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Damage to Spinach Chloroplasts Induced by Dark Preincubation with Ferricyanide [1, 2, 3]

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While ferricyanide has been used as an electron acceptor for light-induced electron flow in chloroplasts since the early work of Hill and Scarisbrick (14), an oxidant this strong might be expected to have additional effects. Indeed, Lumry and Spikes (21) in a careful kinetic analysis of the Hill reaction, found that ferricyanide when used at concentrations above 1 mM seemed to inhibit the light step(s) of the Hill reaction, and to stimulate the limiting dark reaction in a manner which we would now suspect of being related to uncoupling. A rather large number of studies have been carried out in which ferricyanide was used to effect a dark oxidation of one or another component of the electron transport chain, either in chloroplasts (1, 4, 13, 16, 17, 18, 20, 22, 24) or in bacterial chromatophores (7, 8, 11, 20).

We noticed that preincubation of chloroplasts with ferricyanide in the dark, at the concentrations ordinarily employed to measure the Hill reaction, appeared to have a deleterious effect on the subsequent rate of photoreduction. The present work is an attempt to explore this and related phenomena more systematically.

A preliminary account of this work has appeared (5).

Materials and Methods

Chloroplasts were prepared from commercial spinach by grinding briefly in a Waring blendor in STKM[5]. The resulting homogenate was filtered through cheesecloth and centrifuged at 1200 × g in a refrigerated centrifuge. The chloroplast pellet was resuspended, and either used without washing or washed once in fresh STKM.

In experiments where chloroplasts were preincubated with ferricyanide solutions and washed prior to assay, 3 ml aliquots of untreated chloroplast sus-

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[5] Abbreviations include: STKM, 0.4 M sucrose, 0.02 M tris-HCl, pH 8.0, 0.01 M KCl, 0.001 M $MgCl_2$; TCPIP, trichlorophenolindophenol; CMU, *p*-chlorophenyl dimethyl urea; PMS, phenazine methosulfate; P^{32}, radioactive isotope of phosphorous as orthophosphate, FeCN-$K_3Fe(CN)_6$.

pension were pipetted into plastic centrifuge tubes, each containing 35 ml of the preincubation solutions. Chloroplasts and media were generally incubated for 20 minutes at 4° in complete darkness. The chloroplasts were then collected by centrifugation, resuspended in fresh STKM, and washed once prior to assay. All operations connected with chloroplast preparation and storage were carried out at temperatures of 0 to 4°.

In other experiments where the Hill reaction was measured in the same suspension used for ferricyanide preincubation, the reaction mixture containing chloroplasts and ferricyanide was made up and test tubes loaded in room light of 10 to 15 ft-c. The preincubation condition was started by turning off the light (or adjusting the light intensity to some known level), almost simultaneously adding trichloroacetic acid to the O-time control tubes.

In the standard assay for ferricyanide reduction, 0.1 ml aliquots of washed chloroplasts at a known concentration were pipetted into a series of 12 × 75 mm test tubes, and then 2.3 ml of reaction mixture added as quickly as possible to each by means of a repeating syringe (Clay-Adams Aupipette). The reaction mixture, containing 40 μg of chlorophyll, 40 μmoles tris-HCl (pH 8.0), 140 μmoles of KCl and 1.5 μmoles of $K_3Fe(CN)_6$ in a total volume of 2.4 ml, was illuminated for 1.5 to 5 minutes at room temperature. The incident light intensity was usually about 2000 ft-c, provided by a 100 w tungsten lamp, and filtered through 10 cm of a solution containing acid ferrous ammonium sulfate. Lower light intensities were achieved by interposing metal screens of known percent transmission. At the end of the reaction 0.6 ml of 12 % trichloroacetic acid was added, the chloroplasts removed by centrifuging, and the OD determined at 420 mμ to estimate changes in ferricyanide concentration. In some experiments, small amounts of ferrocyanide were measured by the method of Avron and Shavit (3) using 1,10 phenanthroline as the chelating agent.

Reduction of trichlorophenol indophenol (TCPIP) was performed similarly, in a reaction volume of 3.0 ml containing 0.1 μmole of TCPIP. OD was measured at 625 mμ directly in the cuvette without denaturing the chloroplasts, and the millimolar extinction coefficient was taken to be 25. Reaction time for dye reduction was usually 30 seconds.

Reduction of NADP was measured in a 1.0 ml reaction volume, containing 25 μmoles of tris-HCl (pH 8.0), 25 μmoles of KCl, 0.2 to 0.5 μmole of NADP at pH 8.0, and spinach ferredoxin (photosynthetic pyridine nucleotide reductase) as indicated in the text. When phosphorylating reagents were used, these consisted of 5 or 18 μmoles of phosphate, 0.33 μmole of ADP at pH 8.0, and either 0.67 or 2 μmoles of $MgCl_2$, with or without added P^{32}. Absorbancy was determined at 340 mμ before and after illumination, and compared to a dark control. With reduced TCPIP as electron source rather than water the reaction mixture was the same, with the addition of 0.05 μmole of TCPIP, 6 μmoles of ascorbate at pH 8.0, and 0.02 μmole of CMU.

Phosphorylation with PMS as cofactor was measured in 2.4 ml reaction volumes, containing Tris and KCl as above, and in addition 4 to 5 μmoles of ADP at pH 8.0, 10 μmoles of $MgCl_2$, 0.15 μmole of PMS and 13 to 40 μmoles of potassium phosphate at pH 8.0, containing radioactive phosphate with 10^5 to 10^6 cpm. Uptake of P^{32} into organic form was measured by a slight modification of the Lindberg and Ernster procedure (19). Modifications included replacing the silicotungstic acid with 12 % trichloroacetic acid and increasing somewhat the amount of ammonium molybdate in order to complex larger amounts of phosphate. The residual water layer was pipetted onto a planchet, dried and counted.

Fluorescence excitation spectra of chloroplasts were determined by Dr. W. Butler, U.S.D.A., Beltsville as previously described (6). Chlorophyll was measured by the procedure of Arnon (2). Spinach ferredoxin (previously called PPNR) was prepared by the method of San Pietro (23) through the Dowex-bentonite step. The Dowex-bentonite supernatant was then purified and concentrated by chromatography on diethylaminoethylcellulose (Whatman DE). Protein concentrations were estimated from the OD of the solutions at 280 and 260 mμ using enolase as a standard. Redox potential determinations were made using a Beckman Model G pH-meter, with a platinum electrode replacing the glass electrode.

ADP, NADP, PMS, and Tris were from the Sigma Corporation; ascorbate and 1,10 phenanthrolene were purchased from the Fisher Scientific Company; TCPIP (practical) from Eastman Kodak; and P^{32} from Squibb.

Results

Low Ferricyanide Concentrations. Figure 1a shows the rates of ferricyanide photoreduction by chloroplasts preincubated in the dark at room temperature, either with or without ferricyanide at 0.5 mM. An inhibition due to the preincubation with ferricyanide is evident. In various experiments the inhibition ranged from 20 to 50 %.

Onset of this inhibition is quite rapid, with most of the effect accomplished during the first 4 minutes at room temperature (fig 2). More extended experiments, at 0°, show a slightly more complex picture (data not shown). The initial fast inactivation is followed by a slower one that is seen only when the assay is run in the presence of either phosphorylating reagents or uncoupling reagents. This continuing slow inactivation (from 25 % at 10 min to 35 % at 60 min) is accompanied by a slow uncoupling effect which increases the basal rate. The 2 together counteract each other to produce a spurious equilibrium in measurements of the basal rate.

Figure 1b shows that the inhibition is observed equally well if the dark period (3 min, in this experiment) is interposed in the middle of the ferricyanide Hill reaction. On the other hand, in every

experiment, once a rapid rate of the Hill reaction had been established in the light, no diminution of activity was seen during time periods sufficient to cause inactivation in the dark. This suggested that illumination protects the chloroplasts from damage by ferricyanide, a fact demonstrated directly in figure 2. The light intensity needed to give complete protection was found to be only 200 μw per sq cm, or approximately 10 ft-c (fig 3). This intensity drives the Hill reaction at only 2 % of its maximal rate, and is itself one-twelfth the intensity needed for 50 % of the maximal rate (see also fig 5).

The effects of ferricyanide described so far are not shared by ferrocyanide at the same concentration, or by TCPIP.

Higher Ferricyanide Levels. If ferricyanide concentrations of 50 mM are used the damage is more extensive (table I and fig 2) with inhibition rapidly reaching 70 % and continuing up to 90 %. Also, some of the inhibitory effect is shared by ferrocyanide

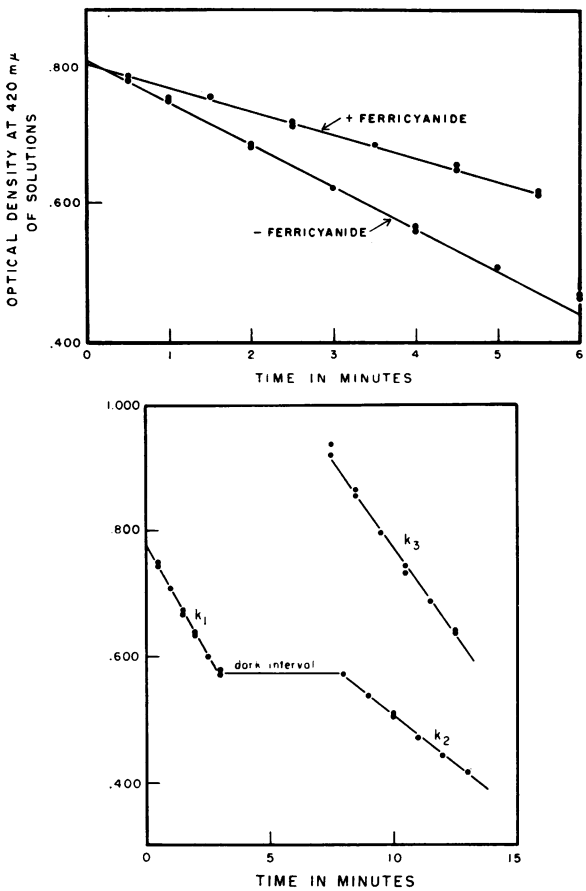

FIG. 1a (*above*). Inhibition of ferricyanide reduction by dark preincubation with ferricyanide. Chloroplasts and reaction mixture as described in Methods section were incubated in the dark at room temperature for 5 minutes with ferricyanide added either before or just after the dark incubation period. The entire 18 ml mixture was exposed to light, and duplicate 2.4 ml aliquots removed at the times shown for assay of residual ferricyanide at 420 mμ. The concentration of chloroplasts was 17 μg per ml, of ferricyanide was 1.0 mM. The calculated rates were 279 and 153 μmoles per mg chlorophyll per hour, respectively for control and pretreated chloroplasts in this treatment.

FIG. 1b (*below*). Reaction conditions as above. In section k_1 chloroplasts and reaction mixture were exposed to light without dark preincubation. This tube kept in the dark for 3 minutes, then illumination and withdrawal of samples was continued in section k_2. Section k_3 represents the absorbancy of samples removed from a mixture to which ferricyanide had been added only just before the second illumination of the first tube. The slopes of the lines calculate out to the following rates in μmoles per mg chlorophyll per hour: k_1, 315; k_2, 140; k_3, 252.

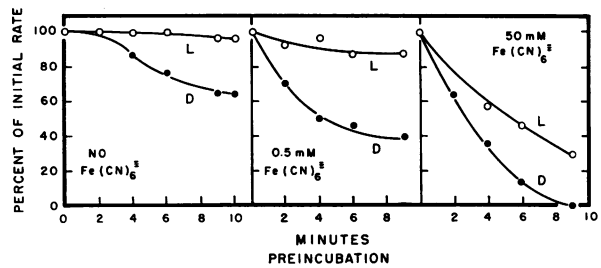

FIG. 2. Time course for onset of inhibition by ferricyanide. Six test tubes containing the standard reaction mixtures with phosphorylating reagents were placed in a rack shielded by black shutters, in an almost completely dark room. At stated time intervals before illumination a 0.10 ml of chloroplast suspension was injected into each tube with a Hamilton microliter syringe. In the series without ferricyanide, the reagent was added just before illumination to all tubes simultaneously by means of a battery of 6 syringes, 1 ml each, held in a supporting rack with fuse clips. Ferricyanide concentration was 0.5 mM, final illumination time 1.5 minutes.

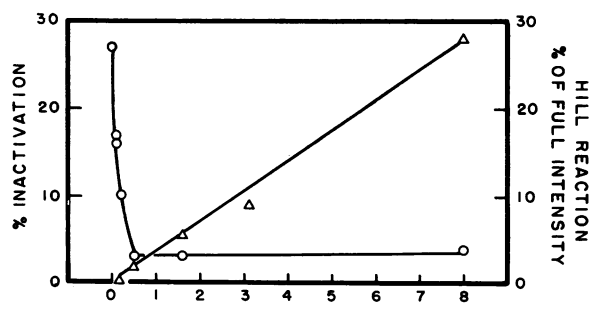

FIG. 3. Light intensity curve for protection of chloroplasts from damage by ferricyanide. The reaction mixtures were made up as indicated in Methods, without a wash between preincubation and exposure to high intensity light. Preincubation at various light intensities was for 3 minutes, followed by 1.5 minutes bright light. The Hill reaction at preincubation light intensities was estimated from the extent of reduction in 2 tubes removed just prior to the high intensity illumination period. Full light intensity was 4×10^4 μw/cm^2, or about 2000 ft-c $\triangle - \triangle$ Hill reaction; $\bigcirc - \bigcirc$ protection.

Table I. *Extent of Inactivation by 0.5 or 50 mM Ferricyanide and by 40 mM Ferrocyanide*

The control and 0.5 mM ferricyanide preincubation mixtures contained 0.1 M NaCl in addition, to make their osmotic strength equivalent to that of the 50 mM ferricyanide solution. The third set of values were taken from a second experiment using ferrocyanide as well. All incubations at 0° with the chlorophyll concentration at 22 μg/ml, at pH 8 with tris KCl. Other conditions as in Materials and Methods.

Preincubation mixture FeCN conc	Time min	Basal	Inhibition %	+ Pi*	Inhibition %
0	15	188**	...	273	...
0.5 mM		127	32	178	35
50 mM		75	60	81	70
0	45	190	...	300	...
0.5 mM		122	36	162	46
50 mM		31	84	31	90
0	15	237	...	...	
50 mM		85	64	...	
40 mM ferrocyanide		155	35	...	

* Refers to the presence of phosphorylation reagents.
** μmoles ferricyanide reduced per mg chlorophyll per hour.

when used at the same concentration; thus the effect is probably due to characteristics of the molecule not directly related to oxidation-reduction properties. And both the extra inhibition by ferricyanide as well as that due to ferrocyanide, require a pH of 8 or more for full expression (fig 4). The inhibitory effect of lower concentrations of ferricyanide is relatively independent of pH between 6.0 and 8.5. The pH curve shown in figure 4 also contains 2 isolated points, showing apparent stimulation by 50 mM ferricyanide, when applied in the dark at pH 6.0. This is due to uncoupling (see below), and shows up as a stimulation because the assays were performed in the absence of phosphorylating reagents. The fact that an increase over the control rate can be seen also emphasizes that damage by 50 mM ferricyanide

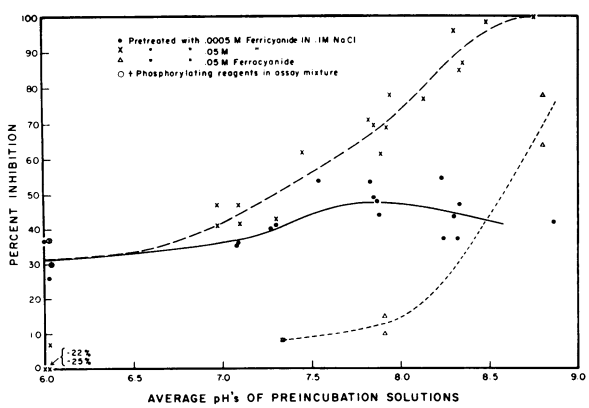

FIG. 4. Effect of preincubation pH on inhibition by ferricyanide. Chloroplasts were preincubated for 20 minutes at 0° in media of composition and average pH as described in the figure, collected by centrifuging, washed, and assayed for Hill reaction activity under standard conditions. The curve represents results from 12 different experiments.

is much less extensive at the lower pH. Light does not prevent the damage caused by 50 mM ferricyanide (see fig 2).

It can be noted that in the few experiments attempted, chloroplasts previously broken in hypotonic media responded in the same way to either 0.5 or 50 mM ferricyanide as did whole chloroplasts.

Site of Damage. No shift of the pH optimum for ferricyanide reduction was found with the ferricyanide-treated chloroplasts, in contrast to the results with some uncoupling treatments (15). However, the inhibition tended to be greater at higher pH in the assay medium, suggesting a partial uncoupling.

In terms of the response to light intensity, 0.5 mM ferricyanide has obviously affected a limiting dark step only, whereas 50 mM ferricyanide pretreatment inhibits equally at all light intensities and is therefore affecting both a limiting light and a limiting dark step (fig 5). In rather brief attempts at determining the action spectrum for ferricyanide reduction, no apparent shift in sensitization could be detected following treatment with either 0.5 or 50 mM ferricyanide.

With the cooperation of Dr. Butler, a fluorimetric analysis of control and treated chloroplasts was made to determine whether ferricyanide pretreatment prevented carotenoids or chlorophylls from transferring their excitation energy to some "sink" or active site. Such an effect would manifest itself in an altered fluorescence excitation spectrum of chlorophyll a (6).

No change in the fluorescence excitation spectrum was found due to pretreatment with 0.5 mM ferricyanide. A generalized increase of about 12% was observed in the case of the 50 mM pretreatment, indicating a general disconnecting of photosynthetic pigments from their quenching centers (active sites). But again, no specific change in the action spectrum for fluorescence was observed. This is strong, albeit negative, evidence that no specific pigments are barred

from participation in effective light absorption due to ferricyanide pretreatment.

Both TCPIP and NADP photoreduction are affected a bit more than ferricyanide photoreduction by the 50 mM ferricyanide pretreatment, and somewhat less by the 0.5 mM concentration (table II). (These effects of ferricyanide damage on NADP photoreduction did not show up until sufficient quantities of spinach ferredoxin were supplied in the reaction mixture. At rate-limiting quantities of ferredoxin, the inhibitions are not seen.) By contrast, the reduction of NADP with the ascorbate-TCPIP dye couple as electron donor is either not affected or is (occasionally) enhanced by these ferricyanide pretreatments (table II).

Phosphorylation with PMS as cofactor was affected very little by pretreatment with 0.5 mM ferricyanide (table III). Correspondingly, the P/2e ratio for phosphorylation accompanying ferricyanide reduction was not at all affected. On the other hand, 50 mM ferricyanide pretreatment inhibited PMS-supported phosphorylation severely, and caused a significant depression in the P/2e ratio during ferricyanide

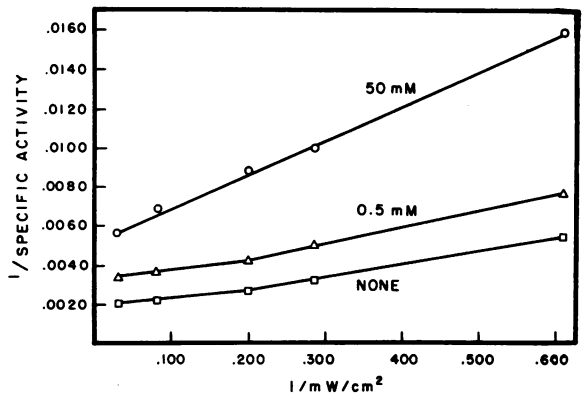

FIG. 5. Rate of the Hill reaction as a function of light intensity, with chloroplasts pretreated with 0.5 or 50 mM potassium ferricyanide. Light intensities were measured with a silicone diode photocell, calibrated against an Eppley thermopile. Reactions were run for 3 minutes in cuvette immersed in a constant temperature water bath at 25°.

Table II. *Effect of Dark Ferricyanide Pretreatment on Reduction of FeCN, TCPIP, and NADP*

Rates are shown in μmoles substrate reduced per mg chlorophyll per hour. With TCPIP as electron donor, it was kept reduced with ascorbate, and CMU was added as indicated in Methods section. 0.1 M NaCl was present in both the 0 mM and 0.5 mM preincubation solutions.

Electron acceptor	Electron donor	Pi	FeCN Pretreatment 0 mM Rate	0.5 mM Inhibition %	50 mM Inhibition %
FeCN	H_2O	—	151	37	48
FeCN	H_2O	+	236	39	52
TCPIP	H_2O	—	240	23	73
FeCN	H_2O	—	209	45	59
NADP	H_2O	—	107	19	66
NADP	H_2O	+	162	29	77
FeCN	H_2O	—	234	35	59
NADP	TCPIP	—	32	(72)*	(75)*
NADP	TCPIP	+	64	(16)*	(20)*

* Rate stimulations.

Table III. *Effect of Ferricyanide Pretreatments on Phosphorylation*

In the preincubation, 0 and 0.5 mM ferricyanide contained also 0.1 M NaCl.

Redox dye	Pi	Preincubation conc mM	FeCN Reduction Rate	Inhibition %	Phosphorylation Rate	Inhibition %	P/2e
FeCN	—	0	197*	...	...	...	...
		0.5	130	34	...	...	...
		50.0	33	83	...	...	...
FeCN	+	0	269	...	148*	...	1.09
		0.5	167	38	89	40	1.07
		50.0	39	85	17	89	.87
PMS	+	0			306	...	...
		0.5			256	16	...
		50.0			113	63	...

* μmoles product formed per mg chlorophyll per hour.

photoreduction when the gross electron flow rate was inhibited 85 %.

The response to added ammonium chloride was the same in photoreduction by control chloroplasts and in those treated with 0.5 mM ferricyanide. After 50 mM ferricyanide preincubation, however, the concentration of NH_4Cl needed for maximum stimulation of the Hill reaction was shifted from 5×10^{-4} down to 1.5×10^{-4} M; and the amount of stimulation was much less than for the controls (data not shown).

Nature of the Damage. Inhibition by ferricyanide was found not to be reversed by washing, by illumination, or by the addition of reducing reagents. The inhibition was, however, prevented by the simultaneous presence of ferrocyanide, leading to a lowered redox potential. The relation between redox potential of the preincubation solution and extent of subsequent damage is shown in figure 6. In these experiments the total ferri- plus ferrocyanide was kept constant at 10 mM. The preincubations were performed at pH 7.2, however, where there is very little difference between the effects of 0.5 and 50 mM ferricyanide, so the results pertain primarily to the low ferricyanide inactivation process. Control experiments showed that even the lowest ferricyanide concentration used in figure 6 (.033 mM) would have caused full inactivation in the absence of added ferrocyanide; thus the results do not simply reflect a ferricyanide concentration curve.

The curves obtained in experiments such as figure 6 resemble that of a redox titration for a compound with a mid-point potential of +0.38 v. The slope of the curve suggests involvement of 2 or 3 electrons in the reaction.

Evidence for the oxidation of some internal chloroplast component by ferricyanide during preincubation was found from the observation of ferrocyanide production in the dark (table IV). A distinctly reproducible difference between control and ferricyanide-treated chloroplasts was found, with the pretreated chloroplasts producing from 0.08 to 0.12 μmole less ferrocyanide per μmole of chlorophyll. This number sets an upper limit on the number of molecules whose oxidation may have led to the change in biochemical status of the chloroplasts.

A decrease in absorbancy in the blue region was noted in 80 % acetone extracts of ferricyanide pretreated chloroplasts (fig 7). This decrease occurred only under aerobic conditions, although the inactivation proceeded to exactly the same extent whether chloroplasts were incubated anaerobically or aerobically (data not shown). Following anaerobic incubation of the chloroplasts with ferricyanide, ascorbate was added immediately to reduce the ferricyanide. In this way the results were not due to the continuing effect of residual ferricyanide during the period between the preincubation and the actual reaction measurement. The absorbancy decreases showed peaks at 440 and 470 mμ, with a shoulder at 420 mμ. These data suggests that we had observed an oxida-

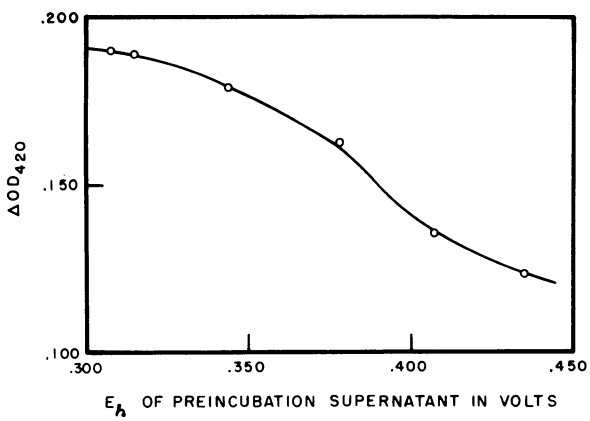

FIG. 6. Effect of redox potential of preincubation media on chloroplast inactivation. Chloroplasts were preincubated in media with the following composition and redox potential as measured both before and after the incubation period:

No.	Ferricyanide conc	Ferrocyanide conc	E_h Initial	E_h Final
	M	M		
1	0.005	0.005	0.441	0.435
2	0.0025	0.0075	0.415	0.407
3	0.001	0.009	0.385	0.378
4	0.00033	0.0099	0.356	0.344
5	0.001	0.010	0.332	0.315
6	0.000033	0.010	...	0.308

Each reaction mixture contained, in addition, 0.1 M NaCl and 0.02 M Tris-HCl, pH 7.2. The pH of the preincubation media was 7.13 or 7.14 in almost every case; being 7.10 in solution 1 and 7.18 in solution 6 at the end of the incubation.

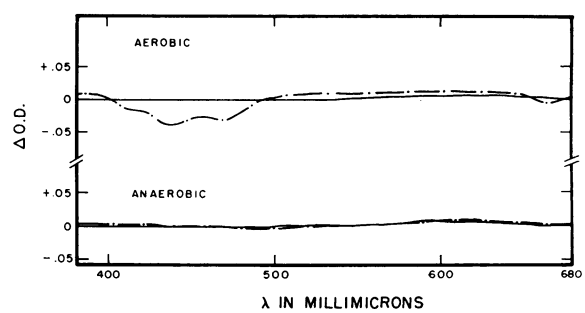

FIG. 7. Carotenoid loss under aerobic but not anaerobic conditions. Chloroplasts containing 1 mg of chlorophyll were preincubated as in table VI, collected by centrifugation, and the pellets extracted 3 times with 100 % methanol (13 ml total) and 2 times with diethyl ether (3 ml total). The combined extracts were made up to 50.0 ml in a volumetric flask with 100 % methanol. Each extract was placed into a cuvette with ground glass stopper for measurement of difference spectra, which are shown using the ferricyanide-treated in the sample position and the controls in the reference position of Spectronic 505 recording spectrophotometer. Duplicate chloroplast samples in each case gave identical difference spectra.

tive destruction of carotenoid compounds due to dark incubation with ferricyanide.

When the loss of carotenoids occurred, it was closely correlated with the inactivation process described above. Thus there was a positive and fairly close correlation between the amount of absorbancy loss and the extent of inactivation, when these were aroused by ferri/ferrocyanide solutions of varying redox potential (data not shown). The light intensities needed to prevent inactivation by 0.5 mM ferricyanide also prevented loss of blue absorbancy (data not shown). As would be expected from the oxidation-reduction characteristics, 50 mM caused no greater decrease in blue absorbancy (or indeed any further spectral shifts) than did 0.5 mM ferricyanide.

Treatment with 50 mM ferricyanide, but not that with 0.5 mM, causes the release of a small but significant amount of soluble protein from whole chloroplasts suspended in 0.40 M sucrose (table VII). This amounts to 0.4 mg protein per mg chlorophyll, or 20 % of the total released by osmotic shock. Greater protein loss is not accompanied by any perceptible breaking up of the chloroplasts into smaller particles, as shown by other differential centrifugation experiments. Indeed it can be seen that the 50 mM ferricyanide also replaces sucrose, to a small extent, as an osmotic protective reagent when the sucrose is absent. Some extra protein (0.15–0.20 mg/mg chlorophyll) is released by 50 mM ferricyanide from chloroplasts previously subjected to osmotic shock (data not shown). Other experiments showed that either equivalent or up to 7 times higher concentrations of NaCl did not cause extra protein release; hence this effect is not simply a high ionic strength extraction.

Discussion

The present work has attempted to explore some of the complexities of the damage caused to chloroplasts by incubation with ferricyanide ions. Half mM ferricyanide caused, very rapidly, a partial inhibition of the Hill reaction, and this was prevented by dim illumination. The light intensities effective in this function are considerably lower than those needed for half saturation of the known light-driven reactions of chloroplasts.

The inhibition imposed by 0.5 mM ferricyanide is almost certainly due in the first place to an oxidation of one of the chloroplast components. The effect is not shared by ferrocyanide, and some dark reduction of the added ferricyanide can be seen (table IV). It is also consistent for light to protect against inactivation, since illumination will affect the redox state of chloroplast electron carriers, and may be able to keep the crucial one(s) in a reduced condition in spite of the presence of ferricyanide.

Observations by many workers indicate that ferricyanide may be reduced by chloroplasts (17, 18, 20, 22) or chromatophores (7, 8, 11) in a dark step. Muller et al., for example, reported some results obtained by chemical oxidation in the dark of chlorophyll a in intact chloroplasts with ferricyanide (22). And Kok used ferricyanide to oxidize the pigment P-700 in chloroplasts in the dark, and thus duplicated the effect of far-red light (17, 18).

The shape of the curve demonstrating inactivation as a function of the redox potential (fig 6) is what might be expected if some internal oxidizable material, with a mid-point potential of +0.38 v, were the primary site of attack of ferricyanide. Components with potentials near this level include cytochrome f, at +0.36 v (13) and plastocyanine at +0.38 v (16).

However, the present effect, a 20 to 50 % inhibition of photoreduction, is apparently irreversible. Oxidation of cytochrome f (13), plastocyanine (16), the compound(s) exhibiting dark- or light-induced absorption difference spectral changes (7, 18, 24), or that giving an ESR signal in light or when oxidized by ferricyanide (1, 20; see also 4) are known to be easily reversible. Even if one of them is the primary point of attack for ferricyanide, therefore, the data require the postulation of a second and irreversible step before the final result. It seems likely that increasing redox potentials of the external solution maintain an increasing proportion of the target redox molecules in the oxidized state, permitting thereby greater destruction of the final components.

The fact that only a partial inhibition is effected

Table IV. *Dark Reduction of Ferricyanide by Treated Chloroplasts*

After the usual preincubation (with 0.10 M NaCl present except with 50 mM FeCN) chloroplasts were resuspended for 20 minutes in complete darkness with 1.5 mM ferricyanide in the usual photolysis reaction mixture. Following that, in the first column, the chloroplasts were centrifuged out before trichloroacetic acid was added to the supernatant. This column shows the ferrocyanide free in solution formed during the second incubation. In the last column trichloroacetic acid was added to the chloroplasts in the solution; the higher values show that trichloroacetic acid releases extra reducing materials from the chloroplasts. The middle column shows ferrocyanide formed when chloroplasts are destroyed by trichloroacetic acid first, before the ferricyanide is added.

Preincubation FeCN conc mM	Hill reaction	Ferrocyanide formed in dark by		
		Chloroplast supernatant*	Denatured Chloroplast*	Untreated Chloroplast*
0	250	0.38	1.85	2.06
0.5	166	0.26	1.72	1.93
50.0	104	0.19	1.72	1.83

* Numbers are μmoles ferrocyanide produced per μmole chlorophyll.

by 0.5 mM ferricyanide suggests either that 2 separate electron pathways contribute to the Hill reaction, or that only 1 pathway exists, but it is only partially accessible to ferricyanide. In general, photoreactions involving compounds other than ferricyanide (table II, III) seem to be less inhibited than is photoreduction of ferricyanide itself by the 0.5 mM ferricyanide pretreatment, while the converse is true for 50 mM ferricyanide pretreatment. This is more readily explainable by a hypothesis involving 2 electron pathways having somewhat different specificities, with one being susceptible and the other resistant to inhibition by low ferricyanide concentrations.

The nature of the component finally destroyed is not known. Although most experiments showed a correlation between loss of activity and loss of a carotenoid, the carotenoid compound cannot be responsible for the lost activity in view of its retention under anaerobic conditions (fig 7) where the same activity losses occur as aerobically. The oxidation of chloroplast carotenoids, enhanced by ferricyanide among other reagents, was previously demonstrated by Friend and Nakayama (9). It seems likely that the conditions which initiate inactivation studied here are the same as those that initiate the oxidation of carotenoids by O_2, in view of the series of correlations between the 2, such as protection by dim light and relation to the redox potential of the solution. Although some carotenoids have been destroyed, fluorescence excitation studies by Butler showed that energy transfer from carotenoids to chlorophyll was not affected by ferricyanide pretreatments (unpublished data).

The site of inhibition by 0.5 mM ferricyanide is likely to be in or near the mechanism for evolution of oxygen. This is supported by the failure to inhibit NADP reduction from reduced TCPIP as electron donor (table II) and the relatively mild effect on PMS-supported phosphorylation (table III). It is of some interest therefore that the kinetics of the resulting Hill reaction with respect to light intensity show that only a dark step has been made more limiting (fig 5). Almost all other reagents or treatments which inhibit oxygen evolution appear to affect the light step as well (10).

The extra inhibition of the Hill reaction caused by the combination of 50 mM ferricyanide and a pH of 8 or above is probably due to considerations involving the charge on the ferri- or ferrocyanide anion. The inhibitory site can again be placed on the oxygen evolution pathway, because of the lack of inhibition of electron flow from reduced TCPIP to NADP (table III). In this case, however, the more familiar result is found of apparent inhibition of a limiting light step, probably superimposed over the effect of the lower concentration (fig 5). An additional difference from the inhibition produced by 0.5 mM ferricyanide is the lack of protection by light (fig 2). Finally, the release of extra protein (table VII) is specific for the higher ferricyanide concentrations.

The action of high ferricyanide concentrations in causing uncoupling is very likely identical with the uncoupling by high concentrations of anions already described by Good (12). The uncoupling here seems more prominent at lower pH (fig 4).

Apart from any theoretical implications of the work, our results may suffice to explain discrepancies in the literature concerning the ferricyanide Hill reaction. Assay procedures which include preincubation of chloroplasts for as little as 2 minutes in darkness with even the lowest ferricyanide levels, or in room light with the 10 to 50 mM ferricyanide, can be expected to cause one or more of the aberrations described here.

Summary

Three effects of ferricyanide on isolated spinach chloroplasts have been distinguished experimentally, and characterized. A) Ferricyanide (0.5 mM) causes a partial inactivation of the Hill reaction, apparently at a dark step associated with the oxygen evolution site. This inactivation is not dependent on pH over a broad range, and is prevented by sufficient ferrocyanide. The inactivation varies with redox potential in such a way as to suggest that the initial site of attack is an internal component with an E'_0 of + 0.39 v. Light at about 10 ft-c prevents the inactivation completely. Oxygen is not needed, although in air loss of some carotenoid is associated with the inactivation. B) Ferricyanide (50 mM) causes a

Table V. *Release of Soluble Protein from Chloroplasts as Affected by Osmotic Shock and by Ferricyanide*

Chloroplasts containing 3 mg of chlorophyll were added to 30 ml of 0.01 M NaCl, with sucrose and/or ferricyanide at the concentrations shown. After incubation for 20 minutes in the dark at 0°, the chloroplasts were spun out at 12,000 × *g*. Protein in the supernatant solutions was precipitated by 2% trichloroacetic acid with the aid of freezing and thawing, collected by centrifuging, washed once, then dissolved in 0.1 M NaOH–2% Na_2CO_3. Protein was estimated from the OD at 260 and at 280 mμ, as in (16).

Sucrose conc	Ferricyanide concentration				
	Experiment I			Experiment II	
M	0 mM	0.5 mM	50 mM	0 mM	50 mM
0	2.03*	1.83	1.31	2.20	1.47
0.20	0.28	...	0.57	0.29	0.53
0.40	0.18	0.21	0.61	0.27	0.60

* mg protein released per mg chlorophyll.

more extensive inactivation of the Hill reaction, inhibiting (in addition to the above) a light step associated with oxygen evolution. This larger degree of inactivation is not prevented by light, is accomplished by ferrocyanide equally well, and proceeds to a greater extent at pH 8 and above. A greater extractability of protein appears to accompany this effect. C) Both ferri- and ferrocyanide have uncoupling effects on chloroplasts. The uncoupling is rapid in 50 mM ferricyanide and slow in 0.5 mM. The uncoupling action is more evident at pH 6 than at pH 8.

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