1953), and it is not reversed with BAL as is arsenite (Fluharty and Sanadi, 1960). CCP derivatives do not readily undergo oxidation-reduction and do not alter the cyanide, antimycin A, or amytal sensitivity of mitochondrial respiration.

The aminothiol blocking of CCP action is particularly characteristic and may represent a clue to the chemical basis of CCP uncoupling. Among other uncoupling agents examined, only 1,1,3-tricyano-2-aminopropene (Eberts, 1960; Carboni, U. S. Pat. 2,719,861, 1955) was similarly counteracted by aminothiols. The resemblance of the 1-terminal end of this material to the dicyanomethylene group of CCP suggests direct involvement of this function in the uncoupling and aminothiol-bonding properties of both types of compounds.

Finally, the inhibition of the photophosphorylation system by the CCP class of uncouplers is unlike the behavior of most of the above-mentioned agents and may help in elucidating similarities between the oxidative and photosynthetic phosphorylation pathways.

It seems curious that the protective agents against CCP uncoupling, *i.e.*, 1,2- and 1,3-aminothiols, are also well-known protective agents against ionizing radiation *in vivo* (Bacq, 1957; Patt, 1958). Whether this implies any similarity in underlying mechanisms remains highly speculative. It is conceivable that short-term, acute radiation damage is primarily a mitochondrial phenomenon involving alteration of reactive sites similar to those bound by CCP agents during uncoupling.

ACKNOWLEDGMENTS

Several experiments described here were made with the help of Dr. R. A. Goldsby of this department. I am also indebted to Drs. E. Racker, I. C. Gunsalus, and A. W. Galston for valuable discussions and suggestions in the course of this work, and to Dr. B. C. Pressman for helpful criticism.

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# Oxidation States of Manganese Hematoporphyrin IX in Aqueous Solution*

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Manganese hematoporphyrin IX and its dimethyl ester have been prepared, and the most stable form of these complexes in air is shown by elemental analyses and magnetic susceptibility measurements to be one in which manganese is in the 3+ oxidation state. Three other oxidation states are demonstrated to be reversibly obtainable in aqueous alkaline solution, and oxidation-reduction potentials are reported for the system $Mn^{III}/Mn^{II}$ hematoporphyrin IX between *p*H 6.7 and 13.6 and the system $Mn^{IV}/Mn^{III}$ hematoporphyrin IX between *p*H 9.9 and 13.6. Both systems show evidence for a redox-linked proton function in $Mn^{III}$ hematoporphyrin IX with a $pK'$ value of 12, which was verified by spectrophotometric determination, and it is suggested that the equilibrium reflected involves the loss of a proton by a coordinated water molecule. Data on $E_m$ *versus* *p*H for $Mn^{IV}/Mn^{III}$ hematoporphyrin IX indicate the presence of two redox-linked proton functions in $Mn^{IV}$ hematoporphyrin IX having $pK'$ values below 10. It is suggested that these reflect the loss of a proton by each of two water molecules, one in coordination position 5 and the other in coordination position 6. Two redox-linked proton functions were determined in $Mn^{II}$ hematoporphyrin IX having $pK'$ values of 6.9 and 12.8, and the latter constant was assigned to the loss of a proton by a coordinated water molecule. The results are related to existing data on metalloporphyrin systems, and it is pointed out that $Mn^{II}$ hematoporphyrin IX is a strong reductant whereas $Mn^{IV}$ hematoporphyrin IX is a very strong oxidant, a fact of possible interest in photosynthesizing systems.

Three transition elements, manganese, iron, and copper, are present in green plant material capable of performing the primary events characteristic of photosynthesis (Park and Pon, 1963). There is some information about the kinds of complexes in which iron is found, for example, cytochromes (Davenport and Hill, 1952; Hill and Bonner, 1961; Lundegardh, 1962) and, more recently, photosynthetic pyridine nucleotide reductase (San Pietro, 1961; Horio and Yamashita,

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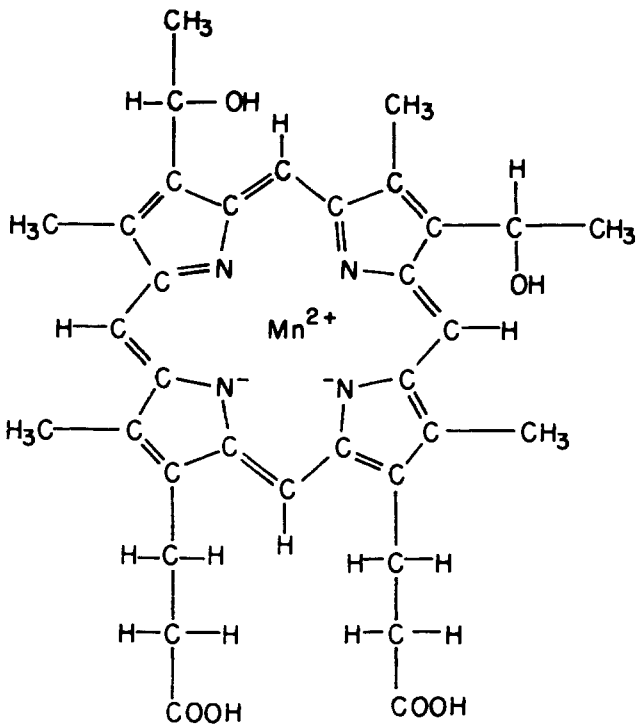
1962) and ferredoxin (Mortenson *et al.*, 1962; Valentine *et al.*, 1962; Tagawa and Arnon, 1962). A little is known about the material with which copper is associated, for example, plastocyanin (Katoh, 1960; Katoh *et al.*, 1961; Katoh *et al.*, 1962), but almost no information is available concerning the molecular environment of manganese. Important to our understanding of the role of these metals in photosynthesis is a knowledge of the physical and chemical properties exhibited by a variety of metal chelate complexes of the type that might exist in biological material. Since one of the most commonly found structures, particularly in photosynthetic units, is the porphyrin ring, manganese complexes with this structure have been chosen for the present study. This report deals with two water-soluble manganese porphyrin systems, manganese hematoporphyrin IX and its dimethyl ester (see Structure I).




*Mn$^{II}$ HEMATOPORPHYRIN IX*

Structure I

(The oxidation state of manganese is arbitrarily written as 2+)

As one of the many studies of metalloporphyrin systems carried out in the laboratory of W. M. Clark (Clark *et al.*, 1940; Shack and Clark, 1947; Cowgill and Clark, 1952), Taylor prepared manganese mesoporphyrin IX and studied its properties in water containing 20% pyridine (Taylor, 1940). From potentiometric data he found that two oxidation states of manganese can exist in this complex. Efforts to examine a reversible system in the absence of added ligand molecules, such as pyridine, were without success. Large amounts of aggregation were thought to be the cause of the problems encountered. In the study presented here, manganese hematoporphyrin IX was found to be sufficiently soluble in water that difficulty of this sort was not a limiting factor even in the absence of added ligand molecules.

## Materials

*Porphyrins.*—Ten grams of hematoporphyrin IX dihydrochloride (California Corporation for Biochemical Research, Los Angeles, Calif.) were esterified in 1500 ml absolute methanol saturated with dry hydrogen chloride. After 6 hours of reaction at 0°, the ester was extracted with chloroform and residual hydrogen chloride was removed from the chloroform by washing with 0.5 N $NH_4OH$. The pigment was concentrated and then purified on a silica gel column (Marshall *et al.*, 1949; Lucas and Orten, 1951). Eight grams of this purified hematoporphyrin IX dimethyl ester was dissolved in 200 ml glacial acetic acid and 5 g of pulverized manganese diacetate added. The vessel was loosely stoppered and the solution stirred magnetically in the dark, and the course of the reaction was followed spectrophotometrically. The temperature was about 20°. On the basis of the absorption spectrum, 99+% of the hematoporphyrin IX dimethyl ester was converted to a manganese complex in the first 6 hours (see Fig. 1). After 22 hours total reaction time, the acetic acid was removed by evaporation at about 15° under reduced pressure and the residue was taken up with a small amount of water. The pigment was dried in a desiccator over $P_2O_5$ and KOH and separated from excess manganese diacetate by four extractions with benzene. The benzene extract containing the manganese hematoporphyrin IX complex was evaporated to dryness, and the residue was taken up in a small amount of water and again evaporated to dryness over $P_2O_5$ and KOH. Analysis of this material as monoacetate monohydrate may be found in Table I.

Table I

Elemental Analysis of Manganese Hematoporphyrin IX Compounds[a]

| | $Mn^{III}$HmdiMe Monoacetate Monohydrate | | $Mn^{III}$HmdiMe Monochloride Monohydrate | | $Mn^{III}$Hm Monohydrate | |
|---|---|---|---|---|---|---|
| Element | Found | Calcd. | Found | Calcd. | Found | Calcd. |
| C | 60.9 | 60.3 | 59.2 | 58.9 | 61.4 | 61.1 |
| H | 5.9 | 6.0 | 5.9 | 5.8 | 5.6 | 5.5 |
| N | 7.5 | 7.4 | 7.6 | 7.6 | 8.2 | 8.4 |
| Cl | — | 0 | 5.0 | 4.8 | 0.34 | 0 |
| Mn (determined as $Mn_3O_4$) | 7.5 | 7.3 | 7.0 | 7.5 | 8.4 | 8.2 |

[a] MnHm and MnHmdiMe are used for the manganese hematoporphyrin IX complex and its dimethyl ester, respectively. When the oxidation state of the manganese is known, it is indicated as, for example, $Mn^{III}$Hm. HmdiMe is used for metal-free hematoporphyrin IX dimethyl ester.

The chloride derivative of manganese hematoporphyrin IX dimethyl ester was prepared by precipitation from aqueous solution with potassium chloride. The acetate derivative was dissolved in water, solid KCl was added to a concentration of 1 M, and the precipitated pigment was collected on a Büchner funnel. Such precipitation was repeated three times and the resulting solid was dried and taken up in benzene as above. The analysis of this material as the monochloride monohydrate is given in Table I.

Manganese hematoporphyrin IX was prepared from 2 g of the chloride salt of the dimethyl ester by alkaline hydrolysis in 30 ml of 0.5 M KOH for 2 hours at room temperature. The pigment was purified by precipitation from solution at *p*H 4, then redissolved in a minimal amount of KOH, and again precipitated by adding

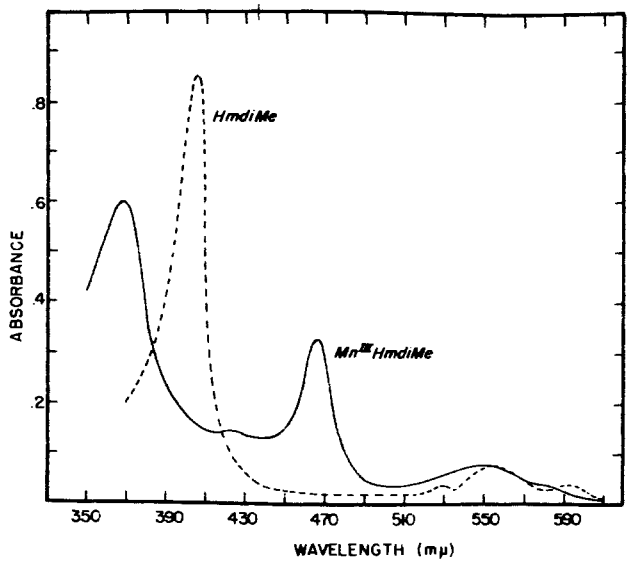
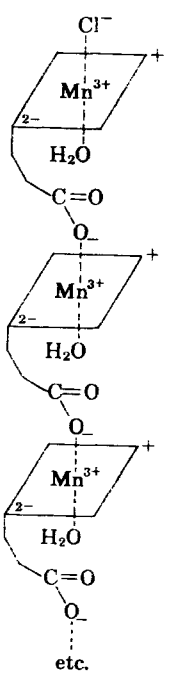



FIG. 1.—Spectra of hematoporphyrin IX dimethyl ester ($6 \times 10^{-6}$ M) and manganese hematoporphyrin IX dimethyl ester ($8 \times 10^{-6}$ M) in glacial acetic acid. 1-cm cuvet; room temperature. See footnote to Table I for abbreviations.

HCl to *p*H 4. It was dissolved in absolute methanol and recovered by evaporation after filtration. This step was repeated four times and the product was suspended in 2 liters of water, collected by filtration, and dried over $P_2O_5$ and KOH in vacuum. Analysis of what is assumed to be the monohydrate is shown in Table I. From Table I it is apparent that much less than one chloride per molecule is present. If this complex is $Mn^{III}Hm$,[1] as the absorption spectrum indicates, then the net positive charge on the molecule must be balanced by an anion; if it is not chloride, it must be either hydroxide or a carboxylate group of one of the propionic acid side-chains. A kind of aggregate which would explain the low chloride content can be pictured as shown in Structure II, where the rhombus




Structure II

represents the porphyrin ring and side-chains, with the exception of the two propionic acid groups. The 2− written inside the rhombus represents the charge distribution on the pyrrole nitrogen atoms of the porphyrin ring, and the 3+ indicates the oxidation state of the manganese. The single + written outside of the ring gives the net charge on the metal-conjugated system only and does not include those charges on coordinated ligands in positions 5 and 6, which are written above and below the plane of the porphyrin, nor does it include those charges on the propionate side-chains.

*Reagents.*—Hydrogen peroxide (30%, Baker's Analyzed; our assay, 8.4 M, by permanganate method) and sodium hypochlorite (commercial bleach; assay, 0.35 M) were appropriately diluted for use. Potassium iridic chloride (K and K Laboratories, Jamaica, N.Y.), potassium ferricyanide, potassium ferrocyanide, sodium hydrosulfite, methyl viologen (K and K Laboratories, Jamaica, N.Y.), and the potassium salts of the phosphate and borate buffers employed were used without further purification. Deionized and subsequently glass-distilled water was used for all solutions. The gases, oxygen, nitrogen, and argon, were acquired from Pacific Oxygen Co., and each was reported to be 99.99% pure. Each gas had been water pumped and dried so that no oil vapor contamination was possible.

## Apparatus and Methods

Absorption spectra were recorded with a Cary model 14 M spectrophotometer. Apparatus and techniques for oxidation-reduction potentiometry were patterned after those described by Harbury (1957). A Beckman Model 76 expanded-scale *p*H meter, a Beckman Glass Electrode with Type E2 glass, and a Beckman calomel electrode with fiber junction were used for *p*H measurements. The apparatus used for magnetic susceptibility measurements on small amounts of solid samples has been described by Cunningham (1961). A standard Gouy balance (Varian Associates, 4-inch magnet) was employed with larger solid samples and solutions. Electron paramagnetic resonance measurements were made with a reflection-type spectrometer which employed 3-cm microwaves and 100 kilocycle per second magnetic field modulation.

Calculations of oxidation-reduction potentials were made with the aid of equation (1), where $E_h$ = the

$$E_h = E_m + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]} \qquad (1)$$

potential at any percentage reduction, referred to the standard hydrogen electrode; $E_m$ (Clark, 1960) = the potential at 50% reduction; $R$ = the molar gas constant; $T$ = the absolute temperature; $F$ = the number of coulombs per faraday; $n$ = the number of electrons transferred per equivalent; [Ox] = the concentration of oxidant; and [Red] = the concentration of the reductant. $E_m$ may be determined by measuring $E_h$ as a function of the ratio [Ox]/[Red]. In the present work this ratio has been calculated both from the volume of titrating agent delivered and from the absorption spectra recorded. In the case of manganese III and II equilibria, $E_h$ was measured directly, and in the case of manganese IV and III equilibria, $E_h$ was established by calculation from the known ratio of ferri- to ferrocyanide, used in excess to establish a particular $Mn^{IV}/Mn^{III}$ ratio. Independent measurements of $E_m$ for the ferri/ferrocyanide couple were made at the concentrations and *p*H values employed for each individual experiment.

When the ratio [Ox]/[Red] was determined from

[1] See abbreviations given in footnote *a* for Table I.

absorption spectra, equation (2) was used, where $A'_{red}$

$$\frac{[Ox]}{[Red]} = \frac{A'_{red} - A_{obs}}{A_{obs} - A'_{ox}} \quad (2)$$

= the absorbance displayed when the material is fully reduced; $A'_{ox}$ = the absorbance displayed when the material is fully oxidized; and $A_{obs}$ = the absorbance measured at any percentage of reduction.

Important to the measurement of equilibrium potentials is the demonstration that the system measured shows complete reversibility. In the case of the manganese III and II conversion, both oxidative and reductive titrations were performed, and measurement of spectra before and after such titration revealed little, if any, loss of material. For the manganese IV to III conversion a large excess of ferri- and ferrocyanide was employed and no differences were observed whether the ferri or the ferro species was added first. In most cases, a set of isosbestic points was conspicuous in the spectra accompanying oxidation or reduction; this is the best evidence obtained to show that only two species are involved in the equilibrium.

Titrations of systems having well-established $E_m$ values (*e.g.*, FMN) were carried out periodically for further assurance that all components were performing properly.

## Results

*Apparent Molar Absorptivity vs. Concentration.*—Simple metalloporphyrin compounds are known to exhibit large aggregation effects in aqueous solution, as witnessed by observed deviations from Beer's Law (Clark *et al.*, 1940; Shack and Clark, 1947; Maehly and Akeson, 1958), and by the exhibition of high particle weights in diffusion studies (Shack and Clark, 1947; Haurowitz, 1938; Zeile and Reuter, 1933). One of the first objects of this study was to determine whether monomeric manganese hematoporphyrin IX can exist in aqueous solution at concentrations that would permit the studies contemplated. In Figure 2*A* is shown a plot of the molar absorptivity *versus* log concentration for $Mn^{III}$HmdiMe acetate in methanol and in water. Behavior similar to that in methanol was also observed for ethanol and chloroform solutions. The lack of dependence of $\epsilon'$ upon concentration may mean that $Mn^{III}$HmdiMe is monomeric in alcohol and chloroform solutions. It is of course possible that aggregates could be forming without effect upon the absorption spectrum, but this is not likely. Another possibility is that a distinct polymeric unit, such as a dimer, exists and is very stable. However, molecular weight estimates[2] in ethanol and chloroform ($4 \times 10^{-3}$ M in $Mn^{III}$HmdiMe) give no indication of the existence of aggregates.

Included in Figure 2 are similar plots for $Mn^{III}$Hm, $Mn^{IV}$Hm, and $Mn^{II}$Hm at *p*H 13. Whereas $Mn^{II}$Hm follows the same general form as $Mn^{III}$HmdiMe in water, $Mn^{III}$Hm and $Mn^{IV}$Hm show no significant variation with concentration in the range from $10^{-3}$ to $10^{-6}$ M. Conversion of $Mn^{III}$Hm to $Mn^{IV}$Hm or $Mn^{II}$Hm will be discussed later.

*Magnetic Susceptibility.*—From the elemental analyses it would appear that the stable form of these manganese hematoporphyrin complexes in air is one in which the oxidation state of the manganese is 3+. If this is so, the magnetic susceptibility of these compounds should correspond to an even number of unpaired electrons: four for a square-planar configuration of the $dsp^2$ type or two for an octahedral complex of the $d^2sp^3$ type. Table II lists the results of susceptibility measurements of solid $Mn^{III}$Hm and its dimethyl ester at three different temperatures. A plot of $1/\chi_m$ *vs.* $T$ passes through the origin so that $\theta$ in the Curie-Weiss susceptibility equation is zero. The magnetic moments for these samples are compared in Table III with the theoretical value for four unpaired electrons. Also given in Table III are data for aqueous solutions ($t$ = 22.5 ± 1°) of $Mn^{III}$Hm and $Mn^{III}$HmdiMe as well as for three oxidation states (II, III, and IV) of MnHm and two of their pyridine complexes. All $Mn^{III}$ and $Mn^{II}$ complexes show good agreement with the existence of four and five unpaired electrons per molecule, respectively. However, the $Mn^{IV}$Hm derivative gives a value in agreement with only one unpaired electron rather than three. It should be kept in mind that aggregation probably exists in all of these solutions at the very high concentrations (0.05 M) used for measurements.

*Electron Paramagnetic Resonance.*—The existence of unpaired electrons in the manganese complexes might be expected to give rise to distinct electron paramagnetic resonance signals. Although the sensitivity of the x-band spectrometer employed was sufficient to detect $10^{-6}$ M $Mn^{2+}$ ion in aqueous solution with a signal-to-noise ratio of about 5 to 10, attempts to observe signals in solutions of MnHm or MmHmdiMe at concentrations below $10^{-2}$ M were unsuccessful for each of the three oxidation states II, III, and IV. At higher concentrations of $Mn^{III}$HmdiMe, broad signals (total width, approximately 1000 gauss) centered at $g$ = 2 were observed (Fig. 3*A*). The failure to see an

Table II
Magnetic Susceptibility Measurements

| Sample | Temperature (°K) | $\chi_m$[a] $10^{-6}$ cgs |
|---|---|---|
| $Mn^{III}$HmdiMe | 296 | 8,950 |
| | 230 | 11,680 |
| | 77 | 34,400 |
| $Mn^{III}$Hm | 296 | 9,560 |
| | 230 | 12,400 |
| | 77 | 36,600 |

[a] These numbers have been corrected for the diamagnetic contribution of the porphyrin by estimation of this value from Pascal's constants at 20°; the value of 330 was used in the case of $Mn^{III}$HmdiMe and 300 for $Mn^{III}$Hm.

Table III
Magnetic Moments for Manganese Hematoporphyrin IX Samples[a]

| Sample | $\mu$[b] B.M. | Unpaired Electrons[c] |
|---|---|---|
| $Mn^{III}$HmdiMe (solid) | 4.7 | |
| (water solution) | 4.9 | |
| $Mn^{III}$Hm (solid) | 4.8 | 4 |
| (0.1 M KOH in water) | 4.9 | |
| $Mn^{III}$Hm in 20% pyridine in 0.1 M KOH ($H_2O$) | 4.9 | |
| $Mn^{II}$Hm in 0.1 M KOH in water | 5.8 | 5 |
| $Mn^{II}$Hm in 20% pyridine in 0.1 M KOH ($H_2O$) | 5.9 | |
| $Mn^{IV}$Hm in 0.1 M KOH in water | 2.0 | 1 |

[a] The temperature for these experiments was 22.5° ± 1°. [b] All values have been corrected for diamagnetic contributions of the porphyrin as in Table II. [c] The number of Bohr Magnetons corresponding to unpaired electrons on a spin only basis is as follows: 1.7, 2.8, 3.9, 4.9, and 5.9.

[2] Determinations based on an osmometer method were made by the Microchemical Analytical Laboratory, University of California, Berkeley.

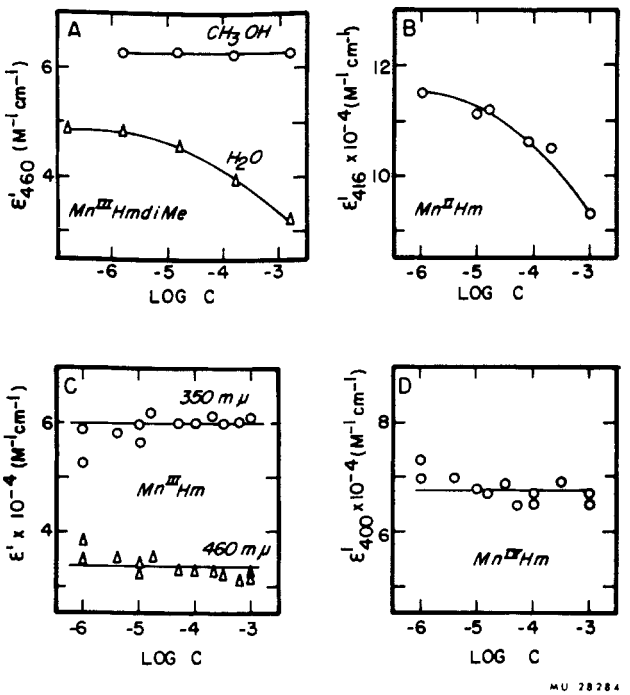



FIG. 2.—Apparent molar absorptivity *vs.* concentration for manganese hematoporphyrin IX complexes. Room temperature; cuvets having 0.05-mm to 10-cm path length were employed. *A*, $Mn^{III}$HmdiMe monohydrate monoacetate in $CH_3OH$ and water; no buffer or salt present; absorbance measured at 460 mμ. *B*, $Mn^{II}$Hm in 0.1 M KOH in water; absorbance measured at 416 mμ. *C*, $Mn^{III}$Hm in 0.1 M KOH in water; absorbance measured at 350 and 460 mμ. *D*, $Mn^{IV}$Hm in 0.1 M KOH in water; absorbance measured at 400 mμ. Experimental points have been corrected for a small amount of irreversible change.

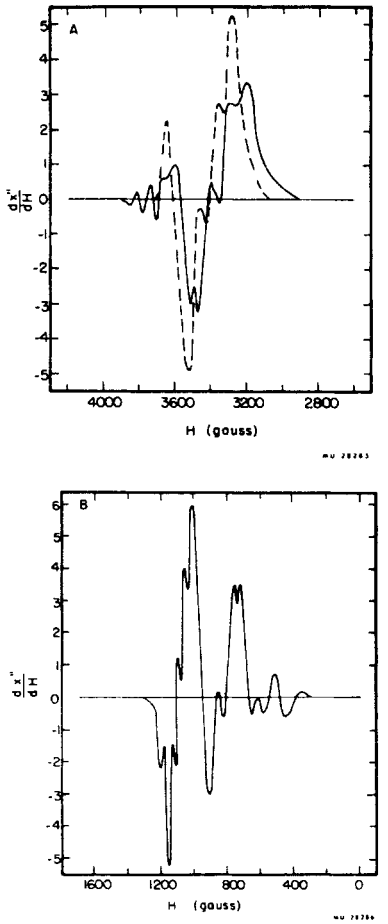



FIG. 3.—Electron paramagnetic resonance derivative spectra of manganese hematoporphyrin IX complexes. $dx''/dH$ is the derivative of the power absorption; a thin quartz cell was used for solutions. *A*, 0.05 M $Mn^{III}$HmdiMe in methanol (solid line) and 1:1 pyridine and methanol (broken line); 25 gauss modulation. *B*, 4.6 mg of $Mn^{III}$Hm solid at −125° with 3 gauss modulation; not shown is a small signal at about $g = 2$ ($H = 3500$ gauss).

electron paramagnetic resonance signal which corresponds quantitatively with the measured paramagnetic susceptibility of these manganese compounds suggests that the electron paramagnetic resonance signal which is observed is not a monomolecular property but a property of aggregates.

A large single crystal of manganese hematoporphyrin IX has not yet been obtained, but it was found that a sample tube filled with crystals of $Mn^{III}$Hm always had a large broad signal (total width, approximately 1000 gauss) at high $g$ values (approximate $g = 6$, Fig. 3*B*). A small signal can also be observed near $g = 2$, and the relative sizes and shapes of the two signals can be varied by twisting the sample tube through 90°, indicating some degree of crystal orientation in the tube. On the other hand, no signal has yet been observed in solid $Mn^{III}$HmdiMe, but only relatively small crystals have been used.

*Redox Titrations.*—Reductive titration of $Mn^{III}$Hm with a solution of sodium dithionite or the semiquinone of methyl viologen resulted in the formation of a $Mn^{II}$-Hm complex. Figure 4 shows a representative set of absorption curves recorded in the course of such a titration. The concentration of the reducing solution was determined beforehand by titrating a standard potassium ferricyanide solution. All values of $E_m$ are based on complete titrations carried out with concurrent measurement of potentials and spectra. Figure 5 shows the potentials corresponding to the absorption spectra of Figure 4. Dilute samples were used in order to favor the monomeric state; the concentration in different titrations ranged from $5 \times 10^{-6}$ to $2 \times 10^{-5}$ M. Equilibrium between the electrodes and solution

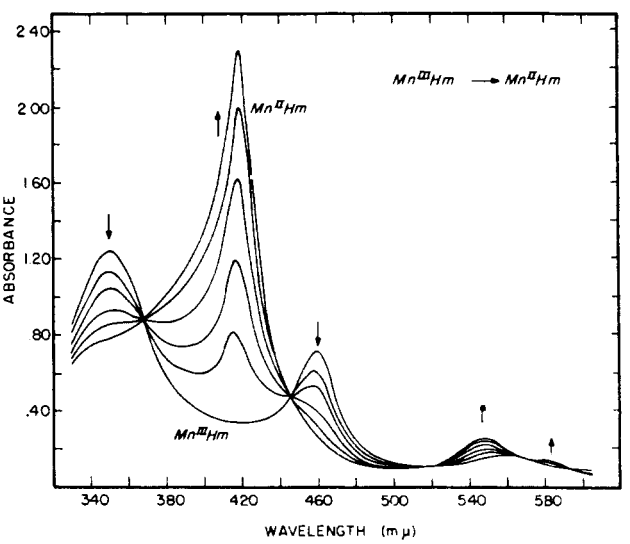



FIG. 4.—Spectra of $Mn^{III}$Hm and $Mn^{II}$Hm recorded in the course of a reductive titration. MnHm, $1.8 \times 10^{-5}$ M; *p*H 12.90; reducing agent, sodium dithionite, $10^{-2}$ M; temperature 22.5 ± 1°; 1-cm cuvet. Curves shown with increasing per cent reduction: 0%; 24.1%; 42.6%; 66.4%; 84.9%; 100%.

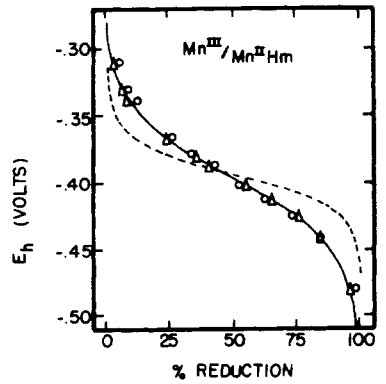



FIG. 5.—Reductive titration of $Mn^{III}Hm$. MnHm, $1.8 \times 10^{-5}$ M; *p*H 12.90; temperature 22.5 ± 1°; reducing agent, sodium dithionite. Solid line, theoretical curve for $n = 1$, $E_m = -0.393$ volt; broken line, theoretical curve for $n = 2$, $E_m = -0.393$ volt; O, experimental points with percentage of reduction calculated from volume of reducing agent added; Δ, experimental points with percentage of reduction calculated from spectra shown in Figure 4.

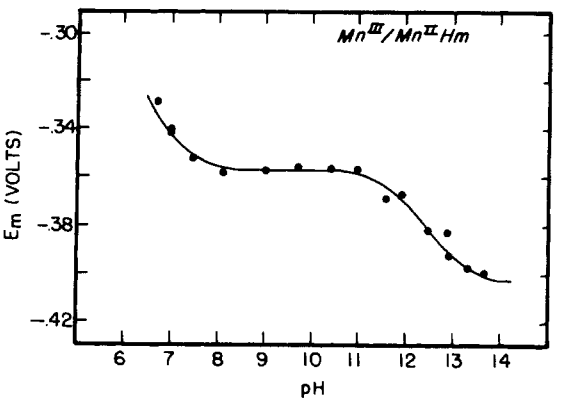



FIG. 6.—*p*H dependence of the oxidation-reduction potentials of half-reduced $Mn^{III}Hm$. Temperature 22.5 ± 1°; ●, experimental values; the solid line represents equation (3) with $K'_{o1} = 1.00 \times 10^{-12}$, $K'_{r1} = 1.26 \times 10^{-7}$, $K'_{r2} = 1.59 \times 10^{-13}$, and $E_x = +0.051$ volt.

was established rapidly, usually in a matter of seconds along the central portion of a titration curve, and the results obtained agree well with those predicted for a reversible one-electron oxidation-reduction system.

$E_m$ values (Clark, 1960) for the system $Mn^{III}/Mn^{II}Hm$ have been determined over a range of *p*H from 6.7 to 13.63, and the results are summarized in Table IV. Using the method of Clark and Cohen

$$E_m = E_x + \frac{RT}{F} \ln \frac{(H^+)^2 + K'_{r1}(H^+) + K'_{r1}K'_{r2}}{(H^+) + K'_{o1}} \quad (3)$$

(1923), equation (3) was found to be the simplest relationship between $E_m$ and *p*H consistent with the data at hand. $K'_{o1}$ is an apparent equilibrium constant for a redox-linked proton function[3] in $Mn^{III}Hm$ and $K'_{r1}$ and $K'_{r2}$ are apparent equilibrium constants for redox-linked proton functions in $Mn^{II}Hm$. $E_x$ is the potential which the half-reduced system would have at *p*H 0 if there were no other net redox-linked proton functions at values of *p*H below those covered in this study (*i.e.*, *p*H < 6.7). In Figure 6 the experimentally determined values of $E_m$ are plotted against *p*H and compared with the curve described in equation (3) upon assignment of the constants $E_x = +0.051$ volt, $K'_{o1} = 1.00 \times 10^{-12}$, $K'_{r1} = 1.26 \times 10^{-7}$, $K'_{r2} = 1.59 \times 10^{-13}$. Differences in observed and calculated values of $E_m$ are given in Table IV. Assignment of acid-base equilibrium underlying the above constants will now be considered.

[3] Whenever a molecule possesses two or more different functions (*e.g.*, dissociation of protons and donation or acceptance of electrons) belonging to different groups in the molecule, there is the possibility of an interdependence of the functions due to interaction between the groups. If such interaction occurs, one may conveniently speak of the functions as linked and refer to the groups as linked groups (after Wyman, 1948).

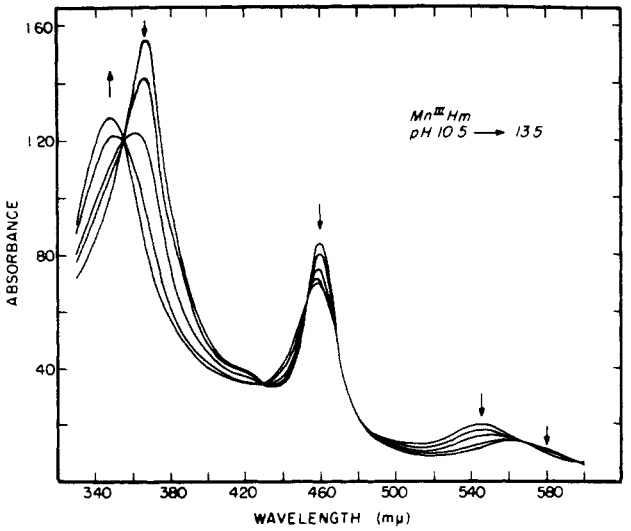



FIG. 7.—Change with *p*H in the absorption spectrum of $Mn^{III}Hm$ ($2 \times 10^{-5}$ M). 0.05 M borate and phosphate buffers employed; ionic strength adjusted to 0.30 with KCl in all cases; 1-cm cuvet. Values for curves from lower to higher *p*H: 10.50; 11.49; 11.95; 12.48; 13.42.

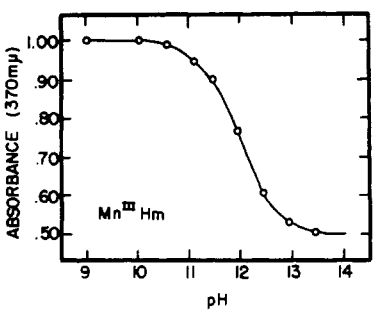



FIG. 8.—Spectrophotometric determination of $pK'$ for $Mn^{III}Hm$. Same conditions as in Figure 7; absorbance measured at 370 mμ. Solid line, theoretical curve for $pK' = 11.95$ in equation (4); O, experimental values.

Examination of the change with *p*H in absorption spectrum of $Mn^{III}Hm$ also reveals the function with $pK'_{o1} = 12$. Figure 7 shows these changes, and a plot of *p*H *vs.* absorbance at 370 mμ is shown in Figure 8. The results obtained agree well with those predicted for a reversible one-proton dissociation

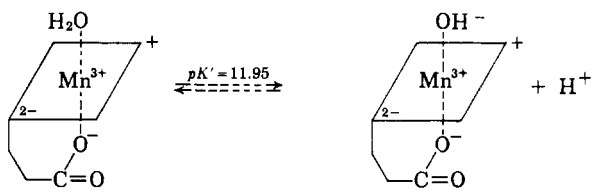
$$pH = pK' + \log \frac{[A^-]}{[HA]} \quad (4)$$

(equation 4), where $[A^-]$ is the concentration of the base, $[HA]$ is the concentration of the conjugate acid, and $K'$ is the apparent dissociation constant. By analogy with $Fe^{III}$ porphyrin systems, the proton equilibrium underlying this $pK'$ is assigned to reaction 1.

TABLE IV
$E_m$ VALUES FOR $Mn^{III}/Mn^{II}Hm$ SYSTEM[a]

| pH | Buffer[b] | $E_m$ Observed (volt) | $E_m$ Calcd.[c] (volt) | $E_m$ Observed Minus $E_m$ Calcd. (volt) |
|---|---|---|---|---|
| 6.70 | I | −0.328 | −0.333 | +0.005 |
| 7.00 | I | −0.340 | −0.342 | −0.002 |
| 7.00 | I | −0.341 | −0.342 | −0.001 |
| 7.51 | I | −0.353 | −0.349 | +0.004 |
| 8.10 | II | −0.358 | −0.356 | +0.002 |
| 9.00 | II | −0.357 | −0.357 | 0 |
| 9.70 | II | −0.357 | −0.357 | 0 |
| 10.39 | II, III | −0.356 | −0.357 | +0.001 |
| 10.95 | III | −0.357 | −0.358 | −0.001 |
| 11.55 | III | −0.369 | −0.364 | +0.005 |
| 11.90 | III | −0.367 | −0.370 | −0.003 |
| 12.46 | IV | −0.384 | −0.380 | +0.004 |
| 12.85 | IV | −0.383 | −0.391 | −0.008 |
| 12.90 | IV | −0.393 | −0.392 | +0.001 |
| 13.18 | IV | −0.398 | −0.395 | +0.003 |
| 13.63 | IV | −0.400 | −0.401 | −0.001 |

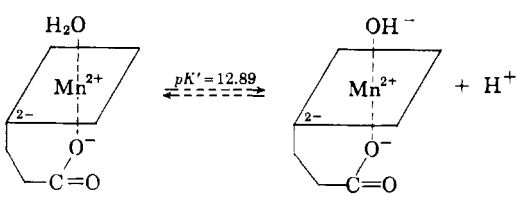
[a] The temperature for these experiments was 22.5 ± 1°. [b] I, $KH_2PO_4$-$K_2HPO_4$; II, $H_3BO_3$-$KH_2BO_3$; III, $K_2HPO_4$-$K_3PO_4$; IV, KOH. [c] According to equation (3) with the following constants: $E_x = +0.051$ volt, $K'_{01} = 1.00 \times 10^{-12}$, $K'_{r1} = 1.26 \times 10^{-7}$, $K'_{r2} = 1.59 \times 10^{-13}$.




*Reaction 1*

Examination of the change with *p*H in the absorption spectrum of $Mn^{III}$HmdiMe reveals changes completely analogous with those found for the free acid. However, in this case the $pK'$ was found to be 11.3. Thus, the role of the negative carboxylate groups is apparent in conferring upon the metal less attractiveness to hydroxide.

A similar study of the change with *p*H in the absorption spectrum of $Mn^{II}$Hm is shown in Figures 9 and 10. The data are consistent with those predicted for a reversible one-proton dissociation with $pK'$ value of 12.89. As with the manganese III derivative, the proton equilibrium involved is attributed to reaction 2.




*Reaction 2*

Another possible interpretation of the data is that the absorbance change is the result of aggregation at higher *p*H due to the concomitant increase in ionic strength. Included in Figure 10, however, are two experiments carried out at higher ionic strength. Although there is a significant effect, the change is small in comparison to the observed changes with *p*H.

The proton equilibrium responsible for $pK'_{r1}$ in Figure 6 has not been examined spectrophotometrically because of irreversible changes in $Mn^{II}$Hm that occur

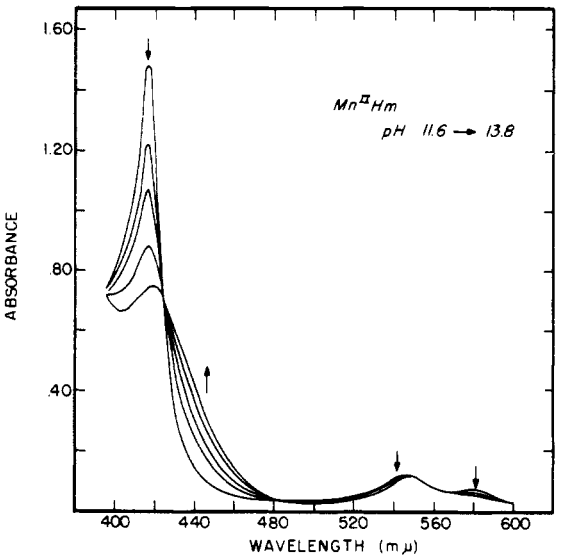



FIG. 9.—Change with *p*H in the absorption spectrum of $Mn^{II}$Hm ($1.3 \times 10^{-5}$ M). 0.05 M borate and phosphate buffers employed below *p*H 12.5; KOH was used above pH 12.5; no attempt was made to control ionic strength. Values for curves from lower to higher *p*H: 11.56; 12.70; 12.92; 13.20; 13.76.

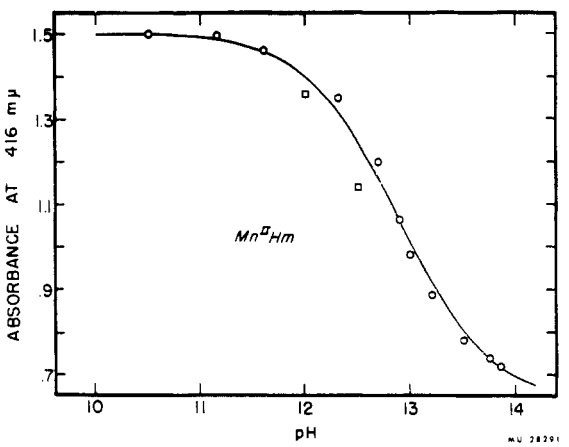



FIG. 10.—Spectrophotometric determination of $pK'$ for $Mn^{II}$Hm. ○, experimental values with conditions the same as given in Figure 9. □, two experiments in 0.5 M phosphate buffer. Absorbance was measured at 416 mμ. Solid line, theoretical curve for $pK' = 12.89$ in equation (4).

with increasing rapidity below *p*H 7. A comparison (Table V) of the absolute value of $E_m$ for the ester with that of the acid reveals a difference of about 70 mv in the range in which both are *p*H independent (*i.e.*, *p*H 7.5 to 10). This agrees closely with a similar difference observed previously with esterified and nonesterified iron porphyrin peptides (Loach and Harbury, to be published; Harbury *et al.*, 1962), and indicates a difference in the role of the carboxylate ester and the acid in determining the redox potential of the $Mn^{III}/Mn^{II}$ or $Fe^{III}/Fe^{II}$ couple.

Oxidative titration of $Mn^{III}$Hm at *p*H 13 with one equivalent of sodium hypochlorite or potassium ferricyanide resulted in the formation of a higher oxidation state (Fig. 11). Addition of somewhat more than one equivalent of sodium hypochlorite had no further immediate effect, but a large excess brought about spectrophotometric changes which were found to be irreversible and eventually resulted in total loss of

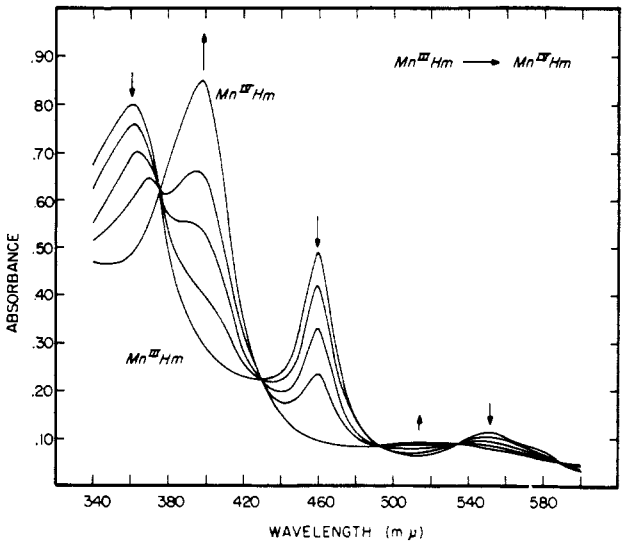



FIG. 11.—Spectra of $Mn^{III}Hm$ and $Mn^{IV}Hm$ recorded in the course of an oxidative titration. MnHm, $1 \times 10^{-5}$ M; *p*H 11.90; temperature 22.5 ± 1°; oxidizing agent, sodium hypochlorite.

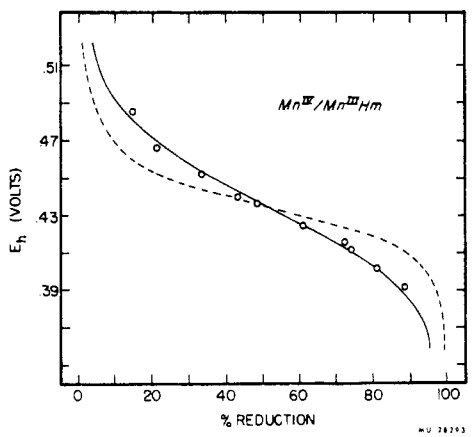



FIG. 12.—$E_m$ determination of $Mn^{IV}/Mn^{III}Hm$. MnHm, $1 \times 10^{-4}$ M; *p*H 11.55; temperature 22.5 ± 1°; oxidizing agent, $K_3Fe(CN)_6$; reducing agent, $K_4Fe(CN)_6$. Solid line, theoretical curve (equation 1) for $n = 1$, $E_m = 0.436$ volts; broken line, theoretical curve for $n = 2$, $E_m = 0.436$ volts; O, experimental points with percentage of oxidation calculated from spectra. Total concentration of ferri/ferrocyanide was 0.01 M in all cases.

absorption in the visible regions of the spectrum. The relative stability of the porphyrin to such strong oxidants as hydrogen peroxide and sodium hypochlorite is quite striking, since most simple iron porphyrin systems are rapidly "bleached" in the presence of these oxidants.

Attempts to measure $E_m$ values for this transition to a higher oxidation state are complicated by a number of factors. First, the material represented by $Mn^{IV}Hm$ in Figure 11 returns spontaneously to $Mn^{III}Hm$ by just standing in a cuvet for 1 or 2 hours in the dark. White light was observed to catalyze the conversion. This spontaneous reduction of the pigment indicates that something in solution must be oxidized. Any destructive oxidation of manganese porphyrin complex at concentrations as low as $10^{-5}$ M can be ruled out, since the absorbance of the material from 350 to 900 mμ as $Mn^{III}Hm$ is unchanged before and after its oxidation. Although there is some possibility that trace reductants may contaminate the buffers used,

TABLE V

$E_m$ VALUES FOR $Mn^{III}/Mn^{II}$HmdiMe AND $Mn^{III}/Mn^{II}$Hm IN 20% ETHANOL[a]

| Sample | *p*H | Buffer[b] | $E_m$ Observed (volt) | $E_m$ Calcd.[c] (volt) | $E_m$ Observed Minus $E_m$ Calcd. (volt) |
|---|---|---|---|---|---|
| MnHmdiMe | 6.91 | I | −0.274 | −0.268 | +0.006 |
| MnHmdiMe | 7.13 | I | −0.265 | −0.268 | −0.003 |
| MnHmdiMe | 7.75 | I,II | −0.268 | −0.268 | 0 |
| MnHmdiMe | 9.20 | II | −0.268 | −0.268 | 0 |
| MnHm | 9.61 | II | −0.336 | −0.336 | 0 |

[a] The temperature for these experiments was 22.5 ± 1°. [b] Buffers as in Table IV. [c] Assuming $E_m$ shows no *p*H dependence through the region studied.

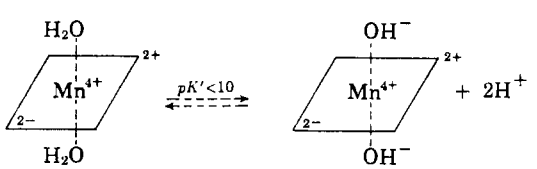
the oxygen atoms of water or of the carboxylate groups are probably oxidized to the peroxide or oxygen level (Calvin *et al.*, 1962).

A severe limitation to direct measurement of potentials of $Mn^{IV}/Mn^{III}Hm$ equilibria is the lack of reproducible response by the gold-plated platinum electrodes being used. No mediators or other electrodes were tried. Instead, $E_m$ values were estimated over the *p*H range 9.9 to 13.6 by use of an excess of the ferri/ferrocyanide couple; the results are shown in Table VI. Figure 12 shows the potentials corresponding to the percentage of oxidation of $Mn^{III}Hm$ calculated from the absorption spectrum change; the results agree well with a one-electron oxidation-reduction system. The values given in Table VI at higher and lower *p*H were determined on the basis of one or two experiments at each *p*H value and not from complete titration curves. $E_m$ values obtained over the *p*H range studied are consistent with equation (5) as representing the simplest relationship (Clark and Cohen, 1923) between $E_m$ and *p*H, where $K'_{r1}$ is an

$$E_m = E_z + \frac{RT}{F} ln\,[(H^+) + K'_{r1}] - 2.303\,\frac{RT}{F}\,pH \quad (5)$$

apparent equilibrium constant for a redox-linked proton function in $Mn^{III}Hm$. $E_z$ is the potential which the half-reduced system would have at *p*H 0, assuming no net redox-linked proton functions at values of *p*H below those covered in this study (*i.e.*, *p*H < 9.9). Differences in observed and calculated values of $E_m$ are given in Table VI. For calculated values, the constants in equation (5) were assigned the values $E_z = 1.788$ volts, $K'_{r1} = 1.00 \times 10^{-12}$.

Once again the redox-linked proton function in $Mn^{III}Hm$ is apparent, and the presence of a slope of −0.117 volt per *p*H unit for the $E_m$ *vs.* *p*H curve below *p*H 12 dictates that two protons are added to $Mn^{IV}Hm$ along with an electron to form $Mn^{III}Hm$. The simplest interpretation of these proton functions in $Mn^{IV}Hm$ is indicated by the equilibrium shown in reaction 3.[4]




*Reaction 3*

[4] The reaction is written as a concurrent loss of protons by two coordinated water molecules purely for the sake of simplicity of presentation.

TABLE VI
$E_m$ VALUES FOR $Mn^{IV}/Mn^{III}Hm$ SYSTEM[a]

| pH | Buffer[b] | $E_m$ Observed (volt) | $E_m$ Calcd.[c] (volt) | $E_m$ Observed Minus $E_m$ Calcd. (volt) |
|---|---|---|---|---|
| 9.90 | II | 0.635 | 0.626 | +0.009 |
| 10.10 | II | 0.603 | 0.602 | +0.001 |
| 10.60 | II, III | 0.535 | 0.546 | −0.011 |
| 10.70 | II, III | 0.543 | 0.532 | +0.011 |
| 10.70 | II, III | 0.527 | 0.532 | −0.005 |
| 10.98 | III | 0.509 | 0.502 | +0.007 |
| 11.25 | III | 0.461 | 0.472 | −0.011 |
| 11.43 | III | 0.450 | 0.452 | −0.002 |
| 11.55 | III | 0.436 | 0.439 | −0.003 |
| 11.78 | III | 0.415 | 0.418 | −0.003 |
| 11.88 | III | 0.404 | 0.408 | −0.004 |
| 12.33 | IV | 0.377 | 0.369 | +0.008 |
| 12.60 | IV | 0.356 | 0.350 | +0.006 |
| 13.04 | IV | 0.325 | 0.320 | +0.005 |
| 13.32 | IV | 0.306 | 0.304 | +0.002 |
| 13.59 | IV | 0.281 | 0.288 | −0.007 |

[a] The temperature for these experiments was 22.5 ±1°. [b] Buffers are the same as given in Table IV. [c] According to equation (5) with the following constants: $E_z = +1.788$ volts, $K'_{r1} = 1.00 \times 10^{-12}$.

Addition of excess potassium ferricyanide to very alkaline solutions (approximately 10% KOH) of $Mn^{IV}$-Hm resulted in the appearance of a new species whose absorption spectrum is shown in Figure 13. The spectrum of $Mn^{IV}Hm$ could be obtained from this material once again by addition of enough potassium ferrocyanide to lower the redox potential of the medium to about 0.4 volt. Compounds having similar spectra have been observed as transitory intermediates when a strong oxidant is first added to $Mn^{III}Hm$ at lower pH values (pH 10 to 13). These observations suggest that a higher oxidation state than $Mn^{IV}$ (perhaps $Mn^{V}$) may exist in very alkaline solution and may play a role as an intermediate in reactions at lower pH values. Since this complex can be formed by addition

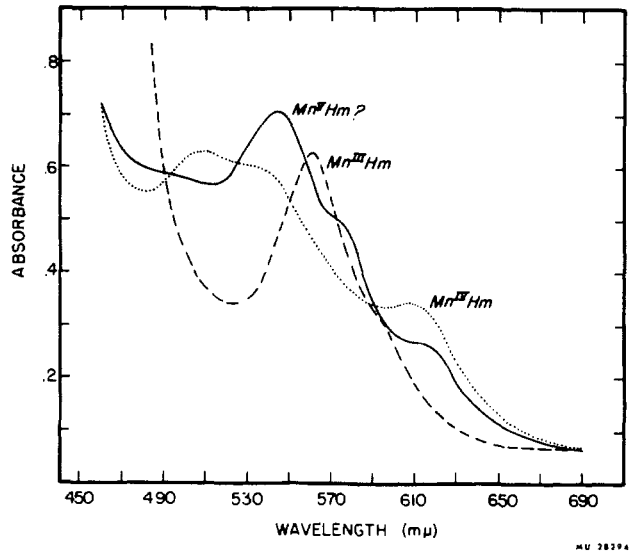
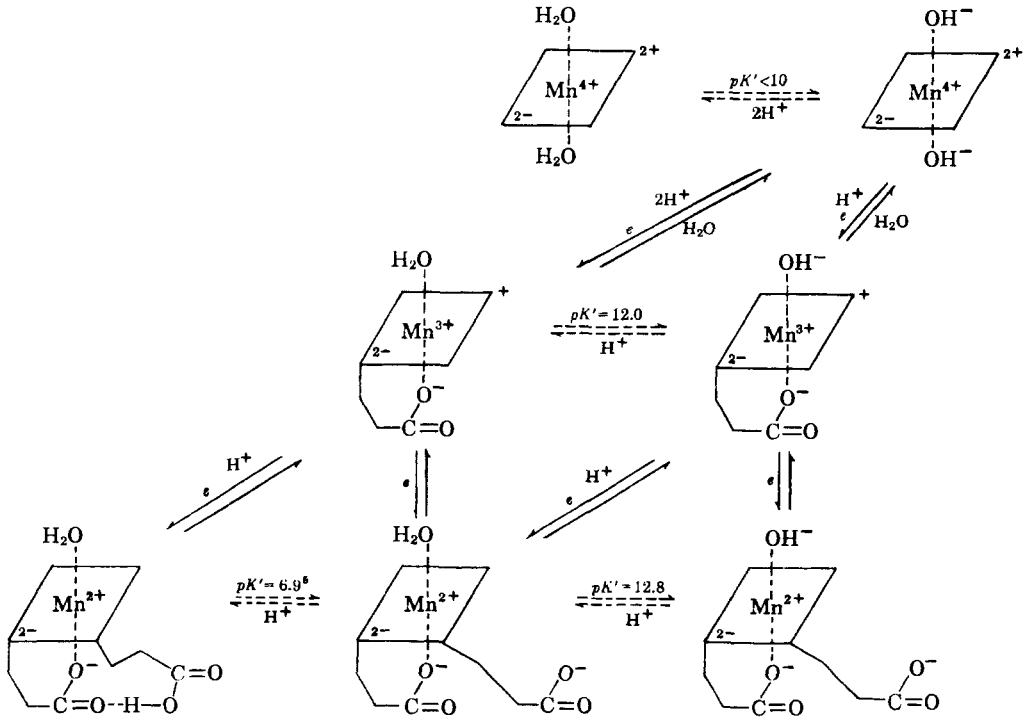



FIG. 13.—Spectra of $Mn^{III}Hm$ (da hed line), $Mn^{IV}Hm$ (dotted line), and a higher oxidation state (solid line) MnHm, 1 × $10^{-4}$ M; approximately 10% KOH in water; room temperature; oxidizing agent, $K_3Fe(CN)_6$.

of $K_3Fe(CN)_6$ in very alkaline solution, or possibly by NaClO at pH 12 (followed, however, by irreversible decomposition of the pigment at this pH), the couple (perhaps $Mn^{V}/Mn^{IV}Hm$) may have a redox level somewhat higher than $Mn^{IV}/Mn^{III}Hm$ between pH 12 and 14.

## DISCUSSION

Figure 14 summarizes the $E_m$ vs. pH relationships for three oxidation states of manganese hematoporphyrin IX in aqueous solution. The equilibria involved may be written schematically as shown in reaction 4, where horizontal equilibria involve only protons and vertical equilibria involve only electrons, while diagonal equilibria involve both.




*Reaction 4*

[5] Only one of the many possible structures has been written to account for this pK'.

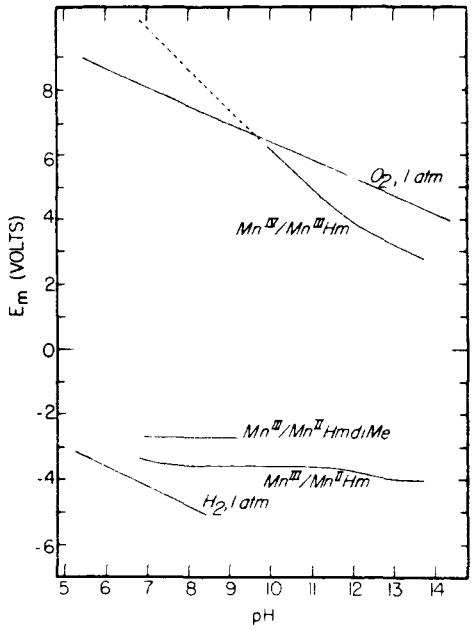
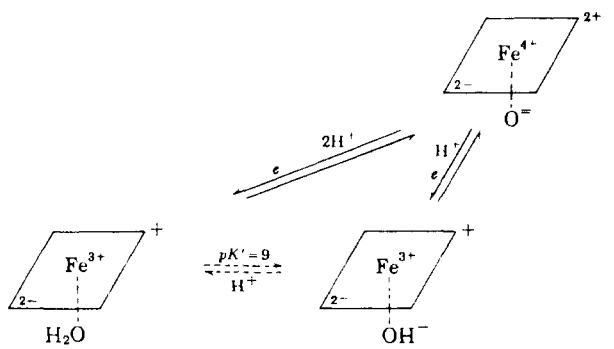



FIG. 14.—Summary of $E_m$-$p$H relationships.

At $p$H 7, $Mn^{III}Hm$ is a good reducing agent and could reduce DPN or TPN ($E_m$ = −0.32 or −0.34 volt, respectively; Rodkey, 1959; Rodkey and Donovan, 1959), providing a mechanism existed for the reaction. Since the association of a protein with such a manganese porphyrin could easily change the $E_m$ value at $p$H 7 by several hundred millivolts, as proteins associated with iron protoporphyrin IX do, an important function of manganese in some photosynthetic material could be due to its role as a manganese porphyrin-protein complex whose 2+ oxidation state is an excellent reducing agent.

The characterization of a stable $Mn^{IV}$ complex is the first example of a simple metalloporphyrin system in which such a high oxidation state has been examined under reversible conditions. George (1953a,b,c) and George and Irvine (1954a,b; 1955) examined higher oxidation states of iron porphyrins complexed with certain proteins. They found that one equivalent of a strong oxidant, such as $K_2IrCl_6$, could be added to a solution of an $Fe^{III}$ porphyrin complex (for example, metmyoglobin) and the formation of a new species could be observed. Subsequent addition of a reducing agent such as ascorbic acid resulted in the restoration of the $Fe^{III}PP$ ($Fe^{III}$ protoporphyrin IX) species. This conversion of $Fe^{III}PP$ to a higher oxidation state was shown to be accompanied by the liberation of two protons at $p$H 7 and one proton at $p$H 12. They suggested that the equilibria shown in reaction 5 obtain,




*Reaction 5*

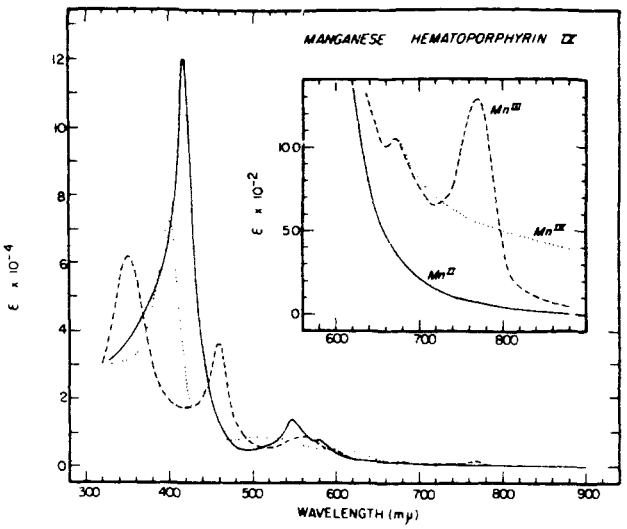



FIG. 15.—Spectra of three oxidation states of MnHm in alkaline solution at $p$H 13.

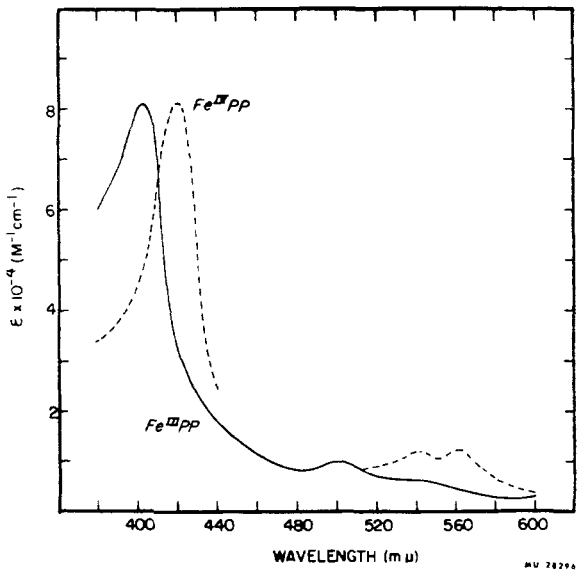



FIG. 16.—Spectra of $Fe^{IV}PP$ and $Fe^{III}PP$ complexes of horseradish peroxidase in aqueous solution (George, 1953a, b).

where groups of protein may occupy the fifth coordination position. The $E_m$/$p$H relationship was shown to be consistent with the above equilibria, the slope of the curve being −0.120 volt/$p$H unit at $p$H 4 and −0.060 volt/$p$H unit at $p$H 11. The similarity of these results with the present data for manganese porphyrin systems is striking.

Extrapolation to $p$H 7 of the $E_m$ *vs.* $p$H curve for $Mn^{IV}/Mn^{III}Hm$ (Fig. 14) is subject to some uncertainty, since equilibria involving other redox-linked proton functions in the range of $p$H below 10 have not been studied. However, it is apparent that $Mn^{IV}Hm$ would be a very strong oxidant at $p$H 7, strong enough to liberate molecular oxygen from water if there were a mechanism for the reaction. Thus, the importance of manganese in sustaining the capacity of biological material to carry out photosynthesis (Kessler, 1955; Possingham and Spencer, 1962) could be due to its role as a manganese porphyrin-protein complex whose 4+ oxidation state can oxidize oxygen of water to the molecular oxygen level.

Of the three transition elements (Mn, Fe, Cu) present in green plant material, iron (George, 1953a,b,c; George and Irvine, 1954a,b; 1955), and manganese porphyrin systems have been shown to exhibit high oxidation states which are quite strong oxidants. Although the light-minus-dark absorbance curves from photosynthesizing material have often shown a maximum at 400 mμ and minima at 425 mμ and 550 mμ attributed to oxidation of "cytochrome" type pigments from $Fe^{II}$ to $Fe^{III}$ states, at least two other possible interpretations fit the data. Figure 15 compares the absorbance of $Mn^{II}Hm$, $Mn^{III}Hm$, and $Mn^{IV}Hm$, and Figure 16 compares the spectra of $Fe^{III}PP$ and $Fe^{IV}$-PP complexes (George, 1953b). A transition of $Mn^{II}Hm$ to $Mn^{IV}Hm$ would be difficult to distinguish from a transition of $Fe^{II}PP$ to $Fe^{III}PP$ merely on the basis of absorbance changes at 400, 425, and 550 mμ. Also, a transition from $Fe^{IV}PP$ to $Fe^{III}PP$ would involve a decrease in absorbance at 425 and 550 mμ as well as an increase at 400 mμ.

The results of these experiments with model manganese porphyrin systems should stimulate a more thorough search for compounds that may undergo similar reactions in biological material capable of producing molecular oxygen from water.

## Acknowledgments

We wish to thank Dr. B. D. Cunningham for the use of his equipment for making magnetic susceptibility measurements, and Dr. G. M. Androes for many helpful discussions concerning the electron paramagnetic resonance studies.

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