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Effect of Hydrophobic Environments and Reducing Agents on the Oxidation of Protoporphyrinogen IX

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The oxidation of protoporphyrinogen IX (Protogen) to protoporphyrin IX (Proto IX) is the last common step in the biosynthesis of heme and chlorophyll.[1] This six-electron oxidation is catalyzed by protoporphyrinogen oxidase (Protox) (EC 1.3.3.4) *in vivo* and can readily occur also nonenzymatically.[2] A variety of diphenyl ether compounds such as oxyfluorfen and acifluorfen are commonly used as effective herbicides and it is generally accepted that Protox is the primary target of photodynamic diphenyl ether herbicides.[3,4] The biochemical basis for the herbicidal effect has been shown to be the competitive inhibition of Protox by diphenyl ether compounds.[5,11] Paradoxically, the inhibition of Protox leads to massive accumulation of Proto IX, the product of enzymatic reaction instead of the substrate.[6] It has been demonstrated by numerous people that phytotoxic herbicidal effect of diphenyl ether herbicides is directly related to the abnormal accumulation of Proto IX which is a well known strong photosensitizer in the presence of light and molecular oxygen.[4,7]

However, the mechanism by which Protogen is converted to Proto IX and the reason for the accumulation of Proto IX *in vivo* are not clear. Since the putative Protox is known to be localized in the plastid envelope,[8] inhibition of the enzyme by diphenyl ether compounds would lead to the accumulation of substrate initially, which could be exported from the plastid through the cytosol to the plasma membrane and the oxidation would occur enzymatically by diphenyl ether resistant Protox-like peroxidase and/or nonenzymatically by autooxidation in the membrane. Our previous results indicated that the rate of nonenzymatic autooxidation of Protogen *in vitro* was highly dependent on the hydrophobic nature of reaction medium.[10] In a continuous effort to address the role of nonenzymatic autooxidation for the accumulation of Proto IX in diphenyl ether treated plants, we investigated effects of reducing agents, ionic strength, and ethyl alcohol on the oxidation of Protogen in enzymatic and nonenzymatic reaction conditions.

Etioplasts from barley (*Hordeum. Vulgare* L.) and wheat (*Triticum Aestivum* L.) were obtained as reported previously.[10] The substrate of Protox was prepared by reduction of Proto IX as previously described.[12] The rate for the oxidation of Protogen was measured following the procedure of Sherman *et al.*.[13] The relative rates of Protogen oxidation at various concentrations of dithiothreitol (DTT) and glutathione (GSH) is shown in Figure 1. and Figure 2, which clearly indicates that the rate of Proto IX formation can be remarkably inhibited by increasing concentration of DTT and GSH in both

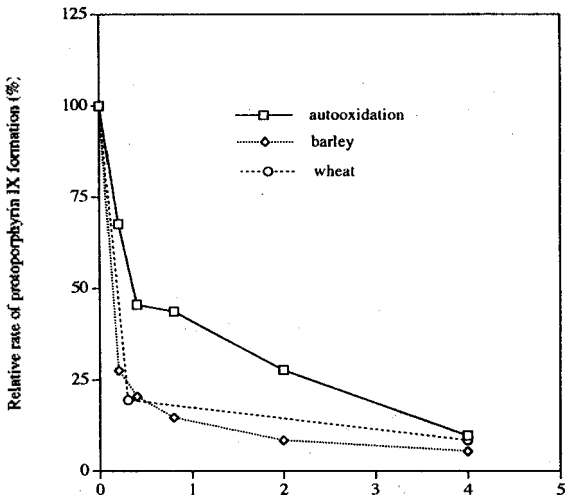

Figure 1. Effect of DTT on the rate of Protogen oxidation. The rates of Protogen oxidation were measured at various concentrations of DTT and calculated by leastsquare method using datapoints for the initial 5 minutes. Reaction mixture contained 100 mM HEPES (pH 7.5), 5 mM EDTA, 1% Tween-20, and 120 μL of etioplast extract (0.62 mg of protein) in 3 mL. The reaction was strated by adding 300 μL of 200 μM substrate. Fluorescence intensity was monitored using spectrofluorometer at 626 nm with excitation at 395 nm. Rates of autooxidation (□) and enzymatic oxidation (barley (◇), wheat (○)) were expressed relative to those at zero concentration of DTT.

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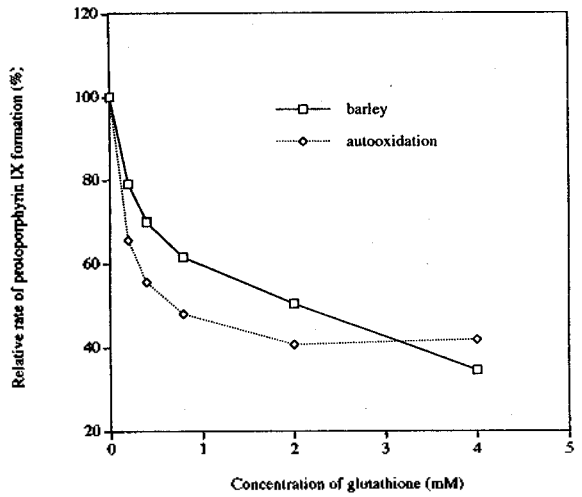

Figure 2. Effect of GSH on the rate of Protogen oxidation. The rates of Protogen oxidation were measured at various concentrations of GSH as in Fig. 1. Rates of autooxidation (◇) and enzymatic oxidation (barley (□)) were expressed relative to those at zero conecentration of GSH.

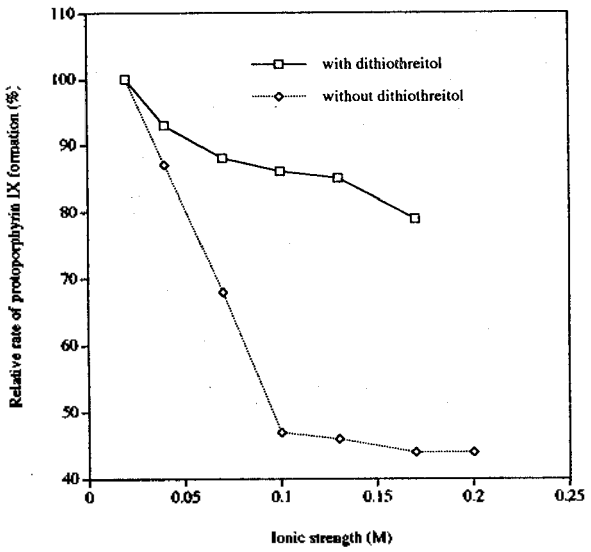

Figure 3. Effect of ionic strength on the autooxidation of Protogen in the Absence or Presence of DTT. The rates of Protogen oxidation were measured at various ionic strengths as in Fig. 1. Ionic strength was adjusted by adding appropriate amount of sodium chloride. Rates of autooxidation in the absence (◇) or presence (□) of dithiothreitol (2 mM) were expressed relative to those without added sodium chloride.

enzymatic and nonenzymatic oxidations. S. Iwata *et al.*[9] reported that the treatment of plant species with oxyfluorfen induced the accumulation of GSH and peroxidative cellular damage could be protected in the presence of GSH. Therefore, cellular reducing agents such as GSH may play a major protective role in diphenyl ether (DPE)-treated plants and the inhibitory effect of reducing agents may likely be attributed to the general property of the oxidation of Protogen.

Since our previous result[10] indicated that the autooxidation rate of Protogen could be enhanced in hydrophobic reaction

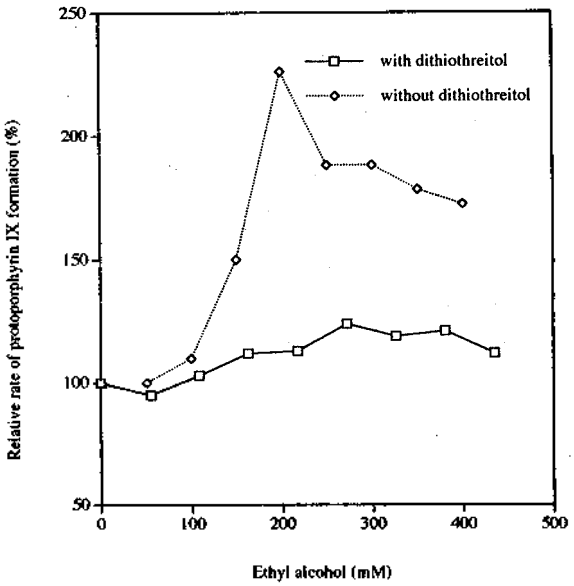

Figure 4. Effect of ethyl alcohol on the autooxidation of Protogen in the absence or presence of DTT. The rates of Protogen oxidation were measured at various concentrations of ethyl alcohol as in Fig. 1. Rates of autooxidation in the absence (◇) or presence (□) of dithiothreitol (2 mM) were expressed relative to those in the absence of ethyl alcohol.

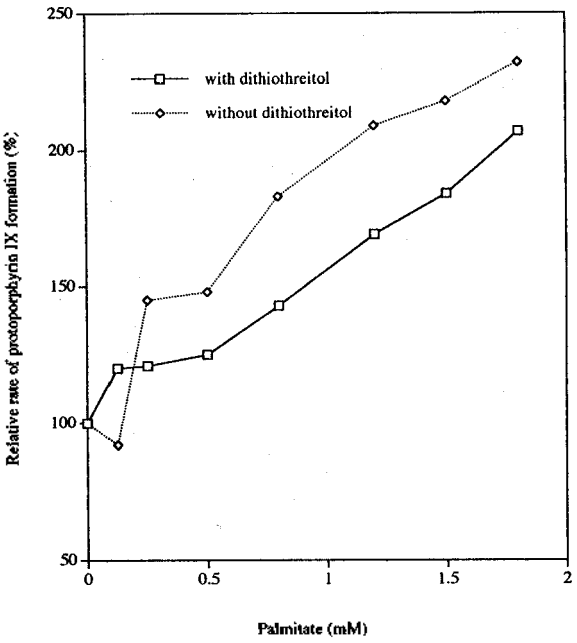

Figure 5. Effect of palmitate on the autooxidation of Protogen in the absence or presence of DTT. The rates of Protogen oxidation were measured at various concentrations of palmitate as described in Fig. 1. Rates of autooxidation in the absence (◇) or presence (□) of dithiothreitol (2 mM) were expressed relative to those in the absence of palmitate.

medium such as low ionic strength and ethyl alcohol, we decided to examine the effect of DTT on the autooxidation of Protogen at various ionic strengths and concentrations of ethyl alcohol.

As shown in Figure 3 and Figure 4, the dependence of autooxidation rate upon ionic strength and ethyl alcohol was more evident in the absence of DTT and the acceleration of the oxidation of Protogen by hydrophobic environment became relatively minor in the presence of DTT. When palmitate was used as the hydrophobic stimulator, the rate enhancement was so large that the counterbalance by DTT was negligible (Figure 5).

Since Proto IX is known to be more hydrophobic than Protogen,[14] the product-like transition state of the oxidation of Protogen would be stabilized by hydrophobic environments and this stabilization is more favourable in the absence of DTT. Therefore, the autooxidation of Protogen would be slow in hydrophilic and reductive condition as in cytosol and fast in hydrophobic and oxidative condition as in plasma membrane. In conclusion, the autooxidation of Protogen initially accumulated by the inhibition of Protox in diphenyl ether treated plants would be hindered in cytosol and the accumulated Protogen would likely be transported to the plasma membrane as an unprocessed form to undergo facilitated enzymatic and/or nonenzymatic autooxidation in the plasma membrane.

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Orthocyclophanes. 6. Hexamethoxy Derivatives of [1$_6$]Orthocyclophane

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In recent years, we have been involved in developing a new branch of orthocyclophane chemistry, the family of [1$_n$] orthocyclophanes ([1$_n$]OCPs). Since the methylene groups in a bisbenzylic position can be easily transformed into other functionalities, the [1$_n$]OCPs are expected to be precursors to novel macrocyclic ionophores having interesting binding properties.[1] In previous investigations, we developed general synthetic routes[2] to the parent [1$_n$]OCP cycles and their functionalization on the aromatic rings.[3] We also found that the oxidation of the bisbenzylic carbons resulted in new families of crown compounds, either starands[4] or ketonands[5] depending on n. The present paper provides a general synthetic route to further modification of the [1$_6$]OCP by introduction of functional groups to the aromatic rings.

One of the starting materials, dibromide **1** was prepared from acid-catalyzed Friedel-Crafts alkylation[6] of 4-bromoveratrole with 1,2-benzenedimethanol in AcOH/H_2SO_4. The other key compound, an aromatic dialdehyde **2**, was prepared according to the literature procedure.[3] Generation of the [1$_6$] OCP cycle was accomplished by treatment of of an aromatic dibromide **1** in dry THF with n-BuLi at −13 ℃ to give the corresponding dilithio reagent, followed by condensation with an aromatic dialdehyde (**2**, 1,2-dimethoxy-4,5-bis(2′-formylbenzyl)benzene) and succesive hydrolytic workup, to give a cyclic diol **3**. Because it turned out difficult to isolate and purify, crude diol **3** was oxidized directly with PCC to the corresponding cyclic dione, hexamethoxy[1$_6$]OCPdione **4**. Clemmensen reduction of **4** did not give the hexamethoxy [1$_6$]OCP **5**, though dimethoxy- and tetramethoxy[1$_6$]OCPs[3] could be obtained by Clemmensen reduction. The reduction of **4** was successfully carried out by the literature procedure of Ono *et al.*,[7] heating at reflux temperature with a mixture of $NaBH_4$ and $AlCl_3$ in THF to give rise to the hexamethoxy [1$_6$]OCP **5**. Whereas the even-numbered [1$_n$]OCPs are generally insoluble in conventional organic solvents presumably due to the molecular symmetry, the hexamethoxy derivative **5** could be extracted into EtOAc without difficulty from the reaction mixture to give crystalline solid after chromatographic purification. The structure of both **4** and **5** was unambiguously confirmed by HRMS, ^{1}H NMR, ^{13}C NMR, and IR spectroscopy. (see experimental section)

Treatment of a solution of **5** in CH_2Cl_2 with BBr_3 at 0 ℃, followed by stirring at rt, provided hexahydroxy[1$_6$]OCP (**6**). Since the phenolic OH functions can be easily modified, the hexahydroxy cyclophane (**6**) could serve as a precursor to other classes of novel crown compounds.

In summary, we developed a convenient route to the synthesis of hexamethoxy[1$_6$]OCP (**5**). Since the methoxy functions in **5** can be demethylated to provide the corresponding