Inhibition of Photosynthetic Electron Transport by Azaphenanthrenes

Walter Oettmeier and Rolf Grewe

Abteilung Biologie, Ruhr-Universität Bochum

(Z. Naturforsch. 29 c, 545–551 [1974]; received June 4, 1974)

Inhibitors, Photosynthetic Electron Transport, Azaphenanthrenes

Various mono- and diazaphenanthrenes were prepared and assayed for their activity as inhibitors of photosynthetic electron flow in isolated chloroplasts in order to get more insight into the mechanism of action of the well known inhibitor *o*-phenanthroline = 1,10-diazaphenanthrene. The results show that 1-, 4- and 5-azaphenanthrene are only slightly less active than 1,10-diazaphenanthrene. In the case of the different diazaphenanthrenes, 1,4-, 1,7- and 5,6-diazaphenanthrene exhibited somewhat lower activity than 1,10-diaphenanthrene, whereas 2,9- and 4,7-diazaphenanthrene were completely inactive. Substitution at C-atoms of 1,10-diazaphenanthrene leads to an increase in activity in the case of the 4- and 7-position, regardless of electropositive or electronegative substituents, whereas substitution at the 2-, 3-, 5-, 6-, 8- and 9-position leads to a decreased activity. The ability of 1,10-diazaphenanthrene to form iron complexes seems to be of little relevance to the inhibitory activity on photosynthetic electron transport. This follows also from the fact that other strong iron complexing agens, like 2,2′-bipyridine or 8-hydroxyquinoline, are not inhibitory.

1,10-diazaphenanthrene * ("*o*-phenanthroline") has been introduced by Warburg and Lüttgens [2] in 1946 as the first inhibitor of photosynthetic electron transport. Its site of inhibition has been demonstrated to be close to photosystem II [3, 4]. At the same site a great variety of substances are acting as inhibitors [5], some of which are used as powerful herbicides [6].

Extensive work has been produced to study the relationship of chemical structure to biochemical inhibitory activity in the case of the herbicidal compounds and a basic structural element, responsible for a photosystem II inhibitor, has been developed. Such relationship has not been established for azaphenanthrenes, except for the recent work of Satoh [7] on some derivatives of 1,10-diazaphenanthrene. Actually, the chemical structure of 1,10-diazaphenanthrene did not fit easily in the class of the other inhibitors. The iron complexing activity of 1,10-diazaphenanthrene has been considered instead, which seemed to be supported from studies with bacterial systems, where an endogeneous iron sulfur protein seems to be inhibited [8, 9].

This report aimes to elucidate structure-activity relationships of azaphenanthrenes. The results of this paper show that the presence of only one nitrogen in the phenanthrene system (monoazaphenanthrene) already caused inhibitory activity. Introduction of a second nitrogen into the aromatic moiety led to an increase in activity only in the case of 1,10-diazaphenanthrene. Among various substituted 1,10-diazaphenanthrenes tested, substitution in the 4- or/and 7-position by electropositive (+I) or electronegative (−I) substituents further increased activity almost tenfold as compared to the parent 1,10-diazaphenanthrene.

From this, a first relationship to the other chemical classes of inhibitors of photosystem II can be developed.

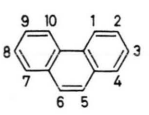
Materials and Methods

E. Merck AG, Darmstadt, W. Germany, was the source of the following compounds: Acridine·HCl, 2,2′-bipyridine·2 HCl, quinoline, isoquinoline, 4-azaphenanthrene (5,6-benzoquinoline), 1,10-diazaphenanthrene·HCl·H_2O (*o*-phenanthroline), 2,9-dimethyl-1,10-diazaphenanthrene·HCl (neocuproine),

Requests for reprints should be sent to Dr. W. Oettmeier, Abteilung Biologie der Ruhr-Universität, *D-4630 Bochum*, Postfach 2148.

Abbreviations: AQ, anthraquinone-2-sulfonic acid; asc, Na-ascorbate; DAD, diaminodurene; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

* Nomenclature of compounds will follow Allen [1] and denominate nitrogen derivatives of phenanthrene as azaphenanthrenes. The position(s) of nitrogen(s) will be numbered according to the formula.



4,7-diphenyl-1,10-diazaphenanthrene (bathophenanthroline), 4,7-diphenyl-1,10-diazaphenanthrene-disulfonic acid disodium salt (bathophenanthroline-disulfonic acid disodium salt). The following substances were obtained from Aldrich, Europe: 1-azaphenanthrene (7,8-benzoquinoline), 5-azaphenanthrene (phenanthridine), 5,6-diazaphenanthrene (benzo-(c)-cinnoline) and 3,4,7,8-tetramethyl-1,10-diazaphenanthrene (3,4,7,8-tetramethyl-*o*-phenanthroline).

The following azaphenanthrene were synthesized according to the literature cited (a review on synthetic methods leading to azaphenanthrenes has been given by Allen [1]): 1-azaphenanthrene-1-oxide [10], 1,7-diazaphenanthrene [11], 4,7-diazaphenanthrene [11, 12], 1,4-diazaphenanthrene [13], 2,9-diazaphenanthrene [14], 1,10-diazaphenanthrene-1-oxide [15, 16], 1,10-diazaphenanthrene-1-methiodide [17], 3,8-dimethyl-1,10-diazaphenanthrene [18], 2-chloro-1,10-diazaphenanthrene [17], 4-chloro-1,10-diazaphenanthrene [15], 5-chloro-1,10-diazaphenanthrene [19, 20], 4,7-dichloro-1,10-diazaphenanthrene [21], 5-nitro-1,10-diazaphenanthrene [22], 4,5-diazafluoren-9-one [23]. In many cases, chromatography on aluminium oxide, neutral or acidic, activity III, turned out to be an excellent purification step. Identity and purity of the above synthesized compounds were checked by melting point, elementary analysis and NMR-data. For reasons of better solubility, free bases were converted into their hydrochlorides.

For determination of the apparent acid-base dissociation constants pK_a*, $5 \cdot 10^{-3}$ M solutions of the hydrochlorides of azaphenanthrenes were titrated potentiometrically under nitrogen at 20 °C by 10^{-2} M NaOH. pK_a*-values for some of the above mentioned compounds are listed in [25], but these values have been obtained by currently differing methods and at varying temperatures. For these reasons, own values are used in the tables.

For estimation of the partition coefficient P, the UV-spectrum of a $2.5 \cdot 10^{-5}$ M solution of the heterocyclic base in 0.02 M Tris buffer, pH 8.0, was recorded between 200 and 340 nm in order to find out a suitable absorption maximum and to determine the optical density at the maximum. To 4 ml of the above solution were added 4 ml of CCl_4, and the mixture intensively agitated on a whirlmix for 1 min. After a short centrifugation, the phases were separated. The optical density at the absorption maximum was recorded with 0.02 M Tris buffer, pH 8.0, treated in the same way, as the reference. The partition coefficients are expressed as the ratio of the difference in absorbance before and after extraction over the remaining absorbance after extraction.

Spinach chloroplasts have been prepared according to Nelson *et al.* [25] (AQ-reduction) or Whatley *et al.* [26], washed three times, frozen in liquid nitrogen and stored until use (ferricyanide- and DCIP-reduction).

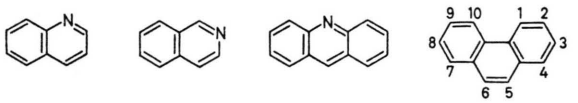
Inhibitory activity of azaphenanthrenes was assayed as well in photosynthetic ferricyanide reduction as in DCIP-reduction. For ferricyanide photoreduction, the medium contained in 2 ml 40 μmol Tris buffer, pH 8.0, 5 μmol NH_4Cl, 2 μmol $K_3[Fe(CN)_6]$ and chloroplasts with 15 μg chlorophyll. The reaction mixture was illuminated in Corex centrifuge tubes for 8 min by white light (intensity $8 \cdot 10^4$ erg sec^{-1} cm^{-2}). The reaction was terminated by addition of 0.2 ml 20% trichloroacetic acid. After filling up to a final volume of 3 ml, the mixture was centrifuged for 10 min at $2000 \times g$ and the optical density of the supernatant recorded at 405 nm. In every experiment as controls served an assay without inhibitor and a dark control. For DCIP-photoreduction, the reaction mixture contained in 2 ml 40 μmol Tris buffer, pH 8.0, 5 μmol NH_4Cl, 0.12 μmol DCIP and chloroplasts with 5 μg chlorophyll. Optical density at 578 nm was measured before and after 4 min illumination (white light, intensity $8 \cdot 10^4$ erg sec^{-1} cm^{-2}). p_i50 ($-\log_{10}$ of molar concentration of inhibitor giving 50% inhibition of ferricyanide or DCIP-photoreduction) was determined graphically from a series of different concentrations of inhibitor by extrapolation to 50% inhibition.

Photosynthetic AQ-reduction was carried out in Warburg vessels at 15 °C under illumination for 15 min with white light 30 000 lx. Oxygen consumption (due to an autoxidation of photosynthetically reduced AQ) was followed manometrically. The medium contained in μmoles in a volume of 3 ml Tris buffer, pH 8.0, 80, $MgCl_2$ 10, ADP 10, inorganic phosphate 10, NaN_3 0.3, AQ 0.1 and chloroplasts with 0.2 mg chlorophyll. When AQ was photoreduced at the expense of DAD/asc as donor system, 0.2 μmol DAD, 20 μmol asc and 0.01 μmol DCMU were added to the medium too.

Results

For detection of basic structural elements, responsible for the action of 1,10-diazaphenanthrene as an inhibitor of photosynthetic electron transport, at first heterocyclic bases with only one nitrogen were considered (Table I). Quinoline as well as the isomeric isoquinoline were completely inactive. Both linear and angular connection of a third aromatic residue to quinoline in all cases rendered ef-

Table I. Inhibition of photosynthetic *Hill*-reaction by some mononitrogen heterocyclic compounds.



Quinoline Isoquinoline Acridine Phenanthrene

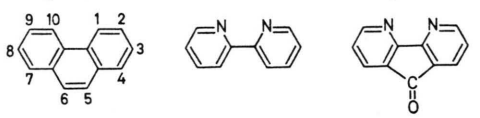
Substance	Position of nitrogen	p_I50	Dissociation constant pK_a *	Partition coefficient *P*
quinoline	—	<2	4.9	2.5
isoquinoline	—	<2	5.4	3.3
acridine	—	3.8	5.0	>100
1-azaphenanthrene	1	4.6	3.6	7.2
1-azaphenanthrene-1-oxide	1	3.8	2.8	8.4
4-azaphenanthrene	4	3.6	4.6	6.3
5-azaphenanthrene	5	3.7	4.3	20

fective inhibitors. 1-Azaphenanthrene is the most potent one, only sightly less active than 1,10-diazaphenanthrene (Table II; p_I50 5.1). Basicity of the tested compounds is about in the same range; the same is true for the partition coefficients *P*, with the exception of acridine, which is highly lipophilic. Blockage of nitrogen in 1-azaphenanthrene lowers p_I50 as well as pK_a*.

The effect by introduction of a second nitrogen into monoazaphenanthrenes on photosynthetic electron transport is shown in Table II. Only substitution in the 1,10-position causes an increase in inhibitory activity, whereas substitution in the 1,4, 1,7 and 5,6 positions renders diazaphenanthrenes less active than 1-azaphenanthrene and finally, substitution in the 2,9- and 4,7-position yields completely inactive compounds. The lipophilicity of the isomeric diazaphenanthrenes is comparably similar, but there are marked differences in basicity. Whereas 1,10- and 5,6-diazaphenanthrene are monobasic (because of the close neighbourhood of nitrogens), the others are dibasic with pK_{a1}* at about 2 – 3 and pK_{a2}* between 4,1 and 4,9. 2,2′-Bipyridine seems to have some mutual structural elements with 1,10-diazaphenanthrene, but is completely inactive. A carbonyl-bridge in the 3,3′-position of 2,2′-bipyridine (*i. e.* 4,5-diazafluoren-9-one) brings back inhibitory activity.

Since 1,10-diazaphenanthrene proved to be the best inhibitor on photosynthetic electron transport of all isomeric unsubstituted diazaphenanthrenes so far tested, first the influence of +I (electron-donating) substituents on p_I50 values was examined. As demonstrated in Table III, a drastic decrease in activity occurs upon methyl-substitution in 2,9-position and a less pronounced decrease upon

Table II. Inhibition of photosynthetic *Hill*-reaction by diazaphenanthrenes and some related compounds.

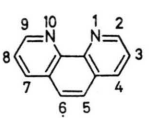


Phenanthrene 2,2′-bipyridine 4,5-diazafluoren-9-one

Substance	Position of nitrogens	p_I50	Dissociation constant pK_a *	Partition coefficient *P*
1,10-diazaphenanthrene	1.10	5.1	4.9	1.2
5,6-diazaphenanthrene	5.6	3.8	2.7	20
1,7-diazaphenanthrene	1.7	4.0	2.8/4.7	9.1
1,4-diazaphenanthrene	1.4	3.7	a	a
2,9-diazaphenanthrene	2.9	<2	3.1/4.8	1.4
4,7-diazaphenanthrene	4.7	<2	2.3/4.1	1.8
2,2′-bipyridine	—	<2	4.4	10.0
4,5-diazafluoren-9-one	—	4.0	b	1.7

a Low yield in synthesis did not allow determination, b determination has not been possible because of low solubility in H_2O.

Table III. 1,10-Diazaphenanthrenes with electropositive (+I)-substituents as inhibitors of photosynthetic *Hill*-reaction.

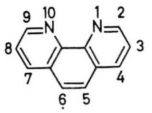


Substituents	p_I50	Dissociation constant pK_a *	Partition coefficient *P*
2,9-dimethyl-	<3	5.7	7.2
3,8-dimethyl-	4.4	5.1	4.6
3,4,7,8-tetramethyl-	5.5	5.8	1.2
4,7-diphenyl-	4.9	4.8	0.1
4,7-diphenyl-disulfonate-	3.2	5.2	<0.05
1-methiodide	<3		<0.05
1-oxide	3.2	6.7	<0.05

methyl-substitution in 3,8-position. p_I50 values are also lowered by blockage of nitrogen (formation of the N-oxide or N-methiodide) and by introduction of a sulfonic acid group into the molecule. In the case of the last three compounds, the partition coefficient P of almost zero indicates a very low lipophilicity. 3,4,7,8-Tetramethyl-1,10-diazaphenanthrene, however, proved to be more active than the unsubstituted parent compound.

Chlorine substitution also demonstrates the unequality of positions in the 1,10-diazaphenanthrene moiety (Table IV). Substitution in the 2- and

Table IV. 1,10-Diazaphenanthrenes with electronegative (—I)-substituents as inhibitors of photosynthetic *Hill*-reaction.



Substituents	p_I50	Dissociation constant pK_a *	Partition coefficient P
2-chloro-	4.8	4.0	5.3
4-chloro-	5.6	4.2	4.8
5-chloro-	3.9	4.0	6.3
4,7-dichloro-	5.8	a	>100
5-nitro-	2.8	3.6	2.0

a Determination has not been possible because of low solubility in H_2O.

5-position respectively, diminishes p_I50, whereas substitution in the 4-position and double substitution in the 4- and (symmetrical to it) 7-position increase p_I50. 4,7-Dichloro-1,10-diazaphenanthrene was the most potent inhibitor of all 1,10-diazaphenanthrenes so far examined; it also stands out by a very high lipophilicity. Similar to chlorination in the 5-position, also nitration at the same position decreases activity. As has to be expected, basicity of all compounds of Table IV is lowered by the strongly electron withdrawing -I-substituents.

As already quoted, 1,10-diazaphenanthrene inhibition in photosynthetic electron transport was located near photosystem II [3, 4]. To ensure the same site of inhibition for other azaphenanthrenes characteristic representatives are compared in Table V in their effect on photosynthetic AQ-reduction at the expense of water as well as at the expense of DAD/asc as donor systems. As can be seen from the results in Table V, at concentrations, where electron

Table V. Inhibition of photosynthetic anthraquinone-2-sulfonate reduction by azaphenanthrenes at the expense of water or diaminodurene/ascorbate as donor systems.

Substance	Concn. [M]	System $H_2O \rightarrow AQ$ (control: 44 μmol O_2/mg chl./h) [% inhibition]	System DAD/asc→AQ (control: 96 μmol O_2/mg chl./h) [% inhibition]
1-azaphenanthrene	$2 \cdot 10^{-4}$	89	0
4-azaphenanthrene	10^{-3}	100	0
5-azaphenanthrene	10^{-3}	100	0
1,7-diazaphenanthrene	10^{-3}	54	0
5,6-diazaphenanthrene	10^{-3}	72	4
1,10-diazaphenanthrene	10^{-4}	77	0
4,7-diphenyl-1,10-diazaphenanthrene	10^{-4}	91	9
3,4,7,8-tetramethyl-1,10-diazaphenanthrene	10^{-4}	82	7
4,7-dichloro-1,10-diazaphenanthrene	10^{-4}	91	7
4,5-diazafluoren-9-one	10^{-3}	69	5

flow from water to AQ is highly inhibited, no or almost no inhibition occurs under conditions, where only photosystem I is acting. It should be noted, however, that at higher concentrations some azaphenanthrenes also inhibit photosystem I activity and in addition, some azaphenanthrenes uncouple.

Discussion

The inhibition site of 1,10-diazaphenanthrene is long known and has been localized close to photosystem II between the quencher of photosystem II and plastoquinone [3, 4]. As demonstrated in results, various mono- and diazaphenanthrenes are powerful inhibitors of photosynthetic electron transport. Although the inhibition site of azaphenanthrenes has not been exactly located, from the results in Table V is clearly obvious that they are inhibitors of photosystem II too.

The inhibition site of 1,10-diazaphenanthrene is common to a variety of substances, like ureas, phenylcarbamates, acylanilides and derivatives of five- and six-membered heterocyclic ring systems (for reviews see [5, 6, 27]). The structural element

$$=\overset{|}{C}-N<$$

where the sp^2-carbon atom may be bound to oxygen or a substituted nitrogen, was elucidated as being

responsible for inhibitory activity [5, 27] and it is assumed that this element is bonded to a special protein amid group in the thylakoid membrane [5].

On the first view, this structural element also seems to occur in azaphenanthrenes. The nitrogen atom in azaphenanthrenes, however, is part of an aromatic π-electron system, which it is not in case of the compounds mentioned above.

As a first basic result, the incorporation of only one nitrogen atom into an aromatic system consisting of at least three rings, leads to active compounds (acridine, azaphenanthrenes), whereas an aromatic two ring system proved to be inactive (quinoline, isoquinoline). From the isomeric monoazaphenanthrenes tested, 1-azaphenanthrene exhibited the highest p_I50 value. Substitution at the nitrogen in 1-azaphenanthrene by formation of the N-oxide lowers activity. The same effect is known for several classes of herbicides, where substitution at the nitrogen decreases activity as well.

As a second basic result, diazaphenanthrenes different from 1,10-diazaphenanthrene (5,6-, 1,4- and 1,7-diazaphenanthrene) are inhibitors too, whereas others like 2,9- and 4,7-diazaphenanthrene are completely inactive. p_I50 values of 5,6-, 1,4- and 1,7-diazaphenanthrene are about in the same range (3,7 – 4,0) and correspond to those of monoazaphenanthrenes. In their behaviour as inhibitors, they can indeed be considered as monoazaphenanthrenes. For example, in 5,6-diazaphenanthrene, the two nitrogen atoms may be regarded as a unity (5,6-diazaphenanthrene is only monobasic and only forms a monohydrochloride) and it resembles 5-azaphenanthrene. In 1,4- and 1,7-diazaphenanthrene, the two nitrogen atoms are on different sides of the molecule and by binding to the active center only one nitrogen is involved, *i. e.* they are acting as monoazaphenanthrenes.

From all isomeric diazaphenanthrenes, 1,10-diazaphenanthrene stands out by its high inhibitory power. As already quoted in the case of monoazaphenanthrenes, substitution of a carbon atom in 1-position by nitrogen leads to the most potent inhibitors. Double substitution in the 1- and 10-position (identical and symmetrical to 1-position) gives rise to a rather unique molecular structure, which is reflected in some chemical properties: 1,10-diazaphenanthrene, despite its two spatial separated nitrogen atoms, is only monobasic and forms a monohydrochloride and a mono-N-oxide [1].

Rigidity and planarity of the aromatic system may be considered as a further condition for inhibitory activity of azaphenanthrenes. 2,2′-Bipyridine, which has the same molecular architekture like 1,10-diazaphenanthrene and only lacks the 3,3′-ethylene bridge, turned out to be completely inactive. The inactivity of 2,2′-bipyridine is ascribed to free rotation of the two pyridine rings around the σ-bond, which connects the aromatic moieties, whereas rotation is impossible in 1,10-diazaphenanthrene. If rotation in 2,2′-bipyridine is blocked by substitution in the 3,3′-positions, for example by a carbonyl brigde as in 4,5-diazafluoren-9-one, a rigid and planar system is established again, and inhibition activity comes back.

In contrast to the qualitative treatment of inhibition activity by mono- and isomeric diazaphenanthrenes, the results in the case of substituted 1,10-diazaphenanthrenes will be considered in a more quantitative way. Quantitative treatment of inhibition activity, expressed as p_I50-values, has been performed in the case of several herbicides, which act as inhibitors of photosystem II, by the extrathermodynamic approach (Hansch-approach) [28]. The principle of this approach rests on the assumption that the change of the biological activity is correlated with the change of measurable molecular or substituent parameters. Especially basicity pK_a^* and partition coefficient P were extremely useful as parameters [6]. In several classes of substances, these two parameters alone allowed calculation of p_I50 values, which were in good agreement to those actually determined [6, 29]. Because of the low number of substituted 1,10-diazaphenanthrenes tested, a Hansch approach was not performed, but dissociation constants pK_a^* and partition coefficients have been determined.

In substituted 1,10-diazaphenanthrenes, certain substitutions cause as well increase as decrease of activity. Besides basicity and partition coefficient, also steric reasons have to be taken into account for interpretation of results. Thus, loss of activity by substitutions in the 2-, 3-, 8- and 9-positions is surely due to steric reasons, because basicity and lipophilicity of the corresponding methyl-substituted 1,10-diazaphenanthrenes do not differ markedly from the parent compound. Extremely low lipophilicity of 4,7-diphenyl-1,10-diazaphenanthrene-disulfonate and 1,10-diazaphenanthrene-1-oxide and -1-methoiodide, however, account for their pro-

nounced drop in activity. In the case of 3,4,7,8-tetramethyl-1,10-diazaphenanthrene, p_I50 is rised compared to 1,10-diazaphenanthrene, where steric hinderance is overcome by substitution in the 4- and 7-positions. Substitutions in these positions generally increase activity.

The latter interpretation is strongly supported by the results of Satoh [7]. Although our p_I50 values and those of Satoh are not comparable (for example 1,10-diazaphenanthrene: Our value 5.1; Satoh's 4.4 [7]), because he only follows the onset slope of DCIP-photoreduction, we agree qualitatively. The pronounced suitability of the 4,7-positions for increase in activity by substitution is further supported by the result that 4,7-dimethyl-1,10-diazaphenanthrene proved to be the most effective inhibitor in Satoh's experiments (increase in p_I50 by 1.5 units) [7]. Substitution in the 5- or 6-position is less favourable, as it decreases activity [7].

For several herbicides, acting as inhibitors of photosynthetic electron transport, it is known that halogen substitution leads to an increase in activity. The same is true for certain chlorinated 1,10-diazaphenanthrenes. The three isomeric chloro-1,10-diazaphenanthrenes tested exhibit almost identical basicity and partition coefficients. Consequently, the decrease in activity of the 2-chloro-derivative again has to be ascribed to steric reasons and also the decreasing of activity by substitution in 5- or 6-positions has been already stressed. In contrast, substitution in the favoured 4- and 7-positions brought about the expected increase in activity and 4,7-dichloro-1,10-diazaphenanthrene turned out to be the most potent inhibitor of all azaphenanthrenes tested. It also stands out by a very high lipophilicity.

Since 1,10-diazaphenanthrene is known to form stable complexes with several metal ions, it is often assumed that its inhibitory action in photosynthesis is due to the complexation of iron containing components of the photosynthetic electron transport chain. In bacterial photosynthesis, 1,10-diazaphenanthrene acts inhibitory by shifting the midpoint potential of the primary acceptor, a bound ferredoxin. This shift is assumed to be due to an iron-sulfur-1,10-diazaphenanthrene-complex of reduced ferredoxin [8, 9]. As to the inhibition of photosynthetic electron transport by 1,10-diazaphenanthrene in the region of photosystem II, it is concluded that this inhibition has nothing to do with the iron chelating properties of 1,10-diazaphenanthrene. This opinion is based on the following observations. 8-Hydroxyquinoline, a strong chelating compound too (stability constant $\beta_2 = 15.0$ [30]), is not inhibitory [8]. Thes ame is true for 2,2′-bipyridine ($\beta_3 = 17.3$ [30]), which has almost the same complexing activity as 1,10-diazaphenanthrene ($\beta_3 = 21$ [30]). Finally, mono- and diazaphenanthrenes, which cannot form stable iron complexes, are inhibitors of photosynthetic electron transport as well.

We are indebted to Prof. Dr. A. Trebst for many helpful discussions and to Bundesministerium für Forschung und Technologie for financial support.

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