

## Macromolecular Hemochromes: The System Ferroprotoporphyrin IX-Polylysine in Aqueous Medium

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Light-absorption spectra of complexes between reduced iron-protoporphyrin IX ( $2 \times 10^{-5}$  M) and various polylysine samples (up to  $2 \times 10^{-3}$  residue molar) were measured in the range 380–630  $m\mu$  in deaerated aqueous solutions of pH 12 and at 27°. The absorption spectra obtained were very similar to well-known ferrohemochrome spectra of proteins or of complexes of protoheme with low-molecular-weight nitrogenous bases at high concentrations. In contrast to analogous complexes of trivalent iron, there was no fundamental spectral difference, under optimum conditions, between complexes involving either poly- $\alpha$ ,L-lysine or poly- $\alpha$ ,DL-lysine. A 0.04 M L-lysine monomer did not produce a ferrohemochrome, but 0.01 M *n*-butylamine formed it to a large extent. The hemochrome band at 558  $m\mu$  of the synthetic complex ferroprotoporphyrin IX-poly- $\alpha$ ,L-lysine had its maximum extinction at pH 12.1. In the absence of air and at room temperature, reduction of the trivalent iron-protoporphyrin IX in the presence of poly- $\alpha$ ,L-lysine by either excess sodium ascorbate or dithionite led to the same spectral data. The synthetic macromolecular ferrohemochromes were autoxidizable. The integrated absorption of the various light-absorption bands was significantly higher for synthetic complexes involving higher molecular weight poly- $\alpha$ ,L-lysine ( $\overline{DP} = 700$ ) than for those of lower molecular weight ( $\overline{DP} = 70$ ).

It has been known for a long time that the prosthetic group iron-protoporphyrin IX forms distinct octahedral complexes under certain conditions, in particular with nitrogenous substances [(1-5) and refs. cited therein]. These are generally termed hemochromes or hemochromogens. Many hemochromes of FoP<sup>1</sup> have two characteristic light-absorption bands in the visible region with maxima at about 550–560  $m\mu$  ( $\alpha$ -band) and 520–530  $m\mu$  ( $\beta$ -band), respectively, while the Soret band positions are usually near 420–430  $m\mu$ . Low-spin values have been measured for many of these complexes involving strong-field ligands [see, for example, ref. (4), pp. 61–66]. Mammalian cytochrome *c* at neutral pH constitutes a

naturally occurring hemochrome [ref. (1), p. 349, and refs. cited]. Most ligands of protoheme hemochromes investigated so far were small molecules (1, 3). Recently, hemochromes of FiP with synthetic polyamino acids, in particular PLL (6-8) and poly-L-histidine (6, 9), have been described which, as hemoprotein models, are considered to be structurally closer to the native complexes than low molecular weight analogues. The polylysine also allowed for effective coordination of primary amino groups to the porphyrin iron (7). The present investigation deals with the formation and properties of hemochromes of divalent iron-protoporphyrin IX with either PLL or PDLL in aqueous medium in which the ligands are considered to originate from  $\epsilon$ -amino groups which constitute part of the macromolecular structure. It will be shown that, as in the case of trivalent heme iron, typical hemochromes are formed at relatively low concen-

<sup>1</sup> Abbreviations used: FiP, ferriprotoporphyrin IX; FoP, ferroprotoporphyrin IX (in both substances coordinated ligands not specified); PLL, poly- $\alpha$ ,L-lysine; PDLL, poly- $\alpha$ ,DL-lysine;  $\overline{DP}$ , average degree of polymerization.

trations of the polyamino acids, although there are significant differences in the conditions of complex stability in both valence states of the iron. A preliminary report of this work has been presented (10). Formation of a ferrohemochrome spectrum had been observed previously on sealing an aqueous solution of the trivalent iron complex FiP-PLL in a high vacuum (7) and also for FoP-PLL at pH 8.5 (11). Very recently, hemochromes of heme *a* with polylysine have been reported which were considered as models of cytochrome oxidase (12).

#### MATERIALS AND METHODS

*Protohemin (chloro-ferriprotoporphyrin IX)*. Twice-crystallized Sigma (St. Louis, Missouri) bovine hemin was further recrystallized and analyzed as described previously (7). The crystals were stored in a desiccator in the dark at 2–5°. After dissolving 10–20 mg of protohemin in 100 ml of 0.01N aqueous NaOH, which took about 2 hours at room temperature, the stock solutions were stored in the dark and in air at 2–5°. Under these conditions, the light-absorption spectrum of FiP remained constant for about 2 weeks [mM at room temperature of absorption maxima in the region 300–650 m $\mu$  (see also ref. 13): 385 m $\mu$ , 61  $\pm$  2; 490–493 m $\mu$ , 6.4  $\pm$  0.1; 610–612 m $\mu$ , 4.6  $\pm$  0.1]. The band at 490–493 m $\mu$  seemed to be most sensitive to changes. Care was taken to exclude CO<sub>2</sub> and direct light from all solutions containing the heme.

*Poly- $\alpha$ ,L-lysine-HBr and poly- $\alpha$ ,DL-lysine-HBr* were obtained from the Biophysics Department, Weizmann Institute, and the Yeda Co., Rehovot, Israel, respectively. These poly- $\alpha$ -amino acids, which were prepared from the corresponding  $\epsilon$ ,*N*-carbobenzyloxy- $\alpha$ ,*N*-carboxy-lysine anhydrides by conventional polymerization in solution using amines as initiators (14), were treated and characterized as described previously (7). Two PLL samples were used: Sample PLL-H ( $\overline{DP}$  = 700) and Sample PLL-L ( $\overline{DP}$  = 70) as estimated on the parent carbobenzyloxy compounds by viscosity measurements in *N,N*-dimethylformamide which were calibrated by sedimentation-diffusion data. After recarbobenzyloxylation of the PLL samples (15), practically the same molecular weights as for the parent carbobenzyloxy compounds were obtained by the above method. Thus the average molecular weight of these PLL samples did not change significantly upon removal of the carbobenzyloxy groups by HBr. The parent  $\epsilon$ ,*N*-carbobenzyloxy compound of PDLL (1:1 copolypeptide) had an estimated  $\overline{DP}$  of 190.

Stock solutions of the polyamino acids [(1–2)  $\times$  10<sup>-2</sup> M] in water were stored near 0°. Their concentrations were checked by micro-Kjeldahl determinations of nitrogen. In all cases, concentrations of the polyamino acids are given in monomolar units (moles amino acid residues per liter).

*L-Lysine-HCl*. A commercial preparation was dried *in vacuo* at room temperature.

*n-Butylamine*. A B.D.H. product was distilled and the middle fraction was taken.

*L-Ascorbic acid*. Both N.B.C. (Cleveland, Ohio) and B.D.H. (Poole, England) analytical grade products were used. Titration with 2,6-dichlorophenolindophenol indicated over 99% purity, even when titrated immediately after bringing solutions to pH 12. Under the experimental conditions used in this work (see below), 95% of the original amount was titrated after two hours in alkaline solution and in an atmosphere of argon. In air, and under otherwise similar conditions, only 80% of the ascorbic acid remained after one hour.

*Sodium dithionite* was a commercial B.D.H. product. In some experiments, a N.B.C. preparation was used.

*Argon* was 99.8% according to the manufacturers (Chemicals and Phosphates Ltd., Haifa, Israel).

*Nitrogen* was 99.9% (Matheson Co., New Jersey). It was passed through a bubbler containing 10% aqueous NaOH to remove residual CO<sub>2</sub>.

Other chemicals were analytical grade. Double distilled water was used and its conductivity was checked frequently.

*pH measurements*. Two pH meters were used: (a) Photovolt (New York) with Beckman entire pH range glass electrodes; and (b) Radiometer (Copenhagen, Denmark) Titrator TTT1a with B-type glass electrodes (low Na-ion error).

*Light-absorption spectra*. Both Beckman DU and DB spectrophotometers were used. In the DU instrument the cell compartment was kept at constant temperature by circulation of water from a thermostat. The absorbance read by the instruments was checked frequently by standard alkaline chromate solutions (16), and the wavelength scale by a mercury lamp. The absorbance in the range 400–600 m $\mu$  of mixtures of either PLL and ascorbate or PLL and dithionite as used under the usual experimental conditions of this work was found to be negligible. Distilled water was therefore used as reference in all cases. The absorbance of the bands at 428 and 558 m $\mu$ , respectively, of the ferrohemochrome FoP-PLL (see below) was found to be independent of slit width in the range of measurements of 0.02–0.1 mm. If not indicated otherwise, reproducibility of absorbance maxima was within about 5%.

$\epsilon_{\text{mM}}^{\lambda}$  = decadic extinction coefficient at a wavelength  $\lambda$  ( $m\mu$ ) in  $\text{mm}^{-1} \text{cm}^{-1}$ , calculated per gram-atom of heme iron.

### Procedure

Solutions of FiP and polyamino acid or any other component were mixed at room temperature and adjusted to the desired pH by aqueous NaOH. Aqueous solutions of the reducing agent were brought close to the desired pH. They were used not later than 15 minutes after their preparation. Two principal methods of reduction were employed:

(A) A reaction vessel consisted of a round-bottomed flask and an optical cell of 1-cm light path as a side arm. Four ml of the solution containing FiP (and a complexing component) was placed into the round-bottomed flask together with a magnetic stirrer. An alkaline solution of the reducing agent (ascorbic acid in most cases), 0.01–0.03 ml, was introduced into the optical cell by means of an Agla syringe fitted with a micrometer. The reaction vessel was then connected to a vacuum line and the solutions were degassed by repeated cycles of freezing, pumping, thawing, and admitting argon at a pressure close to one atmosphere while stirring the solution. The reaction vessel was detached from the vacuum line, the main solution was brought to a desired temperature in a thermostat and mixed with the reducing agent in the cell, and the latter was placed into the spectrophotometer; precautions were taken to exclude light from the cell compartment. The absorbance at 558  $m\mu$  was followed until a constant value was obtained (see below) and the complete spectrum was then measured. Immediately afterward, the pH was recorded and taken as the value prevailing at the time of the spectral measurements. This value was always lower than the pH before reduction.

(B) The solution containing FiP and other nonreducing components was brought to the temperature of measurement in an optical cell (1.0 cm light path) and argon was bubbled through it for several minutes. Some grains of solid dithionite were then dissolved in the solution by rapid stirring. After reaching a constant absorbance and recording the spectrum a small additional quantity of dithionite was added. No further significant change in absorbance could be observed in most cases. pH determination and changes were as in Method A.

### RESULTS

*Spectra of FoP in the absence of bases.* Reduction of FiP at pH 12 by Method A (excess ascorbate) did not lead to reproducible re-

sults. The absorption spectrum in the visible range was diffuse (Soret band at about 390  $m\mu$ ). Better reproducibility was obtained by Method B (dithionite) [ $\epsilon_{\text{mM}}^{560} \simeq 6$  (max.) under conditions given for Fig. 1]. Both FiP and FoP are considered to be aggregated in alkaline aqueous medium under the conditions of this work (see, for example, refs. 19, 21, 4).

*L-Lysine.* Under the conditions given for Fig. 1 ( $2 \times 10^{-5}$  M FoP) with dithionite as reducing agent, and up to 0.04 M lysine, no complex formation could be ascertained.

*Complexes of FoP with polylysine.* Typical light-absorption spectra obtained under favorable conditions (see below) at pH 12 upon reduction of systems involving FiP and various samples of polylysine are presented in Fig. 1. In all cases, the  $\alpha$ -band has its maximum absorption at 558  $m\mu$ , the  $\beta$ -band at 528  $m\mu$ , and the Soret band at 428–429  $m\mu$ . The corresponding  $\epsilon$ -values, however, obtained by dividing the measured absorbance per centimeter by the molar concentration of total heme, are significantly different for complexes involving an excess of either high or low molecular weight PLL (see Fig. 2). For PLL-H:  $\epsilon_{\text{mM}}^{558} = 35.0 \pm 0.7$ ;  $\epsilon_{\text{mM}}^{528} = 15.7 \pm 0.7$ ;  $\epsilon_{\text{mM}}^{429} = 137 \pm 3$  (arithmetic means of 30, 30, and 10 experiments, for each maximum, respectively, involving both Methods A and B. Maximal deviations are indicated). For PLL-L:  $\epsilon_{\text{mM}}^{558} = 29.0 \pm 1$ ;  $\epsilon_{\text{mM}}^{528} = 13.0 \pm 0.5$ ;  $\epsilon_{\text{mM}}^{428} = 110 \pm 4$  (15, 15, and 3 experiments, respectively). The ratio of  $\epsilon^{\alpha}/\epsilon^{\beta}$  is  $2.2 \pm 0.2$  for both PLL systems. The complex involving PDL has absorption values close to those observed for PLL-L:  $\epsilon_{\text{mM}}^{558} = 29.0 \pm 1.5$ ;  $\epsilon_{\text{mM}}^{528} = 13.3 \pm 0.2$ ;  $\epsilon_{\text{mM}}^{428} = 105 \pm 2.5$  (all from 3 experiments). Again,  $\epsilon^{\alpha}/\epsilon^{\beta} = 2.2 \pm 0.2$ .

*Reproducibility of spectral data under modified conditions.* The system FoP-PPL-H was measured under the conditions given for Fig. 1 with the following changes introduced: (a) addition of 0.01 M sodium carbonate; (b) rigorous exclusion of air from all solutions prior to the regular deaeration procedure described above (Methods A and B); (c) reduction by ascorbate (Method A) in an atmosphere of nitrogen; (d) Method A was used to introduce PLL-H into the side arm together with the ascorbate prior to evacua-

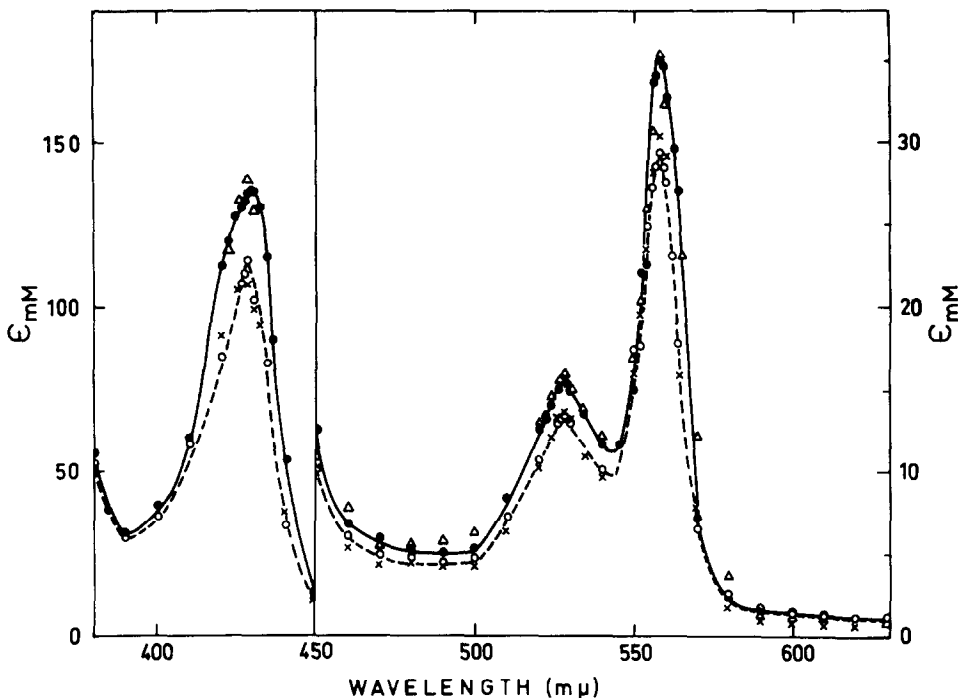


Fig. 1. Light-absorption spectra in water of reduced iron-protoporphyrin-polylysine complexes. Temp.,  $27 \pm 1^\circ$ ; pH 12.0–12.1; anaerobic conditions. ● FoP  $2.0 \times 10^{-5}$  M; PLL-H  $8.5 \times 10^{-4}$  M; reducing agent: dithionite.  $\Delta$  FoP  $1.4 \times 10^{-5}$  M; PLL-H  $8.5 \times 10^{-4}$  M; reducing agent: ascorbate. ○ FoP  $2.0 \times 10^{-5}$  M; PLL-L  $2.4 \times 10^{-3}$  M; reducing agent: dithionite.  $\times$  FoP  $2.0 \times 10^{-5}$  M; PDLL  $8.8 \times 10^{-4}$  M; reducing agent: dithionite. All reducing agents were in large excess of FoP.

tion and filling with argon; (e) the ascorbate was added to FiP in the flask, and PLL-H was placed in the optical cell; (f) FiP was reduced by dithionite at pH 12, and after 15 minutes a solution of PLL-H at pH 12 was added.

In all cases except (c), the  $\epsilon$ -values obtained for the  $\alpha$ - and  $\beta$ -bands were identical with those of Fig. 1, within the limits of error. Lower values obtained in case (c) may have been due to impurities (residual oxygen) in the nitrogen used. Apparently, the order of addition of complex components or reducing agent had no influence on the final values of absorption maxima of both  $\alpha$ - and  $\beta$ -bands.

*Dependence of FoP-PLL complex formation on time and temperature.* Under the conditions given for Fig. 1, reduction by dithionite (estimated  $10^{-3}$  M) led to constant  $\epsilon$ -values within several minutes of its addition. No further change could be observed up to 96 hours. Upon reduction under similar con-

ditions but at  $3^\circ$ ,  $\epsilon_{\text{mM}}^{558}$  was 33.6 after 10 minutes (PLL-H).

Again under the conditions given for Fig. 1, upon reduction by excess ascorbate (minimum concentration required was  $4 \times 10^{-4}$  M) about 40 minutes elapsed before attainment of the final value of  $\epsilon^{558}$ . Both  $\epsilon^{558}$  and  $\epsilon^{528}$  then remained constant up to 20 hours at least. Intermediate and final spectra had isosbestic points at about 590, 490, and 400  $m\mu$ , respectively.

When the reduction by ascorbate was carried out at temperatures between 20 and  $40^\circ$ , the same final  $\epsilon$ -values were obtained in each case. Below  $20^\circ$ , however, lower final values resulted and the time required to reach a constant value was longer. For example, at  $3^\circ$ ,  $\epsilon_{\text{mM}}^{558} = 26.2$  and  $\epsilon_{\text{mM}}^{528} = 13.3$  were maximum values reached after about 100 minutes under the conditions given for Fig. 1, in the presence of PLL-H. If brought to  $27^\circ$  from low temperatures, the  $\epsilon$ -values

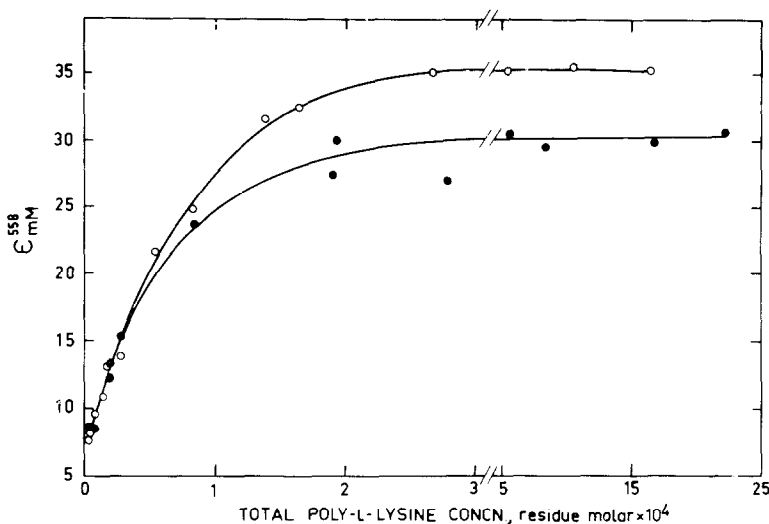


FIG. 2. Spectrophotometric titration of FoP with PLL. Total FoP  $2.0 \times 10^{-5}$  M; pH 12.1  $\pm 0.1$ ; temp.  $27 \pm 0.5^\circ$ ; reducing agent: dithionite (Method B).  $\circ$  PLL-H;  $\bullet$  PLL-L.

corresponding to the higher temperatures were obtained within several minutes. On subsequent cooling back to a lower temperature (e.g.,  $7^\circ$ ), however, the  $\epsilon$ -values remained practically unchanged. Thus, some kind of inhibition of the reduction by ascorbate seems to occur when the reduced complex is prepared at lower temperatures.

*Effect of added salt.* Reduction of the system FiP-PLL (either PLL-H or PLL-L) by dithionite under the conditions given for Fig. 1 and in the presence of 1 M NaCl resulted in the same band positions and corresponding  $\epsilon$ -values as were obtained in the absence of added salt.

*Complex formation at varying concentrations of PLL.* Results are summarized in Fig. 2. For the saturated complex, an average mole ratio of lysine residues:bound heme of about 12:1 is estimated for both PLL-L and PLL-H, which is very similar to that obtained from the analogous titration of the trivalent iron complex FiP-PLL (7). With increasing concentrations of PLL, higher equivalent concentrations of NaBr will be present owing to neutralization of the PLL-HBr. These are not considered to influence the results (see Effect of Salt). For spectra measured at different concentrations of PLL-H, isosbestic points were obtained near 570, 500, 450, and 405  $m\mu$ , respectively.

*pH dependence.* Each point in Fig. 3 was obtained in a separate reduction experiment. A maximum value of  $\epsilon^{558}$  is found at pH 12.1. Reproducibility at various pH values as well as agreement between both methods of reduction were fair. However, the final absorption values were found to depend considerably on the mode of preparation of the complex. When the trivalent complex was prepared and reduced at  $\text{pH} \leq 10$  and then brought to pH 12 by concentrated NaOH, the maximum absorption values were considerably lower than those obtained at pH 12 by starting from about pH 12.5–11.5, if all other conditions are kept constant. The data of Fig. 3 refer to the regular conditions where the pH was higher before than after reduction (see Procedure).

*Dilution of complex.* Experiments were conducted at an initial FoP concentration of  $2.0 \times 10^{-5}$  M, at pH 12.1,  $27 \pm 0.5^\circ$ , and using dithionite (Method B). Up to fourfold dilution of the reduced complex with aqueous NaOH of pH 12 at excess of either PLL-H or FoP (see Fig. 2) did not result in any significant change in  $\epsilon^{558}$ . Thus Beer's Law is obeyed and the complex does not appear to dissociate under these conditions. Dilution of either complex component while keeping the other constant resulted in values of  $\epsilon^{558}$  which corresponded to those expected

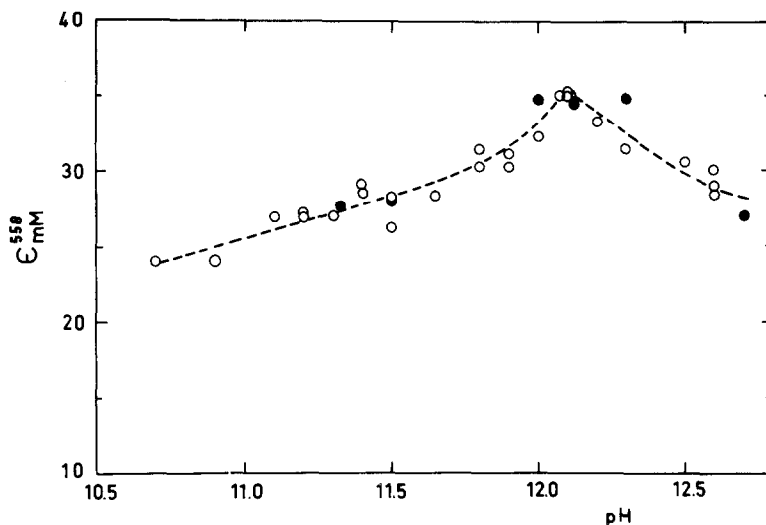


FIG. 3. pH dependence of  $\epsilon^{558}$ . FoP  $2 \times 10^{-5}$  M; PLL-H  $(8-10) \times 10^{-4}$  M; temp.  $27 \pm 1^\circ$ . ○ Reduction by  $(4-5) \times 10^{-4}$  M ascorbate (Method A). ● Reduction by excess dithionite (estimated of the order  $10^{-3}$  M) (Method B).

for a particular mole ratio of FoP:PLL-H according to Fig. 2, provided  $\epsilon^{558}$  were unaffected by dilution. Thus, reversibility of the system is indicated under these conditions, since similar  $\epsilon$ -values could be obtained directly by mixing more dilute solutions.

All solutions used for dilution were deaerated by argon prior to their use.

*Autoxidation of the reduced complex.* When a limited amount of air was admitted to a complex involving  $1 \times 10^{-3}$  M PLL-H and which was reduced by ascorbate (Method A) at optimum conditions (Fig. 1),  $\epsilon^{558}$  decreased at first but increased again with time provided an excess of ascorbate was present. Cycles of increase and decrease of absorption at 558 m $\mu$  could be repeated until  $\epsilon_{\text{mM}}^{558}$  remained below 10. The final spectra obtained were different from those measured for the trivalent iron system FiP-PLL-H under analogous conditions. This indicates irreversible reactions which may take place upon reoxidation of the complex.

Attempts to measure the oxidation-reduction potential of the complex were unsuccessful so far. The system was sluggish even in the presence of a mediator and results were not reproducible.

*n-Butylamine.*  $2 \times 10^{-5}$  M FiP in 0.01 N NaOH and at  $27^\circ$  was reduced with dithionite (Method B) in the presence of 1.0 M

aqueous *n*-butylamine. Spectra were similar to those obtained with low molecular weight PLL-L, though there was a shift of about 4 m $\mu$  to shorter wavelengths in all maxima ( $\epsilon_{\text{mM}}^{554} = 30.5$ ;  $\epsilon_{\text{mM}}^{524} = 13.4$ ;  $\epsilon_{\text{mM}}^{424} = 105$ ). In the presence of 0.01M *n*-butylamine:  $\epsilon_{\text{mM}}^{562} = 26.3$ ;  $\epsilon_{\text{mM}}^{531} = 13.2$  (for the effect of *n*-propylamine, see ref. 3, p. 74).

## DISCUSSION

*Complex ligands.* In summarizing the effects of the various variables investigated on complex formation between the prosthetic group iron-protoporphyrin IX and polylysine, the conditions given for Fig. 1 are considered optimal at the FoP concentration given. According to the spectral type obtained, this complex may clearly be classified as a ferrohemochrome (1-5). (For recent and short reviews on theoretical aspects of these spectra see, for example, refs. 4, 17). *Per analogiam* to other complexes of a similar spectral type it may be assumed that two  $\epsilon$ -amino groups of the polylysine side chains provide the ligands on coordination positions 5 and 6 of the porphyrin iron (1-5, 18). This octahedral complex may be expected to be diamagnetic (3, 8).

*Steric requirements for complex formation and stability.* There is practically no spectral difference between ferrohemochromes of

PDLL and low molecular weight PLL formed under similar conditions (Fig. 1). In contrast, two different complexes ("red" with PLL and "green" with PDLL of  $\overline{DP} = 190$ , respectively) were found at pH 11 in the case of trivalent iron-protoporphyrin (6, 7), where an ordered structure of the polyamino acid was considered necessary for formation of the "red" complex (6, 7). [It may be assumed for steric reasons, that PDLL has a random-coil structure at pH 12 while PLL is helical above about pH 10 and "randomly coiled" at lower pH (see refs. 6, 7 and refs. cited therein)]. In the present ferrohemochromes the absence of a spectral difference could be explained by greater stability in the pH range considered of the ferrohemochrome as compared with the ferri-complexes (see refs. 19, 11; 1, p. 182), so that the ferrohemochrome is formed with PDLL even under less favorable steric conditions. Also, the formation of a "green" complex seems to depend on the hydroxyl ion coordinated to the trivalent protoheme iron (20; 21, for pyridine as ligand) which is absent in the divalent iron complex in the pH range considered (see, for example, refs. 21; 4, p. 48).

A full discussion of the mode of binding and of the factors affecting stability of "red" hemochromes involving trivalent iron has been presented previously (7, 8) and could be applied in the present case, at least for complexes involving helical PLL: In this case it has been postulated that the two  $\epsilon$ -amino groups coordinating to the heme iron originate from two helices (8) and that cooperative binding such as hydrogen bonding and/or certain entropy terms contribute to the overall stability of the complex (7). Previously, hemochrome formation with lysine residues in polylysine has been reported to be more effective than with lysine monomer (11, 18). Also, ferrohemochrome formation of FoP with some alkali-denatured proteins was found to occur more readily than with basic amino acids (22; see also ref. 23 and refs. cited). A better comparison between the macromolecule and its monomer with regard to hemochrome formation is offered by the use of primary amines of low molecular weight. As the present data show, 0.01 M *n*-butylamine forms some hemochrome with FoP, but under similar conditions, 0.04 M L-

lysine does not. This difference may be due to electrostatic repulsion between the carboxylates of FoP and of L-lysine (7). It remains to be shown to what extent ferrohemochromes formed with macromolecules are more stable than those with the corresponding monomeric bases.

*pH dependence.* The pH optimum (Fig. 3) of formation of the ferrous complex is about 1 pH unit higher than that of the ferric complex as measured for  $7 \times 10^{-5}$  M FiP (7). This difference may be due to more effective competition between hydroxyl ion and a nitrogenous ligand in the ferric complex. The decrease in the absorption maximum of the ferrous complex in the range pH 10-12 is more difficult to explain. The increasing charge on the  $\epsilon$ -amino groups of polylysine [intrinsic  $pK_a = 10.4$  at room temperature (24)] may interfere with complex formation at pH < 10 (electrostatic dissociation of complex; ligand inactivation by protonation).

*Effect of salt.* The ferrohemochrome differs from the trivalent iron system by the absence of a spectral effect at high concentrations of salt. As above, both increased complex stability, and absence of coordinated OH<sup>-</sup> in the ferrous system may account for this result.

*Effect of molecular weight.* It appears from Fig. 1 (also Fig. 2) that the light absorption in all bands is significantly higher for the higher molecular weight polyamino acid. The ratio *r* of the integrated absorption bands (apparent oscillator strengths) of PLL-H ( $\epsilon_1$ -values) and PLL-L ( $\epsilon_2$ ), respectively,

$$r = \frac{\int \epsilon_1 d(1/\lambda)}{\int \epsilon_2 d(1/\lambda)}$$

is found to be 1.075 for the combined  $\alpha$ - and  $\beta$ -bands and 1.19 for the Soret band. In the presence of excess PLL-L it is unlikely that this effect results from a significant fraction of unbound heme (Fig. 2). This may therefore be considered to constitute a hyperchromic effect due to optical interactions of FoP molecules arrayed between helical molecules of PLL-H as in the case of the analo-

TABLE I  
SELECTED  $\lambda_{\max}$  AND  $\epsilon_{\max}^{\lambda}$  VALUES<sup>a</sup> OF SOME NATURAL AND SYNTHETIC FERROHEMOCHROMES OF FO P IN AQUEOUS MEDIUM

Substance	Conditions <sup>b</sup>	Soret-band max.			$\beta$ -band max.			$\alpha$ -band max.		
		$\lambda(\text{m}\mu)$	$\epsilon_{\text{mM}}$	Ref.	$\lambda(\text{m}\mu)$	$\epsilon_{\text{mM}}$	Ref.	$\lambda(\text{m}\mu)$	$\epsilon_{\text{mM}}$	Ref.
Cytochrome <i>c</i> <sup>c</sup> (horse heart)	pH 6.8	416	129.1	25	520.5	15.9	25	550.2	27.7	25
Cytochrome <i>b<sub>2</sub></i> <sup>d</sup> (baker's yeast)	neutral pH	423	232	3(p. 509)	528	18.6	3(p. 509)	556.5	38.8	3(p. 509)
Denatured human globin-FoP	pH > 12				528	14.8	26	558	29.1	26
Pyridine-FoP	20 vol. % pyridine + alkali	418.8	157	27	526.5	17.0	27	558	30.6	27
PLL-H-FoP	See Fig. 1	429	137		528	15.7		558	35.0	
PLL-L-FoP	See Fig. 1	428	110		528	13.0		558	29.0	
Poly-L-histidine-FoP	pH 10	425	115	9	529	12	9	560	19	9

<sup>a</sup> Units [(mM Fe)<sup>-1</sup> (cm)<sup>-1</sup>] assumed where these not stated explicitly in original reference.

<sup>b</sup> For further details, see references.

<sup>c</sup> At least part of the shift to shorter wavelengths is due to saturation of vinyl groups of the heme by thioether linkages (26, 1, 28).

<sup>d</sup> Contribution of flavin to the absorption considered to be small (3, p. 509).

gous ferric complexes (7). Since PDLL (1:1 copolymer) (Fig. 1) probably has a random conformation for steric reasons, the absence of the above effects would not be surprising.

**Reduction.** A large excess of reducing agent is always required for the present system. This may be due, at least in part, to the rapid oxidation of the reducing agent by residual oxygen in solution. It is assumed that no changes other than reduction of the iron are taking place on the prosthetic group during the course of reduction. This is in accordance with the fact that two different reducing agents lead to the same spectrum of the reduced complex.

**Comparison with other ferrohemochromes.** A comparison of the selected spectral data of Table I confirms both the hemochrome nature and the strong binding properties of the synthetic macromolecular models. No definite conclusions with regard to the nature of the binding groups in the various proteins can be drawn from the models because of the similarity of spectra obtained with different ligands and because of the different possible

states of protonation of a given ligand in the proteins as compared with the synthetic complexes under the conditions measured.

#### REFERENCES

1. LEMBERG, R., AND LEGGE, J. W., "Hematin Compounds and Bile Pigments," Chapter V, p. 159. Wiley, (Interscience), New York (1949).
2. PAULING, L., "The Nature of the Chemical Bond," 3rd edition, p. 165. Cornell Univ. Press, Ithaca, New York (1960).
3. FALK, J. E., LEMBERG, R., AND MORTON, R. K., (eds.), "Haematin Enzymes," pp. 1-172. Pergamon Press, Oxford (1961).
4. FALK, J. E., "Porphyrins and Metalloporphyrins." Elsevier, Amsterdam (1964).
5. MARTELL, A. E., AND CALVIN, M., "Die Chemie der Metallchelateverbindungen." p. 335. Verlag Chemie, Weinheim (1958).
6. BLAUER, G., *Nature* **189**, 396 (1961).
7. BLAUER, G., *Biochim. Biophys. Acta* **79**, 547 (1964).
8. BLAUER, G., AND EHRENBERG, A., *Acta Chem. Scand.* **17**, 8 (1963).
9. TOHJO, M., AND SHIBATA, A., *Arch. Biochem. Biophys.* **103**, 401 (1963).



10. BLAUER, G., AND YONATH, A., *Israel J. Chem.* **3**, 90 p. (1965-1966).
11. AKOYUNOGLU, J. H. A., OLCOTT, H. S., AND BROWN, W. D., *Biochemistry*, **2**, 1033 (1963).
12. KING, T. E., YONG, F. C., AND TAKEMORI, S., *Biochem. Biophys. Res. Commun.* **22**, 658 (1966).
13. HOGNESS, T. R., ZSCHEILE, F. P., JR., SIDWELL, A. E., JR., AND GUZMAN BARRON, E. S., *J. Biol. Chem.* **118**, 1 (1937).
14. KATCHALSKI, E., AND SELA, M., *Advan. Protein Chem.* **13**, 243 (1958).
15. YARON, A., AND BERGER, A., *Biochim. Biophys. Acta* **69**, 397 (1963).
16. NATIONAL BUREAU OF STANDARDS, Letter Circular 1017, p. 15 (1959).
17. PHILLIPS, J. N., in "Comprehensive Biochemistry" (M. Florkin and E. H. Stotz, eds.), Vol. 9, p. 34. Elsevier, Amsterdam (1963).
18. KEILIN, J., *Nature* **187**, 365 (1960).
19. DAVIES, T. H., *J. Biol. Chem.*, **135**, 597 (1940).
20. BLAUER, G., AND ROTTENBERG, H., *Acta Chem. Scand.* **17**, S216 (1963).
21. SHACK, J., AND CLARK, W. M., *J. Biol. Chem.* **171**, 143 (1947).
22. KEILIN, J., *Nature* **165**, 151 (1950).
23. KAJITA, A., UCHIMURA, F., MIZUTANI, H., KIKUCHI, G., AND KAZIRO, K., *J. Biochem.* **46**, 593 (1959).
24. KATCHALSKY, A., SHAVIT, N., AND EISENBERG, H., *J. Polymer Sci.* **13**, 69 (1954).
25. MARGOLIASH, E., AND FROHWIRT, N., *Biochem. J.* **71**, 570 (1959).
26. DRABKIN, D. L., *J. Biol. Chem.* **146**, 605 (1942).
27. GALLAGHER, W. A., AND ELLIOTT, W. B., *Biochem. J.* **97**, 187 (1965).
28. MORTON, R. K., *Rev. Pure Appl. Chem. (Australia)* **8**, 161 (1958).