The Haem-Globin Linkage

2. ARTIFICIAL MYOGLOBINS FROM HAEMATINS LACKING FREE CARBOXYL GROUPS

By J. E. O'HAGAN* AND P. GEORGE

John Harrison Laboratory of Chemistry, University of Pennsylvania, Philadelphia, U.S.A.

(Received 20 May 1959)

In the preceding paper (O'Hagan, 1960) it was shown that artificial haemoglobins could be prepared from aetiohaemin III, dimethylprotohaemin IX, dimethylmesohaemin IX, tetramethylcoprohaemin III and coprohaemin III. These studies have been extended for the first three pigments to the preparation of artificial myoglobins.

MATERIALS AND METHODS

Haemins. The preparations of aetiohaemin III, dimethylprotohaemin IX and dimethylmesohaemin IX were the same as used for the preceding paper.

Horse ferrimyoglobin. The method of Theorell (1932) as modified by Lawrie (1951) was used. Three precipitations with $(\text{NH}_4)_2\text{SO}_4$ were performed, the solutions dialysed free of sulphate and stored in polythene bottles at -10° with 2% NaCl added, as suggested by Hanania (1953).

Apomyoglobin. This was prepared by the method of Theorell & Åkeson (1955). Into the cups of a refrigerated centrifuge at -15° were placed tubes each containing 40 ml. of acetone-HCl (2 ml. of M-HCl/l. of acetone) at the same temperature. A portion (2 ml.) of 1% (w/v) ferrimyoglobin solution was injected into each tube, the tubes were stoppered and shaken, allowed to stand for a few minutes until the protein had coagulated and then were spun at 4000 *g* for 5 min. The supernatant haemin solution was then decanted, the tubes were inverted on filter paper to drain for 5 min., and then 2 ml. of ice-cold water was added to each and the apomyoglobin dissolved. The apomyoglobin solution from eight tubes was pooled, placed in a cellulose dialysis tube (1½ in. diam.) and dialysed in the cold at 1° against dilute NaHCO_3 solution (50 mg./l.), the equipment described by O'Hagan (1959*b*) being used. A small amount of denatured protein was precipitated, and was removed by high-speed centrifuging (16 000 *g* for 10 min.); by contrast, globin solutions showed heavy precipitation at this stage. The apomyoglobin solutions were stored at 1° and could be kept for 3 weeks. The concentration of protein was determined by u.v. spectrophotometry at 280 μm , with the E_{mM} of 13 obtained by Hanania (1953). There was some indication that this figure may have been too low (P. George & J. E. O'Hagan, unpublished work) but this would not have affected the present work since the protein was used in excess of the haemins. A small trace (not more than 1.5%) of unsplit myoglobin was present.

Use of more acid for splitting or longer contact with the acetone-HCl removed more haematin but more denaturation of the protein occurred.

Recombination of haematin with apomyoglobin. To ensure that all haematin capable of combination did so, the apomyoglobin was used in excess. In a typical experiment, 0.3 ml. of freshly prepared 1.5 mM-haemin solution (9.78 mg. of protohaemin + 1 ml. of 0.1 N-NaOH to dissolve, with 9 ml. of water added) was introduced with rapid mixing into 10 ml. of 0.15 mM-apomyoglobin solution in phosphate buffer (pH 7.0, *I* 0.05). The mixture was set aside for 1 hr. before reading.

Fluoride derivatives. These were prepared by adding NaF in considerable excess. To 2 ml. of the ferrimyoglobin solutions was added 1 ml. of a filtered saturated solution of NaF ($\sim 1\text{M}$).

Spectrophotometry. A Beckman model DU spectrophotometer with photomultiplier attachment was employed, the extinction scale was checked against the copper sulphate and cobalt sulphate standards of Gibson (1950) and the wavelength scale against a mercury arc.

Hydrogen-ion concentration measurements. A Beckman model G pH meter, standardized against the phthalate, phosphate and borate buffers described in British Standard 1647 (1950), was employed.

RESULTS

Recombined ferrimyoglobin. The Soret peaks of native and recombined ferrimyoglobin are shown in Fig. 1, and indicate that about 96% of the haematin had recombined when added in 0.01 N-NaOH to excess of apomyoglobin in phosphate buffer (pH 7.0, *I* 0.05).

The clearest indication of recombination to an active haemoglobin was demonstrated by conversion into the fluoride complex. This avoids the use of dithionite, which partly denatures the protein. Fluoride does not combine with haematin or ferrihaemalbumin [where linkage of haematin is through the carboxyl groups (O'Hagan, 1955, 1959*b*)]. The curves obtained are shown in Fig. 2, again indicating about 96% recombination.

Combination of aetiohaemin with apomyoglobin. In this case combination was achieved by adding the aetiohaemin in methanol to the apomyoglobin solution in phosphate buffer at 0° .

* Present address: Biochemistry Dept., Red Cross Blood Transfusion Service, Brisbane, Queensland, Australia.

Fig. 1. Absorption spectra showing effect of addition of protohaematin to apomyoglobin in phosphate buffer, pH 7.0, I 0.05. (a) Apomyoglobin, (b) haematin in 0.01 N -NaOH; (c) after addition of haematin to excess of apomyoglobin; (d) original ferrimyoglobin. Recombination was as described in the text.

Fig. 2. Absorption spectra of myoglobins in phosphate buffer, pH 7.0, I 0.05. (a) Native ferrimyoglobin; (b) recombined ferrimyoglobin; (c) native ferrimyoglobin fluoride; (d) recombined ferrimyoglobin fluoride. Recombination was as described in the text. Fluoride was added to 0.3 M concentration.

Fig. 3. Absorption spectra of aetiomyoglobin derivatives in phosphate buffer, pH 7.0, I 0.05. Ferri-, —; ferro-, — — —; carboxy-, - - - -; 3 ml. of 68 μM -apomyoglobin + 0.116 mg. of aetiohaemin in 0.1 ml. of methanol. Dilution, 1:10 with buffer for Soret-region measurements; 1 cm. cell.

Fig. 4. Absorption spectra of dimethylprotomyoglobin derivatives in phosphate buffer, pH 7.0, I 0.05. Ferri-, —; ferro-, — — —; carboxy-, - - - -; 3 ml. of 68 μM -apomyoglobin + 0.134 mg. of dimethylprotohaemin in 0.1 ml. of methanol. Dilution, 1:10 with buffer for Soret-region measurements; 1 cm. cell.

Fig. 5. Absorption spectra of dimethylmesomyoglobin derivatives in phosphate buffer, pH 7.0, I 0.05. Ferri-, —; ferro-, — — —; carboxy-, - - - -; 3 ml. of 68 μM -apomyoglobin + 0.134 mg. of dimethylmesohaemin in 0.1 ml. of methanol. Dilution, 1:10 with buffer for Soret-region measurements; 1 cm. cell.

The ferri-, ferro- and carboxy-compounds had absorption curves with peaks corresponding to those described by Theorell & Åkeson (1955) for protomyoglobin, but were displaced as would be expected. The spectra for the artificial myoglobin derivatives are shown in Fig. 3. The aetiohaemin and apomyoglobin were $68\text{ }\mu\text{M}$ in the final solution, the solution being diluted 1:10 with the phosphate buffer for the Soret-band measurements.

Combination of dimethylprotohaemin and dimethylmesohaemin. Artificial myoglobins from these pigments were obtained in a similar fashion at the same concentration as for aetiohaemin. The curves are shown in Figs. 4 and 5.

Fig. 6. Absorption spectra of dimethylmesomyoglobin derivatives in phosphate buffer, pH 6.2, I 0.05. Ferri-, —; fluoride, ——. Concn., $\sim 60\text{ }\mu\text{M}$ -dimethylmesomyoglobin; fluoride was added to 0.3M concn.; 1 cm. cell.

Fig. 7. Absorption spectra of dimethylmesomyoglobin derivatives in phosphate buffer, pH 8.0, I 0.05. Ferri-, —; fluoride, —; carboxy-, ——. Concn., $\sim 30\text{ }\mu\text{M}$ -dimethylmesomyoglobin; fluoride was added to 0.3M concn., 1 cm. cell.

The solutions of the ferrimyoglobins of Figs. 3–5 were set aside for 4 hr. at 22° before measurement and conversion into the other derivatives.

Fluoride compounds of ferridimethylmesomyoglobin. Since the curves from dimethylmesomyoglobin appeared sharper than those from the others, this compound was used for further studies, the combination with fluoride at pH 6.2 and 8.0. Figs. 6 and 7 clearly show that combination had taken place in both instances.

DISCUSSION

The tendency for apomyoglobin to be more stable than globin and to be more readily cleared of denatured material allowed the use of phosphate buffer solutions, made these studies much easier than those reported in the preceding paper (O'Hagan, 1960) and allowed investigation of the fluoride complexes.

Rosenfeld & Surgenor (1950) found that haematin in 0.01N-sodium hydroxide lost the ability to form ferrihaemalbumin to the extent of 7% in 1 hr. This probably explains why about 4% of the haematin used here did not combine with the apomyoglobin, as can be seen from Figs. 1 and 2.

With aetiohaemoglobin derivatives (O'Hagan, 1960) it was noted that the absorption curves had peaks that were less sharp than those from the corresponding haemoglobins (proto- and meso-) with free carboxyl groups. A similar lessened intensity of extinction was observed here with the aetio-, dimethylproto- and dimethylmeso-myoglobin derivatives. This might suggest the absence of a linkage which in the normal haemoproteins enhances the extinction.

The slightly sharper peaks for the derivatives of dimethylmesohaemin might suggest that more complete combination had occurred. The small hump at about $630\text{ m}\mu$ in the carboxydimethyl-protomyoglobin suggests that there was some free haematin present, as does the flatness of the curve for the ferric derivative. That the ester had not been hydrolysed during the combination experiments was shown for dimethylmesomyoglobin by splitting off the haematin with acetone-hydrochloric acid. The haematin was found to be insoluble in alkali, indicating that the carboxyl groups were still esterified.

The fluoride compounds are most interesting since free haematin does not give fluoride complexes under similar experimental conditions. This specific reactivity of the compounds from substituted haematin provides convincing proof, like the oxygenation of the corresponding artificial ferrohaemoglobins, that combination has occurred between the haematin and the protein. Furthermore, it is strong evidence for the formation of an iron-protein bond. These fluoride reactions are also

significant because, in similar experiments with artificial ferrihaemoglobins, denatured protein was precipitated, making it impossible to judge whether a reaction had occurred.

Rossi-Fanelli & Antonini (1957) have shown that deuterohaemin can also combine with apomyoglobin to give an active oxymyoglobin, but the oxygen affinity was much higher, though the Bohr effect was similar to that of the native myoglobin. Clearly, as with haemoglobin, variation of the side chains influences the reactivity towards oxygen and it would appear that protohaem represents the most suitable prosthetic group for the physiological activity of haemoglobin and myoglobin.

SUMMARY

1. Artificial myoglobins have been prepared from aetiohaemin III, dimethylprotohaemin IX and dimethylmesohaemin IX.

2. The spectra for the ferric, ferro and carbon monoxide complexes of these myoglobins are given.

3. Fluoride complexes of ferridimethylmesomyoglobin can be formed at pH 6.2 and 8.0, providing further evidence that combination through the haematin iron had taken place.

We are indebted to the U.S. National Science Foundation for a Research Grant (G2309) in support of this work, and one of us (J. E. O'H.) is grateful for study leave from the Red Cross Blood Transfusion Service, Brisbane, to enable this work to be undertaken.

REFERENCES

- Gibson, K. S. (1950). In *Analytical Absorption Spectroscopy*, p. 186. Ed. by Mellon, M. G. New York: John Wiley and Sons Inc.
- Hanania, G. I. H. (1953). Ph.D. Thesis: University of Cambridge.
- Lawrie, R. A. (1951). *Nature, Lond.*, **167**, 802.
- 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. (1960). *Biochem. J.* **74**, 417.
- Rosenfeld, M. & Surgenor, D. M. (1950). *J. biol. Chem.* **183**, 663.
- Rossi-Fanelli, A. & Antonini, E. (1957). *Arch. Biochem. Biophys.* **72**, 243.
- Theorell, H. (1932). *Biochem. Z.* **252**, 1.
- Theorell, H. & Åkeson, A. (1955). *Ann. Acad. Sci. fenn. Ser. A II*, **60**, 303.

Fatty Acids of Human Blood

By V. S. PATIL AND N. G. MAGAR

Department of Biochemistry, Institute of Science, Bombay 1, India

(Received 24 June 1959)

Peters & Man (1943) studied the fatty acid composition of human blood. Brun (1939) and Kirk (1938) observed that esterified and free cholesterol in blood cell was different from that in plasma in healthy individuals. Studies by Chevallier, Manuel & Rouillard (1951) and Evans, Waldron, Oleksyshyn & Riemenschneider (1956) have indicated that the partition of the unsaturated fatty acids in blood cells was quite different from that in plasma. Alkali-isomerization spectrophotometric methods (Hammond & Lundberg, 1955; Luddy, Barford & Riemenschneider, 1958) have been employed to determine the polyunsaturated fatty acids of human blood plasma, the component lipids of human plasma and atheroma. Very few workers have, however, analysed the component fatty acids of human blood cells, plasma and whole blood. This paper describes an investigation into the component lipids and fatty acids of whole blood, plasma and blood cells of healthy men, alkali-isomerization spectrophotometric methods, iodine

values of fatty acids and low-temperature crystallization techniques being utilized.

EXPERIMENTAL

From each of the normal five subjects in the post-absorptive state (8–10 hr.) about 250 ml. of blood was drawn via the antecubital vein into heparinized syringes. The blood was transferred to a sterile bottle to which had been added 50 ml. of a solution containing 3.0% of sodium citrate, 0.85% of sodium chloride and 2.0% of sodium sulphathiazole. Afterwards the whole solution was lyophilized and the bottle kept evacuated.

The ages of the above-mentioned five subjects were from 30 to 35 years. Their calorific and fat intakes per day per man were 2500–2640 and 55–65 g. respectively.

Another seven normal healthy men selected were from 33 to 40 years of age. Their calorific and fat intakes per day per man were 2268–2400 and 43–71 g. respectively. From each of the seven subjects in the post-absorptive state, about 40 ml. of blood was taken in a bulb containing sodium oxalate. Plasma and cells were separated as described by Evans *et al.* (1956).