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Crystalline Leghemoglobin

IX. Artificial Leghemoglobins

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Artificial leghemoglobins have been prepared from mesohemin IX, deuterohemin IX, hematohemin IX, etiohemin III, pyrrohemin XV, pheophorbide a_5 hemin, chlorin a_6 hemin, and a mesoporphyringlobin complex from mesoporphyrin IX. The apoprotein used in all the experiments was that of the slow component of leghemoglobin. All recombination reactions were performed in phosphate buffer, pH 7.0, μ equal to 0.1. The qualitative spectra for the artificial ferrileghemoglobins formed and those of their fluoride complexes are given. The spectral changes obtained with fluoride provides evidence of a combination through the hemin iron. The spectral differences observed with the 6,7-dipropionate hemins with different groups in positions 2 and 4 indicate that those groups have a function in combining the hemins to the apoprotein. The poor spectral extinctions as well as the low recombination yield obtained with etiohemin III, lacking carboxyl groups, stress the importance of the propionate groups in the recombination reaction. One propionate group seems to be enough to form a stable recombination compound, which was shown by the spectral changes of pyrrohemin as well as pheophorbide a_5 hemin on combination. The specificity requirements for a hemin to recombine with apoleghemoglobin have been discussed.

Artificial hemoglobins have been prepared for the first time 40 years ago, when meso- and deuterohemins were combined with globin.[1] Unnatural hemoglobins and their derivatives have since then been studied more closely by several workers.[2–7]

Leghemoglobin (Lhb) has been split with acid acetone and the apoleghemoglobin (apoLhb) obtained recombined with protohemin IX to give a compound with properties identical to those of native leghemoglobin.[8,9]

In order to elucidate the extent to which protohemin, the natural prostetic group of Lhb, can be exchanged for other hemins, it was decided to study the formation of artificial leghemoglobins by combining apoLhb with different hemins derived from protohemin as well as from chlorophyll. It was hoped that this investigation would also give information on the site of combination on the apoprotein as well as on the groups of hemin, which are essential for a stabile compound to be formed.

METHODS AND MATERIALS

Hydrogen ion measurements were performed at 20°C with a Radiometer PHM3 pH-meter, which was standardized against a phthalate buffer.

Paper chromatography. The chromatography of the hemins was performed on silicon-treated paper in a water-propanol-pyridine (5.5:0.1:0.4) system as described by Chu and Chu.[10] The purity of the porphyrin preparations was checked by chromatography in a 2,6-lutidine-water (1:1) system according to Kehl and Stich.[11]

Spectrophotometric measurements. A Beckman Recording spectrophotometer model DK-1 was used in most experiments.

Pyridine hemochrome was determined according to Paul *et al.*[12]

Chemicals. Acetic acid p.a. (Merck) was frozen, thawed and finally distilled.[13] Chloroform p.a. (Merck) was washed with a 1 % (w/v) K_2CO_3 solution and dried over $CaCl_2$. Ether was treated with $FeSO_4$, washed and dried over $CaCl_2$.[14] Formic acid was purified by refluxing with dried $CuSO_4$ for 30 min, distilled under reduced pressure and crystallized at 0°C.[13]

Leghemoglobin. The two main components of Lhb were prepared as described previously.[15]

Apoleghemoglobin. The apoprotein of the slow component of Lhb was prepared as before by acid acetone splitting.[16] Globin and apoleghemoglobin have been used in parallell to express the apoprotein of the slow component of Lhb.

Crystalline protohemin IX was a commercial preparation from Sigma, the purity of which was checked by chromatography on silicon treated paper.

Protoporphyrin IX (1,3,5,8-tetramethyl-2,4-divinyl-porphin-6,7-dipropionic acid) was prepared by a method of Morell and Stewart.[17]

The porphyrin preparation obtained gave the following absorption spectra: in chloroform, I, 633; Ia, 605; II, 578; III, 544; IV, 508 nm (decreasing order of intensity IV, III, II, I, Ia) and in 5 % (w/v) HCl, I, 602 and II, 557 nm (order of intensity II > I). These values compared well with those found in the literature.[13]

Mesoporphyrin IX (1,3,5,8-tetramethyl-2,4-diethyl-porphin-6,7-dipropionic acid) was prepared according to Muir and Neuberger [18] from protoporphyrin by catalytic reduction using a Pd-catalyst of polyvinyl type as described by Rampino and Nord.[19] The original procedure was altered for a protoporphyrin amount of 5 mg. The final purification of the preparation obtained was performed on thin layer chromatograms in a benzene-methanol (9:1) system containing formic acid (0.3 M). The mesoporphyrin containing silica gel was eluted with methanol.[20]

The porphyrin preparation obtained gave the following absorption spectra: in chloroform, I, 622; Ia, 596; II, 568; III, 534; IV, 499 nm (decreasing order of intensity IV, III, II, I, Ia) and in 5 % (w/v) HCl, I, 598 and II, 554 nm (order of intensity II > I), all values were in good agreement with those of the literature.[13]

Mesohemin IX. The insertion of iron into mesoporphyrin in order to obtain mesohemin was performed by using the ferrous sulphate method of Morell and Stewart,[17] originally worked out for removal of iron. The absorption spectra of the preparation were as follows: in acetone, I, 630; II, 575; III, 530; IV, 505 nm (decreasing order of intensity III, IV, I, II) and the pyridine hemochrome, α-band at 547 and β-band at 518 nm, respectively. The spectra compared well with those given in the literature. [13] The degree of mesohemin formation was decided on the basis of thin layer chromatography in a chloroform-methanol (9:1) system containing formic acid (0.3 M) worked out for the analysis of free porphyrins.[20]

Deuteroporphyrin IX (1,3,5,8-tetramethyl-porphin-6,7-dipropionic acid) was prepared by heating protohemin with resorcinol at 170—180°C for 20 min.[21] The removal of iron was performed by treating the crude reaction mixture directly with iron powder, acetic acid and HCl.[22]

The porphyrin preparation obtained gave the following absorption spectra: in chloroform, I, 621; Ia, 595; II, 566; III, 533; IV, 498 nm; in ethyl acetate, I, 620; Ia, 594; II, 565; III, 530; IV, 497 nm (decreasing order of intensity IV, III, II, I, Ia) and in 5 % (w/v) HCl, I, 591 and II, 549 nm (order of intensity I < II). The values compared well with those found in the literature.[13]

Deuterohemin IX. The iron was incorporated into deuteroporphyrin in a similar way as in the case of mesohemin and the purity of the preparation obtained was checked in the same manner as mesohemin.

The preparation gave the following absorption spectra: in ether, I, 629; II, 569; III, 530; IV, 504 nm and in acetone, I, 630; II, 572; III, 530; IV, 505 nm (decreasing order of intensity III, IV, I, II) and the pyridine hemochrome, α-band at 545 and β-band at 516 nm, respectively. The values compared well with those found in the literature.[13]

Hematoporphyrin IX (1,3,5,8-tetramethyl-2,4-dihydroxyethylporphin-6,7-dipropionic acid) was a commercial preparation of hematoporphyrin dihydrogen chloride (Fluka). The preparation gave an absorption spectrum, which indicated it to contain considerable amounts of 2(4)-vinyl-4(2)-hydroxyethyl-deuteroporphyrin. The preparation was purified according to Porra and Jones [23] by dissolving it in 0.1 N NH_4OH and by extracting with hydrochloric acid to remove the hydroxymonovinyldeuteroporphyrin. The final purification was performed by thin layer chromatography on acidic silica gel in a system of toluene-isopropanol (1:1).[20]

The absorption spectra of the preparation was as follows: in chloroform, I, 627; Ia, 600; II, 571; III, 528; IV, 499 nm (decreasing order of intensity: IV, III, II, I, Ia) and in 5 % (w/v) HCl, I, 595 and II, 551 (order of intensity II > I). The values compared well with those found in the literature.[13]

Hematohemin IX was prepared according to the procedure used for the preparation of mesohemin and the purity of the preparation was checked in respect to free porphyrin by thin layer chromatography in the benzene-methanol system as used above. The absorption spectra of the preparation were as follows: in acetone, I, 630; II, 580; III, 535; IV, 510 nm (decreasing order of intensity III, IV, I, II) and the pyridine hemochrome, α-band at 549 and β-band at 519 nm, respectively — values in good agreement with those found in the literature.[13]

Etioporphyrin III (1,3,5,8-tetramethyl-2,4,6,7-tetraethylporphin) was prepared as described by Schumm.[24] 150 mg of protohemin IX was heated in 30 g of liquid paraffin at 340°–360°C for 90 min. The removal of iron was performed by treating the crude preparation with phenol-oxalic acid. The preparation was diluted with water, the porphyrin taken into ether, washed with aqueous HCl and precipitated with solid KOH. The dry precipitation was finally dissolved in HCl, extracted into chloroform and evaporated to dryness.

The preparation obtained gave the following absorption spectra: in dioxan, I, 620; Ia, 595; II, 567; III, 528; IV, 496 nm (decreasing order of intensity IV, III, II, I, Ia); in ether, I, 623; Ia, 609; III, 528; IV, 497 nm (decreasing order of intensity IV, III, II, I, Ia) and in 5 % (w/v) HCl, I, 588 and II, 547 nm (order of intensity II > I). All values are in good agreement with those found in the literature.[13,25]

Etiohemin III. The insertion of iron into etioporphyrin was performed according to the ferrous acetate-acetic acid method of Warburg and Negelein.[2]

The absorption spectra of the preparation obtained were as follows: in ether, I, 637; II, 594; III, 536; IV, 505 nm (decreasing order of intensity III, IV, I, II) and the pyridine hemochrome, α-band at 547 and β-band at 518 nm, respectively.

Pheophorbide a_5 (1,3,5,8-tetramethyl-4-ethyl-2-vinyl-9-oxo-10-carbmethoxy-phorbin-7-propionic acid). A dry powder was prepared from spinach leaves,[26] from which the two pheophorbides *a* and *b* were isolated according to Stoll and Wiedemann. [27] The two forms of pheophorbide were separated by partition between aqueous HCl and ether.[25]

The pheophorbide a_5 preparation gave the following absorption spectra: in dioxan, I, 666; Ia, 608; II, 560; III, 536; IV, 506 nm (decreasing order of intensity I, IV, III, Ia, II) in good agreement with values given in the literature.[13]

Pheophorbide a_5 *hemin*. The iron complex of pheophorbide a_5 was prepared according to Zaleski.[28] The spectra of the preparation obtained were as follows: in chloroform, 634 nm; in pyridine, I, 649 and II, 590 nm (order of intensity I, II), and pyridine hemochrome, α-band at 654 and β-band at 594 nm, respectively.

Chlorin a_6 (1,3,5,8-tetramethyl-4-ethyl-2-vinyl-chloron-6-carboxylic acid-γ-acetic acid-7-propionic acid) was obtained by treating pheophorbide a_5 with methanolic alkali.[29] 100 mg pheophorbide a_5 in 4 ml pyridine was pipetted into boiling methanol containing KOH. Chlorin a_6 formed was extracted into ether and further into 3 % HCl. The procedure was repeated several times. The preparation gave the following absorption spec-

trum: in dioxan, I, 664; Ia, 612; II; 561; III, 530; IV, 500 nm (decreasing order of intensity I, IV, III, Ia, II). The values are in good agreement with those of the literature.[13]

Chlorin a_6 *hemin* was prepared according to Zaleski.[28] The preparation gave the following spectra: in chloroform, I, 675; II, 622 nm (order of intensity I < II); pyridine hemochrome, α-band at 630 and β-band at 576 nm, respectively.

Pyrroporphyrin XV (1,3,5,8-tetramethyl-2,4-diethyl-porphin-7-propionic acid) was prepared by heating an alkaline solution of pheophorbide a_5 in a bomb tube.[25] 98 mg dry pheophorbide a_5 was weighed into a silver tube containing 4 ml of 30 % KOH in methanol (w/w). The silver tube was enclosed in a bomb tube, which was kept for 8 h at 185°C. The content of the silver tube was centrifuged after cooling. The precipitate was washed with methanol and dissolved in 5 % HCl. After dilution with water the porphyrin was extracted with ether.

The supernatant obtained after the centrifugation was treated according to Treibs and Wiedemann.[30] The solution was neutralized with diluted HCl and the pyrroporphyrin was taken into ether. The ether phases were pooled and washed with water. The spectra of the pyrroporphyrin preparation obtained were as follows: in acetone, I, 619; Ia, 595; II, 566; III, 527; IV, 495 nm and in ether, I 624; Ia, 594; II, 565 – 570; III, 530; IV, 498 nm (decreasing order of intensity IV, III, II, I, Ia). The maxima differ slightly from those given by Fischer and Orth.[25]

Pyrrohemin XV. The iron complex of pyrroporphyrin was prepared by the ferrous acetate-acetic acid method of Warburg and Negelein.[2] The preparation gave the following spectra: in ether, I, 629; II, 581; III, 534; IV, 503 nm (decreasing order of intensity III, IV, I, II); the pyridine hemochrome, α-band at 545 and β-band at 515–519 nm, respectively.

Recombination of hematin with apoleghemoglobin. To ensure a hematin recombination as quantitative as possible, apoLhb was used in excess. In a typical experiment 0.1 ml of 1.5 mM hematin in 0.01 N NaOH was rapidly mixed into 3 ml of 0.15 mM apoprotein in a pH 7.0 phosphate buffer, μ equal to 0.1 at room temperature. The recombination was studied in two pairs of cuvettes. Those forming the control pair consisted of one cuvette with hemin, the other with globin, both in the same buffer. The sample pair consisted of one cuvette with hemin and globin in the same buffer solution, the other one only with buffer. A spectrophotometric reading was made immediately after adding hemin into the globin solution and after 2 h of incubation.

Fluoride derivatives. These were prepared by adding 1 ml of saturated (~ 1 M) NaF solution into 2 ml of the buffered (pH 7.0) recombined leghemoglobin solution.

RESULTS

Mesoleghemoglobin. On combination of mesohemin with apoLhb a slightly coloured precipitation was formed and was removed by centrifugation. The reaction between mesohemin and apoLhb appeared to be instantaneous. Fig. 1 shows the spectrum of ferrimesoLhb and its fluoride complex. The slightly lower extinction values of the recombination product as compared with those of uncombined apoLhb plus mesohemin evidently depends on losses of mesohemin by removal of the coloured precipitate. The spectrum of mesoLhb corresponds to that of protoLhb,[31] but the peaks were slightly displaced as expected. The ferriprotoLhb maximum at 625 nm was displaced to 617 nm in ferrimesoLhb. A strong increase of the extinction of the Soret band was observed along with a shift of maximum from 388 nm to 394 nm. The fluoride complex of ferrimesoLhb showed a strong maximum at 587 nm, the maximum of the fluoride complex of ferriprotoLhb being at 605 nm.[32] No fluoride complex was formed with mesohemin alone under the same experimental conditions.

Hematoleghemoglobin. The combination reaction between hematohemin and apoLhb was very fast. Also in this experiment a precipitation was formed and

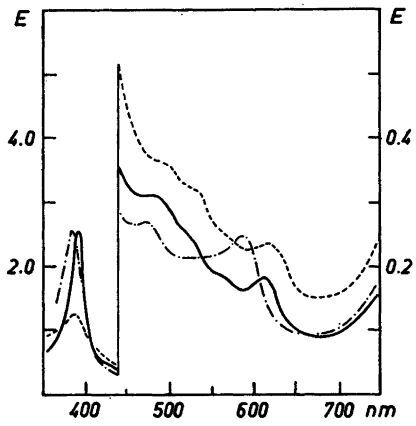

Fig. 1. The qualitative spectrum of ferrimesoLhb (———), that of its fluoride complex (—·—·), and that of uncombined mesohemin plus apoLhb (----) in phosphate buffer pH 7.0. The hemin concentration in the reaction mixture was ~48 μM. The ratio apoLhb/hemin was equal to 3. The final spectra were taken 2 h after mixing apoLhb and mesohemin at room temperature.

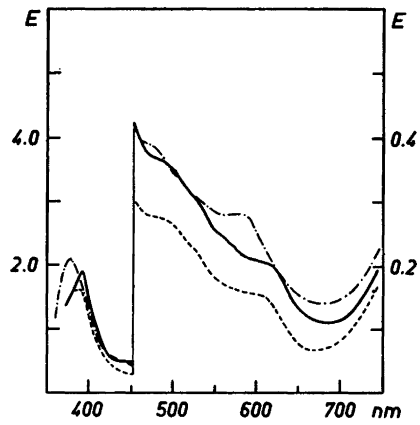

Fig. 2. The qualitative spectrum of ferrihematoLhb (———), that of its fluoride complex (—·—·), and that of uncombined hematohemin plus apoLhb (----). The experimental conditions are identical to those described in Fig. 1.

centrifuged out. Fig. 2 shows the qualitative absorption spectrum of ferrihematoLhb and that of its fluoride complex. FerrihematoLhb showed a maximum at 615 nm. The Soret band shifted from 389 to 401 nm on combination. The fluoride complex of ferrihematoLhb had a maximum at 594 nm. Hematohemin itself gave no fluoride complex under similar experimental conditions. The general appearance of the spectrum of ferrihematoLhb differs considerably from that of ferrimesoLhb with very diffuse maxima and minima.

Deuteroleghemoglobin. In Fig. 3 is shown the spectrum of ferrideuteroLhb and that of its fluoride complex. The recombination reaction was also very fast in this reaction. The reaction solution was slightly opalescent and could not be clarified by centrifugation. The ferrideuteroLhb showed a diffuse maximum at 608 nm. An increase in the Soret band was noticed with a shift of the maximum from 383 to 386 nm, upon combination. The fluoride complex of ferrideuteroLhb had a maximum at 575 nm. No fluoride complex was formed with deuterohemin under similar experimental conditions. In general, the spectrum of ferrideuteroLhb is very similar to that of ferrihematoLhb.

Etioleghemoglobin. The combination between etiohemin and apoLhb was achieved by adding 0.1 ml of 1.5 mM etiohemin in methanol to 3.0 ml 0.15 mM apoLhb in phosphate buffer at 0°C. A solution prepared by adding 0.1 ml of the methanolic etiohemin solution into 3.0 ml phosphate buffer at 0°C was used as a control. After reaching room temperature the two solutions were both centrifuged at 18 000 *g* for 20 min in order to remove uncombined etiohemin. About

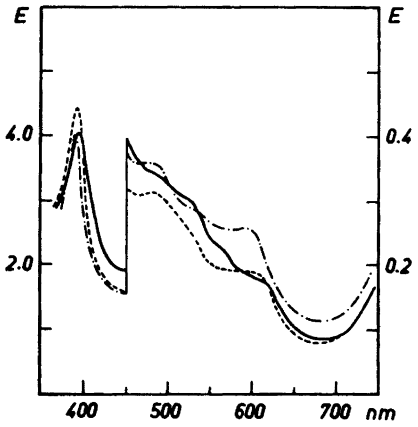

Fig. 3. The qualitative spectrum of ferrideuteroLhb (———), that of its fluoride complex (—·—·), and that of uncombined deuterohemin plus apoLhb (- - - -). The experimental conditions are identical to those described in Fig. 1.

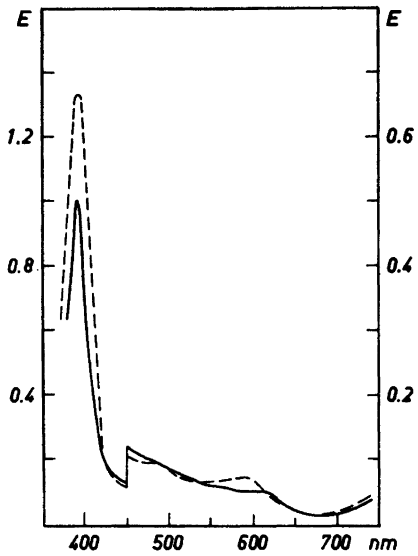

Fig. 4. The qualitative spectrum of ferrietioLhb (———), that of its fluoride complex (— — —). The hemin dissolved in methanol was combined with apoLhb at 0°C and after reaching room temperature centrifuged at 18 000 *g*. The spectra were taken 2 h after mixing etiohemin with apoLhb at 0°C.

97 % of the etiohemin in the recombination experiment was removed by centrifugation as compared with the amount of etiohemin still remaining in solution in the control solution after centifugation (the calculation was performed on the basis of the extinction values of the Soret bands of the two solutions). Fig. 4 shows the qualitative spectrum of ferrietioLhb and that of its fluoride complex. The ferrietioLhb showed a very diffuse maximum at 610 nm. The Soret band was found to shift from 385 to 394 nm on combination. The fluoride complex of ferrietioLhb showed a maximum at 590 nm.

O'Hagan [33] and O'Hagan and George [34] found also a poor recombination of etiohemin with globin of hemoglobin and myoglobin as well. They assumed the low reactability of etiohemin with globin to depend upon its low solubility in aqueous solutions. However, this assumption cannot explain our findings of etiohemin being stable in phosphate buffer without apoprotein, only a small fraction being centrifuged off at 18 000 *g*. When apoLhb in phosphate buffer was added to such a stable etiohemin buffer solution neither recombination occurred, not even after an incubation of 24 h, nor was any precipitation observed. This seems to indicate that etiohemin is able to combine with apoLhb only if it is added to the buffered apoprotein solution dissolved in methanol. It is assumed that only this form of etiohemin, evidently the monomer, is able

to combine with the groups available in the apoprotein. The unspecific combination with the protein might cause the precipitation of the protein.

Because of difficulties in detecting components with slightly deviating properties in amounts of some percent of the main compound with spectroscopic and chromatographic methods available at the moment, the possibility should not be overlooked that some other isomer or even other related compound present in the etiohemin preparation might react with apoLhb instead of etiohemin III.

Pheophorbide a_5 *leghemoglobin.* The recombination reaction between pheophorbide a_5 hemin and apoLhb seemed to be very fast. The spectra in Fig. 5 are obtained after centrifugation of ferripheophorbide a_5 leghemoglobin and its fluoride complex. Ferripheophorbide a_5 Lhb showed a strong maximum at 651 nm. A strong increase of the extinction of the Soret band was observed with a shift from 386 to 400 nm on combination. The fluoride complex of ferripheophorbide a_5 Lhb showed maxima at 698 and 585 nm, respectively. No formation of fluoride complex was obtained with pheophorbide a_5 hemin alone.

Chlorin a_6 *leghemoglobin.* The combination reaction between chlorin a_6 hemin and apoLhb was markedly slower than that observed with all the other hemins. It seems that the recombination reaction in this case is sterically hampered by the carboxyl groups. The combination reached its completion in 2 h. Fig. 6 shows the spectrum of ferrichlorin a_6 Lhb and that of its fluoride complex. Ferrichlorin a_6 Lhb exposed a strong maximum at 642 nm. The Soret

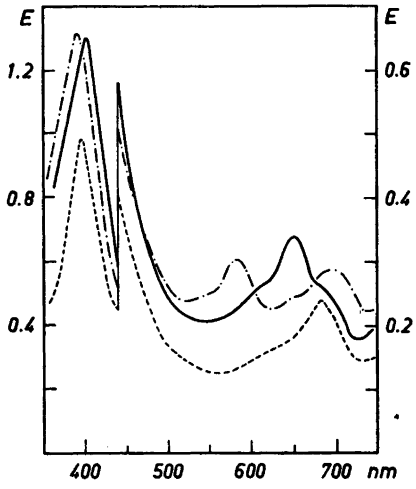

Fig. 5. The qualitative spectrum of ferripheophorbide a_5 Lhb (———), that of its fluoride complex (——·——), and that of uncombined pheophorbide a_5 hemin plus apoLhb (- - - -). The experimental conditions are identical to those described in Fig. 1.

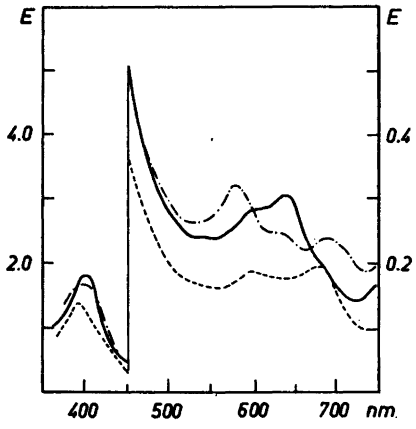

Fig. 6. The qualitative spectrum of ferrichlorin a_6 Lhb (———), that of its fluoride complex (——·——), and that of uncombined chlorin a_6 hemin plus apoLhb (- - - -). The experimental conditions are identical to those described in Fig. 1.

band was shifted from 394 to 405 nm on combination. The fluoride complex of ferrichlorin a_6 Lhb showed two maxima at 688 and at 579 nm, respectively. No formation of fluoride complex was observed with chlorin a_6 hemin alone.

Pyrroleghemoglobin. Because of its low solubility pyrrohemin was first dissolved in pyridine (25 μl) and the volume made up to 175 μl with 0.01 N NaOH giving a 1.5 mM solution in respect to pyrrohemin. The recombination reaction was very fast as could be decided by the colour change from brown to red. A precipitate was formed and centrifuged out; a slight opalescence, however, still remained in the solution. Fig. 7 shows the spectrum of ferripyrroLhb and that of its fluoride complex. FerripyrroLhb showed a main maximum at 515 nm with a smaller peak at 555 nm. The pyrrohemin maximum at 615 nm seemed to decrease on combination. An increase of the Soret band was observed on combination, the shift of the maximum being somewhat questionable. The fluoride complex of ferropyrroLhb showed a maximum at 521 and 555 nm, respectively. No fluoride complex was obtained with pyrrohemin alone.

Mesoporphyrin-globin complex. Mesoporphyrin combined rapidly with apoLhb, shown immediately by the colour change from brown to redviolet. The spectrum of the mesoporphyrin-apoLhb complex is shown in Fig. 8. The

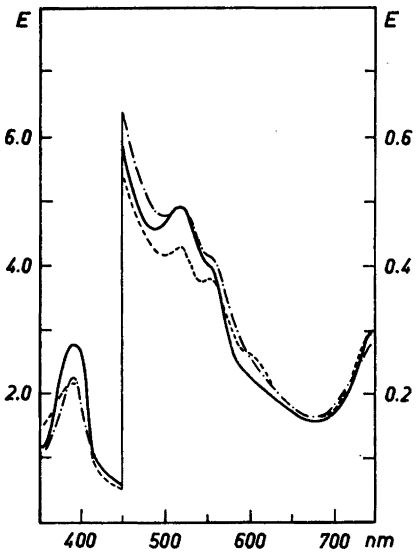

Fig. 7. The qualitative spectrum of ferripyrroLhb (———), that of its fluoride complex (—·—·—), and that of uncombined pyrrohemin plus apoLhb (----). The hemin, dissolved in pyridine and diluted with 0.01 N NaOH to a concentration of 1.5 mM, was combined with apoLhb at room temperature, and the mixture was centrifuged. The final spectra were taken 2 h after the mixing of pyrrohemin and apoLhb.

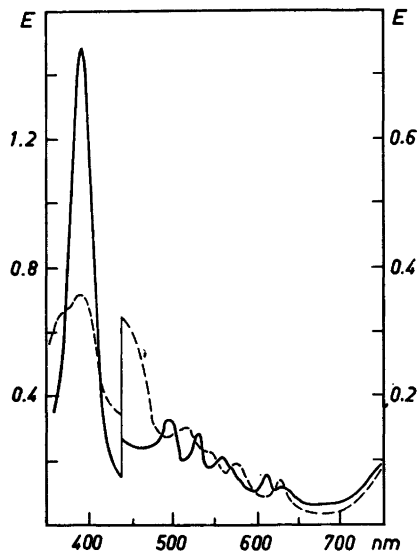

Fig. 8. The qualitative spectrum of mesoporphyrin-globin complex (———), and that of uncombined mesoporphyrin plus apoLhb (----). The experimental conditions are identical to those described in Fig. 1.

mesoporphyrin-apoLhb combination product exposed the following maxima: I, 630; II, 611; III, 558; IV, 530; V, 499 nm. A strong increase of the Soret band was observed on combination with a shift of maximum from 386 to 395 nm.

DISCUSSION

No crystallographic data are available on leghemoglobin and the recombination studies performed cannot be correlated with an exact picture of the apoprotein molecule. However, it is assumed that the general principles, which characterize a heme-protein as a hemoglobin, can be found in recent crystallografic studies on myoglobin [35-37] and hemoglobin.[38]

The first group of recombination experiments were performed in order to elucidate the extent of which different substituents in positions 2 and 4 of the porphyrin ring may influence the combination reaction between hemin and apoLhb. The spectrum of mesoLhb was found to clearly differ from those of hemato- and deuteroLhb:s, evidently indicating a stability difference between these compounds. In myoglobin the 2,4-vinyl-groups are involved in hydrophobic bonds with non-polar residues. Analogously, it is assumed that similar hydrophobic bonds are necessary to combine a hemin to apoLhb in order to form a stabile recombination product. No hydrophobic bonds could be formed with the hydrophilic 2,4-hydroxyethyl groups of hematohemin and non polar residues.

The effect of changes in the two propionate groups in positions 6 and 7 was also studied in several recombination experiments in order to elucidate their influence on the combination between hemin and apoLhb. The propionate groups have been supposed to play an important role in combining the heme to globin.[39] However, Kendrew [37] has recently questioned the role of the propionate groups in this respect.

Etiohemin having methyl groups instead of propionate groups in positions 6 and 7 was found to combine with apoLhb only to about 3 % of the hemin available. The recombination product exhibited a spectrum with diffuse maxima and minima indicating a labile compound. The recombination with pyrrohemin with one propionate group in position 7 seemed to proceed in good yield, although the combination product was somewhat labile. However, the recombination product obtained with pheophorbide a_5 hemin, which also has one propionate group in position 7, was stable. This difference between the two Lhb:s might depend on a difference in solubility in aqueous solutions, pheophorbide a_5 hemin being highly soluble as compared with pyrrohemin.

From these findings it is evident that a propionate group in position 7 strongly increases the recombination of a hemin with apoLhb. If this depends on the propionate group forming a salt linkage with apoLhb or on its giving a hydrophilic character to one part of the porphyrin ring, is difficult to decide on the basis of the experimental data available. In myoglobin the carboxyl group of the propionic acid residue in position 7 is free and that of the propionate group in position 6 forms salt linkages with groups in apomyoglobin.

The hemins studied formed all recombination products which gave complexes with fluoride indicating a specific ironprotein linkage. The secondary

role, which iron seems to play in binding hemin to apoLhb, was shown by the recombination of mesoporphyrin with apoLhb. This clearly illustrates prominence to the side chains of the porphyrin rather than to the iron on combining heme to apoLhb. The iron-protein bond seems to have importance only for the reactivity of the iron atom .

Recent rotatory dispersion studies [40,41] on myoglobin and apomyoglobin have shown that the removal of heme from myoglobin is accompanied by a comformational alteration reducing the content of α-helix. It is estimated that an unwinding to about 15 residues occurred. The addition of 1 mole of hematin to the globin resulted in restoration of the α-helix to that of native metmyoglobin. An identical restoration of the α-helix content was found by combining apomyoglobin with protoporhyrin.[42]

No rotatory dispersion studies have been performed with Lhb and its apoprotein, but it is very probable — if any conclusions can be drawn from the findings in this paper — that a somewhat similar conformation change occurs also with Lhb when the heme group is removed or when the Lhb is restored by combining heme with apoLhb.

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