actual experiment employed 5.284 mmoles of $(CH_3)_2NP(CH_3)_2$ and 7.912 mmoles of HCl. The combination occurred in a 50-ml. vertical reaction tube attached to the high-vacuum system, with slow warming from −100° to room temperature. The volatile product now had physical properties indicative of purity; for example, the molecular weight of the 80% saturated vapor was measured as 97.9; calcd., 96.5.

Numerous earlier attempts to make $(CH_3)_2PCl$ by the thermal dissociation of $(CH_3)_3PCl_2$—a method reported by Plets for other R_2PCl compounds[7]—had led only to a non-volatile brown solid, HCl, PCl_3 and all of the chloromethanes. Once, however, we obtained a yield of $(CH_3)_2PCl$ representing a little less than 2% of the phosphorus. It was identified by its mol. wt. (obsd., 97.2; calcd., 96.5), its vapor tension of 34 mm. at 0° and a chloride analysis (found, 37.1%; calcd., 36.7).

Physical Properties of Dimethylchlorophosphine.—The vapor tensions of pure solid $(CH_3)_2PCl$, shown in Table II, determined the equation $\log_{10}p_{mm.} = 12.1408 - 2887/T$.

TABLE II

VAPOR TENSIONS OF SOLID $(CH_3)_2PCl$

t (°C.)	−39.8	−34.0	−27.4	−18.6	−12.2$_5$	−5.5
$p_{mm.}$ (obsd.)	0.57	1.19	2.44	6.31	11.92	22.62
$p_{mm.}$ (calcd.)	0.59	1.17	2.47	6.31	11.91	22.63

It was more difficult to obtain consistent measurements for the liquid, which slowly and irreversibly formed a slightly soluble non-volatile solid on standing. After each measurement the apparatus could be cleared by flaming *in vacuo* to form volatile materials whose constitution cannot yet be inferred. The vapor tension values shown in Table III determine the equation $\log_{10}p_{mm.} = 7.844 - 1722/T$. This would indicate the normal b.p. as 71°, but since the Trouton constant (22.9 cal./deg. mole) is a little above normal, the true b.p. probably is nearer to 73°.

TABLE III

VAPOR TENSIONS OF LIQUID $(CH_3)_2PCl$

t (°C.)	0.00	2.4	9.4	15.3	19.8$_3$	21.7	33.1
$p_{mm.}$ (obsd.)	34.6	39.5	56.6	74.9	91.5	99.5	168.7
$p_{mm.}$ (calcd.)	34.7	39.4	56.2	74.9	92.6	100.9	166.5

From the two vapor tension equations the heat of fusion is calculated as 5.33 kcal./mole and the m.p. as −2.0°. The directly observed melting range was −1.4 to −1.0°.

Aminolysis of Dimethylchlorophosphine.—Samples of $(CH_3)_2PCl$ (0.437 mmole) and $(CH_3)_2NH$ (0.947 mmole) were measured as gases and allowed to warm together from −78° to room temperature during 8 hr. The excess amine was distilled through a trap at −78° and measured as 0.071 mmole, meaning that 0.876 mmole of the amine had combined (calcd., 0.876). The volatile product trapped out at −78° had a vapor tension of 12.1 mm. at 0°, corresponding to pure $(CH_3)_2NP(CH_3)_2$; weight 42.2 mg., or 0.402 mmole (92.0% of the expected 0.437). The non-volatile product was analyzed for Cl^-: found, 0.437 mmole, strictly in accord with the equation

$$(CH_3)_2PCl + 2(CH_3)_2NH \longrightarrow (CH_3)_2NP(CH_3)_2 + (CH_3)_2NH_2Cl$$

This result is a further confirmation of the formula $(CH_3)_2PCl$. It appears that 8% of the expected $(CH_3)_2NP(CH_3)_2$ remained with the $(CH_3)_2NH_2Cl$—in what form cannot be judged.

Ammonolysis of Dimethylaminodimethylphosphine.—An initial micro-scale experiment showed that ammonia attacks $(CH_3)_2NP(CH_3)_2$ fairly rapidly at room temperature, displacing more than $2(CH_3)_2NH$ per NH_3 used up, and forming polyphosphino-amines. For more accurately quantitative results, 2.3725 g. (22.57 mmoles) of $(CH_3)_2NP(CH_3)_2$ and 53.2 mg. (3.13 mmoles) of NH_3 were heated for 22 hr. in a 100-ml. sealed tube at 46°. Then the volatile components were separated by high-vacuum fractional condensation, with U-traps at 0, −23, −112 and −196°. The recovery of NH_3 was 0.50 mmole (used, 2.63) and of $(CH_3)_2NP(CH_3)_2$, 16.60 mmoles (used, 5.97 mmoles). The dimethylamine weighed 268.4 mg. (5.96 mmoles; mol. wt. 45.3 *vs.* calcd. 45.1; vapor tension 556 mm. at 0° *vs.* known value, 562). The yield of almost pure $[(CH_3)_2P]_2NH$ was 273.5 mg. (1.995 mmoles); and after very thorough refractionation, the pure product melted in the range 39–40° and showed a vapor tension of 4.1 mm. at 30° (literature values, m.p. 39.5° and 4.13 mm. at 30°).[4] Thus the overall reaction balance would require that the slightly volatile oily liquid residue contain 1.98 mmoles of $(CH_3)_2P$ groups and 0.635 N, reasonably interpreted as 0.65 mmole of $[(CH_3)_2P]_3N$. The stoichiometry of the whole experiment is well represented by

$$9(CH_3)_2NP(CH_3)_2 + 4NH_3 \longrightarrow 3[(CH_3)_2P]_2NH + [(CH_3)_2P]_3N + 9(CH_3)_2NH$$

The recovery of some ammonia after exposure to a great excess of the aminophosphine suggested that the reaction is sensibly reversible.

Another approach to this complex equilibrium system was tried in an experiment wherein 3.44 mmoles of $(CH_3)_2NP(CH_3)_2$ and 0.479 mmole of $[(CH_3)_2P]_2NH$ reacted partially during 20 hr. at 105–130° to form 0.564 mmole of $(CH_3)_2NH$ and 0.216 mmole of $[(CH_3)_2P]_3N$. The presence of 1.1 mg. (0.068 mmole) of NH_3 again indicated the reversibility of the original ammonolysis.

LOS ANGELES 7, CALIFORNIA

(7) V. M. Plets, Dissertation, Kazan, 1938, cited by G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley and Sons, New York, N. Y., 1950, p. 47.

[CONTRIBUTION NO. 1467 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Hemoglobin Studies. I. The Combination of Carbon Monoxide with Hemoglobin and Related Model Compounds

BY JUI H. WANG, AKITSUGU NAKAHARA[1] AND EVERLY B. FLEISCHER

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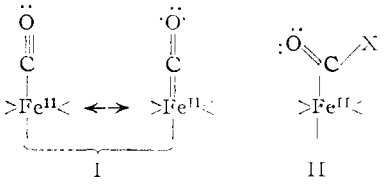
The nature of binding of carbon monoxide to hemoglobin, heme and Fe(II)-dicysteinate was studied by means of infrared and magnetic measurements. The formation constant of carbonmonoxyheme was determined and compared to that of carboxyhemoglobin. A possible cause for the unusual resistance of hemoglobin derivatives toward oxidation by molecular oxygen was proposed.

Although it has long been well-known that hemoglobin and myoglobin combine reversibly with molecular oxygen, whereas aqueous solutions of free heme do not, it appears that no satisfactory explanation has yet been proposed to account for this important difference in their chemical property. The mere formation of a coördination bond between a donor group of globin and the ferrous iron in heme is clearly inadequate to prevent the irreversible oxidation of the latter by molecular oxygen, for the ferrohemochromogens formed by combining heme with ammonia, pyridine, imida-

(1) On leave from the Department of Chemistry, Osaka University, Nakanoshima, Osaka, Japan.

zole, etc., are readily oxidized to the ferrihemochromogens when aqueous solutions of the former are shaken with air. In the absence of oxygen, heme and ferrohemochromogens do combine reversibly with carbon monoxide. But these carbonmonoxyheme compounds are oxidized rapidly when their aqueous solutions are exposed to air. By analogy, it has been conjectured that heme also combines with molecular oxygen, but the resulting "oxyheme" is so unstable in aqueous solutions, that it immediately goes over to the ferric state.[2] It is of interest to know whether the greater stability of carboxyhemoglobin and oxyhemoglobin is due to the difference in the primary valence force which binds the diatomic molecule to the respiratory pigment or due to secondary interactions which may exist in the protein molecule.

For example, the binding of carbon monoxide to hemoglobin has been suggested by Pauling[3] to be represented by structure I.



I II

But because of the existence of bridge carbonyl groups in iron enneacarbonyl[4] and iron tetracarbonyl,[5] one may wonder if the binding of carbon monoxide to hemoglobin could be represented by structure II where X represents an unknown donor group of the globin molecule. Although the weight of opinion favors structure I, it is desirable to disprove structure II by means of a single unambiguous experiment. Measurement of the infrared absorption spectrum could serve this purpose. It may also yield information regarding the nature of binding of carbon monoxide to heme in carbonmonoxyheme as compared to that in carboxyhemoglobin.

Infrared Studies

The infrared absorption spectra of crystalline carboxyhemoglobin, carbonmonoxyheme–pyridine complex and dicarbonmonoxy-Fe(II)-dicysteinate were measured. The experimental methods are outlined below.

Preparation of Crystalline Oxyhemoglobin and Carboxyhemoglobin.—Three times recrystallized oxyhemoglobin was prepared from fresh horse blood by the method described by Keilin and Hartree.[6] The carboxyhemoglobin was made by reducing a concentrated solution of the three times recrystallized oxyhemoglobin with a very slight excess of sodium dithionite, $Na_2S_2O_4$, and then equilibrating the solution with carbon monoxide. The final crystalline samples of carboxyhemoglobin and oxyhemoglobin were obtained by recrystallization from the aqueous solution in a rotary vacuum evaporator at about 2°.

Preparation of Crystalline Dicarbonmonoxy-Fe(II)-dicysteinate.—The disodium salt of dicarbonmonoxy-Fe(II)-dicysteinate, $Na_2[Fe(SCH_2CHNH_2COO)_2(CO)_2]\cdot xH_2O$, was prepared according to the method of Schubert[7] in the complete absence of air.

Preparation of Crystalline Carbonmonoxyheme–Pyridine Complex.—This was prepared in the absence of air in an apparatus similar to that described by Schubert.[7] One gram of hemin, Fisher (recrystallized) was dissolved in 40 cc. of pyridine, reduced by a slight excess of sodium dithionite. Carbon monoxide was then passed through the solution, and the product was precipitated by diluting the mixture with saturated aqueous solution of sodium chloride. The crystalline ppt. was filtered, washed and dried in the absence of air.

Measurement of Infrared Spectra.—The above three compounds were made into KBr-disks for infrared measurements with a Perkin–Elmer Model 21 IR-spectrometer. After the infrared measurements the carboxyhemoglobin and oxyhemoglobin disks were dissolved in water. The water solutions showed the same visible absorption bands as the original proteins. On the other hand, the carbonmonoxyheme–pyridine complex was so unstable that its infrared spectrum changed rapidly when the KBr disk was exposed to air.

The infrared spectra of crystalline carboxyhemoglobin and carbonmonoxyheme–pyridine complex between 1500 and 3000 cm.$^{-1}$ are shown in Fig. 1. It may be noticed in Fig. 1 that both carboxyhemoglobin and carbonmonoxyheme–pyridine complex showed a sharp absorption peak at 1970 cm.$^{-1}$. Since infrared spectra of oxyhemoglobin and hemochromogen, respectively, did not show this absorption peak, this peak can be safely assigned to the stretching vibration of the bound CO molecule. The observed frequency, 1970 cm.$^{-1}$, is consistent with Pauling's structure I, but definitely rules out structure II which predicts a corresponding frequency in range 1700–1800 cm.$^{-1}$. Furthermore, the fact that both carboxyhemoglobin and carbonmonoxyheme–pyridine complex possess a sharp absorption peak at 1970 cm.$^{-1}$ shows that there is no difference in the primary valence force which binds the CO molecule to the Fe(II) in these two compounds.

The infrared spectrum of dicarbonmonoxy-Fe(II)-dicysteinate also showed a sharp absorption peak at 1970 cm.$^{-1}$. In addition, there are two other peaks at 2025 and 2030 cm.$^{-1}$, respectively. These frequencies cannot yet be assigned definitely.

The infrared spectra of the complexes formed by the diethyl ester of carbonmonoxyheme with pyridine and imidazole, respectively, in ether solution were examined. Each of these also contained a sharp absorption peak at 1970 cm.$^{-1}$.

Magnetic Studies

The magnetic properties of hemoglobin, oxyhemoglobin and carboxyhemoglobin were carefully studied by Pauling and Coryell.[8] In this work, the magnetic susceptibilities of carbonmonoxyheme, Fe(II)-dicysteinate complex and dicarbonmonoxy-Fe(II)-dicysteinate complex, respectively, were measured.

Apparatus for Magnetic Susceptibility Measurements.—A Gouy apparatus with a compensation vessel was used. The compensation vessel, 30 cm. long and 0.70 mm. o.d., was suspended from one arm of an air-damped Sartorius microbalance. The maximum variance of the microbalance

(2) See R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949, p. 184.

(3) L. Pauling, "Haemoglobin," (edited by F. J. W. Roughton and J. C. Kendrew), Interscience Publishers, Inc., New York, N. Y., 1949, p. 60.

(4) R. K. Sheline and K. S. Pitzer, THIS JOURNAL, **72**, 1107 (1950).

(5) R. K. Sheline, *ibid.*, **73**, 1615 (1951).

(6) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (*London*), **B117**, 1 (1935).

(7) M. Schubert, THIS JOURNAL, **55**, 4563 (1933).

(8) L. Pauling and C. D. Coryell, *Proc. Natl. Acad. Sci. U. S.*, **22**, 159, 210 (1936).

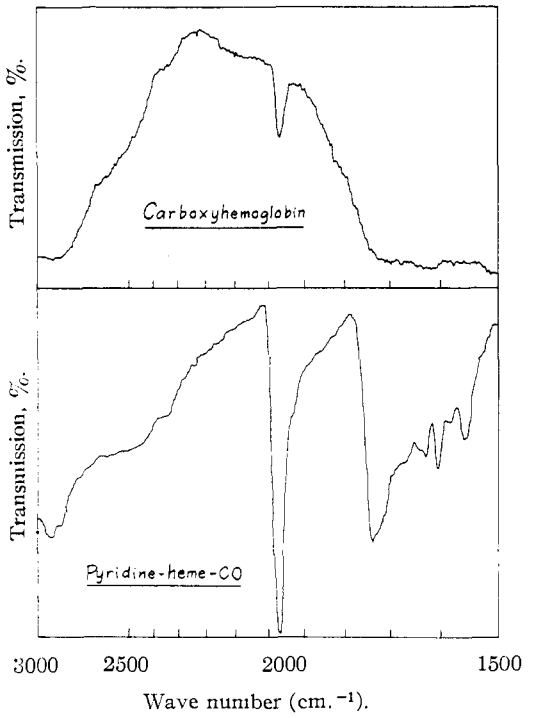


Fig. 1.—Infrared spectra of carboxyhemoglobin and carbonmonoxyheme–pyridine complex.

was 3 micrograms. The magnetic field, about 12,000 gauss, was produced by a Varian Model V-4004 Four Inch Electromagnet with Two Inch tapered poles.

Magnetic Susceptibility Measurements.—The compensation vessel was calibrated with air and oxygen-free water. Both ends of the vessel were sealed with a rubber cap. In general, the solution was injected into the upper compartment, which was prefilled with an inert gas, by means of a hypodermic syringe. The lower compartment was filled with the corresponding solvent medium. The average probable error of the measured paramagnetic susceptibilities by the present procedure is about 2.5%. In order to check the present procedure for systematic errors, the total molar susceptibility of potassium ferricyanide in aqueous solution was determined. The measured value x_{mol} = 2195 c.g.s. units is only 0.7% higher than the accepted value of 2180 c.g.s. units.

The total molar susceptibility of Fe(II)-dicysteinate in aqueous solution was found to be 9256×10^{-6} c.g.s. unit at about 20°. The molar diamagnetic susceptibility was estimated from Pascal's additivity rule to be -151.4×10^{-6} c.g.s. unit. This gave a molar paramagnetic susceptibility of 9407×10^{-6} c.g.s. unit and a permanent magnetic moment of 4.74 B.M. This corresponds to four unpaired electrons per Fe(II). Pauling and Coryell[8] found that the magnetic moment of both hemoglobin and heme showed the existence of four unpaired electrons per Fe(II), but that oxyhemoglobin and carboxyhemoglobin were diamagnetic. Our measurements showed likewise that both carbonmonoxyheme and dicarbonmonoxy-Fe(II)-dicysteinate are diamagnetic.

Thus, these magnetic studies supplement the infrared data described above in showing that there is no detectable difference in the nature of the primary bonds which bind the CO molecule to hemoglobin heme and Fe(II)-dicysteinate, respectively.

Equilibrium Data

It may be speculated that perhaps because of the existence of some secondary interactions in carboxyhemoglobin, the CO molecule is more firmly held to hemoglobin than to heme, and consequently carboxyhemoglobin is more resistant to oxidation by air than carbonmonoxyheme. In order to investigate this possibility, the equilibrium constant for the reaction between carbon monoxide and heme was determined spectroscopically and compared to that for hemoglobin.

Because of the pronounced difference in absorption spectra of heme and carbonmonoxyheme solutions, respectively, in the range 500–600 mμ, the equilibrium constant for the reaction between carbon monoxide and heme can be readily determined by spectroscopic and manometric measurements.

If the reaction is

$$\text{CO} + \text{heme} \rightleftharpoons \text{heme-CO}$$

then the equilibrium constant is

$$K = \frac{[\text{heme-CO}]}{[\text{heme}]p_{CO}} \qquad (1)$$

and the degree of saturation is

$$X \equiv \frac{[\text{heme-CO}]}{[\text{heme}] + [\text{heme-CO}]} = \frac{Kp_{CO}}{1 + Kp_{CO}} \qquad (2)$$

A typical measurement is shown in Fig. 2. Here a 10^{-4} M solution of pure heme was prepared by dissolving weighed amount of hemin in 0.0020 N aqueous KOH solution. This was reduced with a slight excess of sodium dithionite, $Na_2S_2O_4$, in the absence of air. The spectrum of this solution was taken by means of a Cary spectrophotometer. This is shown as curve I in Fig. 2. The solution was then

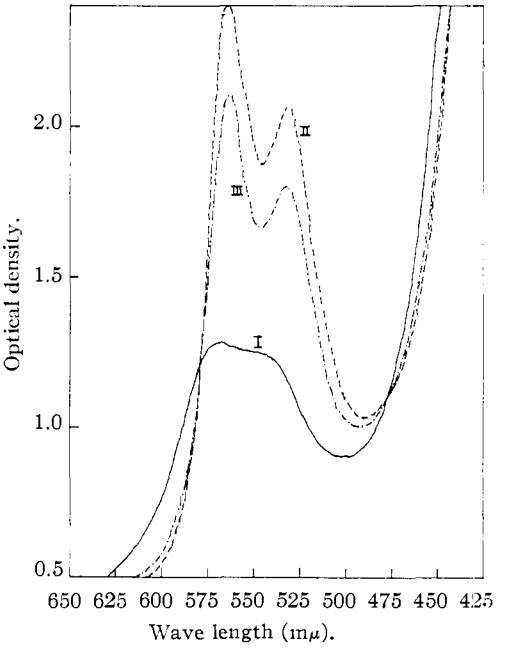


Fig. 2.—A typical spectroscopic measurement for determining the equilibrium constant for the reaction of carbon monoxide with heme: curve I, pure heme; curve II, pure CO–heme; curve III, an equilibrium mixture at p_{CO} = 0.64 cm.

equilibrated with alkaline pyrogallol-washed carbon monoxide at a definite partial pressure with frequent shaking. After one hour the spectrum of this equilibrium mixture was taken. This is shown as curve III in Fig. 2. The solution was then equilibrated with carbon monoxide at 1 atm. pres-

sure, this converted all the heme into heme-CO. The spectrum of the final solution is shown as curve II in Fig. 2. All operations were carried out in the complete absence of air. It may be noticed from Fig. 2 that the three curves intersect at only two points. This is possible only when oxidation to hematin during the equilibration process is completely eliminated, since hematin has a very different spectrum in this range.

The pH of the final solution, as measured immediately after opening the equilibration cell varied from 9.5 to 10.5. However, it gradually dropped to a stationary value between 5 to 6 after being exposed to air for several minutes. It was found that in the range pH 9 to 10.5, the equilibrium was independent of pH. At low pH values, the solution became cloudy, and erratic results were obtained.

If D_1, D_2 and D_3 represent the optical density read from curves I, II and III, respectively, in Fig. 2 at a given wave length, we have

$$X = \frac{D_3 - D_1}{D_2 - D_1} \tag{3}$$

and hence

$$K = \frac{X}{(1 - X)p_{CO}} = \frac{\left(\frac{D_3 - D_1}{D_2 - D_1}\right)}{\left(1 - \frac{D_3 - D_1}{D_2 - D_1}\right)p_{CO}} \tag{4}$$

On the other hand if the equilibrium is

$$2CO + \text{heme} \rightleftarrows \text{heme}(CO)_2$$

then similar considerations lead to

$$K' = \frac{\left(\frac{D_3 - D_1}{D_2 - D_1}\right)}{\left(1 - \frac{D_3 - D_1}{D_2 - D_1}\right)p^2_{CO}} \tag{5}$$

and similarly for other reaction orders.

For each equilibration experiment the degree of saturation, X, was calculated from the measured optical densities at 564, 547 and 534 mμ, respectively. The average value was used to compute the equilibrium constant. For example, for the measurement illustrated in Fig. 2, the degrees of saturation computed from these three wave lengths are 0.734, 0.678 and 0.705, respectively. Using the average value 0.706 and P_{CO} = 0.59 cm., we find K = 3.8 from equation 4.

The results of the equilibration measurements are summarized in Table I.

TABLE I

DETERMINATION OF THE EQUILIBRIUM CONSTANT FOR THE REACTION BETWEEN HEME AND CARBON MONOXIDE

[Heme] + [Heme-CO] = 1.0×10^{-4} M; pH 9.6-10.5

p_{CO}, cm.	Temp., °C.	% Saturation, X	Equilibrium constant K from eq. 4	Equilibrium constant K' from eq. 5
0.13	25.2	35	4.1	31
.18	27.8	42	4.1	23
.64	22.0	71	3.8	5.9
.70	24.6	72	4.0	5.7
.88	23.4	78	4.0	4.6
1.29	21.8	85	4.5	3.5
1.62	25.4	86	4.1	2.9
1.65	26.2	86	4.1	2.8
			Av. 4.1 ± 0.2	

We may conclude from the values in Table I that the equilibrium between carbon monoxide and heme in aqueous solution corresponds to the simple reaction in which one CO molecule combines with each heme to form carbonmonoxyheme. If the partial pressure of carbon monoxide is expressed in atm., the above value for K should be multiplied by 76. This gives

$$K = (3.1 \pm 0.14) \times 10^2 \text{ atm.}^{-1}$$

Because of the presence of heme–heme interaction in hemoglobin, the equilibrium between the latter and CO cannot be satisfactorily described by a single equilibrium constant. However, an approximate comparison between carboxyhemoglobin and carbonmonoxyheme can be made by considering the half-saturation pressures. Thus, for hemoglobin at pH 7.4, the half-saturation pressure for CO is about 0.006 cm., whereas the average value of K for heme in Table I gives 0.24 cm. for the half-saturation pressure. Thus, the half-saturation pressure for the combination of carbon monoxide with hemoglobin is only about 1/40 as large as that for heme. Consequently we may conclude that the CO molecule is not held much more firmly to hemoglobin than to heme.

Although there are no quantitative data on the relative rates of oxidation of carboxyhemoglobin and carbonmonoxyheme, respectively, by molecular oxygen, the fact that aqueous solutions of carbonmonoxyheme are instantaneously oxidized on exposure to air whereas carboxyhemoglobin solution can be kept for weeks in contact with air indicates that the two rates probably differ by a factor of 10^8 or even more. Therefore, the above results show that the difference in the free energies of formation of carboxyhemoglobin and carbonmonoxyheme, respectively, cannot be the major factor which is responsible for high resistance of carboxyhemoglobin toward oxidation by molecular oxygen.

A Possible Cause of the Stability of Hemoglobin Derivatives

If it is assumed that the initial step in the oxidation of heme by molecular oxygen involves the formation of "oxyheme" followed by the decomposition of the latter to ferriheme and O_2^- or HO_2, one would expect the subsequent reduction of the O_2^- or HO_2 by other heme molecules to be comparatively fast. Consideration of coulombic interactions would predict the decomposition of "oxyheme" to ferriheme and O_2^- or HO_2 to be slow in media of low dielectric constant. Thus, if one assumes that hemes in hemoglobin molecule are not completely exposed to water but are partially covered with hydrophobic groups of the protein, one would expect the decomposition of oxyhemoglobin to methemoglobin and O_2^- or HO_2 to be much slower than the corresponding process for a freely exposed "oxyheme." On the other hand, one would expect the dissociation of such a partially covered "oxyheme" back to heme and molecular oxygen to be practically unhindered, although the decomposition to products with net charge or high polarity would be effectively retarded by the low dielectric constant of the local environment.

There is indeed considerable experimental sup port to the hypothesis of partially covered hemes in the hemoglobin molecule. For example, neither imidazole nor pyridine combines with hemoglobin, although both form stable hemochromogens with free heme.[9] St. George and Pauling[10] found that although the combining powers of heme with ethyl isocyanide, isopropyl isocyanide and t-butyl isocyanide are essentially the same, hemoglobin

(9) See, for example, p. 173 of ref. 2.

(10) R. C. C. St. George and L. Pauling, *Science*, **114**, 629 (1951).

combines far more strongly with ethyl isocyanide than with *t*-butyl isocyanide, corresponding to a factor of 200 in equilibrium constants. These facts led them to the conclusion that the hemes in hemoglobin are buried within the protein molecule. Recently Lein and Pauling[11] found similar results on myoglobin and concluded that even in myoglobin, which is a 9 Å. thick disk-shaped molecule, the heme is buried within the protein molecule. While the exact location of heme in hemoglobin and myoglobin is still undecided, these results do suggest that in these molecules each heme is partially covered by some protein groups on the same side of the combining site.

The behavior of cyanide ion toward hemoglobin and free heme, respectively, is even more striking. The CN^- ion is isoelectronic with the CO molecule. It has been shown above that the affinity of hemoglobin at *p*H 7.4 for CO is about 40 times stronger than that of free heme. Cyanide ion forms stable complexes with free heme. For example, the equilibrium constant for the reaction

$$CN^- + \text{heme} \rightleftharpoons \text{heme-}CN^-$$

was found to be about 2.5×10^5 at *p*H 10.6.[12] But on the contrary, the affinity of hemoglobin for cyanide ion is very small. The corresponding equilibrium constant was estimated to be only about 1, even though the complex, hemoglobin cyanide, was shown to be diamagnetic.[13]

Since cyanide ion and carbon monoxide molecule have almost identical size and shape, the observed contrasting behavior of hemoglobin and free heme toward carbon monoxide and cyanide ion, respectively, cannot be explained in terms of simple steric factors. But if it is assumed that the heme in hemoglobin is covered with hydrophobic side chains of the globin, then the free energy of formation of the CN^--complex for hemoglobin should be greater than that for an exposed heme by an amount equal to the electrical work required to bring the charged cyanide ion from an environment of higher to one of lower dielectric constant. If the average dielectric constant of the surroundings of the combining site in hemoglobin is considerably lower than time for free heme, this work could be sufficient to account for the difference in the formation constants of the two cyanide complexes.

Experiments in Non-aqueous Solutions

Benzene and ethyl ether solutions of the hemochromogen formed between pyridine and the diethyl ester of heme were prepared and studied. As expected from the above considerations, these hemochromogen solutions were very stable. Even prolonged shaking with pure oxygen gas and water did not result in a detectable amount of oxidation to the Fe(III)-state. Bubbling carbon monoxide gas through the solutions replaced one of the pyridine molecules in the hemochromogen by CO. The resulting CO–heme–pyridine complex is also very resistant to oxidation by molecular oxygen. Bubbling pure oxygen through the latter solution merely removed the carbon monoxide and reconverted the complex to dipyridine–hemochromogen. A typical experiment is illustrated in Fig. 3. The cycling can be carried out repeatedly.

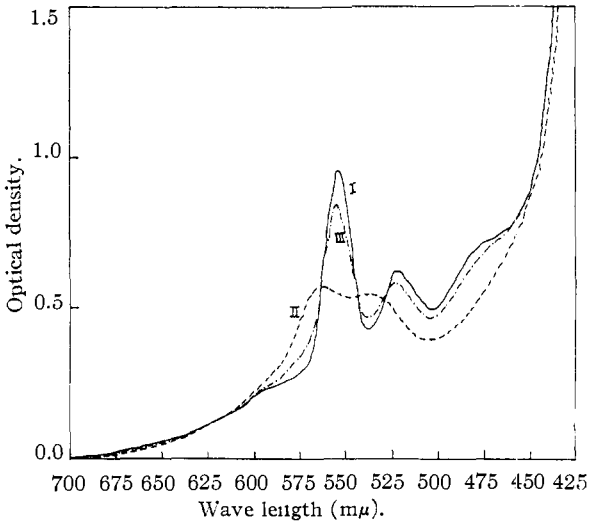


Fig. 3.—Spectroscopic study of the complexes of the diethyl ester of heme in benzene solution: curve I, spectrum of dipyridine-hemochromogen diethyl ester dissolved in benzene (soln. 1); curve II, spectrum obtained after bubbling CO through soln. 1 for 5 min. (soln. 2); curve III, spectrum obtained after bubbling pure O_2 through soln. 2 for 10 min.

Preparation of the Diethyl Ester of Hemin.—0.1 g. of hemin, Fisher (recrystallized), was dissolved in 25 cc. of absolute ethanol containing about 0.2 g. of KOH. The mixture was centrifuged to remove a small amount of insoluble residue. Ten cc. of trifluoroacetic acid was mixed with the solution and the mixture refluxed overnight. The reaction mixture was cooled to room temperature and then evaporated to dryness in a rotary vacuum evaporator at room temperature. The residue was taken up in acetone and centrifuged to remove a small amount of insoluble residue. The acetone solution was again vacuum-evaporated to dryness to remove the last trace of excess trifluoroacetic acid. The product was insoluble in water, but freely soluble in acetone, pyridine, ether, etc.

Preparation of the Complexes of the Diethyl Ester of Heme.—The hemin ester was dissolved in a small amount of pyridine, reduced by a minimum amount of $Na_2S_2O_4$ dissolved in a drop of water, and then extracted into ether or benzene. Alternatively, the hemin ester could be dissolved in an ether or benzene solution of pyridine first, then the solution was reduced by shaking with an aqueous $Na_2S_2O_4$ solution.

Acknowledgment.—This work was supported in part by a research grant (USPHS-RG-4483) from the Division of Research Grants, Public Health Service.

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(11) A. Lein and L. Pauling, *Proc. Natl. Acad. Sci. U. S.*, **42**, 51 (1956).

(12) See p. 189 of ref. 2.

(13) F. Stitt and C. D. Coryell, THIS JOURNAL, **61**, 1263 (1939).