[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF THE JOHNS HOPKINS UNIVERSITY]

Preparation and Properties of Imidazole Ferro- and Ferriprotoporphyrin Complexes[1]

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Imidazole ferro- and ferriprotoporphyrin have been prepared in the crystalline state and some of their properties studied. Imidazole ferroprotoporphyrin crystals were found to possess the property of combining reversibly with oxygen. This fact supports the postulate that in hemoglobin the iron is attached to the imidazole histidine nitrogen of globin. It was shown that the reaction between imidazole and ferriprotoporphyrin proceeds very slowly in the presence of water, which might be attributed to the greater energy required to break the bond between the iron and the oxygen in the water–porphyrin complexes present in water solution. Attempts made to prepare the heme imidazole complex in water solution were unsuccessful.

In the study of the structure of hemoglobin most investigators have arrived at the conclusion that the imidazole group of histidine is involved both in the oxygenation equilibrium and in the union of globin to the prosthetic group, heme.

On the basis of this conclusion, it seems reasonable to believe that the simple imidazole complex of heme should have some properties similar to those of hemoglobin. However, it has not been noted that this complex shows the most important property of hemoglobin: the reversible attachment of oxygen gas.

An investigation on the preparation and properties of the imidazole complex of both ferro- and ferriprotoporphyrin was undertaken with the objective of finding conditions under which the ferroprotoporphyrin–imidazole complex could combine reversibly with oxygen, as hemoglobin does, without oxidation to the ferric state.

Imidazole Ferriprotoporphyrin.—Imidazole ferriprotoporphyrin is a compound formed by the combination of two moles of imidazole with one mole of ferriprotoporphyrin. It was first prepared in crystalline state by Langenbeck,[2] who proved its composition by chemical analysis. In the present investigation this complex was prepared by a very simple method which consisted of stirring crystals of hemin in an imidazole solution in benzene followed by washing and drying of the product. During this process the steel blue color of the hemin crystals changes to reddish-blue. The composition of the complex so prepared was confirmed by chemical analysis. It is not soluble in neutral or acidic aqueous solutions and in alkaline solutions it dissociates into its components and complexes of different composition are formed, perhaps with interference of water molecules. Imidazole–ferriprotoporphyrin complex may also be prepared in water solution by adding excess imidazole to borate buffer solutions of hemin at *p*H 7.2.

It was observed that small amounts of imidazole have no effect on the absorption spectrum of a hemin solution in borate buffer (*p*H 7.2). However, on standing at room temperature for several days the color of the solution changed to orange-red and a slight precipitate of the same color was formed. This precipitate was the imidazole complex of ferriprotoporphyrin, whose formation appears to proceed at a very slow rate in the presence of water.

The optical density of ten solutions containing the same concentration of hemin and different concentrations of imidazole (10 moles to 100 moles/mole of hemin) was measured at 545 mμ. This wave length was selected because it corresponds to the maximum absorption of the imidazole complex in borate buffer solution in the presence of excess imidazole (*p*H 7.2). The results showed that the solutions containing lower concentrations of imidazole have the same optical density as the hemin solution. On standing overnight a red precipitate, which is the imidazole complex, was observed in the solutions containing 100, 90, 80 and 70 moles of imidazole per mole of hemin. The other solutions changed from day to day and gradually the precipitated imidazole complex appeared in all of them. The last solution, which contained 10 moles of imidazole per mole of hemin, showed the precipitate after a period of three weeks. The extent of the reaction could not be followed spectrophotometrically due to the partial precipitation of the product and the complexity of the changes that take place in the solution during such a long period of time.

The fact that the imidazole–ferriprotoporphyrin complex is formed quickly in benzene and only slowly in water suggests interaction of water in the formation of the complex. The slowness of the reaction may be attributed to the greater energy required to break the bond between the iron and the oxygen in the porphyrin–water complexes present in aqueous solutions.

Imidazole–Ferroprotoporphyrin.—An attempt to prepare imidazole–ferroprotoporphyrin complex in water solution was made by adding imidazole to a borate solution of heme at *p*H 7.4, obtained by the reduction of hemin with $Na_2S_2O_4$. This preparation is readily oxidized to the ferric state but shows no tendency to combine with oxygen gas. Because of the possibility that residues of the reductant–oxidant system used in the preparation might catalyze the air oxidation of the complex it was deemed desirable to prepare the imidazole–heme complex in water solution free of reducing agents. The electrolytic reduction of hemin in aqueous solutions and its coördination with imidazole was thought to be the ideal method for the preparation of the complex in such a state. However, all attempts made to reduce hemin or the imidazole complex by this method failed.

(1) Porphyrin Studies. XIV. Paper XIII, A. H. Corwin and M. H. Melville, THIS JOURNAL, **77**, 2755 (1955). From the doctoral dissertation of Zoila Reyes, The Johns Hopkins University. Presented at the 116th Meeting of the American Chemical Society, Atlantic City, N. J., September, 1949.

(2) W. Langenbeck, *Ber.*, **65**, 842 (1932); *Naturwissen.*, **20**, 124 (1932).

A method for the preparation of the ferroprotoporphyrin complex of imidazole in crystalline form was then sought. Heme was prepared in an all-glass, inert atmosphere apparatus especially designed for it and based on the original experiment of Fischer, Treibs and Zeile[3] and the modified procedure of Corwin and Erdman.[4] In this apparatus pure heme was prepared and coördinated afterwards with imidazole.

The pure imidazole–ferroprotoporphyrin complex is insoluble in water. Attempts were made to coördinate imidazole with heme in water solutions in an inert atmosphere. However, all the preparations were as easily oxidizable as those prepared by the reduction of hemin with reducing agents. In these experiments some coördination took place, as judged by the change in color of the solutions. No criterion was available for testing the completeness of this reaction, however. It is possible that due to the slowness of the reaction, some water complex of heme was still present and that the rapid oxidation was due to this impurity.

Crystalline imidazole–ferroprotoporphyrin is soluble in pyridine and although the position of the bands (545–555 mμ, 510–520 mμ) permit it to be distinguished from the pyridine hemochromogen (550–560 mμ, 520–530 mμ), it was thought desirable to find a different solvent where the properties of the complex could be studied without the interference of another coördinating substance. This was not accomplished, for the ferroprotoporphyrin–imidazole complex was found to be insoluble in all the solvents tried.

The search for reaction conditions which permit oxygenation similar to that of hemoglobin was rewarded by the discovery that the crystalline state possesses the property of absorbing oxygen from the air. The compound so formed decomposes reversibly upon heating and evacuation. Oxygenation of the complex was carried out by passing dry air through weighed samples, packed in special tubes. The extent of oxygenation was determined by weight. This absorption of oxygen is usually accompanied by a change in color from bright red to wine-red. The increase in weight was found in two cases to be equivalent to an absorption of 0.94 mole and 0.97 mole of oxygen per mole of imidazole–ferroprotoporphyrin. Deoxygenation was accomplished by heating in a vacuum at 100° or in a stream of nitrogen at the same temperature. One of the samples that was carried through the cycle again absorbed less oxygen the second time but the reversibility of the reaction was the same.

The fact that imidazole–ferroprotoporphyrin, when prepared in the pure state, is capable of combining reversibly with oxygen supports the postulate that the iron is attached to the histidine imidazole of globin. This experiment shows that the attachment of two imidazole rings to the iron in heme alters its reactivity sufficiently to permit it to combine with oxygen.

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(3) H. Fischer, A. Treibs and K. Zeile, *Z. physiol. Chem.*, **195**, 1 (1931).

(4) A. H. Corwin and J. G. Erdman, THIS JOURNAL, **68**, 2473 (1946).

Experimental

Hemin.—Hemin was prepared by Fischer's[5] method.

Imidazole–Ferriprotoporphyrin.[2]—One hundred thirteen milligrams of imidazole was dissolved in 20 cc. of benzene. Thirty milligrams of hemin was added and the solution was stirred until the steel-blue crystals of hemin were changed to reddish-blue. The crystals were separated by centrifuging, then washed once with benzene, three times with distilled water, once with methanol and dried in vacuum at 100°. The product obtained was a violet powder which was identical in appearance to that obtained by Langenbeck's method. Analysis showed that it contained two moles of imidazole per mole of hematin.

Anal. Calcd. for $C_{40}H_{41}O_5N_8Fe$: N, 14.56. Found: N, 14.58.

Ferroprotoporphyrin (Heme).—Heme was prepared in a nitrogen atmosphere by a modification of the procedures of Fischer, Treibs and Zeile[3] and by Corwin and Erdman.[4] The modification consisted in the elimination of all rubber tubing and substitution of glass to prevent diffusion of oxygen into the system. Glass vessels were arranged in sequence on a standard taper joint so that the manipulations of preparation, filtration, crystallization and filtration for isolation could all be performed under purified nitrogen.

Imidazole–Ferroprotoporphyrin.—This complex was prepared by a procedure analogous to that described for the preparation of the ferriprotoporphyrin complex, except that in this case the reaction was carried out in a nitrogen atmosphere using the same reaction apparatus that was employed for the preparation of heme and substituting heme in place of hemin. After the apparatus had been swept free of air, heme was placed in one reaction vessel and the imidazole solution in oxygen-free benzene in another. The benzene solution was transferred to the heme vessel and stirred for one-half hour by reversing the flow of nitrogen. Afterwards, the suspension was filtered, the crystals washed with benzene, water, methyl alcohol, peroxide-free ether, and dried by the current of nitrogen for two hours. The complex was not soluble in water.

Properties of Imidazole–Ferroprotoporphyrin.—Crystalline imidazole–ferroprotoporphyrin dissolved in anhydrous pyridine gives a pink-yellow solution which shows the characteristic hemochromogen spectrum, the bands appearing at 555–545 mμ (strong) and 520–510 mμ. The color of the solution and the position of the bands facilitated distinguishing it from the pyridine hemochromogen whose pyridine solution exhibited a bright pink color and whose absorption bands appeared at 560–550 mμ (strong) and 530–520 mμ, measured under identical conditions. An anhydrous pyridine solution of imidazole–ferroprotoporphyrin showed the hemochromogen spectrum even after being exposed to air for three days.

When exposed to sunlight the crystalline solid was gradually oxidized. During this process the strong band at 555–545 mμ became weaker and that at 510–520 mμ became broader and more intense. Attempts were made to prepare it in water solution by dissolving heme, prepared by the reduction of hematin with sodium dithionite, in 0.02 *N* potassium hydroxide in a nitrogen atmosphere and then adding an excess of imidazole dissolved in the amount of 0.02 *N* boric acid required to bring the *p*H to 7.4. Samples of the solution obtained showed the typical bands of the reduced hemochromogen but were oxidized very quickly so that the properties of the complex could not be studied in aqueous solution.

Oxygen-carrying Properties of Imidazole–Ferroprotoporphyrin.—When dried air was passed through imidazole–ferroprotoporphyrin crystals placed in a 4 mm. Pyrex tube for a day, the color changed from bright red to wine-red. Since this color resembles that of the imidazole–ferriprotoporphyrin, a sample of it was dissolved in pyridine and its spectrum observed with a hand spectroscope. It showed two bands as the reduced compound but they were sharper and the band at 510–520 mμ was more intense. On standing the solution became oxidized. In some of the preparations no change in color was observed by passing air through the crystals.

To determine whether the change in color was due to the

(5) H. Fischer, *Org. Syntheses*, **21**, 53 (1941).

absorption of oxygen and whether it was reversible as in the oxygen-carrying chelates described by Calvin, Bailes and Wilmarth[6] the following experiment was performed: A Pyrex sample tube with an outside diameter small enough to fit inside a closable absorption tube used for carbon and hydrogen determinations was constructed at one end, and plugged with glass wool at both ends. It was placed inside a test-tube and dried to constant weight in a vacuum at 100°. The weighings were taken with the tube filled with nitrogen. About 20 mg. of imidazole–ferroprotoporphyrin, which had been dried in a vacuum at 100° for six hours, was quickly placed in the sample tube, and this in turn inserted in the absorption tube, which had been previously weighed. Nitrogen was passed through the absorption tube and it was again weighed. A blank determination was run by transferring the sample tube to the drying apparatus and, after drying it for half an hour, replacing it in the absorption tube, passing nitrogen through the assembly, and then weighing it. This weight, within the limits of experimental error, was identical to the preceding one. From this weight and that of the sample tube, the weight of the sample was calculated. Then dry air was passed through the assembly at the rate of 20 cc. per minute for eight hours and, after sweeping it with nitrogen for 15 minutes, its weight was determined; weight of sample, 10.234 mg.; weight after passing in air, 10.644 mg.; increase, 4.01%, calculated on the basis of one mole of oxygen to one mole of imidazole complex, 4.25%. Hence, 0.94 mole of oxygen was absorbed. The sample was then heated for four hours at 100° in a vacuum and weighed again: weight after regeneration, 10.240 mg. In another experiment the increase in weight was 4.12% corresponding to 0.97 mole of oxygen per mole of complex. This sample was deoxygenated by placing it inside of a water condenser heated with steam through which a current of pure nitrogen was passed for six hours. Upon submitting it to a current of air again, it absorbed 3.8% of its original weight of oxygen.

(6) M. Calvin, R. H. Bailes and W. K. Wilmarth, THIS JOURNAL, **68**, 2254 (1946).

Purification of Solvents.—Diethyl ether was dried over calcium chloride and distilled over sodium through a long column in an all-glass apparatus, discarding first and last runnings. It was stored over sodium wire and distilled before use. Methyl alcohol was refluxed four hours over powdered CaO, distilled twice in a nitrogen atmosphere from an all-glass still and stored in a flask maintained at constant nitrogen pressure. Pyridine was dehydrated for ten days over BaO, distilled over BaO in a nitrogen atmosphere, and redistilled. The water and glacial acetic acid used in the preparation of heme were distilled twice in a nitrogen atmosphere and stored in flasks maintained at constant nitrogen pressure.

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[CONTRIBUTION FROM ABBOTT LABORATORIES]

The Reactions of Dibromoalkanes and *o*-(Bromoalkyl)-α-bromotoluenes with *o*-Substituted Anilines. The Synthesis of 1-Arylpyrrolidines and Related Compounds

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The reaction of 1,4-dibromoalkanes with *o*-substituted and *o,o'*-disubstituted anilines yielded only 1-arylpyrrolidines except in one case when a low yield of the substituted 1,4-diaminoalkane was obtained. Similarly, 2-(β-bromoethyl)-α-bromotoluene gave only 2-aryl-1,2,3,4-tetrahydroisoquinolines, while α,α'-dibromo-*o*-xylene gave principally 2-arylisoindolines together with small amounts of substituted α,α'-diamino-*o*-xylenes. 1,3-Dibromopropane and 1,6-dibromohexane yielded no cyclic products from this reaction, even when *ortho* groups were not present in the amine. The reactions of phthalic and homophthalic anhydrides with substituted anilines were also investigated as a route toward alternate syntheses of some of these cyclic amines, and a novel by-product from lithium aluminum hydride reduction of a substituted phthalimide was observed.

The reaction of 1,5-dibromopentane upon *o*-substituted aromatic amines recently was shown to give principally the corresponding 1-arylpiperidines[1] rather than the 1,5-diarylaminopentanes which had been described earlier as the only products.[2] Three of these hindered amines have now been treated with other dibromoalkanes and with two aralkylene dibromides to observe the extent of formation of four-, five- or seven-membered rings.

1,3-Dibromopropane and 1,6-dibromohexane reacted with *o*-toluidine, and with aniline as well, to give only open chain diamines. Although Scholtz reported the formation of a low yield of 1-phenylazetidine[3] from 1,3-dibromopropane and aniline, Veer in more recent work failed to obtain the cyclic product.[4] Von Braun observed similar results using 1,6-diiodohexane and aniline.[5] Aliphatic[6] and aromatic[7] primary amines are known to yield 1-substituted pyrrolidines when treated with 1,4-dibromo- or 1,4-dichlorobutane. The ease of pyrrolidine ring formation from 1,4-dibromobutane seems greater than that of piperidine ring formation from 1,5-dibromopentane, for the latter gives only the open-chain diamine I on reaction with 2,6-dimethylaniline,[1] while 1,4-dibromobutane has now been found to yield 1-(2,6-dimethylphenyl)-pyrrolidine (II) with no evidence of diamine formation. 1-Arylpyrrolidines were also formed as the only products when *o*-toluidine, *o*-anisidine and *m*-anisidine reacted with 1,4-dibromobutane. In the last case, where there is no *ortho* substituent, the yield was significantly higher. Several derivatives of the methoxyphenylpyrrolidines were prepared, including the corresponding phenols.

In their study of the influence of *o*-substitution on the reaction of anilines with 1,4-dibromopentane in solvents, Scholtz and Friemehlt report that *o*-toluidine gives only N,N'-di-(*o*-tolyl)-1,4-diaminopentane[8] (IVa) and conclude that the presence

(1) A. H. Sommers and S. E. Aaland, THIS JOURNAL, **75**, 5280 (1953).

(2) M. Scholtz and E. Wassermann, *Ber.*, **40**, 852 (1907).

(3) M. Scholtz, *ibid.*, **32**, 2251 (1899).

(4) W. L. C. Veer, *Rec. trav. chim.*, **57**, 989 (1938).

(5) J. von Braun, *Ber.*, **43**, 2853 (1910).

(6) R. C. Elderfield and H. A. Hageman, *J. Org. Chem.*, **14**, 605 (1949); D. D. Libman, D. L. Pain and R. Slack, *J. Chem. Soc.*, 2305 (1952); J. G. Erickson and J. S. Keps, THIS JOURNAL, **77**, 485 (1955).

(7) L. C. Craig with R. M. Hixon, *ibid.*, **52**, 804 (1930); O. Wichterle and M. Vavruska, *Chem. Listy*, **46**, 237 (1952); *C. A.* **47**, 4330 (1953).

(8) M. Scholtz and P. Friemehlt, *Ber.*, **32**, 848 (1899).