Biochimica et Biophysica Aact, 333 (1974) 127–135

BBA 46660

ACTION OF SOME DERIVATIVES OF 1,10-PHENANTHROLINE ON ELECTRON TRANSPORT IN CHLOROPLASTS

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(Received July 30th, 1973)

SUMMARY

The effectiveness and the mode of inhibitory action of some derivatives of 1,10-phenanthroline and a few other substances on 2,6-dichlorophenolindophenol (DCIP) photoreduction and fluorescence induction at room temperature in spinach chloroplasts were compared in this paper.

4,7-Dimethyl-1,10-phenanthroline was the most potent inhibitor [pI_{50} ($-\log_{10}$ of molar concentration of inhibitor giving 50 % inhibition), 5.9] examined, and 5-methyl-1,10-phenanthroline (pI_{50}, 4.2) was as potent as 1,10-phenanthroline (pI_{50}, 4.4), in the case of DCIP photoreduction. The order of inhibition by 4,7-dimethyl-1,10-phenanthroline ($n = 2$) was different from that of 1,10-phenanthroline and 5-methyl-1,10-phenanthroline ($n = 1$), under the experimental conditions used.

Fluorescence induction was diminished by 4,7-dimethyl-1,10-phenanthroline or 5-methyl-1,10-phenanthroline with the same pattern as 1,10-phenanthroline, but the effective concentrations varied corresponding to the pI_{50} values for the inhibition of DCIP photoreduction.

Metal ions which form a chelate complex with phenanthroline of high stability constant prevented the inhibition of both DCIP photoreduction and fluorescence induction.

From these results, the structure–activity relations and the mode of inhibition of the photosynthetic electron transport chain by these reagents are discussed.

INTRODUCTION

1,10-Phenanthroline has been shown to act as an inhibitor of photosynthetic electron transport both in plants [1, 2] and in bacteria [3–5]. In bacterial systems, it was suggested that 1,10-phenanthroline blocks electron transport from primary to secondary acceptors [3–5] by causing some changes in the midpoint potential of the primary electron acceptor [5].

The site of action of 1,10-phenanthroline on the electron transport chain in

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

chloroplasts has been studied by analyzing the fluorescence induction phenomena at room temperatures [6–8]. The analysis of the fluorescence induction indicated that the site of action of this reagent is similar to that of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [7, 9]: inhibition of the reoxidation of a postulated electron acceptor, Q [10]. The mechanism of inhibitory action on the electron transport chain and the structure–activity relations for herbicides, e.g. DCMU [10–16], have been studied in some detail, but there are only a few studies on 1,10-phenanthroline at present.

This report aims to further elucidate the mechanisms of action of 1,10-phenanthroline on photosynthetic systems in chloroplasts. A comparison of the effectiveness and the mode of the inhibitory action of some derivatives of 1,10-phenanthroline and a few other substances on DCIP photoreduction and fluorescence induction at room temperature in spinach chloroplasts are discussed. 4,7-Dimethyl-1,10-phenanthroline was shown to be the most potent inhibitor examined (pI_{50}, 5.9), with a somewhat different mode of action from that of 1,10-phenanthroline.

Recently, 1,10-phenanthroline was also shown to have another site of action in Photosystem II [Satoh, K., unpublished].

MATERIALS AND METHODS

Chloroplast fragments were prepared from spinach as described elsewhere [17].

Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan), was the source of 1,10-phenanthroline derivatives and related substances, except for the following: 4,7-dimethyl-1,10-phenanthroline, 5-methyl-1,10-phenanthroline, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline sulfonate (sodium salt), and 4,7-diphenyl-1,10-phenanthroline sulfonate (sodium salt) were from Dozindo Co., Ltd, Research Laboratories (Kumamoto, Japan). Because of their slight solubility in water, certain reagents were dissolved first in ethanol and then diluted (10- –20-fold) into the suspension of chloroplasts. These reagents included 2,9-dimethyl-1,10-phenanthroline, 4,7-dimethyl-1,10-phenanthroline, 5-methyl-1,10-phenanthroline, 4,7-diphenyl-1,10-phenanthroline, 5-nitro-1,10-phenanthroline, and 2,4,6-tris-(2-pyridyl)-*s*-triazine.

Photoreduction of DCIP was followed spectrophotometrically at 610 nm with a recording spectrophotometer (MPS-50L, Shimadzu), equipped with a crossillumination attachment. Actinic light for the reaction was provided by a 500-W xenon arc lamp (OXL-500DV, Ushio) with a cut-off filter (V-R67, Toshiba) and a heat absorbing filter (HA-50, Hoya). The actinic light intensity was $3.05 \cdot 10^4$ ergs $\cdot$ cm$^{-2} \cdot$ sec^{-1} as measured with a radiometer (MPM-I, Japan Spectroscopic Co., Ltd). An interference filter (KL-61, Nihon Shinku Kogaku) in front of the photomultiplier protected it against scatttered actinic light. The reaction mixture for the DCIP photoreduction contained 125 μmoles Tris–HCl (pH 7.2), 0.2 μmole of DCIP (sodium salt), chloroplast preparations equal to 22.5 μg of chlorophyll, and water in a total volume of 5.0 ml. The velocity of the DCIP photoreduction was estimated from the initial slope of the progress curve (reaction time, 20 s).

The experimental setup for the fluorescence measurement was described previously [18]. The light for fluorescence excitation was obtained from a tungsten–halogen lamp (45 W, Sylvania) with a combination of heat absorbing filter (HA-50),

blue filter (V-V40, Toshiba), and interference filter (KL-43, Nihon Shinku Kogaku). The exciting beam irradiated the chloroplast suspension (10 μg chlorophyll/ml) in a cuvette, in a 2-mm path length, at an angle of 45 ° to the normal line of the surface. The light intensity at the surface of the sample was $2.29 \cdot 10^3$ ergs $\cdot$ cm^{-2} $\cdot$ sec^{-1}. A part of the fluorescence emitted from the sample at room temperature (20 °C) at an angle of 45 ° to the incident light was isolated by a Bausch and Lomb grating monochromator (685 nm, half-band width of 5 nm) equipped with a cut-off filter (V-R62, Toshiba). The time-function signal, detected by an EMI 9659QB photomultiplier (spectral response, extended S-20) operated at 960 V, was amplified and recorded on a strip chart servo recorder. In each measurement, a dark adaptation of the sample for 5 min preceeded the recording of the time course of fluorescence intensity changes.

RESULTS

DCIP photoreduction

The percent inhibitions of DCIP photoreduction by spinach chloroplasts as induced by some derivatives of 1,10-phenanthroline and 2,2'-dipyridine are plotted as a function of the inhibitor concentration, in Fig. 1. Because of the lack or the solubility in water of the various substances used, all reactions were carried out in a mixture containing 5 % ethanol, except in the cases of 4,7-diphenyl-1,10-phenanthroline and 5-nitro-1,10-phenanthroline (where the solubility problem is more severe) where the reactions were carried out under 10 % ethanol. The presence of 5 % ethanol in the

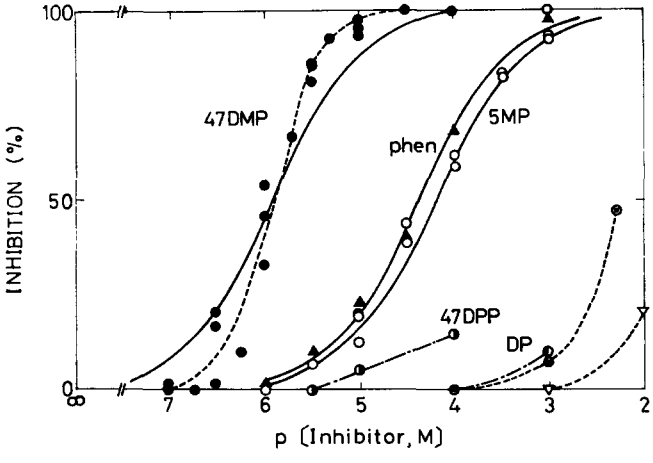

Fig. 1. Concentration dependence of the inhibition of DCIP photoreduction by some derivatives of 1,10-phenanthroline. Reaction mixture for DCIP photoreduction contained 125 μmoles Tris–HCl (pH 7.2), 0.2 μmole DCIP, spinach chloroplasts equal to 22.5 μg chlorophyll, ethanol and water in a total volume of 5.0 ml. The final concentration of ethanol in the reaction mixture was 5 %, except in the case of 4,7-diphenyl-1,10-phenanthroline (ethanol concentration, 10 %). Temperature, 20 °C. Concentration of inhibitors are expressed in $-\log_{10}$ of molar concentrations. ●, 4,7-dimethyl-1,10-phenanthroline (47 DMP); ▲, 1,10-phenanthroline (phen); ○, 5-methyl-1,10-phenanthroline (5 MP); ◑, 4,7-diphenyl-1,10-phenanthroline (47 DPP); ⊙, 4,7-diphenyl-1,10-phenanthroline sulfonate; ▽, 2,9-dimethyl-1,10-phenanthroline; ◐, 2,2'-dipyridine (DP). Curves for the first-order inhibition (—) and for the second-order inhibition (· · ·) are shown in the case of 4,7-dimethyl-1,10-phenanthroline (47 DMP).

TABLE I

INHIBITION OF DCIP PHOTOREDUCTION BY SOME DERIVATIVES OF 1,10-PHENANTHROLINE AND A FEW OTHER SUBSTANCES

The reaction mixture for DCIP photoreduction contained 125 μmoles Tris–HCl (pH 7.2), 0.2 μmole DCIP, spinach chloroplasts equal to 22.5 μg chlorophyll, ethanol and water in a total volume of 5.0 ml. Final concentration of ethanol in the reaction mixture was 5 %, except in the case of 4,7-diphenyl-1,10-phenanthroline and 5-nitro-1,10-phenanthroline (ethanol concentration, 10 %). Temperature, 20 °C. pI_{50}, $-\log_{10}$ of molar concentration of inhibitors giving 50 % inhibition of DCIP photoreduction; pK_a, acid-dissociation constant at 25 °C cited from ref. A; $\log \beta_3$, stability constant of Fe^{2+}–trichelate at 18 or 25 °C cited from ref. B.

Substance	pI_{50}	pK_a	Log β_3	ref. A	ref. B
Phenanthroline derivatives					
2,9-Dimethyl-1,10-phenanthroline	<2	6.15	≪ 4**	19	24
4,7-Dimethyl-1,10-phenanthroline	5.9	5.94	23.1	19	25
2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline sulfonate	<2	5.80*		20	
5-Methyl-1,10-phenanthroline	4.2	5.26	21.9	19	26
4,7-Diphenyl-1,10-phenanthroline sulfonate	<2.5	5.20*		20	
1,10-Phenanthroline	4.4	4.92	21.3	21	27
4,7-Diphenyl-1,10-phenanthroline	<3.5	4.84	21.8	22	28
5-Nitro-1,10-phenanthroline	<3	3.33	17.8*	23	29
Other heterocycles					
Pyridine	<3	5.21			
2,2′-Dipyridine	<3	4.44	16.9		
4,4′-Dipyridine	<3				
1,3-Diazine	<2				
1,4-Diazine	<2				
2,4,6-tris-(2-pyridyl)-*s*-triazine	<3				

* Temperature of the measurement is not specified.
** Stability constant of Fe^{2+}–monochelate.

reaction mixture had no appreciable effect on the rates or the percent inhibitions in the cases of 1,10-phenanthroline, 4,7-diphenyl-1,10-phenanthroline sulfonate, and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline sulfonate (reagents easily soluble in water) Ethanol, at 10 %, stimulated the reaction rate by about 2 %, but showed no effect on the percent inhibition. The noteworthy facts brought out in this experiment are as follows: (1) 4,7-dimethyl-1,10-phenanthroline is the most potent inhibitor among phenanthroline derivatives and a few other substances examined (pI_{50}, 5.9). 5-Methyl-1,10-phenanthroline also has nearly equal activity to 1,10-phenanthroline (pI_{50} values of 4.2 and 4.4 for 5-methyl-1,10-phenanthroline and 1,10-phenanthroline, respectively). The other substances showed very small or no activity in the concentration ranges tested; these include 5 phenanthroline derivatives and 6 other nitrogen-containing heterocyclic compounds i.e. pyridine, dipyridines, diazines, and a triazine. The pK_a values at 25 °C, the stability constants of Fe^{2+}–trichelates at 18 or 25 °C, and the pI_{50} values for the DCIP photoreduction estimated in this study at 20 °C, for these reagents, are summarized in Table I (see Discussion). (2) The second point of interest is concerned with the order of inhibition (n), as expressed in an equation

$H = G^n/[(I_{50})^n + G^n]$;

where H is the inhibition at a given concentration of inhibitor, I_{50} is a constant corresponding to the concentration of inhibitor at which 50 % inhibition occurs, and G is the concentration of inhibitor. The mode of inhibition of DCIP photoreduction by 4,7-dimethyl-1,10-phenanthroline under these experimental conditions was of the second order ($n = 2$); this is contrasted with the first-order character ($n = 1$) of the inhibition caused by 1,10-phenanthroline or by 5-methyl-1,10-phenanthroline.

Incubation of chloroplasts at 20 °C with 4,7-dimethyl-1,10-phenanthroline for 60 min did not change the percent inhibition, indicating practically non-progressive inhibition as in the case of 1,10-phenanthroline.

Fluorescence induction

Fig. 2 shows the effects of 4,7-dimethyl-1,10-phenanthroline on the transient changes in chlorophyll fluorescence at the onset of illumination in spinach chloroplasts. Upon illumination of chloroplasts without inhibitor, the fluorescence gradually increased in two phases from its initial level (F_0) to a steady state level (F_∞). According to Duysens and Sweers [10], this reflects a reduction in fluorescence quencher, Q. On addition of 4,7-dimethyl-1,10-phenanthroline, the F_0 level gradually increased and the rate of the second phase decreased with the rise in concentration of the reagent. Finally, at 10^{-4}–10^{-3} M of the reagent, $F_\infty - F_0$ almost disappeared, but not completely. 5-Methyl-1,10-phenanthroline also showed the same pattern of effect as 4,7-dimethyl-1,10-phenanthroline, but its effective concentration was higher than that of the latter. 2,9-Dimethyl-1,10-phenanthroline (10^{-3} M) and 4,7-diphenyl-1,10-phenanthroline ($10^{-4.5}$ M) showed no appreciable effect on the fluorescence induc-

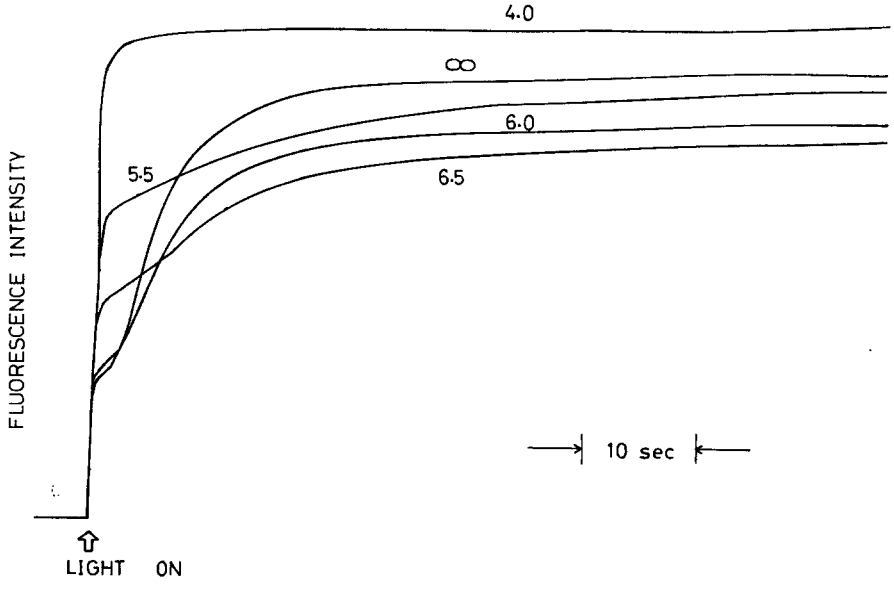

Fig. 2. Fluorescence induction curves at 20 °C in the presence of different concentrations of 4,7-dimethyl-1,10-phenanthroline. The sample contained 5 % ethanol. Number beside each induction curve indicates $-\log_{10}$ of molar concentration of the inhibitor.

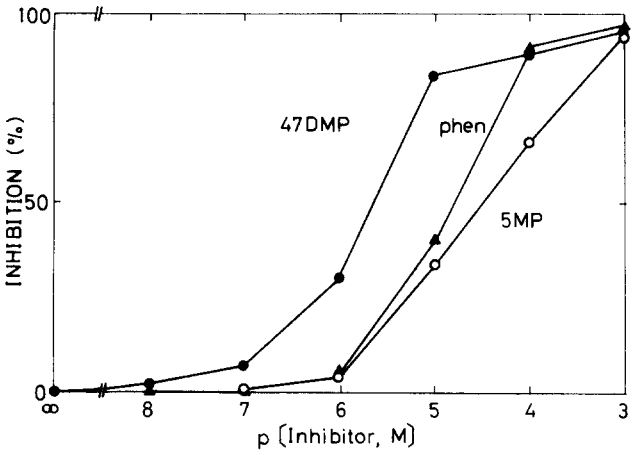

Fig. 3. Concentration dependence of the inhibition of fluorescence induction at 20 °C by 4,7-dimethyl-1,10-phenanthroline. See text for further explanation.

tion. 4,7-Diphenyl-1,10-phenanthroline sulfonate, 2.9-dimethyl-4,7-diphenyl-1,10-phenanthroline sulfonate, and 2,2'-dipyridine at 10^{-4}–10^{-2} M, on the other hand, decreased both the F_∞ level and $F_\infty - F_0$, but the effect was not reversed by the addition of a metal ion which forms a stable chelate complex with phenanthrolines e.g. Ni^{2+} (see a later part of this paper). The effect of 4,7-dimethyl-1,10-phenanthroline and of 5-methyl-1,10-phenanthroline on the fluorescence induction described above is identical with that of 1,10-phenanthroline, indicating that the site of inhibition in the photosynthetic electron transport chain of these reagents is identical with that of 1,10-phenanthroline [6–8].

To describe the fluorescence induction quantitatively, a quantity "work integral" was introduced by Murata et al. [7]. In Fig. 3, the percent inhibition of the values corresponding to the work integral (area above the induction curve, $\int_0^\infty (F_\infty - F_t)dt$, where F_t means fluorescence intensity at a given time, $t = t$) are plotted against inhibitor concentrations. In this plot, the concentration of the inhibitors which cause 50 % inhibition (pI_{50}) of the fluorescence induction was 5.7, 4.8 and 4.5 for 4,7-dimethyl-1,10-phenanthroline, 1,10-phenanthroline and 5-methyl-1,10-phenanthroline, respectively, values which are nearly identical with the pI_{50} values of these reagents in the inhibition of the DCIP photoreduction. As expected, the order of inhibition in the work integral is of a somewhat complex nature as compared with that in the DCIP photoreduction.

Effect of metal ions

The inhibition of DCIP photoreduction and of fluorescence induction by 1,10-phenanthroline, 5-methyl-1,10-phenanthroline, and 4,7-dimethyl-1,10-phenanthroline was reversed either by washing out the additions or by the addition of some metal ions. Fig. 4 shows the effect of 10^{-5} M of 4,7-dimethyl-1,10-phenanthroline on the activity of DCIP photoreduction by chloroplasts under varying concentrations of bivalent metal ions. The presence of Ni^{2+} (either as $NiCl_2$ or as $NiSO_4$), which forms a chelate complex with phenanthroline of a high stability constant, diminishes

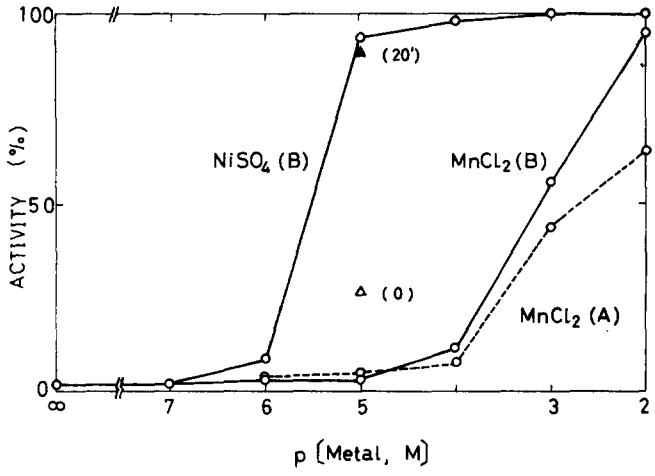

Fig. 4. Reversal of the phenanthroline inhibition of DCIP photoreduction by metal ions. Reaction mixture for DCIP photoreduction contained 125 μmoles Tris–HCl (pH 7.2), 0.2 μmole DCIP, 50 nmoles 4,7-dimethyl-1,10-phenanthroline, spinach chloroplasts equal to 22.5 μg chlorophyll, ethanol (final concentration, 5 %) and water in a total volume of 5.0 ml. Temperature, 20 °C. $NiSO_4$ or $MnCl_2$ was added to the reaction mixture before (B) or after (A) the addition of chloroplasts. The activity of DCIP photoreduction was measured immediately after (△) and 20 min after (▲) the addition of $NiCl_2$.

the inhibitory effect of 4,7-dimethyl-1,10-phenanthroline at concentrations comparable with the inhibitor (see Kautsky et al. [6]), while metal ions of low stability constant, such as Mn^{2+} in Fig. 4, diminish the inhibition at concentrations much higher than that of the inhibitor. The order of the additions of chloroplasts, inhibitor, and metal ion seriously affected the results; addition of a metal ion after incubation of chloroplasts with inhibitor lowered the effect, but incubation at room temperature for a few minutes is sufficient to attain the final value (equal to the value in the case of the incubation of the metal ion with the inhibitor before the addition of chloroplasts) The effectiveness in the reversal of the inhibition of both the DCIP photoreduction and fluorescence induction was in the following order: Ni^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, and Mn^{2+}. Mg^{2+}, Ca^{2+} and Ba^{2+} were almost inactive in both cases.

DISCUSSION

It is usually assumed that the sequestration of trace metals is involved in the phenanthroline inhibition, but the possibility of action without the intervention of a metal should not be ignored at present. The metal ion, if involved in the inhibition, is not present as hydrated ions in the chloroplast membranes or attached by some coordinate bonds to protein (e.g. by sulfur and/or nitrogen linkage) with a low stability constant, for higher concentrations of inhibitor (e.g. 10^{-3} M of 4,7-dimethyl-1,10-phenanthroline) cannot remove the metal ions (reversibility). On the other hand, the interaction of the reactive component in chloroplasts with phenanthroline derivatives is weak compared with that of the component with membranes; the inhibition caused by phenanthroline derivatives can easily be reversed by the addition of relatively

lower concentrations of metal ions which form a chelate complex with a high stability constant.

Structure–activity relations

A given structural change in the ligand, by virtue of steric, electronic, mass and polar effects, can be expected to bring about dramatic changes in the inhibition properties of 1,10-phenanthroline. As described in Table I, the inhibitory activity of phenanthroline derivatives on DCIP photoreduction (and also on fluorescence induction) seems mostly to be parallel with the stability constants of the Fe^{2+}–trichelates of these reagents (and those of the chelate compounds formed by the other bivalent ions such as Co^{2+}, Ni^{2+} etc.), where two different factors have been shown to be concerned: first, the electronic effects of substitutions in 1,10-phenanthroline as revealed by the acid-dissociation constants (compiled in Table I) is one of the primary factors for the inhibition; thus 4,7-dimethyl-1,10-phenanthroline is a more active compound than 1,10-phenanthroline or 5-methyl-1,10-phenanthroline, and 5-nitro-1,10-phenanthroline is nearly inactive compared with 1,10-phenanthroline. The second factor is a steric hindrance or "packing effect" as induced by the introduction of methyl groups at 2,9-positions; this type of steric hindrance has been shown to be responsible for the specificity of the so-called cuprine reagents in analytical chemistry [30] (which form an obstacle to the successive coordination of two or more ligands in those cases, and in addition to this in this case some hindrances due to the local structure of the coordination site can be expected). In addition to these two factors, a different mode of steric hindrance or packing effect seems to arise due to the introduction of phenyl groups at 4,7-positions; this also can be expected to greatly discourage the interaction of ligands with the reactive site (in the cases of 4,7-diphenyl-1,10-phenanthroline and 4,7-diphenyl-1,10-phenanthroline sulfonate). Methyl or phenyl substituents tend to decrease the water solubility of the ligand; partition of the ligand to the lipophilic portion of the chloroplast membranes can expected to be enhanced. The solubility effect as discussed in the case of DCMU [13] does not seem to be a principal factor in the case of phenanthroline, for 2,9-dimethyl-1,10-phenanthroline and 4,7-diphenyl-1,10-phenanthroline were nearly inactive in the inhibition.

Order of the inhibition

Differences in the order of inhibition are evident between 4,7-dimethyl-1,10-phenanthroline and the other reagents. The mechanism of the "second-order" inhibition in general was discussed earlier by Huzisige [31]. The following two types of reaction can be expected: (1) the second order inhibition may arise when bimolecular fixation of the inhibitor at a reaction site occurs. (2) The process involves a number of intermediate steps and the factor which causes the first-order inhibition of some of the intermediate steps involved, may also cause the second-order inhibition. 4,7-Dimethyl-1,10-phenanthroline, if it reacts with metal ion, can easily be expected to cause the first type of inhibition (coordination of two molecules of ligand at a reactive site), but some mechanisms which favor monomolecular coordination (and which still cause the inhibition) must be present in the cases of 1,10-phenanthroline and of 5-methyl-1,10-phenanthroline.

ACKNOWLEDGMENTS

The author is indebted to Professor Hirosi Huzisige (Okayama University) for his valuable discussion and support during the course of this investigation.

Grants from the Ministry of Education (Japan) supported this research.

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