Substituted Deuteroporphyrins. VI. Ligand-Exchange and Dimerization Reactions of Deuterohemins*

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ABSTRACT: Ligand-exchange and dimerization reactions of deuteroporphyrin IX dimethyl ester iron(III) derivatives (deuterohemins) were explored. Ligand-exchange reactions of the acetatodeuterohemin were used to prepare solid monomeric fluoro-, chloro-, bromo-, iodo-, azido-, phenoxo-, and hypophosphitodeuterohemins, which were characterized by elemental analyses, infrared spectra, mass spectra, and molecular weight. Aquation reactions of the deuterohemins, and also autoxidation of deuteroheme, gave a product characterized as a dimer with an Fe–O–Fe linkage. Isotopic substitution with ^{18}O provided strong infrared evidence for such a linkage. Mössbauer parameters were also determined. These data suggest dimers of this type may be encountered frequently in hemins and also in certain hemeproteins (*e.g.*, cytochrome oxidase). Also considered are mechanisms for ligand exchange and dimerization which involve doubly bridged dimeric structures.

The properties and reactions of heme proteins are most readily (and frequently) studied by changes in the properties of their iron(II) porphyrin (heme) or iron(III) porphyrin (hemin) moieties, which are iron complexes of substituted deuteroporphyrins IX. The examination of pure iron porphyrins of verified structure permits the elucidation of substituent, axial ligand, and medium effects which, when coupled with examination of pure heme proteins, can aid in the description of specific protein effects on the iron porphyrin and its ligand(s). Here we report the preparation and structural verification of a series of monomeric hemins derived from deuteroporphyrin. We also report isolation and characterization of a dimeric oxo-bridged species, as well as more tentative evidence for other dimeric hemin structures. From interrelationships among the conditions giving rise to monomeric and dimeric hemins, we propose mechanisms by which ligand exhange occurs and by which monomeric and dimeric forms are interconverted.

Experimental Section

Infrared spectra were obtained with Perkin-Elmer Model 521 and 225 spectrometers. Wave number values are reported with an estimated accuracy of ± 5 cm^{-1}. Electronic spectra were recorded on Beckman Model DK-2 and Cary Model 14 spectrophotometers. Molecular weight determinations were carried out on a Mechrolab vapor-phase osmometer at 30° in chloroform and were calibrated with *o*-terphenyl and deuteroporphyrin dimethyl ester. Molecular weights have an estimated accuracy of $\pm 10\%$, except in the case of iododeuterohemin with an estimated accuracy of $\pm 20\%$ (and acute solubility problems). Room temperature Mössbauer spectra for solid compounds unenriched in ^{57}Fe were obtained by Dr. J. J. Spijkerman. Far infrared spectra were obtained by Professor P. L. Richards. Elemental analyses were performed by Jospeh Walter, Dr. S. N. Nagy, and Schwarzkopf Microanalytical Laboratories. Mass spectroscopy was carried out by the Morgan–Schaffer Corp.

Materials. All solvents and other reagents were reagent grade or equivalent. Chloroform (J. R. Baker, stabilized with *ca.* 0.2% ethanol) was stored over calcium oxide for at least 24 hr prior to use. Benzene was washed with H_2SO_4 and fractionally distilled. Pyridine was stored over KOH, mixed with one-fifth volume of benzene, and fractionally distilled. Fisher-activated alumina (A-540) was used for chromatography. Water-enriched 97 atom % in ^{18}O was purchased from Miles Laboratories. The preparations of deuteroporphyrin IX dimethyl ester (Caughey *et al.*, 1966) and methoxo- and ethoxodeuteroporphyrin dimethyl ester iron(III) (Alben *et al.*, 1968) have been reported.

Preparation of Acetatodeuteroporphyrin IX Dimethyl Ester Iron(III). Ferrous acetate (4.0 g) was added to a solution of deuteroporphyrin IX dimethyl ester (5.0 g) in glacial acetic acid (110 ml) refluxing under nitrogen. The reaction mixture was maintained under reflux for 2.5 hr and then allowed to stand at room temperature in air for 48 hr. Crystals were collected, washed with 30% aqueous acetic acid (100 ml), and dried under vacuum at 55°: yield 5.4 g; acetate ν_{CO} in KBr and in chloroform 1655 and 1635 (sh) cm^{-1}; molecular weight calcd for $C_{34}H_{35}FeN_4O_6$, 652; found in chloroform, 540, 600; most abundant mass spectral peak, *m/e* 592; *m/e* 60 (acetic acid) observed.

Anal. Calcd for $C_{34}H_{35}FeN_4O_6$: C, 62.7; H, 5.4; N, 8.6; O, 14.7. Found: C, 62.7; H, 5.5; N, 8.7; O, 15.4. Found after 2 hr at 148° under vacuum: C, 63.9; H, 5.7; N, 8.8.

However, when crystals of acetatodeuteroporphyrin dimethyl ester iron(III) were washed with water and

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dried under vacuum at 60°, infrared spectra showed only traces of acetate.

Acetatodeuteroporphyrin dimethyl ester iron(III) (100 mg) dissolved in acetic acid (50 ml) was precipitated by addition of water (100 ml). The precipitate was washed with 30% aqueous acetic acid and dried under vacuum at 60°. The infrared spectrum of the precipitate was identical with that of the starting material.

Anal. Found: C, 62.3; H, 5.5; N, 8.5.

Preparations Involving Ligand Exchange in Heterogeneous Media. FLUORODEUTEROPORPHYRIN IX DIMETHYL ESTER IRON (III). A solution of acetatodeuteroporphyrin IX dimethyl ester iron(III) (202 mg) in chloroform (10 ml) was shaken thoroughly with a nearly saturated aqueous solution of sodium fluoride (15 ml). After separation from the aqueous phase, the chloroform solution was passed through a column of solid sodium fluoride, condensed to 4 ml, heated to its boiling point, treated with hot isooctane (15 ml), and allowed to stand for 15 hr at room temperature. Crystals were collected, washed with isooctane, and dried under vacuum at 55°: yield 154 mg; ν_{FeF} in KBr and chloroform, 570 cm^{-1}; molecular weight calcd for $C_{32}H_{32}FFeN_4O_4$, 611; found in chloroform, 580.

Anal. Calcd for $C_{32}H_{32}FFeN_4O_4$: C, 62.9; H, 5.3; F, 3.1; N, 9.1. Found: C, 63.1; H, 5.5; F, 3.2; N, 9.0.

Preparations of this and the following hemins by a similar procedure utilizing benzene instead of chloroform were also successful.

CHLORODEUTEROPORPHYRIN IX DIMETHYL ESTER IRON (III). The procedure was identical with the preparation of the fluoroiron(III) compound except for use of NaCl in place of sodium fluoride. The yield from 402 mg of acetatodeuteroporphyrin dimethyl ester iron(III) was 324 mg; ν_{FeCl} in KBr, 330 cm^{-1}; most abundant mass spectral peak, *m/e* 592; *m/e* 36, 38(HCl) observed.

Anal. Calcd for $C_{32}H_{32}ClFeN_4O_4$: C, 61.2; H, 5.1; Cl, 5.6; Fe, 8.9; N, 8.9. Found: C, 61.5; H, 5.0; Cl, 5.3; Fe, 8.9; N, 8.4.

BROMODEUTEROPORPHYRIN IX DIMETHYL ESTER IRON (III). The procedure was identical with the preparation of the fluoroiron(III) compound except for use of sodium bromide in place of sodium fluoride. The yield from 203 mg of acetatodeuteroporphyrin dimethyl ester iron(III) was 169 mg; molecular weight calcd for $C_{32}H_{32}BrFeN_4O_4$, 672; found in chloroform 660.

Anal. Calcd for $C_{32}H_{32}BrFeN_4O_4$: C, 57.2; H, 4.8; Br, 11.9; Fe, 8.3; N, 8.3. Found: C, 57.2, 56.5; H, 4.6; 5.0; Br, 11.6; Fe, 8.3; N, 8.5.

IODODEUTEROPORPHYRIN IX DIMETHYL ESTER IRON (III). The procedure was identical with the preparation of the fluoroiron(III) compound, except for use of sodium iodide in place of sodium fluoride. The yield from 405 mg of acetatodeuteroporphyrin dimethyl ester iron (III) was 362 mg; molecular weight calcd for $C_{32}H_{32}FeIN_4O_4$, 719; found in chloroform, 870.

Anal. Calcd for $C_{32}H_{32}FeIN_4O_4$: C, 53.4; H, 4.5; Fe, 7.8; I, 17.6; N, 7.8. Found: C, 53.3; H, 4.7; Fe, 7.8; I, 18.9; N, 7.5.

AZIDODEUTEROPORPHYRIN IX DIMETHYL ESTER IRON (III). The procedure was identical with the preparation of the fluoroiron(III) compound, except for use of sodium azide in place of sodium fluoride. The yield from 212 mg of acetatodeuteroporphyrin dimethyl ester iron (III) was 189 mg; azide ν_{N-N} in KBr and chloroform, 2055 cm^{-1}; molecular weight calcd for $C_{32}H_{32}FeN_7O_4$, 635; found in chloroform, 710; most abundant mass spectral peak, *m/e* 592; *m/e* 28 (N_2) more abundant than observed from air leak in other compounds.

Anal. Calcd for $C_{32}H_{32}FeN_7O_4$: C, 60.6; H, 5.1; Fe, 8.8; N, 15.4. Found: C, 60.5; H, 5.4; Fe, 9.1; N, 15.2.

PHENOXODEUTEROPORPHYRIN IX DIMETHYL ESTER IRON (III). The procedure was identical with the preparation of the fluoroiron(III) compound, except for use of sodium phenoxide in place of sodium fluoride. The yield from 203 mg of acetatodeuteroporphyrin dimethyl ester iron(III) was 213 mg; most abundant mass spectral peak, *m/e* 592; *m/e* 94 (phenol) observed.

Anal. Calcd for $C_{38}H_{37}FeN_4O_5$: C, 66.6; H, 5.4; Fe, 8.1; N, 7.7. Found: C, 66.4; H, 5.6; Fe, 7.9; N, 7.7.

HYPOPHOSPHITODEUTEROPORPHYRIN IX DIMETHYL ESTER IRON(III). The procedure was identical with the preparation of the fluoroiron(III) compound, except for use of sodium hypophosphite in place of sodium fluoride. The yield from 203 mg of acetatodeuteroporphyrin dimethyl ester iron(III) was 195 mg.

Anal. Calcd for $C_{34}H_{34}FeN_4O_6P$: C, 58.4; H, 5.2; N, 8.5. Found: C, 58.1; H, 5.2; N, 8.6.

Ligand Exchange in Homogeneous Solution. PARTIAL CONVERSION OF ACETATO INTO METHOXODEUTEROPORPHYRIN DIMETHYL ESTER IRON(III). Acetatodeuteroporphyrin dimethyl ester iron(III) (206 mg) dissolved in chloroform (1 ml) was heated to its boiling point, and hot methanol (2 ml) was added. The solution was cooled and filtered. Crystals were washed with water and dried under vacuum at 50° for 6 hr: yield 176 mg; methoxo ν_{CH} in KBr, 2750 cm^{-1}; methoxo ν_{FeO}, 540 cm^{-1} in KBr. A strong acetate ν_{CO} was observed at 1660 cm^{-1} in KBr.

PARTIAL CONVERSION OF ACETATO INTO ETHOXODEUTEROPORPHYRIN DIMETHYL ESTER IRON(III). Acetatodeuteroporphyrin dimethyl ester iron(III) (103 mg) was dissolved in hot ethanol (100 ml), filtered, evaporated to dryness, redissolved in hot ethanol, cooled, and filtered. The crystals were washed with cold ethanol and dried under vacuum at room temperature for 9 hr; yield 43 mg. A weak acetate ν_{CO} was observed at 1660 cm^{-1} in KBr.

Preparations Leading to Oxo-Bridged Dimer. ALUMINA CHROMATOGRAPHY. Chlorodeuteroporphyrin dimethyl ester iron(III) (500 mg) dissolved in chloroform (30 ml) was added to an alumina column (2 × 35 cm) and washed five times with 30-ml portions of benzene. A small red zone was eluted with 1,2-dichloroethane. The major zone was eluted with chloroform, concentrated under vacuum, precipitated with petroleum ether (bp 30–60°), and dried at 60° under vacuum; yield 410 mg; **1** of Table I. Elemental analysis showed no chlorine present.

Acetatodeuteroporphyrin dimethyl ester iron(III) (400 mg) was treated similarly; yield 320 mg; **2** of Table I.

TREATMENT WITH AQUEOUS SOLUTIONS. Acetatodeuteroporphyrin dimethyl ester iron(III) (412 mg) dissolved in chloroform (20 ml) was shaken four times with 30-ml

TABLE I: Treatment of Deuterohemins Leading to Oxo-Bridged Dimer.

Compd	Original Axial Ligand	Treatment	Calcd (%) C	H	Fe	N	Mol Wt in Chloroform
Calcd for $(C_{32}H_{32}FeN_4O_4)_2O$:			64.0	5.3	9.3	9.3	1201
1	Chloro	Alumina chromatography	63.6	5.2	9.8	9.0	1240
2	Acetato	Alumina chromatography	64.2	5.4	9.0	9.2	1290
3	Acetato	Chloroform–water	64.2	5.8	9.0	9.5	1230
4	Acetato	Chloroform–deuterium oxide	63.9	5.7	9.3	9.3	
5	Acetato	Chloroform–water, then aqueous sodium nitrite	64.2	5.7	8.9	9.3	
6	Acetato	Chloroform–water, then aqueous sodium nitrate	64.2	5.8	8.8	9.4	
7	Acetato	Chloroform–water, then aqueous sodium cyanide	64.0	5.8	9.1	9.4	
8	Acetato	Benzene–aqueous sodium phosphate	64.2	5.6			
9	Acetato	Chloroform–aqueous H_2SO_4	64.0	5.6	8.9	9.0	
10	Chloro	Aqueous pyridine	63.5	5.5	9.2	9.1	1240

portions of water. The chloroform solution was concentrated, precipitated with hot isooctane, collected, washed with cold isooctane, and dried at room temperature for 5 hr; yield 324 mg; **3** of Table I. No significant change in elemental analysis was found after 4 hr under vacuum at 50°.

Acetatodeuteroporphyrin dimethyl ester iron(III) (200 mg) dissolved in chloroform (10 ml) was shaken six times with 5-ml portions of deuterium oxide. The chloroform solution was treated as in the preparation of **3**; yield 143 mg; **4** of Table I.

Acetatodeuteroporphyrin dimethyl ester iron(III) (117 mg) dissolved in chloroform (10 ml) was shaken two times with 50-ml portions of water, then ten times with 10-ml portions of aqueous sodium nitrite. The chloroform solution was treated as in the preparation of **3**; yield 71 mg; **5** of Table I.

Acetatodeuteroporphyrin dimethyl ester iron(III) (102 mg) dissolved in chloroform (10 ml) was shaken with water (100 ml), then seven times with 30-ml portions of aqueous sodium nitrate. The chloroform solution was treated as in the preparation of **3**; yield 42 mg; **6** of Table I.

Acetatodeuteroporphyrin dimethyl ester iron(III) (201 mg) dissolved in chloroform (10 ml) was shaken two times with 30-ml portions of water, then five times with 30-ml portions of saturated aqueous sodium cyanide. The chloroform solution was treated as in the preparation of **3**; yield 70 mg; **7** of Table I.

Acetatodeuteroporphyrin dimethyl ester iron(III) (202 mg) dissolved in benzene (100 ml) was shaken six times with 30-ml portions of aqueous trisodium phosphate. The benzene solution was treated as in the preparation of **3**; yield 103 mg; **8** of Table I.

Acetatodeuteroporphyrin dimethyl ester iron(III) (100 mg) dissolved in chloroform (50 ml) was shaken four times with portions of *ca.* 0.05 M H_2SO_4. The chloroform solution was filtered, evaporated, and dried under vacuum at 60°; yield 80 mg; **9** of Table I.

Chlorodeuteroporphyrin dimethyl ester iron(III) (150 mg) was dissolved in pyridine (15 ml) to which water (50 ml) was then added. The hemin was extracted into chloroform (20 ml), precipitated with petroleum ether, and dried under vacuum at 60°; **10** of Table I.

OXIDATION OF HEME. Dipyridinedeuteroporphyrin dimethyl ester iron(II)·H_2O was prepared by reduction of the acetatoiron(III) compound in the manner of Alben *et al.* (1968), except that instead of extraction of the heme into benzene, the reddish precipitate resulting from addition of water and sodium dithionite to the pyridine solution of hemin was separated by centrifugation, washed rapidly with pyridine–water (1:4, v/v), and dried under vacuum at 40° for 4 hr. In Nujol mull the infrared spectrum exhibited a shoulder at lower wave numbers to the ester ν_{CO}, as well as water absorption, which may indicate that the water present interacts with the ester groups. In KBr pellet, which was subjected to vacuum in preparation, the infrared spectrum was identical with that of the pyridine iron(II) compound with 1.5 pyridines/iron reported by Alben *et al.* (1968).

Anal. Calcd for $C_{42}H_{44}FeN_6O_7$ (dipyridine heme·H_2O): C, 65.5; H, 5.7; N, 10.9; O, 10.4. Found: C, 65.2; N, 5.8; N, 10.9; O, 10.0.

The dipyridinedeuteroporphyrin dimethyl ester iron(II) (40 mg) was dissolved in chloroform (2 ml) and dried under vacuum at room temperature. The infrared spectrum in KBr and Nujol mull was identical with those of oxo-bridged dimeric compounds prepared from iron(III) compounds. No pyridine absorption was found.

The dipyridinedeuteroporphyrin dimethyl ester iron(II) (20 mg) was dissolved in pyridine (0.5 ml) and H_2O-enriched 97 atom% in ^{18}O (0.25 ml) was added. The tube was sealed, kept at 60–70° for 2 hr, and the solvent was removed under vacuum at room temperature. The infrared spectrum in Nujol mull was identical with those of the oxo-bridged dimer, except for a decrease in in-

tensity of the band at 840 cm^{-1} and appearance of an additional band at 780 cm^{-1}.

Results and Discussion

Preparative Methods. Methods for the preparation of pure solid hemins with a variety of axial ligands have not been extensively explored. Chloro- and bromoiron (III) porphyrin esters have been prepared by the classical method of iron insertion in acetic acid saturated with sodium chloride or bromide (Erdman and Corwin, 1947; Alben *et al.*, 1968). Erdman and Corwin (1947) reported preparation of an iodoiron(III) porphyrin ester by the same method but were unable to purify a fluoroiron(III) derivative.[1] Difficulty in obtaining pure homogeneous products by the classical method have been discussed (Paul, 1958; Alben *et al.*, 1968). Chromatography of the iron insertion product and subsequent crystallization under suitable conditions were used in the preparation of chloro-, methoxo-, and ethoxoiron(III) porphyrin esters (Alben *et al.*, 1968).

A new approach to the preparation of hemins was applied to deuterohemin in these studies. In the absence of dissolved salts, iron insertion into deuteroporphyrin dimethyl ester in glacial acetic acid, followed by concentration and cooling, gave the acetatoiron(III) derivative. (Products of hemin synthesis by the classical method may, therefore, contain acetatohemin as a contaminant or even a major component.) Solutions of the acetatoiron(III) compound, when dissolved in chloroform (or benzene), were readily converted into other hemins by shaking with aqueous solutions of the sodium salt of the potential anionic ligand. Fluoro-, chloro-, bromo-, iodo-, phenoxo-, azido-, and hypophosphitohemins were prepared in good yield. Qualtitative experiments utilizing the distinctive electronic spectra of hemins, although not described in detail here, indicated that these hemins were readily interconverted by the same method. However, when water or aqueous solutions of the sodium salts of nitrate, nitrite, phosphate, and cyanide were used, the product was characterized, as a dimer with a single bridging oxygen atom (Table I). The dimer was also readily converted into monomeric hemins by treatment with aqueous solutions of those salts whose anions exchanged with acetate on the acetatohemin. Treatment of the acetato- and chloroiron(III) derivatives with hot methanol or ethanol failed to give complete axial ligand exchange.

Acetatodeuteroporphyrin dimethyl ester iron(III) underwent axial ligand exchange not only when chloroform (or benzene) solutions were washed with aqueous solutions, but also when crystals were washed with water. The acetato axial ligand was not lost when crystals were washed with 30% aqueous acetic acid or when the compound was precipitated from acetic acid solution by addition of water. Crystals were stable at 55° under vacuum but at 148° under vacuum the solid decomposed to a product with an elemental analysis consistent with the oxo-bridged dimeric structure. (Loss of acetic anhydride or acetic acid and ketene could lead to this product.)

We found no evidence in elemental analyses or infrared spectra for a stable solid hydroxohemin, even after washing other hemins with water (and dilute base), although hydroxohemins appear to be necessary intermediates in some of the reactions reported here.

Mass spectral measurements made on several of the compounds reported here provided structural evidence in addition to that obtained by elemental analyses and infrared spectra. Because of low volatility, the direct inlet method was used and samples were heated to *ca.* 200° under vacuum. These conditions were severe enough to remove the axial ligands from all deuterohemins examined (chloro-, azido-, phenoxo-, methoxo-, and ethoxodeuteroporphyrin dimethyl ester iron(III)); the most abundant peak observed at 592 mass units, corresponded to [deuteroporphyrin dimethyl ester iron (III)]$^+$. Methoxomesoporphyrin dimethyl ester iron(III) and chloroprotoporphyrin diethyl ester iron(III) also lost their axial ligands. Although chlorodeuteroporphyrin dimethyl ester iron(III) heated to 180° gave weak peaks at 626–629 (chloroiron (III) species), they diminished in intensity upon heating to 205°, and mass peaks were not generally found at the expected mass number of the molecular ion. The high mass region thus provides evidence for the stability of the iron porphyrin ring system to the reaction conditions used for synthesis, while at low *m/e* values the acetato-, chloro-, phenoxo, methoxo-, and ethoxoiron(III) compounds gave peaks for free protonated axial ligand (acetic acid, HCl, phenol, methanol, and ethanol, respectively). The azidoiron(III) compound gave large amounts of N_2. Loss of anionic axial ligands under mass spectroscopic conditions has been reported for chloromesoporphyrin dimethyl ester iron(III) (Whitten *et al.*, 1966) and other metalloporphyrins (Sadasivan and Fleischer, 1968).

At *m/e* values greater than 592, weak peaks at 606 (and sometimes 620) were observed in all deuterohemin compounds. (The metal-free porphyrin dimethyl ester also showed a weak M + 14 peak at 552 mass units.) Comparison of intensities relative to the 592 peak indicated that the peaks arose from transesterification and not from bound axial ligands or additional substituents (impurities) on the porphyrin ring system. Abnormally high abundances of *m/e* peaks at 606 and 620 relative to the 592 peak (0.75 and 0.16, respectively) were found for ethoxodeuteroporphyrin dimethyl ester iron(III), which had been prepared in the presence of excess ethanol (Alben *et al.*, 1968). However, the abundance of the 606 peak relative to 592 was low (0.02) for methoxodeuteroporphyrin dimethyl ester iron(III), which had been prepared in the presence of excess methanol (Alben *et al.*, 1968). Thus masses of 606 and 620 correspond to monomethylmonoethyl and diethyl ester species, respectively.[2] Transesterification evidently can

[1] The suggestion that fluorohemins are inherently unstable is not supported by our findings. Stable fluoro derivatives of heme proteins have been widely studied as well.

[2] From relative mass peak abundances, 39% monomethylmonoethyl and 8% diethyl ester species were calculated to be present in the ethoxoiron(III) dimethyl ester compound of Alben *et al.* (1968). Correction of the calculated elemental analysis to take transesterification into account gave values of C, 64.3; H, 5.9; N, 8.7. The values found by Alben *et al.* still compare acceptably.

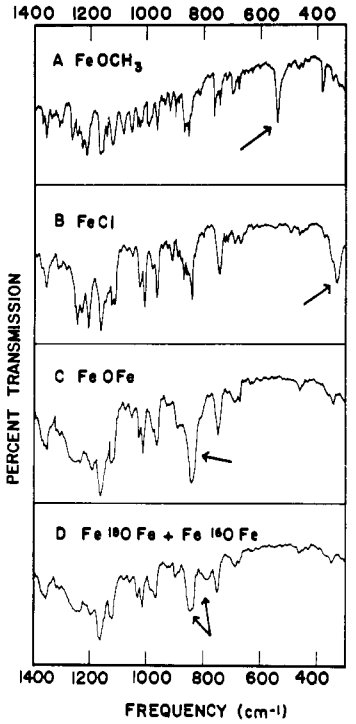


FIGURE 1: Infrared spectra of deuteroporphyrin dimethyl ester iron(III) compounds obtained in KBr pellets with a Perkin-Elmer 225 spectrometer. (A) Methoxodeuteroporphyrin dimethyl ester iron(III); (B) chlorodeuteroporphyrin dimethyl ester iron(III); (C) product of alumina chromatography of chlorodeuteroporphyrin dimethyl ester iron(III) (= oxo-bridged deuterohemin dimer); (D) product of autoxidation of dipyridinedeuteroporphyrin dimethyl ester iron(II) in the presence of $[^{18}O]H_2O$ (= oxo-bridged deuterohemin dimer). Arrows mark (A) ν_{FeO}, (B) ν_{FeCl}, (C) $\nu_{Fe-O-Fe}$, and (D) $\nu_{Fe-^{16}O-Fe}$ at 840 cm^{-1} and $\nu_{Fe-^{18}O-Fe}$ at 780 cm^{-1}.

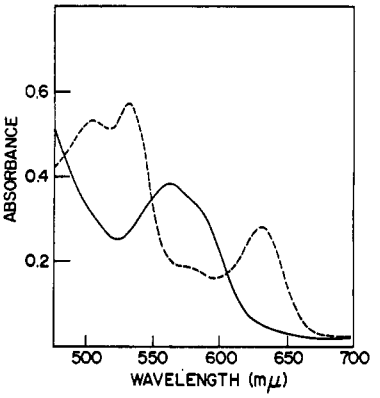


FIGURE 2: Electronic spectra of the oxo-bridged deuterohemin dimer (———) and chlorodeuterohemin (- - -) in chloroform.

occur when the dimethyl ester groups are exposed to ethanol, even that serving as preservative in commercial reagent grade chloroform.

Electronic and Mössbauer spectra for the deuterohemins reported here will be presented in detail in subsequent papers of this series.

Evidence for Oxo-Bridged Dimer Structure. Compounds prepared from deuterohemins by several methods (Table I) gave elemental analyses consistent with the stoichiometry of one oxygen atom per two iron porphyrin esters. Molecular weights for four of the compounds were obtained in chloroform solution; all four were dimers. The compounds showed an absence of infrared absorption attributable to the axial ligands of the parent compounds (Figure 1 illustrates the absence of ν_{FeCl} in the alumina chromatographic product of chlorodeuterohemin.), and appeared identical with one another by electronic and infrared spectra. The visible portion of the electronic spectrum of the dimer is compared with that of chlorodeuterohemin in Figure 2. Treatment of dipyridinedeuteroheme under conditions appropriate for autoxidation gave products with electronic and infrared spectra identical with those of the dimeric compounds prepared from deuterohemins. No ester ν_{CO} splitting indicative of dimerization through the ester groups was observed in KBr pellet, Nujol mull, or chloroform solution. Absorption attributable to ν_{OH} or ν_{FeO} was not detected, and preparation from acetatohemin by washing with water and deuterium oxide gave products with identical infrared spectra in chloroform solution, KBr pellet, and Nujol mull. Infrared evidence was obtained for a single bridging oxygen atom (see below). We propose the structure, $N_4Fe-O-FeN_4$, where N_4Fe is deuteroporphyrin dimethyl ester iron (III), analogous to the structure proposed for the autooxidation product of 2,4-diacetyldeuteroheme (Alben *et al.*, 1968; Fuchsman, 1967).

Direct infrared evidence was obtained for the dimeric structure proposed. Infrared absorption has been observed in the region 800–900 cm^{-1} for a variety of transition metal complexes which appear to be oxo-bridged dimers, and has been attributed to the metal–oxygen–metal asymmetric stretching vibration (Cotton and Wing, 1965; Hewkin and Griffith, 1966; Schugar *et al.*, 1967). Of two iron(III) complexes shown by crystallographic studies to be oxo-bridged dimers (Fleischer and Hawkinson, 1967; Lippard *et al.*, 1967), one absorbed at 795 cm^{-1} and the other at 830 cm^{-1} (Nelson *et al.*, 1966; Schugar *et al.*, 1967). Infrared spectra of the deuterohemin dimer showed a broad intense absorption at 840 cm^{-1}. When the compound was prepared by heme autoxidation in the presence of water enriched in ^{18}O, the 840-cm^{-1} band was partially replaced by an isotopic band at 780 cm^{-1} (Figure 1). The observed frequency of the isotopic peak is close to the value of 760 cm^{-1} calculated by the method of Herzberg (1945) for the effect of isotopic substitution on the asymmetric stretching frequency of a simple linear trinuclear system. The bridging oxygen was exchangeable. The 780-cm^{-1} band disappeared completely when the solid dimer was washed with water or exposed for 3 weeks to atmospheric humidity, and heat treatment of the KBr pellet (100° under vacuum for 1 hr) caused a diminution in the 780-cm^{-1} peak and an increase in the 840-cm^{-1} band. Since the dipyridineiron(II) compound, which lost pyridine even at room temperature and atmospheric

TABLE II: Mössbauer Parameters of Deuteroporphyrin Dimethyl Ester Iron Compounds at Room Temperature.[a]

Axial Ligand and Oxidation State	Chemical Shift[b] (mm/sec)	Quadrupole Splitting (mm/sec)	Rel Intensities[a]	Rel Half-Band Widths[a]
Pyridine Fe(II)	0.61	1.13	1.0	1.0
Chloro Fe(III)	0.51	0.91	0.4	2.5
μ-Oxo Fe(III) dimer[c]	0.55	0.64	1.2	1.1

[a] All spectra were simple doublets. Intensities and half-band widths are relative to the peak at lower chemical shift. [b] Taken with respect to sodium nitroprusside as standard. [c] Compound 3 of Table I.

pressure, was stable to 100° under vacuum when pressed into a KBr pellet, as were the oxo-bridged dimer preparations unenriched in ^{18}O; the decrease in the 780-cm^{-1} band and increase in the 840-cm^{-1} band evidently was not caused by loss from the pellet of an axial or bridging ligand, but instead was caused by oxygen exchange between the bridging oxo atoms and the considerable quantity of water within the pellet. Samples stored for 3 weeks in a sealed vial within a dessicator gave an infrared spectrum identical with that of the initial isotopically enriched product. The oxo-bridged deuterohemin dimer is the first oxo-bridged compound for which an oxygen isotopic shift in the metal–oxygen–metal asymmetric stretching frequency has been reported.[3]

Observed physical properties were consistent with the structure proposed. No zero-field splitting value was observed by far-infrared spectroscopy in the range 0–28 cm^{-1}, although by the same technique zero-field splitting parameters were directly measured for many of the monomeric hemins reported here (Richards *et al.*, 1967). The Mössbauer spectrum of the oxo-bridged deuterohemin dimer was more symmetrical than that of the chloroiron(III) derivative but the quadrupole splitting appeared too small for an iron(II) porphyrin (Table II). Oxygen bridging, with resultant magnetic coupling between the iron atoms of the dimeric hemin, could explain these observations. No mass peak (other than 606) was observed at higher mass than the most abundant 592 peak in the mass spectrum, but the abundance of low mass peaks at 18 (water) and 17 (OH) was greater in the spectrum of the oxo-bridged dimeric deuterohemin than in those of deuterohemins with other axial ligands.

Possible Mechanisms for Ligand Exchange and Dimerization. MECHANISMS INVOLVING MONOMERIC INTERMEDIATES. Ligand exchange without dimer formation could involve a simple displacement reaction (eq 1), or a dissociative sequence of reactions (eq 2 and 3), which might be favored by the presence of water as a polar solvent permitting charge separation. Dimer formation

$$N_4FeY + X^- \rightleftharpoons N_4FeX + Y^- \quad (1)$$

$$N_4FeY \rightleftharpoons N_4Fe^+ + Y^- \quad (2)$$

$$N_4Fe^+ + X^- \rightleftharpoons N_4FeX \quad (3)$$

could occur when no anionic axial ligand besides hydroxide was present or able to bind to the hemin cation (eq 4 and 5). Reversibility of eq 1 or 2 and 3 is necessary

$$N_4Fe^+ + H_2O \rightleftharpoons N_4FeOH + H^+ \quad (4)$$

$$N_4FeOH + N_4Fe^+ \rightleftharpoons N_4FeOFeN_4 + H^+ \quad (5)$$

to explain interconversion of hemins; conversion of the dimer into monomeric hemins could occur by reversal of eq 5, 4, and 2.

MECHANISMS INVOLVING DIMERIC INTERMEDIATES. Dimerization, however, might reasonably be expected to involve dimeric intermediates doubly bridged by either the same ligand (eq 6) or mixed ligands (eq 7).

$$2N_4FeOH \longrightarrow N_4Fe\begin{matrix}H\\O\\O\\H\end{matrix}FeN_4 \longrightarrow N_4FeOFeN_4 + H_2O \quad (6)$$

$$N_4FeOH + N_4FeX \longrightarrow N_4Fe\begin{matrix}H\\O\\X\end{matrix}FeN_4 \longrightarrow N_4FeOFeN_4 + HX \quad (7)$$

These mechanistic possibilities are rendered more plausible by dimerization of methoxo- and ethoxodeuteroporphyrin dimethyl ester iron(III) in concentrated chloroform solution (observed molecular weights *ca.* 1230). A likely structure for the dimers is

$$N_4Fe\begin{matrix}R\\O\\O\\R\end{matrix}FeN_4$$

[3] A pyridinated manganese(III) complex of phthalocyanine has also been shown crystallographically to be an oxo-bridged dimer (Vogt *et al.*, 1967). Its infrared properties, however, are disputed. Although Elvidge and Lever (1959) reported an 820-cm^{-1} band in the same compound after heat treatment, Yamamoto *et al.* (1968) did not find an 820-cm^{-1} band after heat treatment, nor any band sensitive to preparation (by oxidation of a manganese(II) complex) in the presence of water-enriched 30 atom % in ^{18}O.

where R is CH_3 or CH_2CH_3, since alkoxo groups are well known to promote bridge formation. (In the solid in KBr, methoxohemins of deutero-, meso-, and protoporphyrin prepared by Alben *et al.* (1968) exhibited ν_{FeO} bands at 540 cm^{-1} (*cf.* Figure 1). Since in other metal methoxides the ν_{MO} frequency is sensitive to whether the methoxo group is bridging or terminal (Kawasaki and Okawara, 1965) and methoxomesohemin is monomeric in the crystal (Hoard *et al.*, 1965), methoxodeuterohemin and -protohemin may be assumed monomeric in the crystal. No infrared band at 540 cm^{-1} was observed for methoxodeuterohemin in concentrated chloroform solution but solvent absorption at lower and higher wave numbers was too intense to permit location of a new band.)

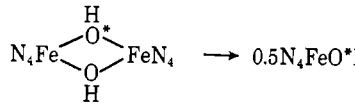
A doubly bridged intermediate could also explain the incorporation of oxygen from water during autoxidation and the exchange of the bridging oxygen atom of the dimeric hemin with water. Cohen and Caughey (1968) proposed that the kinetically implicated species pyr-$N_4FeO_2FeN_4$-pyr, where pyr signifies pyridine, decomposed in the presence of water according to eq 8 and 9 (an asterisk is used to denote oxygen originating in water), incorporating oxygen from both molecular oxygen and water into a monomeric product or intermediate. One half of the bridging atoms would arise

$$\text{pyr-}N_4FeO_2FeN_4\text{-pyr} + 2H_2O^* \longrightarrow 2N_4FeO^*H + H_2O_2 + 2\text{pyr} \quad (8)$$

$$H_2O_2 + 2\text{pyr-}N_4Fe\text{-pyr} \longrightarrow 2N_4FeOH + 4\text{pyr} \quad (9)$$

from water if the oxo-bridged dimer formed as in eq 10.

$$N_4FeO^*H + N_4FeOH \longrightarrow$$



$$0.5N_4FeO^*FeN_4 + 0.5N_4FeOFeN_4 + 0.5H_2O^* + 0.5H_2O \quad (10)$$

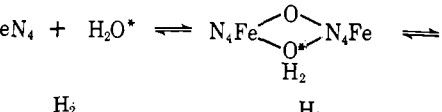
Alben *et al.* (1968) proposed an alternative mechanism for the decomposition of the $N_4FeO_2FeN_4$ species (eq 11 and 12) which would not incorporate oxygen from water.

$$\text{pyr-}N_4FeO_2FeN_4\text{-pyr} \longrightarrow 2\text{pyr-}N_4FeO \quad (11)$$

$$2\text{pyr-}N_4FeO + 2\text{pyr-}N_4Fe\text{-pyr} \longrightarrow 2\text{pyr-}N_4FeOFeN_4\text{-pyr} + 2\text{pyr} \quad (12)$$

Exchange with water would then be required to explain incorporation of ^{18}O from water. The exchange, also observed independently of oxidation, would involve either a dihydroxo-bridged dimer (eq 13) or reversibility of eq 10.

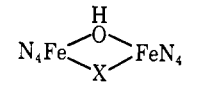
$$N_4FeOFeN_4 + H_2O^* \rightleftharpoons$$



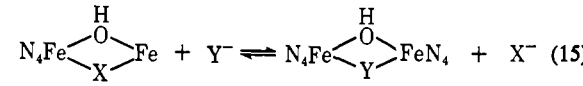
$$N_4FeO^*FeN_4 + H_2O \quad (13)$$

Ligand exchange could also take place through doubly bridged intermediates (eq 14–16). Although

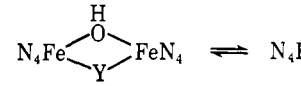
$$2N_4FeX + H_2O \rightleftharpoons$$



$$+ H^+ + X^- \quad (14)$$



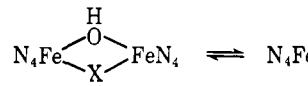
$$(15)$$



$$(16)$$

the collapse of a doubly bridged dimer of the type indicated in eq 16 might be statistically unfavorable compared with a collapse to give the oxo-bridged dimer and HY (eq 7), the ready conversion of the oxo-bridged dimer into monomeric hemins in the presence of aqueous salt solutions favors doubly bridged dimeric intermediates (eq 17 and 18).

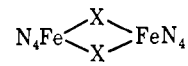
$$N_4FeOFeN_4 + X^- + H^+ \rightleftharpoons$$



$$(17)$$

An intermediate of the type

$$N_4FeOH + X^- \rightleftharpoons N_4FeX + OH^- \quad (18)$$



might also occur. Ligands which did not bind (nitrate, nitrite, phosphate, and sulfate) were bulky and might not be able to participate in doubly bridged structures. (Initial color changes indicated that cyanide was probably bound in solution but was not stable to the conditions of isolation.)

The studies reported here, especially those summarized in Table I, suggest that oxo-bridged dimers may frequently be formed under solvent conditions promoting either hemin aquation or heme autoxidation. The oxo-bridged dimeric structure may also be important in heme proteins containing two or more heme groups capable of direct interaction. The possibility of an Fe–O–Fe linkage in cytochrome *c* oxidase has been discussed (Caughey *et al.*, 1968).

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Properties of Transfer Ribonucleic Acid and Aminoacyl Transfer Ribonucleic Acid Synthetases from an Extremely Halophilic Bacterium*

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ABSTRACT: Aminoacyl transfer ribonucleic acid synthetases have been prepared by precipitation at pH 5, from cell-free extracts of *Halobacterium cutirubrum* in 3.4 M KCl, 0.1 M magnesium acetate, and 0.01 M Tris-HCl buffer (pH 7.6); tRNA was isolated from the pH 5 supernatant by phenol extraction after first reducing the salt concentration. Aminoacylation of transfer ribonucleic acid was found to depend upon adenosine triphosphate, Mg^{2+}, and aminoacyl transfer ribonucleic acid synthetases; it is inhibited by pancreatic ribonuclease and *p*-mercuribenzoate. In the presence of guanosine triphosphate, a supernatant fraction containing transfer enzymes, ribosomes, NH_4^+ and Mg^{2+} ions, the amino acid is transferred from the aminoacyl transfer ribonucleic acid into polypeptide; puromycin is inhibitory. Thus, both reactions are similar to those in nonhalophilic systems except for their ionic requirements: aminoacylation of transfer ribonucleic acid requires specifically 3.8 M KCl and the transfer reaction 3.8 M KCl, 1 M NaCl, and 0.4 M NH_4Cl for maximum activity. Kinetic studies on the formation of aminoacyl transfer ribonucleic acid show that replacing K^+ with Na^+ does not affect the apparent K_m for the amino acid but causes some reduction in the apparent V_{max}. In contrast, replacing K^+ with NH_4^+ does not affect the apparent V_{max}, but considerably increases the apparent K_m for the amino acid. The kinetic data are consistent with the NH_4^+ ion being a competitive inhibitor of the enzyme with respect to the amino acid.

It is suggested that binding of the amino acid to the aminoacyl transfer ribonucleic acid synthetase involves ionic interaction between the α-amino group of the amino acid and an anionic site on the enzyme. The results also indicate that in extremely halophilic bacteria ionic interactions are specific as they must be insensitive to the high ionic strength.

The first enzymic step in the biosynthesis of protein is the activation of the amino acid and its esterification to tRNA. The enzymes responsible are particularly interesting for their ability to recognize individual tRNAs and amino acids. The nature of this specificity is not known. However, Loftfield and Eigner (1967) have suggested that since the aminoacylation of tRNA in *Escherichia coli* is very sensitive to salt concentration,

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