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Inhibition of the Hill Reaction by Tris and Restoration by Electron Donation to Photosystem II[1]

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Abstract. **Experiments in which chloroplasts were washed with tris and tricine buffers at different pH's indicated that the non-protonated (uncharged) form of tris was inhibitory to the Hill reaction while the protonated form of tris and the zwitterionic forms of tricine were non-inhibitory. Buffers analogous to tris and tricine gave similar results. Photoreduction of NADP could be restored to the inhibited chloroplasts by adding the reduced forms of *p*-hydroquinone, *p*-aminophenol, *p*-phenylenediamine, benzidine, semicarbazide, and dihydroxydiphenyl, all of which donated electrons to photosystem II. Photoreduction of ferricyanide was shown with those donor systems (benzidine and semicarbazide) which did not react chemically with ferricyanide. Photophosphorylation was also restored with all of the electron donors except semicarbazide.**

We previously reported that washing chloroplasts with 0.8 M tris, pH 8.0, inhibited the Hill reaction by blocking electron transport between water and PS2 (5). In the present paper, we have examined the inhibitory action of tris further by comparing the effects of washing treatments with tris and tricine at different pH's and by examining the inhibitory effects of another pair of buffers analogous to tris and tricine. The results indicate that the unionized form of tris or the tris analog is inhibitory in the wash treatment while the charged species are noninhibitory.

Our previous work (5) with the tris-washed chloroplasts showed that artificial electron donor compounds could donate electrons to the oxidizing side of PS2. The classical electron donor systems which donated electrons between PS2 and PS1 with DCMU-inhibited chloroplasts could also donate electrons prior to PS2 with tris-washed chloroplasts, but some electron donors, *e.g.*, low concentrations of phenylenediamine (5) or hydroquinone (6), donated electrons only to the oxidizing side of PS2. Electron transport from these latter donors was strongly inhibited by DCMU. In the present paper, we report several additional electron donor compounds which are specific in donating electrons to PS2. The electron donor systems restore photophosphorylation as well as NADP photoreduction to the tris-washed chloroplasts. We also show that ferricyanide can be used as the Hill acceptor with those electron donor systems that do not react directly with ferricyanide.

Materials and Methods

The experimental methods for preparing chloroplasts and washing the chloroplasts and the methods for measuring photoreduction, photophosphorylation and fluorescence yield were the same as those described previously (5). NADP photoreduction under aerobic conditions was measured with an Aminco-Chance Dual Wavelength Spectrophotometer with the measuring wavelengths set at 340 and 374 nm. Ferricyanide photoreduction was measured similarly with the wavelengths set at 420 and 480 nm. Red actinic light of 6.6×10^4 ergs cm^{-2} sec^{-1} was obtained with a tungsten lamp and 645 nm interference filter. The standard reaction medium contained 15 mM tris-HCl, 4 mM KPO_4, 1 mM ADP, 4 mM $MgCl_2$ and 20 mM NaCl at pH 7.8 except where noted. pH measurements on the buffers and reaction media were made at room temperature and the assays were made at room temperature. The chloroplasts were washed in the various buffers at 0°, however, so that the pH during the wash treatment may have been somewhat different from that indicated at room temperature.

Photophosphorylation was measured under anaerobic conditions in 1 cm Thunberg-type cuvettes evacuated and filled with argon 3 times. White light filtered through 7 cm of 1 % $CuSO_4$ solution was the actinic light (10^6 ergs cm^{-2} sec^{-1}). The amount of NADPH formed in the Thunberg cuvette was proportional to the measured increase of absorbance at 340 nm in a Unicam SP800 Spectrophotometer at the end of the reaction. The reactions were stopped by addition of trichloroacetic acid to 7.5 % after the measurement of NADPH and the amount of ATP formed from ADP and radioactive phosphate

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was measured by the method of Nielsen and Lehninger (3) as modified by Avron (1). The ratio of molecules of ATP formed per pairs of electrons transported is expressed as P/e_2 ratios.

Ferredoxin was purified from spinach leaves; DCMU donated by Dr. P. G. Heytler was used as in methanolic solution. Tricine, ADP, NADP, and benzidine were obtained from Calbiochem Company; *p*-phenylenediamine and semicarbazide were products of Eastman Organic Chemicals Company; *p*-hydroquinone was obtained from Aldrich Chemical Company; *p*-aminophenol from K & K Laboratories, Incorporated; *p,p'*-dihydroxydiphenyl from Alfred Bader Chemicals; hydroxylamine from Baker Chemical Company; glycine amide hydrochloride from Mann Research Laboratories; glycylglycine from Sigma Chemicals Company and tris from General Biochemicals.

Results and Discussion

Inhibition. Inhibition of the Hill reaction by washing chloroplasts with tris buffers has been tested with various grades of tris from once recrystallized to 3 times recrystallized. All gave the same results which strengthens the contention that the inhibition was due to the action of tris rather than to an impurity in the tris.

The inhibitory effects of washing chloroplasts with 0.8 M tris, RNH_2, pH 8.0, and 0.8 M tricine, $RNHCH_2COO^-$, pH 8.0 where R is $(CH_2OH)_3C$, are compared in Fig. 1. Washing with tricine gave no inhibition while washing with tris resulted in almost complete inhibition. Hydroquinone plus ascorbate restored most of the NADP photoreduction activity and this restored electron transport was inhibited by DCMU, indicating that electron donation from hydroquinone was prior to PS2. Essentially the same degree of inhibition could be obtained by washing with 0.05 M tris if the pH of the buffer was raised to 8.8 (Fig. 2). The inhibition was not primarily due to pH since washing with 0.05 M tricine, pH 8.8, showed very little inhibition. The pK of tris is 8.3 so that the nonprotonated form, RNH_2, appears to be the inhibitory one in the wash treatment. Winget *et al.* (4) showed that the pH dependence of the ferricyanide Hill reaction in tris buffer had a relatively sharp maximum at pH 8.0 whereas good rates of ferricyanide photoreduction could be obtained in tricine buffer to pH 9.0 or above. The decrease of Hill activity at pH's above 8.0 in tris buffer is due to the inhibitory action of the nonprotonated species of tris. The lack of inhibition of the zwitterion, tricine, and the protonated tris, RN^+H_3, suggests that the charged species are ineffective because they do not interact with or pass through the chloroplast membrane.

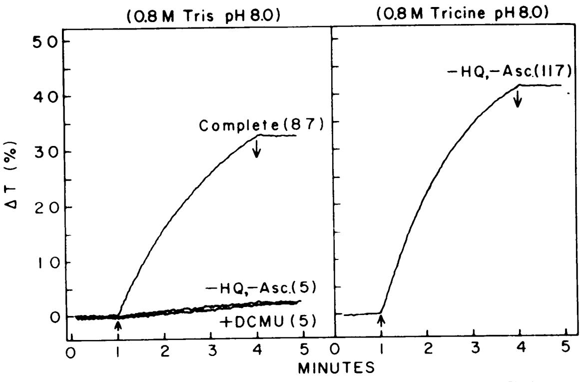

FIG. 1. Photoreduction of NADP with: A) Chloroplasts (10 μg chl/ml) washed with 0.8 M tris, pH 8.0, and: B) Chloroplasts washed with 0.8 M tricine, pH 8.0. Complete reaction medium is the standard reaction medium plus 3 μM ferredoxin and 330 μM NADP for the electron acceptor system and 200 μM hydroquinone and 330 μM ascorbate for the electron donor system. The electron donor system was omitted (—HQ, —Asc) and 1 μM DCMU was added where indicated. Numbers in parenthesis indicate μmoles NADPH formed/mg chl per hr averaged over 3 min irradiation period.

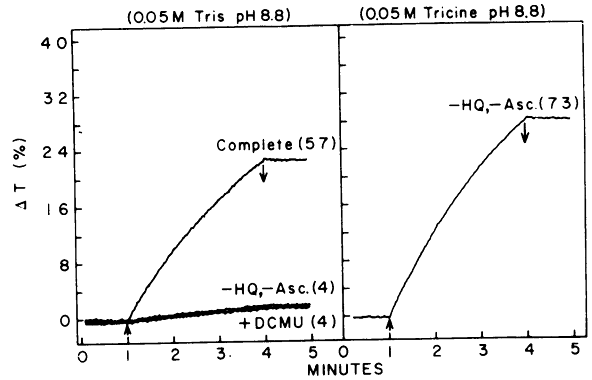

FIG. 2. Same as Fig. 1 except chloroplasts were washed with 0.05 M tris, pH 8.8, and 0.05 M tricine, pH 8.8.

The inhibitory effects of washing with compounds analogous to tris and tricine, glycine amide, $R'NH_2$, and glycylglycine, $R'NHCH_2COO^-$ (R' is NH_2CH_2CO), respectively, are shown in Fig. 3. Washing with 0.8 M glycine amide, pH 8.0, resulted in a

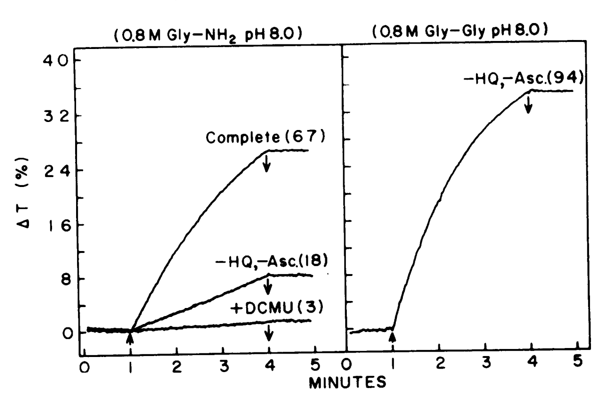

FIG. 3. Same as Fig. 1 except chloroplasts were washed with 0.8 M glycine amide, pH 8.0, and 0.8 M glycylglycine, pH 8.0.

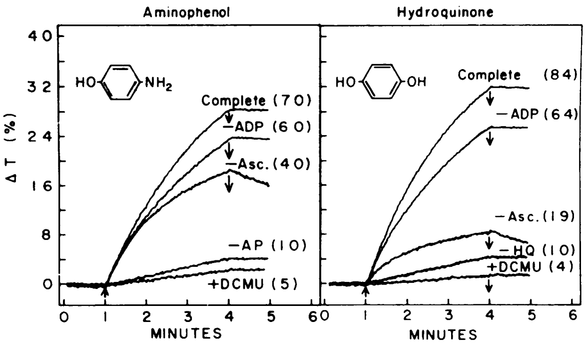

FIG. 4. Photoreduction of NADP by tris-washed (0.8 M, pH 8.0) chloroplasts (10 μg chl/ml). A) With 200 μM aminophenol and 330 μM ascorbate as the electron donor system and B) With 200 μM hydroquinone and 330 μM ascorbate as the electron donor system. Complete reaction media contains standard reaction medium plus electron donor system plus 3 μM ferredoxin and 330 μM NADP. Components omitted where indicated and 1 μM DCMU added where indicated. Numbers in parenthesis indicate μmoles NADPH formed/mg chl/hr averaged over 3 min irradiation period.

marked inhibition, although not as complete as with tris, and washing with 0.8 glycylglycine, pH 8.0, gave no inhibition. These results are consistent with those of Good, *et al.* (2) which showed that zwitterionic buffers were preferable for the Hill reaction.

Electron Donation. A number of compounds which were poor electron donors for DCMU-poisoned chloroplasts were able to restore the NADP Hill reaction and photophosphorylation of tris-washed (washed with 0.8 M tris, pH 8.0) chloroplasts. Typical results for NADP photoreduction under aerobic conditions are shown in Fig. 4 with aminophenol (200 μM AP, 330 μM asc) and hydroquinone (200 μM HQ, 330 μM asc) as the electron donor compounds. The somewhat lower rate of photoreduction in the absence of ADP suggests that the restored electron transport is coupled to phosphorylation. Similar results were obtained with phenylenediamine (33 μM PD, 330 μM asc), benzidine (33 μ BZ, 330 μM asc), dihydroxydiphenyl (100 μM DHDP, 330 μM asc) and semicarbazide (3000 μM SC, 330 μM asc) although the last 3 donor systems gave somewhat lower rates of photoreduction. Ferrocyanide (data not shown) did not serve as an electron donor. Aminophenol acts as a relatively good electron donor for the photoreduction of NADP even in the absence of ascorbate, probably because the oxidized form is a relatively poor electron acceptor for the Hill reaction. With hydroquinone, which is a good electron donor in the reduced form and a good electron acceptor in the oxidized form, the photoreduction of NADP in the absence of ascorbate starts at a reasonable rate but falls off sharply as the quinone, oxidized during electron donation, begins to compete with NADP as the electron acceptor.

The Hill reaction with ferricyanide as the electron acceptor for tris-washed chloroplasts is shown in Fig. 5 with benzidine and semicarbazide as the electron donors. The reaction medium for the ferricyanide reaction was changed from 15 mM tris and 4 mM KPO$_4$ to 50 mM KPO$_4$ (other components and the pH remaining the same) because semicarbazide, for unknown reasons, did not support the photoreduction of ferricyanide in the standard reaction medium (although it did support the photoreduction of NADP). Benzidine served as an electron donor for ferricyanide equally well in either reaction medium. Benzidine and semicarbazide could be used as electron donors for ferricyanide because their reduction potentials are sufficiently positive that the reduced forms do not react appreciably with ferricyanide. Also, these compounds give reasonably good rates of photoreduction in the absence of ascorbate which would have reacted directly with the ferricyanide.

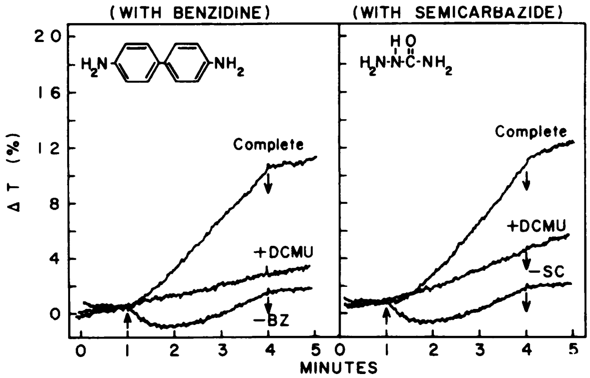

FIG. 5. Photoreduction of ferricyanide by tris-washed (0.8 M tris, pH 8.0) chloroplasts (10 μg chl/ml), A) With 33 μM benzidine and B) With 3000 μM semicarbazide as the electron donor. Complete reaction medium contained 50 mM potassium phosphate, 20 mM NaCl, 4 mM MgCl$_2$, 1 mM ADP, 330 μM ferricyanide and the electron donor. The electron donor was omitted or 1 μM DCMU was added where indicated.

Fluorescence yield measurements were also used previously (5) to indicate electron donation to PS2. Fig. 6 shows the fluorescence yield measurements of tris-washed chloroplasts with the various electron donor systems. The tris-washed treatment eliminates most of the light-induced fluorescence-yield increase which, in normal chloroplasts, is a factor of about 4. The electron donor systems restore a part of the fluorescence of variable yield but to different extents. The restoration of the light-induced fluorescence-yield increase is not a sufficient condition for electron donation to PS2. It was noted previously (5) that the addition of DCMU to tris-washed chloroplasts resulted in the maximal fluorescence yield during illumination, presumably because there were sufficient endogenous electron donors to photoreduce Q if the electron transport out of Q was blocked by DCMU. The large light-induced fluorescence-yield increase observed with hydroxylamine in Fig. 6 may be due

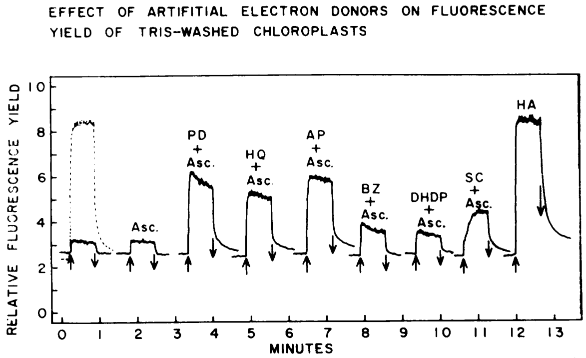

FIG. 6. Relative fluorescence yield of tris-washed (0.8 M tris, pH 8.0), chloroplasts (10 µg chl/ml) (solid line) and normal chloroplasts (10 µg chl/ml) (dashed line). Red actinic light (2.4×10^4 ergs cm^{-2} sec^{-1}) on at upward arrow, off at downward arrow. Standard reaction medium plus 330 µM ascorbate, 33 µM phenylenediamine (PD), 200 µM hydroquinone (HQ), 200 µM aminophenol (AP), 33 µM benzidine (BZ), 100 µM dihydroxydiphenyl (DHDP), 3000 µM semicarbazide (SC) and 3000 µM hydroxylamine (HA) added where indicated.

to a similar block although the magnitude of the effect suggests that hydroxylamine may be donating electrons to PS2 as well.

Photophosphorylation. All of the electron donors tested supported photophosphorylation (see table I) with the exception of the semicarbazide, which may have acted as an uncoupler at the relatively high concentrations required for electron transport. Both photoreduction and photophosphorylation were inhibited by 1 µM DCMU. Photophosphorylation occurred with P/e_2 ratios near unity with hydroquinone, aminophenol and benzidine as the electron donors for NADP photoreduction. The P/e_2 ratio found with phenylenediamine in this experiment was lower than usual; previously (5) we found P/e_2 ratios between 0.66 and 0.95 with this donor. We conclude that the tris-wash treatment is a relatively gentle means of inhibiting electron transport and that good rates of photophosphorylation can be restored with electron donors which donate to PS2. A moderate rate of phosphorylation was also found with electron transport from benzidine to ferricyanide (table II).

Table I. *Photophosphorylation and Photoreduction of NADP by Tris-washed Chloroplasts (30 µg chl/ml) With Various Electron Donor Systems*

Reaction medium is standard reaction medium plus 3 µM ferredoxin, 330 µM NADP and the electron donor system indicated with and without DCMU. Irradiation with white light (10^6 ergs cm^{-2} sec^{-1}) under anaerobic conditions.

Addition (µM)	µmoles formed / mg Chl•hr		P / e_2
	NADPH	ATP	
None	5	1	0.20
HQ(200), Asc. (330)	90	82	0.91
” , ” , DCMU(1)	5	0	0
AP (200), Asc. (330)	89	86	0.97
” , ” , DCMU(1)	5	1	0.20
PD(33), Asc. (330)	63	34	0.54
” , ” , DCMU(1)	9	0	0
BZ(33) Asc. (330)	35	34	0.97
” , ” , DCMU(1)	4	0	0
DHDP(100), Asc. (330)	37	15	0.41
” , ” , DCMU(1)	4	0	0
SC(3000), Asc. (330)	25	4	0.16
” , ” , DCMU(1)	1	0	0

Table II. *Photophosphorylation By Tris-washed (0.8 M Tris, pH 8.0) Chloroplasts (30 µg chl/ml) With 1 mM Ferricyanide As the Electron Acceptor and 67 µM Benzidine As the Electron Donor*

Reaction medium was the standard reaction medium plus the additions indicated.

Addition (µM)	µmoles ATP formed / mg Chl•hr
None	0.0
FeCN (1000)	4.8
FeCN (1000), BZ (67)	22.4
FeCN (1000), BZ (67), DCMU (1)	0.0
BZ (67)	1.0
BZ (67), DCMU (1)	0.2
BZ (67), Asc (330)	1.0

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