in normal oxyhaemoglobin. This may have been due to the presence of some uncombined aetiohaemin or of a compound of the type described by Dalziel & O'Brien. Recombination appeared best at about pH 5.5–6.0, but this may have been because at lower H^+ ion concentrations traces of denatured protein precipitated.

The oxyaetiohaemoglobin appeared more unstable than oxymesohaemoglobin and auto-oxidized more rapidly. That a true oxyhaemoglobin was present was confirmed by the reversion of the two-banded spectrum to the one-banded reduced spectrum when the solution was placed under a vacuum of 5 mm. Hg for 45 min., and the re-appearance of the oxyhaemoglobin type of spectrum on admission of air.

Carboxyaetiohaemoglobin

By reduction of a solution of ferriaetiohaemoglobin with dithionite and the passage of coal gas for a few minutes carboxyaetiohaemoglobin was obtained with absorption curve as shown in Fig. 4.

Fig. 2. Qualitative absorption spectra of aetiohaemoglobin derivatives: ferriaetiohaemoglobin (—), ferroaetiohaemoglobin (---), oxyaetiohaemoglobin (····). pH 5.6; no added buffer.

Fig. 3. Qualitative absorption spectra of ferrimesohaemoglobin (—) and ferriaetiohaemoglobin (---). pH 7.0, no added buffer; solution was clarified by centrifuging at 12 000 *g* for 5 min.

Fig. 4. Qualitative absorption spectrum of carboxyaetio-haemoglobin. pH 7.0, no added buffer.

Dimethylprotohaemoglobin and derivatives

Dimethylprotohaemoglobin and the corresponding dimethylmesohaemoglobin were prepared by dissolving the haemins in methanol to form saturated solutions and adding 5 ml. of such solutions to 50 ml. of 0.34 % globin solution, under the same conditions as for aetiohaemoglobin. The position of the peaks found for the dimethylmesohaemoglobin were the same as those of Haurowitz & Waelsch (1929), and for dimethylprotohaemoglobin they were as follows:

ferridimethylprotohaemoglobin, 631.5 $m\mu$;
ferrodimethylprotohaemoglobin, 561.5 $m\mu$;
oxydimethylprotohaemoglobin, I, 576.8,
II, 541.0 $m\mu$ (Hartridge reversion spectroscopy).

Oxytetramethylcoprohaemoglobin III and oxycoprohaemoglobin III

Combination experiments with tetramethylcoprohaemin III and coprohaemin III gave similar spectra to those obtained with the dimethylmesohaemin, as follows:

oxycoprohaemoglobin III, I, 568; II, 533.5 $m\mu$;
oxytetramethylcoprohaemoglobin III, I, 567;
II, 532 $m\mu$ (Hartridge reversion spectroscopy).

Reduction with sodium dithionite gave the typical broad-banded spectra of the ferrohaemoglobins. Unfortunately precipitation set in on the several occasions when these experiments were carried out, so that it was not possible to obtain a reliable absorption curve, and the small quantity of the haemins available did not permit further attempts.

DISCUSSION

The question whether it is legitimate to draw conclusions from experiments with recombined and artificial haemoglobins, in order to interpret the structure of native haemoglobin, must be considered. There would appear to be abundant evidence that recombination can produce a haemoglobin very closely resembling the native molecule. Roche & Combette (1937) observed resemblance in crystalline form, oxygen affinity, solubility in ammonium sulphate solutions and molecular weight (by osmotic-pressure measurements). Gralén (1939) found the same molecular weight (diffusion measurements), pH-stability diagram and molecular shape (ultracentrifuge sedimentation). However, slight differences showed up on electrophoresis (Gralén, 1939), on splitting with bases (Roche & Combette, 1937) and on exact ultraviolet spectroscopy (Hill & Holden, 1926; Joep, 1949). Joep *et al.* (1949) prepared a crystalline recombined haemoglobin spectroscopically identical with native haemoglobin, and it is very likely that the other differences would have been resolved had their method of preparation been used by earlier workers. In fact, Rossi-Fanelli, Antonini & Caputo (1958b) have claimed to have prepared such recombined haemoglobin, identical with native haemoglobin in absorption spectra, isoelectric point, electrophoretic mobility, sedimentation and diffusion, stability to heat and alkali, haem-haem interaction, Bohr effect and oxygen affinity.

It would therefore appear reasonable to interpret experiments with other haematin in place of protohaematin on the basis that corresponding haemoglobins are formed in those cases where similar spectral-absorption curves are obtained. It should be pointed out, however, that no experiments have yet been reported to show that haemoglobins prepared from these haematin have the same molecular weight as protohaemoglobin, and, since globin (mol.wt. 34 000; Gralén, 1939) dimerizes on recombination with protohaematin, it may well be that some of the side chains effect this change.

Oxyhaemoglobins can be prepared from mesohaemin and haematohaemin (Hill & Holden, 1926), rhodohaemin, diacetyldeuterohaemin, *Spirographis* haemin and pheohaemin b (Warburg & Negelein, 1932), so that it is evident that vinyl groups are not essential for reversible oxygenation, at least *in vitro*. Callaghan & O'Hagan (1949) noticed differences in the rate of reduction and subsequent uptake of oxygen when recombined ferriproto- and ferrimeso-haemoglobin were incubated with ascorbic acid at pH 7.2 in 0.05M-phosphate buffer. The ferriprotohaemoglobin took 15 min. and the meso-compound 90 min. to change to the oxyhaemoglobin. Rossi-Fanelli *et al.* (1958a) found

evidence that the vinyl groups influenced haem-haem interaction, and Rossi-Fanelli & Antonini (1957), working with myoglobin, discovered that deuteromyoglobin had a much higher oxygen affinity than recombined protomyoglobin.

Clearly, the haematin side chains have a marked effect on the oxygenation reaction. One of the problems associated with the formation of the oxyaetiohaemoglobin described above has been the need to use about three times as much dithionite to reduce the aetiohaemoglobin as had been required with the proto- and meso-haemoglobin. This probably represents differences in the oxidation-reduction potential of the iron atom and suggests that the vinyl and propionate groups may interact to provide the ideal supplement to the resonating structure of the porphyrin ring to allow physiological oxygenation to occur. Oxyaetiohaemoglobin at pH 5.6 required 45 min. at 5 mm. Hg to remove most of the oxygen (O'Hagan, 1959b), whereas recombined oxyprotohaemoglobin lost its oxygen in less than 1 min. at the same pressure and hydrogen-ion concentration.

Granick & Gilder (1946) considered that the vinyl groups played a role in the insertion of iron into the porphyrin nucleus, since they found that whereas protoporphyrin, protohaemin, mesohaemin, haematohaemin and deuterohaemin supported the growth of *Haemophilus influenzae*, mesoporphyrin, haematoporphyrin and deuteroporphyrin did not. Since to insert the iron two hydrogens on the pyrrole nitrogens have to be displaced, the vinyl groups may exert influences along the resonating structures in the same way as they affect the oxygen affinity of the haem iron in the haemoglobins and myoglobins. The finding of Granick & Gilder (1946) that esterified haemins and dimethylprotoporphyrin did not support growth may be due simply to their insolubility in the media.

There have been many suggestions that the haem lies in a cleft in the globin molecule. This was implied by Conant (1932-33) and advanced by St George & Pauling (1951), but the ability to form an active oxyhaemoglobin from tetramethylcoprohaemin III, although not conclusive evidence, does appear to support the argument of Keilin (1953) that the haems lie on the surface and not in a cleft or trough into which only haems of the dimensions of protohaem can fit. Of course, another explanation would be that there is a certain amount of flexibility of the polypeptide chains, which may have been displaced sufficiently to allow the larger molecule to enter. In any case, this means that if haemoglobin biosynthesis proceeds from porphyrins or haematin with longer side chains than those of protohaematin, attachment of these precursors to the globin or to its precursor could occur (O'Hagan, 1955).

George & Lyster (1958) have discussed the evidence for a crevice-positioning of the haems in haemoglobin. They consider that the concept of a structure comprising one bond from the protein to the iron is the most satisfactory, with the uncertainty whether a part of the protein shields the other side of the haem from reaction with large ligands. Recently, fresh evidence has suggested (O'Hagan, 1959c) that the residue in the protein responsible for the linkage to the haem iron may be a carboxyl group rather than an imidazole nitrogen, as proposed by Conant (1932-33) and widely accepted.

That active haemoglobins can be formed from aetiohaemin and methylated haemins, although indicating that the essential linkage is through the iron atom, does not mean that the propionate groups are unnecessary for haemoglobin function. Rather, the instability of oxyaetiohaemoglobin suggests that these groups may have an influence in consolidating the molecule and the importance of haem propionate-protein linkage in ferrihaemalbumin (O'Hagan, 1955, 1959b) indicates the possibility of a similar type of linkage in haemoglobin and myoglobin. Blyumenfeldt (1952) considered them of major importance in effecting haem linkage to globin, based on his work on the 'transhaemation reaction', in which the haematin is transferred from haemoglobin to gelatin, on incubation. Hill & Holden (1926), Holden (1937, 1941), Granick (1949) and Lemberg & Legge (1949) consider such linkages possible. The existence of bonds of this type is also implied by George & Hanania (1954).

The poor spectral extinction of the aetiohaemoglobin compared with the mesohaemoglobin derivatives is no doubt related to the similar differences described by Haurowitz (1927), for the corresponding pyridine haemochromes. In chloroform, in which solvent interaction with the propionate groups is unlikely, aetiohaemin and mesohaemin have essentially the same extinction in the visible and Soret region (J. E. O'Hagan, unpublished work).

It is probable that, with mesohaematin, combination of the propionate groups with either basic groups in the protein or pyridine occurs, enhancing the extinction. The extinction of native haemoglobin derivatives correspond much more closely to those of the artificial haemoglobins prepared from mesohaematin than to those from aetiohaematin. This strongly suggests that the normal linkage in haemoglobin is through haem propionate groups in addition to those through the iron atoms.

SUMMARY

1. Artificial haemoglobins capable of reversible oxygenation can be prepared from aetiohaemin III, dimethylprotohaemin IX, tetramethylcopro-

haemin III and coprohaemin III. The work of Haurowitz & Waelsch (1929), who prepared an active haemoglobin from dimethylmesohaemin IX, has been confirmed.

2. The indications are that the linkage essential for oxygenation is through the iron atom but the possibility of linkages through the haem propionate groups, imparting further stability to the linkage and to the whole protein structure, is suggested by the instability and poor spectral extinction of the artificial haemoglobins prepared from haemins lacking free carboxyl groups.

3. That tetramethylcoprohaemin III can give rise to an active haemoglobin lends support to the suggestion that the haems are on the 'surface', and that linkage to the globin of haematin precursors of larger dimension than protohaematin could occur during haemoglobin biosynthesis.

I am most grateful to Dr A. E. Shaw and the Blood Transfusion Committee of the Queensland Division of the Australian Red Cross Society for providing facilities and giving encouragement to further this work. Professor R. Lemberg generously gave advice on the preparation of the haemins. Mrs Teresa Hamilton and Miss Cecily Barnett gave their willing technical assistance.

REFERENCES

- Blyumenfeldt, L. A. (1952). *Dokl. Akad. Nauk. S.S.S.R.* **85**, 1111.
- Callaghan, J. P. & O'Hagan, J. E. (1949). *Aust. J. exp. Biol. med. Sci.* **27**, 275.
- Chu, T. C., Green, A. A. & Chu, E. J. (1951). *J. biol. Chem.* **190**, 643.
- Conant, J. B. (1932-33). *Harvey Lect.* **28**, 159.
- Dalziel, K. & O'Brien, J. R. P. (1957). *Biochem. J.* **67**, 119.
- Fischer, H. & Orth, H. (1937). *Die Chemie des Pyrrols. Pyrrolfarbstoffe*, II Band, pp. 194, 201, 387, 482, 492. Leipzig: Akademische Verlagsgesellschaft m.b.H.
- Fischer, H. & Treibs, A. (1930). *Farbstoff mit Pyrrolkernen*. Quoted in *Handb. Biochem.*, Suppl. Band, p. 114. Jena: Gustav Fischer.
- George, P. & Hanania, G. I. H. (1954). *Nature, Lond.*, **174**, 33.
- George, P. & Lyster, R. L. J. (1958). *Publ. nat. Acad. Sci., Wash.*, no. 557, p. 33.
- Gibson, K. S. (1950). In *Analytical Absorption Spectroscopy*, p. 186. Ed. by Mellon, M. G. New York: John Wiley and Sons Inc.
- Gralén, N. (1939). *Biochem. J.* **33**, 1907.
- Granick, S. (1949). *Blood*, **4**, 404.
- Granick, S. & Gilder, H. (1946). *J. gen. Physiol.* **30**, 1.
- Gray, C. H. & Holt, L. B. (1948). *Biochem. J.* **43**, 191.
- Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
- Grinstein, M. & Watson, C. J. (1943). *J. biol. Chem.* **147**, 671.
- Haurowitz, F. (1927). *Hoppe-Seyl. Z.* **169**, 235.
- Haurowitz, F. (1949). In *Haemoglobin (Barcroft Memorial Conf.)*, p. 53. Ed. by Roughton, F. J. W. & Kendrew, J. C. London: Butterworths Scientific Publications Ltd.
- Haurowitz, F. & Waelsch, H. (1929). *Hoppe-Seyl. Z.* **182**, 82.
- Hill, R. & Holden, H. F. (1926). *Biochem. J.* **20**, 1326.
- Holden, H. F. (1937). *Aust. J. exp. Biol. med. Sci.* **15**, 409.
- Holden, H. F. (1941). *Aust. J. exp. Biol. med. Sci.* **19**, 1.
- Joep, E. M. (1949). In *Haemoglobin (Barcroft Memorial Conf.)*, p. 205. Ed. by Roughton, F. J. W. & Kendrew, J. C. London: Butterworths Scientific Publications Ltd.
- Joep, E. M. & O'Brien, J. R. P. (1945). *Biochem. J.* **39**, 239.
- Joep, E. M., Joep, H. M. & O'Brien, J. R. P. (1949). *Nature, Lond.*, **164**, 623.
- Kehl, R. & Stich, W. (1952). *Hoppe-Seyl. Z.* **290**, 151.
- Keilin, D. (1953). *Nature, Lond.*, **171**, 922.
- Küster, W. & Schlayer, K. (1927). *Hoppe-Seyl. Z.* **168**, 302.
- Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*, p. 240. New York: Interscience Publ. Ltd.
- Mueller, J. H. (1939). *J. Immunol.* **37**, 103.
- Nicholas, R. E. H. & Rimington, C. (1949). *Scand. J. clin. Lab. Invest.* **1**, 12.
- O'Hagan, J. E. (1950). M.Sc. Thesis: University of Queensland.
- O'Hagan, J. E. (1951). *Med. J. Aust.* **2**, 172.
- O'Hagan, J. E. (1955). *Abstr. 3rd int. Congr. Biochem., Brussels*, p. 10.
- O'Hagan, J. E. (1959a). *Aust. J. exp. Biol. med. Sci.* **37**, 307.
- O'Hagan, J. E. (1959b). Ph.D. Thesis: University of Queensland.
- O'Hagan, J. E. (1959c). *Nature, Lond.*, **183**, 393.
- O'Hagan, J. E. & George, P. (1960). *Biochem. J.* **74**, 424.
- Roche, J. & Combette, R. (1937). *Bull. Soc. Chim. biol., Paris*, **19**, 627.
- Rossi-Fanelli, A. & Antonini, E. (1957). *Arch. Biochem. Biophys.* **72**, 243.
- Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1958a). *Abstr. 4th int. Congr. Biochem., Vienna*, p. 18.
- Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1958b). *Arch. Biochem. Biophys.* **30**, 608.
- St George, R. C. C. & Pauling, L. (1951). *Science*, **114**, 629.
- Schumm, O. (1927). *Die Spectrochemische Analyse natürlicher organischer Farbstoffe*. Jena: Gustav Fischer. Quoted by Lemberg, R. & Legge, J. W. *Hematin Compounds and Bile Pigments*, p. 177. New York: Interscience Publications Ltd.
- Schumm, O. (1928). *Hoppe-Seyl. Z.* **178**, 1.
- Schumm, O. (1929). *Hoppe-Seyl. Z.* **181**, 141.
- Warburg, O. & Negelein, E. (1932). *Biochem. Z.* **244**, 9.
- Zaleski, J. (1904). *Hoppe-Seyl. Z.* **43**, 11.