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Uncoupling of Oxidative Phosphorylation by Carbonyl Cyanide Phenylhydrazones. I. Some Characteristics of *m*-Cl-CCP Action on Mitochondria and Chloroplasts

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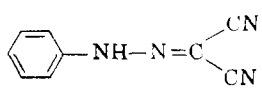
Carbonyl cyanide *m*-chlorophenylhydrazone (m-Cl-CCP), at concentrations between 10^{-7} and 10^{-6} M, is an effective uncoupler of oxidative phosphorylation in plant, animal, and insect mitochondria. While in some respects its action on mitochondria resembles that of 2,4-dinitrophenol—*e.g.*, apparent ATPase of intact mitochondria is stimulated and swelling of resting mitochondria is inhibited—*m*-Cl-CCP also exhibits several unusual characteristics. It does not stimulate or inhibit purified soluble ATPase from mitochondria; its action is specifically blocked by 1,2- and 1,3-aminothiols; and it strongly inhibits photophosphorylation in chloroplasts. The *m*-Cl compound is representative of the class of CCP uncouplers, which appear to be useful tools in phosphorylation studies.

Preliminary reports from this laboratory (Heytler *et al.*, 1962; Heytler and Prichard, 1962) described a new class of uncoupling agents, ring-substituted phenylhydrazones of carbonyl cyanide.



Carbonyl Cyanide Phenylhydrazone (CCP)

Some of these derivatives are at least as effective as the best antibiotic uncouplers (*e.g.*, gramicidin [Hotchkiss, 1944] or valinomycin [McMurray and Begg, 1959]) and represent the most potent synthetic inhibitors of this type reported. Also, as we reported (*loc. cit.*), these materials block photosynthetic phosphorylation, which is rather resistant to most uncoupling agents. The CCP derivatives, therefore, are promising tools in the study of both these energy-conversion mechanisms, and their mode of action is being studied in greater detail. The present report deals with some characteristics of the *m*-chloro derivative (*m*-Cl-CCP), which has relatively high uncoupling activity and appears to be qualitatively characteristic of the class.

* Contribution No. 803.

MATERIALS AND METHODS

The carbonyl cyanide phenylhydrazones were prepared by Dr. W. W. Prichard of this laboratory; details will be published shortly. Briefly, the corresponding aniline is diazotized by conventional means, then coupled with malononitrile in mildly basic solution. The yellow product precipitates upon subsequent acidification and is recrystallized from chloroform or benzene. Buffered solutions of the Tris or sodium salt were used routinely.

Oligomycin was the generous gift of Prof. H. A. Lardy.

Other reagents were used as obtained commercially. Enzymes and most biochemicals were purchased from Sigma Chemical Company in highest routinely available grades.

Mitochondria from several sources were prepared by minor modifications of standard differential centrifugation techniques (Schneider and Hogeboom, 1950). Homogenization in 0.25 M sucrose medium was followed by centrifugation at 1,000 × *g* for 12 minutes to remove debris and at 10,000 × *g* for 20 minutes to sediment

the mitochondria. These were washed once in 0.25 M sucrose. All operations were done at 0–3°.

With plant material, homogenization was effected by grinding with sand in a cold mortar, and reduced glutathione (0.01 M), potassium phosphate (0.1 M, *p*H 7.4), and bovine serum albumin (1–3 mg/ml) were added to the sucrose medium.

Oxygen consumption was measured by standard Warburg manometric technique at 25° or 26° for 15–30 minutes and stopped by rapid chilling followed immediately by centrifugation at 25,000 × *g*.

Phosphorylation was measured by spectrophotometric assay of glucose-6-phosphate produced, based on reduction of TPN in the presence of glucose-6-phosphate dehydrogenase. Aliquots (0.05–0.2 ml) of clear supernatant were diluted to 1.0 ml with reagent containing 70 μmoles Tris (*p*H 7.4), 1 μmole TPN, 7 μmoles $MgCl_2$, and 0.15 units glucose-6-phosphate dehydrogenase. Change in optical density at 340 mμ *vs.* a reagent blank was followed in a Beckman DU Spectrophotometer. Appropriate controls were run to correct for inherent absorbancy of samples. Net $OD_{340} \times 0.173$ = μmoles glucose-6-phosphate in aliquot assayed.

The *m*-Cl-CCP was tested separately for inhibition of the hexokinase and glucose-6-phosphate dehydrogenase systems, in order to check this potential source of error. No inhibitory effect was observed in the concentration range employed in the present studies.

Results

Physical Properties of m-Cl-CCP.—Crystalline carbonyl cyanide *m*-chlorophenylhydrazone is a relatively stable, yellow compound (m.p. 170°) readily soluble in organic solvents but virtually insoluble in water. It is acidic, having a pK_a of 5.95, and forms water-soluble salts. The anion has an absorption maximum at 378 mμ with a molar extinction coefficient of 2.37×10^4. Aqueous solutions at *p*H 7.4 may be kept for a few days, but deterioration has been noted on long storage.

Uncoupling Action on Isolated Mitochondria.—Tables I and II illustrate effectiveness of *m*-Cl-CCP (carbonyl cyanide *m*-chlorophenylhydrazone) as an uncoupler in a variety of mitochondrial systems and with either succinate or DPN-linked substrates. The susceptibilities of all these systems are quite similar. These data were obtained with similar, relatively high concentrations of mitochondrial protein in all assays. The effects of *m*-Cl-CCP on dilute mitochondrial suspensions can be observed at several-fold lower uncoupler concentrations. The increased sensitivity of frozen-thawed heart mitochondria (Table I) is probably due to partial breakdown of the permeability barrier as well as to the lower protein concentration employed.

Succinoxidase activity was depressed in manometric runs by the higher levels of *m*-Cl-CCP. Apparently this is due to increased oxalacetate accumulation, since respiratory inhibition can be prevented by adding cysteine-sulfinic acid to trap oxalacetate. Also, in short-time polarographic determinations of oxygen uptake, stimulation of succinoxidase is observed on treatment of tightly coupled mitochondria with *m*-Cl-CCP, and gradual inhibition sets in only after several minutes.

Respiration of *m*-Cl-CCP–treated mitochondria remains sensitive to cyanide, to antimycin A, and, in DPN-linked oxidations, to amytal. There is no indication that CCP derivatives undergo reversible oxidation-reduction or divert the normal pathway of the electron transport system.

Binding of m-Cl-CCP to Mitochondria.—The uncoupling action of carbonyl cyanide *m*-chlorophenylhydrazone is not readily reversible. Washed mitochondria isolated from tissues of poisoned animals (rat liver) or plants (tomato root) remained completely uncoupled. Also, repeated washing (*e.g.*, three times in 0.25 M sucrose at 3°) of mitochondria treated *in vitro* failed to restore activity. This may be due to secondary degeneration of structure or simply to the difficulty of removing tightly bound uncoupler.

Table I

Uncoupling of Succinoxidase-Linked Phosphorylation of Various Mitochondrial Preparations by *m*-Cl-CCP

The 3-ml system in each Warburg vessel contained, in μmoles/ml: 50 succinate; 17 P_i (*p*H 7.4); 0.3 ADP; 67 glucose; 67 KCl; 3 $MgCl_2$; 0.1 cytochrome *c*; 7 NaF (omitted in plant systems); 17 Kunitz-MacDonald units/ml hexokinase; 0.3–3.0 mg mitochondrial protein. O_2 consumed and glucose-6-phosphate formed were measured.

Source of Mitochondria	P/O (with Succinate)		
	Control	1.6×10^{-7} M *m*-Cl-CCP	1.6×10^{-6} M *m*-Cl-CCP
Rat liver	1.8	1.0	0.02
Mouse liver	1.85	1.4	0.00
Beef heart	1.8	1.1	0.06
Beef heart (frozen-thawed)	1.5	0.1	0.00
Tomato stem	1.3	1.0	0.00
Pea seedlings	1.3	—	0.02
Cauliflower	1.45	0.80	0.09
House fly larvae	1.3	—	0.10

Tracer studies, summarized in Table III, showed that *m*-Cl-CCP is significantly accumulated by mitochondria. Its uptake is depressed by cyanide or cold. The bulk of tagged uncoupler can be recovered, apparently unchanged, by carrier dilution methods, after disruption of the mitochondria with "Triton X-100" and Na_3PO_4. In this experiment, 5.0 mg of carrier *m*-Cl-CCP was added to 15 ml of the alkaline solution containing the lysed, labeled mitochondria. The carrier was dissolved completely with slight warming (50–60°) and allowed to stand for 20 minutes. Upon acidification with HCl, the precipitate was collected, dried, and extracted with boiling benzene. The volume of benzene was then reduced to less than 0.5 ml, and *m*-Cl-CCP crystallized out on cooling. The radioactivity of the crystalline material (2.7 mg recovered) was counted and the specific activity (C^{14}) used to calculate total labeled *m*-Cl-CCP initially present. A small amount (*ca.* 1%) was found to remain bound to a nondialyzable fraction, however.

Effect of m-Cl-CCP on ATPase.—Hydrolysis of ATP both by purified soluble mitochondrial ATPase (Pullman *et al.*, 1960) and by fresh intact rat liver mitochondria (Kielley, 1955) has been measured. The influence of *m*-Cl-CCP, 2,4-dinitrophenol (DNP), and Mg^{++} on these systems is compared in Table IV. In the purified system, which is consistently stimulated by DNP, *m*-Cl-CCP is completely inert; neither stimulation nor inhibition has been obtained over a wide concentration range of this agent. In this respect, the CCP derivatives differ from other uncouplers which have been tested.

The apparent ATPase of intact mitochondria is, by contrast, stimulated by *m*-Cl-CCP to the same extent (but at lower concentrations) as by DNP. Added Mg^{++} was not required in this system. Oligomycin, which blocks DNP-induced ATPase in intact mitochondria (Lardy *et al.*, 1958), similarly blocked *m*-Cl-CCP induced ATPase activity.

TABLE II

m-Cl-CCP UNCOUPLING OF MOUSE LIVER MITOCHONDRIA

Each 3-ml system contained, in μmoles/ml: 50 succinate or 17 β-OH-butyrate; 17 P_i (*p*H 7.4); 0.3 ADP; 67 KCl; 3 $MgCl_2$; 0.1 cytochrome *c*; 7 N_2F; 17 Kunitz-MacDonald units/ml hexokinase; 0.85 mg/ml mitochondrial protein. O_2 consumed and glucose-6-phosphate (G-6-P) formed are expressed in μmoles.

	Succinate			β-OH-Butyrate		
	ΔO_2	ΔG-6-P	P/O	ΔO_2	ΔG-6-P	P/O
Controls	9.7	17.9	1.8	1.55	3.8	2.5
+ 0.08 × 10^{-6} M *m*-Cl-CCP	8.4	14.5	1.7	1.7	3.9	2.3
+ 0.16 × 10^{-6} M *m*-Cl-CCP	9.8	13.4	1.4	1.7	2.8	1.7
+ 0.4 × 10^{-6} M *m*-Cl-CCP	9.2	8.6	0.9	1.6	1.9	1.2
+ 0.8 × 10^{-6} M *m*-Cl-CCP	4.8	2 3	0.5	1.5	0.65	0.4
+ 1.6 × 10^{-6} M *m*-Cl-CCP	4.7	0.0	0.0	1.5	0.0	0.0

Effect on Swelling of Mitochondria.—Swelling of fresh rat liver mitochondria in hypotonic solution was followed by means of turbidity changes at 540 mμ. Typical results are plotted in Figure 1. Swelling induced by ADP or thyroxin is blocked by 10^{-6} M *m*-Cl-CCP, but substrate-induced swelling is somewhat accelerated. These responses are analogous, at least superficially, to those seen with DNP (Lipsett and Corwin, 1959).

Relation of Uncoupling to P_i and ADP Concentrations.—To determine whether the *m*-Cl-CCP anion competes with P_i for a reaction site, uncoupling efficiency was determined in various phosphate-Tris buffer mixtures over a 0.001 to 0.1 M P_i concentration range. No competitive effect was found. Similarly, possible competition with ADP was investigated. Data here are restricted to a narrower, 30-fold concentration range (0.05–1.5 mM), since ADP concentration is rather critical in the assay system. Again, no competitive trend was seen (Table V).

Protective Action of Aminothiols.—The presence of cysteine in the incubation medium at about 0.001 M was found to protect mitochondria completely against the uncoupling action of *m*-Cl-CCP, even in the presence of excess uncoupler (Table VI). This is not simply a sulfhydryl effect; BAL, glutathione, reduced coenzyme A, and many other thiols are inert. Of the compounds tested, only 1,2- or 1,3-aminothiols—*i.e.*, cysteine, cysteamine, homocysteine—show this interesting effect. These materials were found to have no effect upon the uncoupling action of DNP, arsenite, dicumarol, or gramicidin.

Only preventive action has been demonstrable in this system, and attempts to reverse prior CCP uncoupling have failed. The mechanism of protection is currently being studied. An *in vitro* interaction between carbonyl cyanide phenylhydrazones and aminothiols, as evidenced by a shift in absorption spectrum, can be demonstrated under mild conditions and may represent a model for the reaction of CCP compounds with an intramitochondrial site.

Inhibition of Photophosphorylation.—The inhibitory action of low concentrations (10^{-6} M) of *m*-Cl-CCP on cyclic photosynthetic phosphorylation in isolated chloroplasts has been described elsewhere (Heytler and Prichard, 1962) and has since been confirmed in other laboratories (M. Gibbs, 1962, personal communication; D. I. Arnon, 1962, personal communication). The observation is included here because of its possible relevance to the underlying similarity of photosynthetic and oxidative energy-converting mechanisms.

Spinach chloroplasts were isolated, and their

TABLE III

UPTAKE OF C^{14}-*m*-Cl-CCP BY MITOCHONDRIA

Each 2.5-ml system contained, in μmoles/ml: 50 phosphate buffer, *p*H 7.4; 200 sucrose; 0.04% NaCN where indicated; 40 succinate. Incubated at 37° for 10 minutes, then centrifuged at 40,000 × *g* for 10 minutes. Mitochondria washed once in cold 0.25 M sucrose, recentrifuged, dried on aluminum planchets, and counted with "Micromil" window on "Nuclear D-47" detector. Counts were corrected for self-absorption. Line 7 refers to recovery of unchanged *m*-Cl-CCP-C^{14} from mitochondria described in line 6 by means of addition of unlabeled carrier to the lysed preparation and subsequent precipitation and recrystallization as described in text.

		Labeled *m*-Cl-CCP Added (C^{14} cpm)	Net cpm in Washed Mitochondrial Pellet
1	Complete system	2,500	496
2	Complete system + CN^-	2,500	348
3	Complete system (at 0°)	2,500	292
4	Complete system	75,000	12,900
5	Complete system + CN^-	75,000	7,720
6	Complete system (mitochondria finally washed 3 × at 25°)	75,000	1,380
7	Activity recovered in carrier *m*-Cl-CCP (see text)		1,280

TABLE IV

EFFECT OF *m*-Cl-CCP ON ATPase

Values represent averages of 3–4 determinations. Reactions were incubated at 28° and stopped with 2.5% (final) perchloric acid, and P_i was determined. Additions, where indicated (μmoles/ml): 0.1 DNP; 0.0025 *m*-Cl-CCP; 4 μg/ml oligomycin A; 5 $MgCl_2$.

Enzyme	Additions	μmoles ATP Hydrolyzed/5 min.
Intact mitochondria[a]	None	0.23
	DNP	1.31
	DNP + oligomycin (4 mg/ml)	0.30
	m-Cl-CCP	1.26
	m-Cl-CCP + oligomycin (4 mg/ml)	0.31
	Mg^{++}	0.29
	Mg^{++} + DNP	1.32
	Mg^{++} + *m*-Cl-CCP	1.32
Soluble ATPase[b]	None	0.00
	Mg^{++}	0.88
	Mg^{++} + DNP	1.27
	Mg^{++} + *m*-Cl-CCP	0.89

[a] μmoles/ml: 60 histidine buffer, *p*H 7.4; 45 KCl; 5 ATP; 0.3 mg mitochondrial protein/ml. [b] μmoles/ml: 50 Tris-acetate, *p*H 7.4; 6 ATP; 5 phosphoenolpyruvate; 50 mg/ml pyruvic kinase; 0.6 mg ATPase protein/ml.

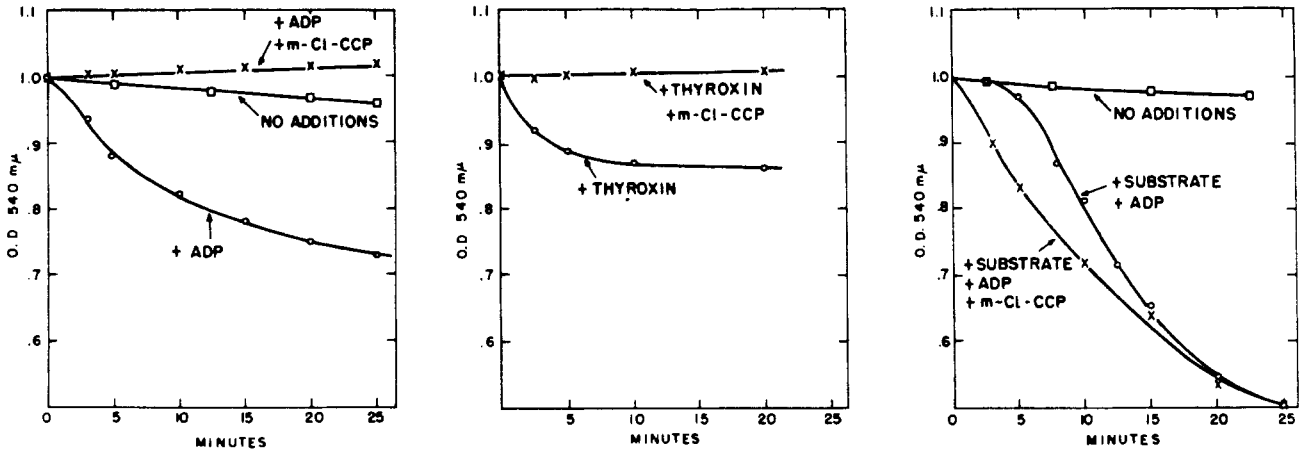


Fig. 1.—Effect of *m*-Cl-CCP on swelling of rat liver mitochondria. 0.05 ml mitochondria was added to 3.0 ml of a medium containing 0.1 M sucrose, 0.01 M Tris buffer (*p*H 7.4), 0.01 M phosphate (*p*H 7.4), and 0.002 M Mg^{++}. Where indicated, *m*-Cl-CCP was present at 10^{-6} M, ADP at 0.001 M, and thyroxin at 10^{-6} M; substrate was 0.01 M glutamate + 0.01 M malate. Optical density at 540 mμ was read at about 3-minute intervals, and solutions were aerated between readings. The mitochondrial suspension was adjusted to give an initial optical density near 1.0, and readings were normalized to this value. Rat liver mitochondria for these runs were prepared from a 0.33 M sucrose homogenate.

phenazine methosulfate-catalyzed photophosphorylation was determined (Jagendorff and Avron, 1958). These preparations esterified 80–100 μmoles phosphate/hour/mg chlorophyll when illuminated by two General Electric "Circline" fluorescent lamps (12 in. diameter) immersed in a Warburg bath. In the presence of 1.6×10^{-6} M *m*-Cl-CCP, phosphorylation was inhibited by 55–60% and at 5×10^{-6} M by 80–85%. Dinitrophenol at 10^{-4} M had no inhibitory effect and even stimulated the system significantly (*ca.* 20%). The latter effect is in agreement with other reports (Wessels, 1959).

Discussion

The above data demonstrate the powerful uncoupling action of carbonyl cyanide phenylhydrazone (*m*-Cl-CCP) as well as some other aspects of its behavior toward mitochondrial systems. While this compound is not as active an agent as several other CCP derivatives, notably the *p*-trifluoromethoxy analog (Heytler *et al.*, 1962; Heytler and Prichard, 1962), it has been used as a convenient and easily prepared standard in this work. Sufficient checks have been made with several CCP compounds to ensure that they are qualitatively similar in action but differ in degree of accessibility to and/or affinity for the sensitive mitochondrial site.

Uncouplers of the CCP class exhibit several characteristics which make them potentially useful in phosphorylation studies. They differ in significant respects from many other uncouplers, suggesting a different characteristic site of attack. Thus, stimulation of apparent ATPase of intact mitochondria and absence of pronounced respiratory inhibition distinguish CCP action from oligomycin-like effects. In their inertness toward purified soluble ATPase, CCP agents differ from DNP, pentachlorophenol, dicumarol, and triiodothyronine (Penefsky *et al.*, 1960). CCP shows no competitive behavior with respect to phosphate ion such as is seen with arsenate (Crane and Lipmann,

Table V

Effect of P_i and ADP Concentrations on Uncoupling by *m*-Cl-CCP

Warburg flasks contained following concentrations (μmoles/ml) in total volume of 3 ml: 17 P_i (*p*H 7.4), except as indicated; 17 β-hydroxybutyrate; 0.3 ADP (except as indicated); 67 glucose; 67 KCl; 3 $MgCl_2$; 0.1 cytochrome *c*; 7 NaF; 17 Kunitz-MacDonald units/ml hexokinase; 0.7 mg protein/ml beef heart mitochondria. In run I, samples 1–3, Tris buffer, *p*H 7.4, was added so that Tris + P_i = 33 mM.

	P/O (Control)	P/O (+0.10 μM *m*-Cl-CCP)	% Uncoupling
Phosphate Conc. (M)			
0.001	1.4	0.8	42
0.003	2.8	1.5	47
0.010	2.4	1.4	42
0.033	2.6	1.4	44
0.10	0.9	0.4	46
ADP Conc. (mM)			
0.05	2.1	0.7	67
0.15	2.3	0.8	65
0.5	2.5	0.8	68
1.5	2.7	0.9	67

Table VI

Protection Against CCP Uncoupling

Warburg flasks contained following concentrations (μmoles/ml): 17 P_i (*p*H 7.4); 50 succinate or 17 glutamate; 0.3 ADP; 67 glucose; 67 KCl; 3 $MgCl_2$; 0.1 cytochrome *c*; 7 NaF; 17 Kunitz-MacDonald units/ml hexokinase; 1 mg mitochondrial protein (mouse liver mitochondria); plus additions as indicated. Total volume, 3.0 ml. Substrate initially in side-arm; all other components placed into main chamber prior to addition of mitochondria.

	P/O
Controls (glutamate)	2.5
+ 10^{-6} M *m*-Cl-CCP	0.2
Same + 1.0 mM cysteine	2.8
Same + 1.0 mM 2-mercaptoethylamine	2.4
Controls (succinate)	1.4
+ 10^{-6} M *m*-Cl-CCP	0.1
Same + 1.0 mM 2-mercaptoethylamine	1.3
Same + 1.0 mM homocysteine	1.3
Same + 1.0 mM homocysteine thiolactone	0.2
Same + 1.0 mM glutathione	0.0
Controls (succinate)	1.6
+ 10^{-5} M *m*-Cl-CCP	0.0
Same + 1.0 mM cysteine	1.1

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.

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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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