effective uncouplers of photosynthetic phosphorylation. The action of ammonium salts are reversible by simply washing the chloroplasts.

It is suggested tentatively that a P/2e ratio of more than 1.0 might be possible for ATP formation coupled to ferricyanide reduction.

This work was greatly expedited by the assistance of Mrs. M. Evans.

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COMPARISON OF FERRICYANIDE AND 2,3',6-TRICHLOROPHENOL INDOPHENOL AS HILL REACTION OXIDANTS [1,2,3]

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With the development of a spectrophotometric assay of the Hill reaction with ferricyanide (9), a comparison between the rates of reduction of ferricyanide and the dye, 2,3',6-trichlorophenol indophenol by intact chloroplast appeared desirable. Previous attempts at such a comparison were hampered by the different methods used for the two oxidants. These differences are minimized by following the reduction of both oxidants spectrophotometrically. Under comparable circumstances, one observes that intact chloroplasts reduce ferricyanide at a much lower rate than that at which they reduce indophenol dye. This difference has been studied and a procedure has been devised which permits chloroplasts to reduce both acceptors at the same rate.

MATERIALS AND METHODS

REAGENTS: 8-Hydroxyquinoline and a,a'-dipyridyl were purchased from the Baker Chemical Company and Fisher Scientific Company, respectively. Triphosphopyridine nucleotide (TPN) was obtained from the Pabst Laboratories. 2,3',6-Trichlorophenol indophenol was a product of Eastmen Organic Chemicals.

PREPARATION OF CHLOROPLASTS: Fresh spinach was obtained at the local market, and whole chloroplasts were prepared from it either by the method of Jagendorf (7) or of Arnon et al (2). When sonicated chloroplasts were used, sonication was performed in

a 10-kilocycle Raytheon sonic oscillator. Freshly prepared chloroplasts were used in all experiments and Hill reaction activity was measured immediately after preparation of the chloroplasts, unless otherwise indicated.

MEASUREMENTS. All spectrophotometric measurements were performed with a Beckman Model B or DU spectrophotometer using 3.0 ml cuvettes having a light path of 1.0 cm. Hill reaction activity measurements with 2,3′,6-trichlorophenol indophenol were performed as described by Jagendorf (7). Measurement of ferricyanide reduction was accomplished as described by Krogmann and Jagendorf (9). As previously reported, the oxidant concentrations chosen for these measurements were 0.08 micromoles (μM) of the dye and 1 μM of ferricyanide per 3.0 ml final volume. Chlorophyll concentrations ranged from 0.003 to 0.016 mg per ml in the final reaction mixture. All Hill reaction activity measurements were made at room temperature (usually 25° C) with appropriate precautions to avoid a temperature rise during illumination. The light intensity was approximately 5000 ft-c in these experiments, unless otherwise noted. The light source was a 150-watt incandescent lamp. Control measurements had shown that dye reduction was dependent on the presence of active chloroplasts, and no direct photoreduction of the dye occurred under these conditions. The reaction mixture was buffered at p.H 7.2 with either 0.05 M TRIS or 0.05 M phosphate buffer, and included 0.01 M NaCl. Photosynthetic phosphorylation was measured according to the Arnon procedure (2) as modified by Jagendorf and Avron (8) with phenazine methosulfate used as the catalytic cofactor. Chloroplast reduction of TPN was measured as described by San Pietro (13). Chlorophyll concentration was determined by the method of Arnon (1).

RESULTS

HILL ACCEPTOR DIFFERENCES USING INTACT CHLOROPLASTS: When rates of ferricyanide and dye reduction in the Hill reaction were compared using thoroughly sonicated chloroplasts, very little difference was observed (9). However, when intact chloroplasts, prepared in 0.05 M phosphate buffer pH 7, 0.4 M sucrose, 0.01 M KCl medium were used, the rate of Hill reaction with ferricyanide was only 30 to 40 % of the rate with dye. An experiment was performed to check the degree of sonication necessary to equalize the rates to the 2 acceptors. Chloroplasts were diluted in the phosphate, sucrose, KCl medium and sonicated for varying intervals of time. The data obtained are presented in figure 1. The initially high dye reduction activity decreased steadily with increasing time of sonication. The initially low rate of ferricyanide reduction first increased with sonication, then decreased. After 120 seconds sonication, the rates to the 2 acceptors were almost equal.

EFFECT OF HYPOTONIC MEDIUM AND OF AGING: It was reasoned that a medium of very low tonicity
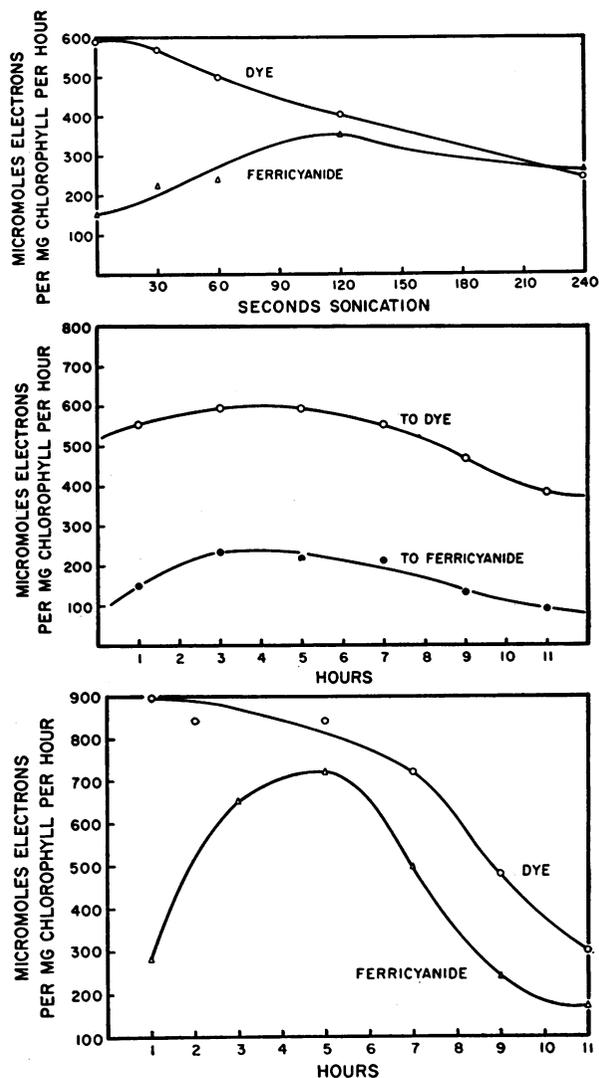

FIG. 1 (top). The effect of time of sonication of chloroplasts on the rates of 2,3′,6-trichlorophenol-indophenol and ferricyanide reduction by the Hill reaction. The chloroplasts were prepared and washed once in the phosphate-sucrose-KCl medium. The chlorophyll concentration during sonication was 0.01 mg per ml. Assays were performed as previously described, using sufficient chloroplast material to give a change in optical density of at least 0.2 after 30 seconds of illumination with saturating light.

FIG. 2 (center). The effect of aging chloroplasts in hypotonic medium on the rates of reduction of 2,3′,6-trichlorophenol indophenol and ferricyanide. The chloroplasts used here were prepared and washed 5 times in 0.35 M NaCl. The curves show the effect of aging chloroplasts in 10^{-3} M phosphate buffer pH 7 at zero degrees on the rate of Hill activity. The chlorophyll concentration of preparations during aging was 0.147 mg per ml.

FIG. 3 (bottom). The effect of aging chloroplasts in 0.35 M NaCl on the rate of Hill activity. Aging in NaCl was carried out at a chlorophyll concentration of 1.17 mg per ml at zero degrees.

might also equalize the rates to the 2 acceptors by causing disruption of the chloroplast structure. Chloroplasts prepared and washed 5 times in 0.35 M NaCl were used in this experiment. The final chloroplast suspension was divided in half, one half centrifuged, and the sedimented chloroplasts resuspended in 10 times the original volume of 10^{-3} M phosphate buffer pH 7. The other half was held as a control. Changes in Hill activity to the 2 acceptors were followed with time. As can be seen in figure 2, decreasing the tonicity of the medium had no immediate effect on ferricyanide reduction. Even prolonged exposure to hypotonic medium did not duplicate the effect of sonication. However, the control samples from the original 0.35 M NaCl suspension showed a 3-fold increase in ferricyanide reducing activity after 5 hours aging at zero degrees (fig 3).

EFFECT OF DILUTION: It soon became apparent that dilution of chloroplasts in NaCl, to a final chlorophyll concentration of approximately 0.01 mg per ml resulted in an immediate increase in the rate at which they could reduce ferricyanide in the light, even when the chloroplasts had been prepared from leaves ground in NaCl. The new rate of ferricyanide reduction approximately equalled the rate of dye reduction. The rate of dye reduction was hardly affected by this dilution process. Maximal values for reduction of the 2 Hill acceptors attained with the spectrophotometric assay of activated chloroplasts are presented in table I.

EFFECT OF HYDROGEN ION CONCENTRATION: If the pH is maintained at neutrality by buffering the NaCl solution used for diluting the chloroplasts, ferricyanide reducing activity remains low (table II). The optimal pH for the increase in ferricyanide reducing ability turned out to be between 6 and 6.3 (fig 4). This pH refers only to the dilution step; the chloroplasts had been prepared in unbuffered NaCl and the assay was performed at pH 7. It should be noted that in the assay procedure the chloroplasts are diluted at least as much as they are in the dilution step.

The neutral pH during the assay presumably prevents activation during the course of the reaction.

The activation of chloroplasts to a high rate of ferricyanide reduction is apparently irreversible. As all assays were performed at pH 7, the activation by exposure to lower pH did not depend on the maintenance of that pH. When chloroplasts were centrifuged from a dilution medium buffered at pH 6.3 and resuspended in a minimal volume of low salt medium buffered at pH 7, the Hill reaction activity to ferricyanide remained high.

EFFECT OF SALT AND CHLOROPLAST CONCENTRATION: The sodium chloride concentration of the diluent was varied in a series of experiments. Sodium chloride concentrations between 0.25 and 0.4 molar proved optimal. Lower concentrations failed to cause activation while exposure of chloroplasts to sodium chloride concentrations higher than 0.4 molar inhibited Hill activity. Inhibition of chloroplast fragments by sodium chloride was observed by Spikes et al who reported 50 % at 0.49 M (14).

Optimal activation required dilution of the chloroplasts to a final chlorophyll concentration of 0.01 mg per ml or less. This aspect was difficult to study quantitatively since activation would occur in the more concentrated suspensions, only at a much slower rate.

EFFECT OF LIGHT INTENSITY: The rate of the Hill reaction was determined as a function of light intensity between 25 and 2000 ft-c, before and after chloroplast activation. The resultant curves are plotted in figure 5, in the form of 1/rate vs 1/light intensity, as suggested by the kinetic treatment of Lumry, Spikes and Eyring (12). In this formulation the slope of the line is related to 1/rate constant for the limiting light reaction, and the intercept is related to

TABLE I

MAXIMAL RATES OF HILL ACTIVITY

PREPARATION	RATES AS μM OF ELECTRONS/ MG CHLOROPHYLL/HOUR	
	TO DYE	TO FERRICYANIDE
Activated chloroplasts avg		
of 6 preparations	912	803
Range	720 to 992	640 to 1219
Intact chloroplasts avg		
of 8 preparations	744	215
Range	583 to 936	120 to 273

Assays were performed as previously described using 10 to 50 μg chlorophyll per assay with a 30-second exposure to saturating light. In all cases, activation of the chloroplasts was achieved by dilution of the preparation in 0.35 M NaCl buffered at pH 6.0 to a final chlorophyll concentration of approximately 0.01 mg per ml.

TABLE II

MAXIMAL RATE OF HILL ACTIVITY ATTAINED UNDER VARIOUS CONDITIONS OF CHLOROPLAST DILUTION

DILUTION TREATMENT	CHLOROPHYLL CONCENTRATION BEFORE ASSAY MG/ML	RATE AS μM OF ELECTRONS/ MG CHLOROPHYLL/HOUR	
		TO DYE	TO FERRICYANIDE
Control	1.2	600	220
Diluted 100 : 1 with 0.35 M NaCl	0.012	720	640
Diluted 100 : 1 with 0.35 M NaCl, 0.01 M phosphate buffer, pH 7.	0.012	630	255
Diluted 100 : 1 with 0.35 M NaCl 0.01 M TRIS pH 7.	0.012	684	276

The chloroplasts used here were prepared and washed once in 0.35 M NaCl prior to dilution. The assays were performed as previously described, using 10 to 50 μg chlorophyll per 3 ml reaction mixture with a 30-second exposure to saturating light.

1/rate constant for the limiting dark reaction. With this analysis it is evident that the dilution treatment has caused a large increase in the limiting dark reaction, but has increased the limiting light reaction only about 50 %.

INHIBITION BY METAL CHELATORS: Activated and control chloroplasts were compared with regard to light dependent ferricyanide reduction (table III). The preparation diluted at pH 6.2 is seen to reduce ferricyanide 3 times as fast as does the control preparation. The 2 sets of chloroplasts were practically identical in their ability to reduce trichlorophenol indophenol. They were then compared in regard to sensitivity to 8-hydroxyquinoline and a,a'-dipyridyl, using indophenol dye as the oxidant. The Hill reaction activity of the treated chloroplasts is much more sensitive to inhibition by chelating agents than is that of the control chloroplasts. Thus the treatment which increases the activity of the chloroplasts in photoreduction of ferricyanide also increases the sensitivity of the chloroplasts to these 2 inhibitors.

CORRELATION WITH PHOTOSYNTHETIC PHOSPHORYLATION: An attempt was made to correlate the 2 levels of ferricyanide reducing ability with the process of photosynthetic phosphorylation. Cyclic phosphorylation was studied, using the experimental procedure of Arnon et al (2) as modified by Jagendorf and Avron (8). Phenazine methosulfate was the catalytic cofactor employed. The data from triplicate experiments are presented in table IV. It can be seen that chloroplasts which show the greatest Hill activity with ferricyanide have the least ability to carry out phosphorylation.

CORRELATION WITH PYRIDINE NUCLEOTIDE REDUCTION: Next a correlation was sought between the ability to photoreduce ferricyanide and pyridine nucleotide. In these experiments, the chloroplasts were saturated with the pyridine nucleotide reducing enzyme from spinach described by San Pietro (13). Chloroplasts were made limiting in the reaction mixture to reveal differences in their abilities to participate in the reduction of TPN. As can be seen in table V,
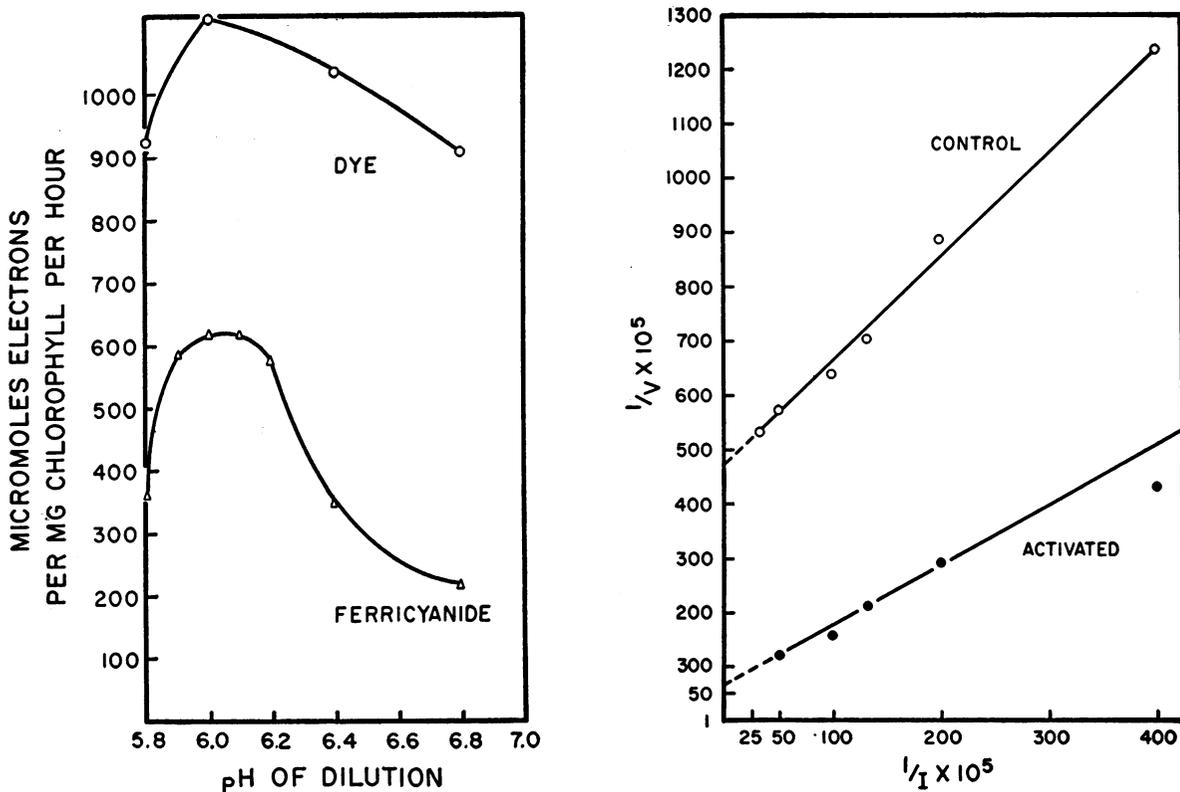

FIG. 4 (left). The effect of pH of dilution pretreatment on the rate of reduction of 2,3',6-trichlorophenol indophenol and ferricyanide by the Hill reaction of whole chloroplasts. The chlorophyll concentration of the diluted chloroplast suspension equals approximately 0.01 mg per ml. Assays were performed as previously described using 10 to 30 μg chlorophyll per 3 ml reaction mixture with a 30-second exposure to saturating light.

FIG. 5 (right). The effect of light intensity on the rate of ferricyanide reduction by chloroplasts before and after activating pretreatment. Assays were performed as previously described. The control chloroplasts were assayed at 30 μg chlorophyll per 3 ml reaction mixture and the activated chloroplasts at 7.5 μg. The upper line (O) is that obtained with control chloroplasts. The lower line (●) was obtained with the same chloroplast preparation diluted 100-fold in 0.35 M NaCl buffered at pH 6. Light intensities were varied by placing the cuvettes at various distances away from the tungsten lamp; the intensity was measured at each distance by a Weston light meter. The units of light intensity are ft-c, those for velocity are μ equivalents of ferricyanide reduced.

TABLE III

CORRELATION OF FERRICYANIDE REDUCING ACTIVITY WITH INHIBITION BY METAL CHELATORS

PREPARATION	HILL REACTION ACTIVITY AS μM OF ELECTRONS/MG CHLOROPHYLL/HOUR	
	TO DYE	TO FERRICYANIDE
Control	652	125
Activated to ferricyanide reduction	688	403

Inhibition of Hill activity

Molar concentration	0.0	1.6×10^{-3}	3.3×10^{-3}	6.6×10^{-3}

8-Hydroxy-quinoline				
Control	0.0	0.0	20 %	92 %
Ferricyanide activated	0.0	29 %	68 %	92 %

a, a'-Dipyridyl				
Control	0.0	0.0	0.0	45 %
Ferricyanide activated	0.0	27 %	48 %	82 %

All assays were performed at 0.003 mg chlorophyll per ml. Comparisons of the effects of inhibitors were made using the 2,3',6-trichlorophenol indophenol assay of Hill activity. Activation was accomplished as in table I.

the dilution process quadrupled the rate of ferricyanide reduction but had little or no effect on the rate of pyridine nucleotide reduction.

DISCUSSION

When the spectrophotometric assay of ferricyanide reduction is used to measure the Hill activity of intact chloroplasts, a large difference in the rate of reduction of ferricyanide as compared with that of the dye 2,3',6-trichlorophenol indophenol is revealed. In both

TABLE IV

CORRELATION OF HILL ACTIVITY TO FERRICYANIDE WITH PHOTOSYNTHETIC PHOSPHORYLATION

PREPARATION	μM Fe $(CN)_6^{-3}$ REDUCED/MG CHLOROPHYLL/HOUR	μM PO$_4$ ESTERIFIED/MG CHLOROPHYLL/HOUR
I Control	172	422
Diluted in NaCl, pH 6	800	220
Diluted in phosphate-sucrose-KCl medium	175	515
II Control	210	500
Diluted in NaCl pH 6	774	57
Diluted in phosphate-sucrose-KCl medium	227	428
III Control	200	510
Diluted NaCl	821	88

assay systems, the oxidant concentrations are sufficiently low so as to avoid their influencing the Hill reaction in the manner noted by Lumry, Spikes and Eyring (12). The reactions are zero order with respect to either dye or ferricyanide, in the concentration range used. Ferricyanide reduction by the Hill reaction of intact chloroplasts occurs at only 20 to 30 % of the rate of dye reduction.

The rate of reduction of ferricyanide is increased by sonic disruption of the chloroplasts. The rate of ferricyanide reduction can be raised to that of dye reduction by the NaCl dilution procedure described here. The dilution procedure has little or no effect on the rate of dye reduction depending on the chloroplasts, while it may increase ferricyanide reduction as much as 5-fold. A less rapid activation of ferricyanide reduction is seen with chloroplasts held in the usual storage conditions of 0.4 M sucrose buffered at pH 7.8. No gross difference could be detected in a microscopic examination of chloroplasts diluted in NaCl, compared to untreated control chloroplasts.

An analysis of the rate of the Hill reaction as a function of light intensity permits one to discern whether a limitation to Hill activity occurs at the photochemical step or at some dark reaction (12). With this analysis it can be seen that the inherent inhibition of the Hill reaction of untreated chloroplasts

TABLE V

CORRELATION OF HILL ACTIVITY TO FERRICYANIDE WITH TRIPHOSPHOPYRIDINE NUCLEOTIDE REDUCTION

PREPARATION	μM Fe $(CN)_6^{-3}$ REDUCED/MG CHLOROPHYLL/HOUR	μM TPN REDUCED/MG CHLOROPHYLL/HOUR
Control	233	75.2
		71.0
Ferricyanide activated	980	62.4
		63.0

Activation is achieved as in table I.

acts at a dark step. This inhibition is released by the NaCl dilution treatment described here.

The accelerating effect on ferricyanide reduction of the treatments described earlier, may be interpreted most easily as a loss of coupling between electron transport and phosphorylation (see (3) and (5)). The loss of ability to accomplish cyclic phosphorylation is consistent with this interpretation. A final proof for it is presented in the accompanying report (10).

Chloroplasts which show maximal rates of ferricyanide reduction also show increased sensitivity to metal chelators as inhibitors (table III, and (11)). This greater sensitivity could be related to a different mechanism for electron transport in uncoupled chloroplasts. Alternatively, the treatments may in some way expose previously inaccessible portions of the electron transport chain to chelating agents.

Bishop, Lumry and Spikes observed an increase in ferricyanide reducing ability of chloroplast frag-

ments in the course of aging at 24° C (6). Although it is likely that a similar interpretation will explain their observations, the leaf material (chard or sugar beet) was different, and they used chloroplasts already fragmented by a Waring blendor. Also it was necessary for the chloroplasts to be prepared under nitrogen for the effect to appear in their study.

The rate of reduction of TPN is not stimulated when ferricyanide reduction increases. Although this is not easy to explain, it is consistent with the fact that TPN reduction, under these conditions, is not stimulated by simultaneous phosphorylation (San Pietro, personal communication).

Indophenol dye reduction is much faster than ferricyanide reduction in the first place, and is not nearly as responsive to the reported treatments in the second place. The inference is that the reduction of indophenol dye is primarily by means of uncoupled electron transport, even in intact chloroplasts. This conclusion is supported by the failure to observe appreciable ATP formation while indophenol dye is being reduced (4).

A significant difference between the indophenol dyes (di- and trichloro) and ferricyanide as Hill oxidants was first observed by Witt, Moraw and Müller (15). By observing the kinetics of disappearance of a light-induced absorption increase at 512 mμ in isolated chloroplasts, they came to the conclusion that the transfer of electrons to indophenol dyes is a simpler and more rapid process than is the transfer to ferricyanide. Witt's conclusion is completely in accord with a rate limiting step in ferricyanide reduction (i.e., the coupling mechanism) which is absent or at least less important in indophenol dye reduction. It is also consistent with one possible mechanism for uncoupled electron transport, i.e., the transfer of electrons to indophenol dye from some component preceding the coupled step. This would amount to a "short circuit" of that part of the electron transport system which is coupled to phosphorylation.

It seems likely that the difference between rates of reduction of indophenol dye and of ferricyanide is one of degree of inhibition by the coupling mechanism. Thus indophenol dye reduction responds to some extent to uncoupling treatment (fig 3 and table II) and is stimulated to some extent by phosphorylating reagents (5). On the other hand some ferricyanide reduction occurs in the absence of phosphorylation even in the best chloroplast preparations now available. Finally, Spikes et al (12) observed stimulation of the Hill reaction at high concentrations of ferricyanide. This stimulation could be accounted for by an uncoupling of electron transport from the phosphorylation mechanism, due to excessive concentrations of ferricyanide.

SUMMARY

1. Freshly prepared intact spinach chloroplasts when illuminated will reduce 2,3',6-trichlorophenol indophenol at appreciably higher rates than ferricyanide.

2. Sonication, aging, or a pretreatment of the chloroplasts involving dilution in 0.35 M NaCl at pH 6 all increase the rate of ferricyanide reduction.

3. Associated with the more rapid reduction of ferricyanide is an increased susceptibility of the chloroplast to metal chelating agents.

4. The possible relation of these effects to the uncoupling of light induced electron transport from phosphorylation is discussed.

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