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ABSTRACT

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1. Conditions have been defined under which 6-thioctic acid, 6T, increases the quantum efficiency of the Hill reaction in Scenedesmus. This increase occurs when the control rate is quinone-limited, under conditions of high light intensity, low quinone concentration, high temperature, and high algal density. When the control rate is inhibited by high concentrations of quinone or photolyzed quinone, 6T results in a further decrease in rate.

2. Fundamental characteristics of the Hill reaction suggest that 6T either increases the rate at which water is separated into reduced and oxidized fragments (quantum conversion agent) or decreases the rate at which these fragments later recombine (hydrogen carrier or quinone diffusion).

3. The variation of the effect with 6T concentration, incubation conditions, pH, quinone concentration, temperature, preillumination of quinone, dark contact time with quinone, light intensity, algal density, sulfhydryl poisons, plant species and other reagents does not permit an unequivocal discrimination between the two possible mechanisms.

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It has recently been proposed (1) that 6-thioctic acid, 6T**, is the primary quantum conversion agent of photosynthesis. By this is meant that the energy liberated when photochemically excited chlorophyll is returned to its ground state is transferred to the five-membered disulfide ring of 6T, labilizing the S-S bond and allowing it to undergo reactions which are energetically not possible in the ground state of 6T. Energy of the quantum absorbed by chlorophyll may be converted into chemical bond energy by this process and hence the term, quantum conversion agent. The reagents with which the activated 6T reacts may either be a hydrogen carrier, or H_2O, or an oxygen carrier, presumably forming either a dithiol, a thiol sulfenic acid, or a disulfide monoxide, respectively. These three possible reactive species may then carry on the oxidation-reduction processes required in natural photosynthesis.

If 6T were operating in the manner described then it should be possible to realize conditions under which synthetic 6T added to the plant could be utilized to increase the rate of quantum conversion and result in a higher quantum efficiency of photosynthesis. The demonstration of such an effect in the Hill reaction (2,3,4) in which the photochemical apparatus is experimentally separated from the carbon dioxide reduction system would provide even more convincing confirmatory evidence for the proposed mechanism of quantum conversion.

Scenedesmus does exhibit such a predicted increase in quantum efficiency when incubated with synthetic 6T prior to the Hill reaction. Unfortunately, we cannot with certainty prove that the 6T is giving the predicted result in the postulated manner, although most evidence is consistent with such a model. The effect is not easily observed for there are many conditions both physical and biological which need to be satisfied simultaneously. We have described below in some detail the minimum number of such conditions which seem to guarantee the reproducibility of the effect.

Materials and Methods

Scenedesmus obliquus and Chlorella pyrenoidosa were grown in continuous culture under conditions described briefly in a previous publication (5). 900 cc. of the culture (pH = 7.1) was harvested every 24 hours leaving 100 cc. as the inoculum, together with 900 cc. fresh nutrient medium (pH = 6.8). The temperature of the culture was maintained for prolonged periods at 18 at 25°C., the algae grown at the latter temperature generally yielding more rapid Hill reaction rates. The carbon dioxide concentration above the mechanically shaken culture vessels was maintained at 4% as continuously recorded by an infrared CO_2 analyzer. Only sterile cultures were used in the experiments. The light incident upon the culture flasks was provided by a bank of Sylvania white 100 watt fluorescent lamps with a radiant energy output of 1.8×10^4 ergs/cm^2/sec. determined bolometrically.

The harvested cells were successively centrifuged (1900 x g) and washed with distilled water from 2 to 4 times. As the Hill activity declines rapidly when the cells are stored in the packed condition, recent experiments have been carried out with but two centrifugations. The packed cells were resus-

pended to the desired algal density, generally 20 mm^3 packed cells/ml., in a buffer (pH = 6.7) made by dissolving 1/30 mole KH_2PO_4, 1/30 mole K_2HPO_4, and 1/100 mole KCl in 1 liter distilled water.. The pH of the algal suspension was identical with that of the original buffer. The packed volume of cells per liter of culture medium was calculated from the observed packed volume/900 cc. of harvest and is termed the culture density, CD. Scenedesmus cells suspended as above retained most of their Hill activity for 24 hours if kept in the dark at 3-5°C. Aliquots of the original culture after harvesting containing a known volume of cells were centrifuged. The packed cells were resuspended in 3 ml. 80% ethanol and heated to boiling for approximately 1 minute, extracting the cell pigments quantitatively. The visible absorption spectrum of the combined extract and two successive ethanol washings was measured with a Cary recording spectrophotometer. The optical density at the 660-670 Å maximum multiplied by the combined volume of extracts and divided by the volume of cells (in mm^3) in the aliquot is termed herein the relative chlorophyll content, RCC. This number may be converted to mmoles chlorophyll a/mm^3 cells by dividing by the molar extinction coefficient of chlorophyll a in ethanol. An approximate value based on ether as solvent is $\varepsilon = 9 \times 10^4$ liter $mole^{-1}$ cm^{-1}. (6)

Quinone was used as oxidant and was freshly purified by sublimation before each experiment, as inhibitory products are produced upon storage, especially when illuminated. Solutions of 1-2 mg. quinone/ml. distilled water were used immediately after preparation. Solutions of dl 6T (yellow cryst.), dl 5T (white cryst.), dl 6DT (oil), and dl 6 MO (oil), were prepared in M/3 phosphate buffer (pH = 6.7) in concentrations of 0.25 - 1.0 mg./0.050 ml.***

Standard Warburg double side-arm manometer flasks were used. The illuminated surface area (bottom) of the vessels was 7.9 - 8.3 cm^2. The flask constants were determined three times with water as described by Umbreit, et al. (7). The experiments were carried out in a thermostat bath which was illuminated through a window in the bottom of the bath by either (1) seven General Electric reflector spots, (2) three 40 watt fluorescents, (3) seven General Electric reflector floods, (4) five General Electric photofloods. Measurements reported herein were made with the reflector floods with an intensity of 1.6×10^5 ergs/cm^2/sec. impinging on the vessels. The fluorescents were not intense enough to yield 6T stimulated rates; the reflector spots did not produce a sufficiently uniform light field; the photofloods were too short-lived for convenience. With the reflector floods the light field was uniform to within 5% throughout the thermostat.

The vessels were prepared for the Hill reaction as follows: 0.20 ml. of 20% KOH was added to the center well (greased lip) containing a 1 cm^2 filter strip. Two ml. of algal suspension was pipetted into the main compartment. To the control algae 100 λ of M/3 phosphate buffer (pH = 6.7) and to the "thioctic algae" 100λ of 6T, etc. in M/3 phosphate buffer was added and let stand with the cells for 10 minutes. One ml. of quinone solution was then inserted into the side arm and the vessels placed in the thermostat in the dark. It is important to add the quinone solution after the 6T as the quinone sublimes directly from the solution in the side arm to cell suspension, preventing the utilization of 6T. After thermal equilibration of the vessels in the thermostat the quinone was tipped into the cell suspension and after 10 minutes the lights were turned on. Early experiments were carried

out in N_2 atmosphere introduced after the 6T had first been incubated with the algae in air, but as there was no difference between the rate in N_2 and air, current experiments are being carried out in air. The rate of shaking in light was about 150 rpm and readings were taken at 2 minute intervals.

In calculating the rate values, R, used herein the microliters oxygen (μl. O_2) evolved was divided by the illumination time in minutes for each two-minute reading. The mean of the three highest of these average rates throughout each experiment is termed R, corresponding most nearly to the "initial rate" found frequently in the literature of the Hill reaction. Frequently the μl. O_2 evolved in the first two minutes was several μl. smaller than in successive two-minute intervals and hence in these cases R was somewhat greater than the initial rate. In general, R values were found to be reproducible with a particular culture to 0.5 μl./min. and differences are not to be considered significant unless the difference, ΔR, is greater than 1.5 μl/min. R values varied considerably from harvest to harvest and interharvest rate comparisons are not significant.

Experimental

Gross character of effect of thioctic acid on the Hill reaction. -

Illuminated Scenedesmus incubated with quinone evolved oxygen at rates approaching 20 μl. O_2/min./40 μl. cells (30 cell volumes/hour). The yield of oxygen corresponded to 91.3% $\pm$ 3 of the stoichiometric yield, considerably higher than reported by earlier observers using cellular material for the Hill reaction (4,8,9,10). Cells incubated with 0.1 - 1.0 mg. 6T prior to incubation with quinone evolved oxygen more rapidly (Fig. 1) but with the same final yield as controls without added 6T. In 91 experiments, for example,

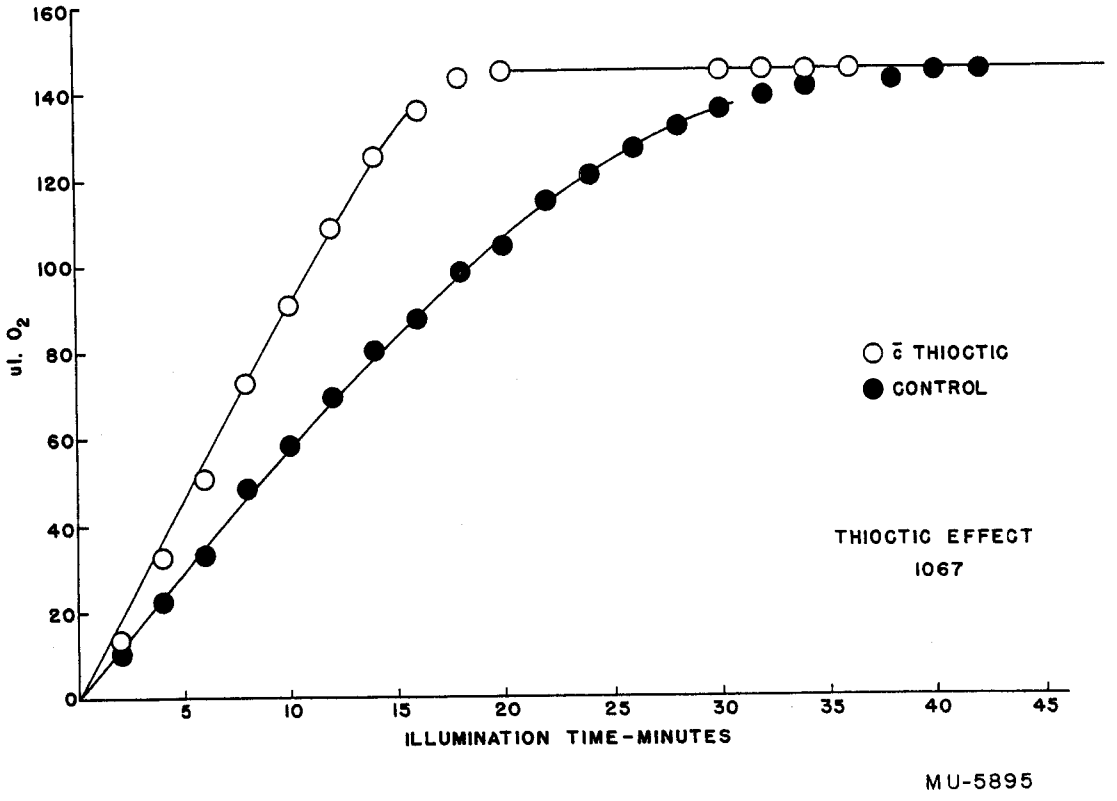

Figure 1

Stimulation of the Hill reaction by 6-thioctic acid. 1.5 mg. quinone, 0.25 mg. 6-thioctic acid, 30 mm^3 Scenedesmus. 15.7° C., aerobic.

enough 6T was added to yield a total of 1821 additional μl. O_2 if 6T were to be reduced to 6DT and act with quinone as a Hill oxidant. Actually 34 μl. less O_2 was evolved in these experiments than in the controls without added 6T. In confirmation of this observation no O_2 was evolved when Scenedesmus incubated with 6T in the absence of quinone was illuminated. Under the same physical conditions Chlorella evolved oxygen with quinone as oxidant with essentially the same rate as Scenedesmus but the rate was not increased by prior incubation with added 6T. In investigating the effect of 6T on Scenedesmus physical conditions were found under which 6T could be either stimulatory, inhibitory or without effect, and these conditions are described in some detail below.

Effect of 6T upon the quantum efficiency of the Hill Reaction. -

A crucial insight into the nature of the rate stimulation by 6T was gained by terminating the illumination while the cells were evolving O_2 at an appreciable rate, cf. Table I. The evolution of O_2 immediately ceased indicating the absence of rate-determining steps involving either the formation and diffusion of O_2 from the cells or the gas-liquid equilibration. The increased R with added 6T thus reflects an actual increase in the quantum efficiency, i.e. that 6T allows light energy which would otherwise be wasted to be used in photochemical oxidation-reduction. The 6T thus increases the quantum yield but not, as seen in the previous experiment, the final yield per milligram quinone. These observations emphasize the importance of the numerical difference between 6T and control rates, i.e. ΔR rather than the proportional difference, i.e. $\Delta R \div$ control rate.

Chemical specificity of the 6T effect. -

Conclusions as to the significance of the rate stimulation depend upon the chemical specificity of 6T. When 0.5 mg. of the six-membered ring isomer of 6T, i.e. 5,8-thioctic acid, was added to the cells (cf. Table II), the stimulation, measured by ΔR, amounted to as much as 2/3 of the effect with 0.5 mg. 6T. However, as the concentration of added 6T and 5T was decreased, the stimulation decreased more rapidly with 5T than 6T so that ΔR was nearly five times as great with 0.125 mg. 6T as with 0.125 mg. 5T, indicating that although 5T was stimulatory, it was only 1/5 as active per milligram at low concentration levels (2×10^{-4} M). That 5T appeared more active proportionately per milligram at high concentration levels (1×10^{-3} M) is attributable to a 6T concentration saturation phenomenon (cf. below). The 6MT which possesses the 5-membered ring structure of 6T was 60-70% as effective as the latter at the 0.25 mg. level. 6DT and 6 MO were inactive, when incubated either in dark or light (Table II). DPN and DPNH were inactive (cf. Table III). 10^{-3} M iodoacetate did not affect either the control or 6T stimulated rate. 10^{-3} M $HgCl_2$ inhibited the control rate somewhat less than the 6T stimulated rate. 10^{-3} M hydroxylamine hydrochloride was completely inhibitory in both control and 6T cells. 1-2 mg. hydroquinone did not affect the rate or yield of O_2 evolution (cf. Table IV).

Effect of 6T concentration on the rate of the Hill Reaction. -

As mentioned above, the effect of 6T upon the rate depends upon the concentration of added 6T. As can be seen from Table V, R increased with increasing 6T up to about 0.1 mg./vessel (2×10^{-4} M) and then became insensitive to the 6T concentration. The two experiments carried out one year apart demonstrate

Table I

Effect of 6T upon the Quantum Efficiency of the Hill Reaction

40 mm^3 Scenedesmus, RCC = 0.58,[1] CD = 2.9,[2] 0.5 mg. 6T

Exper. date	exp. temp.	mg.Q	microliters O_2			
			control		with 6T	
			6M[3] light	6M dark[4]	6M light	6M light ± 6M dark
1-19-54	15.7	1.07	45	43	66	66
		3.08	67	69	77	79
1-19-54	29.6	1.05	61	58	90	90
		3.00	89	89	82	79

(1) Relative chlorophyll content

(2) Culture density

(3) 6 minutes illumination

(4) 6 minutes illumination followed by 6 minutes dark

Table II

Chemical Specificity of the Effect of 6T on the Rate of the Hill Reaction

40 mm^3 Scenedesmus, exp. temp. 15.7°

Exper. date	RCC	CD	mg.Q	R (μl/min.) Control	6T	5T	6MT	6MO[2]	6DT[2]	mg. added 6T	5T	6MT
1-27-54	.58	3.4	0.99	10.5[1] 10.5[1]	19.5[1] 17.6[1]	14.7 12.0[1]	—	—	—	.50 .125	.50 .125	—
1-26-54	.61	3.3	1.04	10.0[1]	16.3[1]	14.3[1]	14.5	—	—	.50	.50	.50
1-15-53	—	3.2	1.00	3.5[1]	6.6[1]	4.8[1]	—	—	—	.78	.81	—
3-10-53	.50	2.8	1.50	6.7[1]	10.0	6.1	—	6.9	6.9[1]	.25	.27	—
3-11-53	.50	2.8	1.51	6.0[3] 7.3[4]	9.0 10.1	—	—	6.4 6.4	6.9 7.0	.25 .25	—	—
4-30-53	—	2.3	1.51	4.7	8.1	—	6.8	—	—	.25	—	.25
4-30-53	—	2.3	1.96	7.0	10.2	—	8.8	—	—	.25	—	.25

(1) Average of duplicates.

(2) 0.25 mg. added when used.

(3) Light incubation for 50 minutes.

(4) Standard dark incubation 50 minutes.

Table III

Effect of Misc. Additives upon the Rate of the Hill Reaction

Exp. temp. 15.7

Exper. date	RCC	CD	mm^3 Scen.	mg.Q	mg.6T	additive	additive	Rate (μl/min.) No addant no 6T	No addant 6T	With addant no 6T	With addant 6T
1-28-54	.74	2.8	40	1.05	.50	$HgCl_2$	10^{-3} M	4.9	9.5	2.4	3.4
						Iodoacetate	10^{-3} M	4.9	9.5	5.7	10.5
						Hydroxyl-amine-HCl	10^{-3} M	4.9	9.5	1.3	0.4
5-1-53	---	2.3	30	1.51	.25	As_2O_3	10^{-3} M	5.4	6.2	5.2	5.9
4-15-53	.62	2.0	30	1.51	.25	DPN	$6x10^{-4}$ M	5.1	7.1	5.0	
						DPNH	$5x10^{-4}$ M	5.1	7.1	4.9	

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Table IV

Effect of Hydroquinone on the Rate of the Hill Reaction

Exp. temp. 15.7°, 30 mm^3 Scenedesmus

Exper. date	CD	mg.Q	R (μl/min.) Control	With 1.0 mg. Hydroquinone	With 2.0 mg. Hydroquinone
7-9-53	2.1	1.65	2.7	2.8	2.6
6-27-53	2.0	1.54	3.8	3.3	3.2

Table V

Effect of 6T Concentration on the Rate of the Hill Reaction

40 mm^3 Scenedesmus, exper. temp. 15.7°

Exper. date	RCC	CD	mg.Q	mg.6T =1	R (μl/min.) Relative 6T Concentration 0	1	1/2	1/4	1/8	1/16
1-26-54	.61	3.3	1.10	.50	9.2[1]	15.7	16.7	13.4[1]	12.1	9.4
1-2-53	—	1.6	1.03	.86	4.6	10.8	10.1	10.1	8.4	6.0

(1) Average of duplicates

the remarkable constancy of the effect of 6T. The natural concentration of 6T in these cells is approximately 1-2 x 10^{-5} mg. 6T per 40 mm^3 Scenedesmus (11) so that a ten-thousand fold excess of exogenous 6T is required under the incubation conditions used to reach maximum stimulation.

Incubation conditions. -

The increase in R by 6T is quite sensitive to certain conditions of the incubation with Scenedesmus. In a typical experiment (Exp. date 3-11-53, 1.51 mg. quinone, 0.25 mg. 6T when added) the control rate was 5.0 μl./min.; with 6T incubated with Scenedesmus in air for 50 minutes followed by 60 minutes in N_2 before illumination in N_2 the rate was 6.8 μl./min.; with 10 minutes air incubation followed by 60 minutes N_2 as above the rate was again 6.8; when incubated 60 minutes in N_2 before illumination the rate was 5.1; when the 6T was pre-mixed with quinone and added together the rate was 5.0; when the 6T was added to the cells in N_2 five minutes after the quinone addition and 10 minutes before illumination, the rate was 4.5 μl./min. Apparently the cells use oxygen to prepare 6T for its stimulatory function. This same effect is evident in Table VI in which it is shown that the control rate was identical in air or commercial nitrogen atmosphere but that oxygen was required during the incubation period.

Table VII compares the effect of 6T upon the rate with identical samples of Scenedesmus one of which was kept at room temperature for 6 hours in diffuse light and the other which was stored at 3° in dark for the same period. Scenedesmus loses much of its ability to carry out the Hill reaction under the former conditions as well as their ability to use 6T as a rate stimulant.

As seen in Table II light or dark incubation made little change in the effect of 6T.

Table VI

Effect of Incubation Conditions on the Rate of the Hill Reaction

40 mm^3 Scenedesmus, exper. temp. 15.7°, 1.04 mg. quinone,

1/2 hour incubation time

			R (μl/min.)			
Exper. date	RCC	CD	air, no 6T	air, with 6T	N_2, no 6T	N_2, with 6T
1-27-54	.58	3.4	15.3[1]	18.1[1]	15.3[1]	14.9[1]

(1) Averages of duplicates.

Table VII

Effect of Storage Conditions on the Rate of the Hill Reaction

40 mm^3 Scenedesmus, 1.54 mg. quinone, exper. temp. 15.7°

			R (μl/min.)			
			Stored 6 hours at room temp.		Stored 6 hours at 3°	
Exper. Date	RCC	CD	Control	With 6T	Control	With 6T
1-8-54	.54	2.9	4.6	4.5	8.1	11.6

Effect of pH. -

To test the effect of pH 100 λ 1 M KOH or 100 λ 1 M HCl were added to the cells prior to 6T incubation, and after the illumination the pH of the suspensions were determined. The rate stimulation was essentially insensitive to pH (cf. Table VIII). In other experiments the pH of the initial buffer, cell suspension before and after illumination with quinone and with or without 6T was found to be within 0.02. That 6T controls the rate by changing the pH is improbable.

Effect of quinone concentration upon the rate of the Hill reaction. -

The particular conditions of light intensity, reaction temperature, and algal density chosen for early experiments resulted in control rates which could be increased by increasing quinone concentration, cf. Table IX. While the rate was frequently nearly first order in quinone concentration between 1 and 2 mg., the rates became zero order in quinone generally between 2 and 4 mg. Beyond 4 mg. quinone per vessel the control rates decreased with increasing quinone, an effect observed previously in the Hill reaction (9,10, 12). The 6T appeared as stimulatory in the quinone-dependent region, had no effect at quinone-saturation, and was inhibitory at quinone-inhibition. The variation in rates between experiments (Table IX) indicates the presence of variables, presumably involving algal culture conditions which are not yet under complete control.

Temperature apparently affects the 6T effect by changing the sensitivity of the control rate to quinone concentration. At higher temperatures the control rate was more highly quinone-limited, as measured by ΔR, and 6T was more highly stimulatory.

Table X shows similar data with Chlorella. As has been observed previously (9,10) the control rates with this organism were quinone-independent or quinone-

Table VIII

Effect of pH on the Rate of the Hill Reaction

Exper. date 1-27-54, 40 mm^3 Scenedesmus

0.5 mg. 6T when used,

1.02 mg. quinone, exper. temp. 15.7°

	R		
Final pH	Control	6T	ΔR
6.17	11.4	14.0	2.6
6.75	10.2[1]	15.4	5.2
7.35	12.5	16.3[1]	3.8

(1) Average of duplicates.

Table IX

Effect of Quinone Concentration and 6T upon the Rate of the Hill Reaction in Scenedesmus

40 mm^3 Scenedesmus, 0.50 mg. 6T added

					R (μl/min.) Relative Quinone Concentration							
					Without 6T added				With 6T added			
Exper. date	RCC	CD	Exp. temp.	mg.Q = 1	1	2	3	4	1	2	3	4
1-18-54	.60	3.0	15.7	.87	6.0	9.5	13.7	12.6	9.5	13.5	13.4	11.4
1-13-54	.69	2.8	15.7	.88	3.9	8.7	12.5	15.0	6.9	12.3	14.1	13.2
1-11-54	.47	3.0	15.7	.88	4.2	8.1	9.4	9.7	9.7	14.4	13.8	13.6
1-9-54	.47	2.9	15.7	1.02	4.9	8.5	10.5	9.8	8.4	11.0	10.6	8.3
1-19-54	.58	2.9	1.2	1.06	2.8	-	3.9	-	2.7	-	4.1	-
1-19-54	.58	2.9	15.7	1.08	5.4	-	8.8	-	8.1	-	9.8	-
1-19-54	.58	2.9	29.6	1.05	10.1	-	15.1	-	17.4	-	14.6	-

Table X

Effect of Quinone Concentration and 6T

upon the Rate of the Hill Reaction in Chlorella

Exper. temp. 15.7°

date	RCC	EC	mm^3 Chlor.	mg. Q = 1	R Realtive Quinone Concentration Controls 1	Controls 2	with added 6T 1	mg.6T
3-5-53	.21	5.3	40	1.01	3.6	2.9	2.6	.50
2-28-53	.50	-	40	1.00	8.6	9.5	6.9	.50
3-1-53	.38	-	40	1.00	6.0	7.0	5.3	.50
2-23-53	.39	1.4	40	1.00	7.8	7.6	5.4	.46
			20	1.00	4.6	4.1	3.2	.46
2-11-53	.42	3.3	100	1.50	9.3	-	9.2	.26
			50	1.50	9.3	-	6.9	.26
2-13-53	-	3.2	40	1.00	8.0	6.9		
			20	1.00	5.3	4.1		
			10	1.00	3.3	2.9		
			5	1.00	2.0	1.1		

inhibited and hence the 6T effect was also either nil or inhibitory. This Chlorella was cultured under as nearly the same conditions of nutrient, CO_2 pressure, light intensity and temperature as Scenedesmus as possible. We are investigating the possibility of extending the range of measurements to conditions under which Chlorella will yield quinone-dependent control rates.

Quinone limited Hill reaction rates have not been reported although to the authors' knowledge Scenedesmus has not been used previously in the Hill reaction with quinone. A possible interpretation of such quinone limitation is that the diffusion of quinone into the cell is rate limiting and this process is accelerated by 6T. Such an interpretation, if the quinone within the cells is never a large fraction of the total quinone in the system, would require that the preliminary incubation of the living, respiring cells specifically with 6T, in some way enhances the subsequent diffusion rate of the quinone (hydroquinone) into (and out of) the dead cells. Alternatively, if the quinone within the cells becomes a large fraction of the total quinone in the system prior to illumination, increasing the time allowed for this process in the dark should increase the effective quinone concentration in the cells and hence the rate. Table XI demonstrates the lack of effect of dark contact time between cells and quinone and makes this interpretation improbable.

Preillumination of Quinone. -

Table XII demonstrates the inhibitory effect of illuminating quinone (9,10) in the side arm of the vessel before mixing with the Scenedesmus. The inhibition selectively reduced the rate with added 6T. The R values do not reflect this fact adequately since R decreased much more rapidly as the reaction proceeded in illuminated Scenedesmus, using preilluminated quinone, with 6T than without. In one hour for example the former evolved 60 μl. O_2 while the latter,

Table XI

Effect of Contact Time with Quinone upon the

Rate of the Hill Reaction

40 mm^3 Scenedesmus, exper. temp. 15.7°

Exper. date	RCC	CD	mg. Q	mg.6T	R (μl/min.) Cells mixed with quinone before illumination: for 22 minutes Control	for 22 minutes with 6T	for 6 minutes Control	for 6 minutes with 6T
1-8-54	0.54	2.9	1.50	0.49	8.8	13.0	8.3	12.8

Table XII

Effect of Preillumination of Quinone on the

Rate of the Hill Reaction

40 mm^3 Scenedesmus, exper. temp. 15.7°, 1.55 mg. quinone

Exper. date	RCC	CD	R (μl/min.) Without preillumination Control	Without preillumination with 6T added	10 minutes preillumination of quinone Control	10 minutes preillumination of quinone with added 6T
1-9-54	0.47	2.9	5.3	13.3	4.3	5.2
			Yield after 1 hour (μl)			
			142	145	111	62

111 μl. This inhibition may be closely related to the inhibitory effect of high quinone concentration and the further inhibition by 6T under such conditions. It will be difficult to interpret the nature of the 6T effect especially as to the comparison between maximum rates with and without 6T until more is learned about the chemistry of the quinone inhibition.

It is important to note that the maximum concentration of the photoproduced inhibitor (Table XII) can be set at 10^{-5} M based upon estimates of intact quinone remaining after the preillumination.

Effect of light intensity upon the rate. -

An extremely important condition for 6T rate stimulation is a very high light intensity. As far as we have been able to discern, no intensity is too high. Table XIII shows this effect by comparing the stimulation, ΔR, with and without a non-selective light filter (calibrated bolometrically) covering the bottom of the Warburg flask. On the average, under the particular experimental conditions, reducing the incident light intensity to 42% reduced ΔR by a factor of 6-fold. In several instances a stimulation at 1.6×10^5 ergs/cm^2 became an inhibitory effect at 0.7×10^5 ergs/cm^2, demonstrating the coexistence of a stimulatory and an inhibitory effect which depend in different ways upon light intensity, quinone concentration, etc. In other words it would seem that 6T definitely raises the "ceiling" of the light intensity-rate curve but that its effect on the lower, linear part of the curve is obscured by an opposing inhibitory effect.

Effect of algal density. -

Because of mutual shading in the rather dense suspensions used (20 mm^3 cells/ml.) and the sensitive relation between light intensity and 6T effect it might be expected that the stimulation would decrease with increasing algal density. Table XIV indicates that this is not the case. Presumably it is the

Table XIII

Effect of Light Intensity on the Rate of the Hill Reaction

40 mm^3 Scenedesmus

Exper. date	RCC	CD	mg.Q	mg.6T	exp. T	R			
						1.6 x 10^5 ergs/cm^2		0.67 x 10^5 ergs/cm^2	
						control	with 6T	control	with 6T
1-18-54	.60	3.0	1.56	.50	29.6	14.2	13.5	9.0	7.0
1-8-54	.54	2.9	1.57	.50	15.7	7.8	12.0	6.6	8.6
4-21-53	-	3.0	1.51	.25	20.7	4.7	7.7	3.0	3.5
4-20-53	-	3.0	1.56	.25	20.7	5.5	9.9	3.5	5.9
4-20-53	-	3.0	1.51	.25	15.7	3.2	6.9	2.6	5.1
8-14-53	-	2.0	1.51	.48	29.5	8.9	17.1	7.4	7.5
2-8-53	.56	2.8	2.00	.49	15.7	12.1	12.6	8.8	6.9
			1.00	.49	15.7	7.9	9.7	6.7	6.9

Table XIV

Effect of Algal Density on the Rate of the Hill Reaction

Exper. temp. 15.7°

Exper. date	RCC	CD	mm^3 Scen=1	mg.Q	mg.6T	R Relative algal density					
						controls			with 6T		
						1	2	4	1	2	4
4-14-53	.62	2.0	20	1.52	.25	5.2	7.7	-	7.4	10.5	-
3-9-53	.50	2.8	10	1.50	.27	3.2	4.5	6.3	3.5	6.8	10.2
3-4-53	.41	3.0	20	1.00	.53	3.3	4.9	-	4.1	6.8	-

quinone/alga ratio which determines the quinone-limitation rather than the quinone molarity and this ratio decreases more rapidly than light/alga resulting in the observed increase in ΔR as density is increased.

Combined effects of light and quinone. -

As seen above, (1) at lower light intensities the rate stimulation by 6T was smaller and the control rates less quinone-limited; (2) at higher quinone concentrations control rates were more light-limited and the 6T stimulation was again smaller and occasionally negative, demonstrating the inhibitory component of 6T effect, cf. Table XV.

Comparison with Photosynthesis. -

Several papers have reported stimulations of the Hill reaction in chloroplast preparations by inorganic ions, such as chloride, as well as organic materials (10, 13,14). However, in such cases the control rates have been very low compared with photosynthesis, coinciding with very low % stoichiometric yields. The maximum rate of the Hill reaction in saturating light, 1 mg. quinone, and neutral phosphate buffer is reported to be nearly the precise value for photosynthesis in Warburg No. 9 buffer for Chlorella (9). This appeared to be the case with Scenedesmus under these conditions (Table XVI), and the 6T stimulated rate was thus above the photosynthetic rate. The correspondence between the control photosynthesis and Hill rates was coincidental since the Hill rate with Scenedesmus was quinone-limited at 1 mg. quinone. The 20% stimulation of photosynthesis requires further investigation to determine whether it is an artifact and/or reproducible effect.

Table XV

Combined Effects of Light and Quinone Concentration

40 mm^3 Scenedesmus, exper. temp. 15.7° C

Exper. date	Light intensity quinone concn.	R (μl/min.)			
		1.6 x 10^5 ergs/cm^2		0.67 x 10^5 /cm^2	
		1 mg. Q	3 mg. Q	1 mg. Q	3 mg. Q
2-8-54	Control	6.6	10.9	4.3	6.6
	6T (0.1 mg.)	8.1	12.0	5.6	6.6
2-4-54	Control	6.3	12.8	4.2	8.5
	6T (0.5 mg.)	12.6	12.8	8.0	6.9
2-4-54	Control	8.2	13.8	6.0	11.3
	6T (0.5 mg.)	13.4	12.2	9.7	7.4

Table XVI

Comparison of the Effect of 6T on the Rates of

Photosynthesis and the Hill Reaction

40 mm^3 Scenedesmus, exper. temp. 15.7°

Exper. date	RCC	CD	mg.Q	mg.6T	R			
					Photosynthesis in Warburg buffer 9		Hill reaction in M/15 PB	
					Control	With 6T	Control	With 6T
2-2-54	.68	2.4	1.04	.50	6.7[1]	8.3[1]	7.0[1]	12.6[1]

(1) Averages of duplicates.

Discussion

In discussing the nature of the effect of 6T upon the Hill reaction several aspects of the reaction itself are pertinent. The necessary characteristics of the mechanism are clear. Light is absorbed by chlorophyll, giving rise to the separation of water into reduced and oxidized fragments, the former leading to quinone reduction and the latter to oxygen evolution. Since no oxygen is evolved in the absence of quinone these oxidized and reduced fragments must be capable of uniting to reform water. Since the yield of oxygen corresponds very nearly to the transfer of two H-atoms from water to quinone, there can be no appreciable side reactions of these oxidized and reduced fragments other than their reunion. The effect which we have observed is that synthetic 6T incubated with Scenedesmus increases the yield of oxygen per unit of absorbed light, and hence the rate, but does not increase the ultimate yield of oxygen (Figure 1). From what has been said above 6T can therefore act either to increase the efficiency with which light is used to separate water into oxidized or reduced fragments or to reduce the rate at which they later reunite. Either mechanism would give rise to the observed increase in quantum efficiency with no change in ultimate yield.

Under the conditions in which 6 T is stimulatory, increasing the quinone concentration also increases the quantum efficiency without increasing the ultimate yield per milligram quinone (Table IX). Presumably quinone acts to prevent the reunion of redox fragments rather than to increase the rate of water photolysis, although there is some indirect evidence that quinone may be intimately associated with the chlorophyll (15,16). The mechanism may involve a competition between quinone and an oxidized water fragment for a reduced water fragment, the quinone being favored by increasing its concentration. At sufficiently high quinone concentrations the quantum efficiency reaches a maxi-

mum value (Table IX) resulting either from the complete prevention of redox reunion, a diffusion or otherwise limited quinone reduction, or a limiting inhibitory effect of quinone. Since the quantum efficiency decreases with increasing quinone concentration beyond the optimum value, such an inhibitory effect does supercede eventually.

If 6T increases the rate of redox separation the observation that at quinone saturation there is no observable 6T stimulation (Table IX) must be explained by a diffusion or otherwise limited quinone reduction, or a limiting inhibitory quinone effect. If either of these explanations were valid then an increase in the rate of redox separation brought about by increased light at quinone saturation rather than increased 6T should not increase the rate of oxygen production, i.e., the yield of oxygen per unit time of illumination. In experiments carried out under conditions in which it is known that increasing the redox separation by increased light does indeed give higher rates (Table XV), the maximum quantum efficiency is not greater with added 6T than without. Taken at face value these experiments would eliminate the possibility that 6T does nothing but increase the efficiency with which quanta are used to separate the redox fragments of water. However, there is evidence that in these experiments 6T increases the inhibitory effect of quinone and this side effect may well mask the true relationships of quinone-saturated rates.

If, on the other hand, 6T acts to prevent the reunion of redox fragments it may act similarly to quinone, i.e. in competition with an oxidized water fragment for a reduced water fragment. If this were the case then we would expect a higher yield of oxygen with 6T than without, corresponding to the transfer of 2 H-atoms from water to 6T, and this is not observed. Hence we must further assume that the reduced 6T in turn reduces quinone and thus acts merely as a hydrogen transfer system. Since 0.1 mg. 6T is about as effective in increasing the quantum efficiency as 1.0 mg. quinone (Table IX) we must assume that 6T is reduced and subsequently reduces quinone, the whole process occurring at approximately 20 times

the rate (on a molar basis) as quinone is reduced directly. Alternatively, 6T may act in this manner at a different point of redox reunion from quinone, i.e., as an intermediate hydrogen carrier not directly involved in quinone reduction. This would seem more reasonable in view of the rapidity with which the presumably non-enzymatic "by-pass" reduction of quinone would be required to proceed.

The stimulation of quantum efficiency by 6T increases with increasing light intensity (Table XIII). This observation is consistent with the action of 6T either in redox separation or hydrogen carrier functions. With increasing light intensity both the quantum conversion and hydrogen transport systems approach their limiting capacity, resulting in increased quantum decay and reunion of redox fragments, and providing 6T with greater opportunity to increase the efficiency. Furthermore, the net 6T effect is the result of a stimulatory action and a smaller inhibitory action which is presumably not light sensitive, so that increases in the stimulatory effect by increased light absorption are made to appear proportionately greater in the net stimulatory effect.

The rise of quantum efficiency produced by 6T increases with increasing temperature (Table IX). At low temperatures the major part of the redox fragments formed reunite rather than form oxygen and hydroquinone. Regardless of whether 6T acts by increasing the rate of redox separation or as a hydrogen carrier we must assume that at low temperatures the redox fragments at another point in the 6T stimulated reaction sequence reunite at a more rapid rate than in the control, to offset in part the increased efficiency of the 6T reaction.

The kinetic data discussed above do not unequivocally discriminate between the action of 6T in redox separation as contrasted with prevention of redox reunion and the question arises as to whether there is any chemical basis

upon which such a decision can be made. The 6T is a cyclic disulfide which exhibits specific redox properties as demonstrated by its role as the coenzyme in pyruvic acid oxidation (17,18). The strain-energy in its 5-membered ring (1, 19) presumably makes it a better oxidant than the 6-membered ring disulfide, 5T, which may explain the greater Hill activity of the 6T. Its mode of action in the Hill reaction may thus involve cyclic oxidation-reduction of the disulfide-dithiol, as in pyruvate oxidation, in acting as a hydrogen carrier although there are several other redox possibilities within this system. An alternative possibility, suggested by Calvin and Barltrop (1) is that the strain energy of 6T would lower the energy required to open the sulfur-sulfur bond so that energy from an excited state of chlorophyll could accomplish the fission. It appears now that the 6T would need to be closely associated with the chlorophyll and that the energy be transformed by a process of internal conversion from electronic to vibrational excitation in the complex. The resulting activated 6T may then react directly with water as proposed by Barltrop, Calvin and Hayes (19) to form a thiol sulfenic acid. This single step would accomplish the redox separation required of photosynthesis and the Hill reaction since the thiol is a good reductant and the sulfenic acid appears to be a reasonably strong oxidant (19). The fact that the dithiol, 6DT, and monoxide, 6MO, are inactive under present conditions in the Hill reaction (Table II) suggests that some other intermediates are involved, or that these are not susceptible to incorporation under the conditions used. Since the relative effectiveness of 6T and 5T is different in pyruvate oxidation from what it has been found to be in the Hill reaction, one would suppose that the rate-limiting step is different in the two cases.

Thus, according to this quantum conversion model, the natural 6T concentration associated with the chloroplast would not be sufficient to convert all of the absorbed quanta at high light intensities and added 6T assists in

this naturally occurring process. Our present knowledge of the chemistry of 6T thus is consistent with quantum conversion and/or hydrogen transport functions.

One important problem is whether the 6T acts to increase the quantum efficiency by competing with a naturally occurring process or by accelerating a process which is otherwise carried out more slowly with a smaller amount of naturally occurring 6T. Studies on the inhibition of the Hill reaction have generally observed a marked lack of sensitivity of the reaction to sulfhydryl reagents such as mercury compounds, iodoacetate, arsenite, etc. (Table III; ref. 20). Taken at face value this would indicate that 6T is not naturally involved in this reaction and that it must act in competition with a natural process. However, it is difficult to determine whether these poisons are able to penetrate to the locus of 6T action, possibly being physically excluded or tied up chemically by other, more easily accessible thiols. That this is a very real possibility is borne out by the fact that when these poisons do not inhibit the control reaction they also do not inhibit even the 6T stimulation which ought to be reduced if the poisons are sulfhydryl active. It is only when the control reaction itself is inhibited that the 6T stimulation is reduced.

It would seem improbable that if 6T were not a natural intermediate in the Hill reaction, metabolic acitivity would be required to prepare the 6T for its stimulatory function (Table VI and Text). We believe this oxygen requirement during incubation is closely related to the binding of the 6T to the chlorophyll molecule or complex to prepare it to internally convert electronic excitation of the chlorophyll to a vibrational excitation and fission of the sulfur-sulfur bond of 6T.

Inhibition of the Hill reaction may be brought about either by a high

quinone concentration (Table IX), photolyzed quinone at lower concentrations (Table XII), or a moderate quinone concentration and added 6T (Table IX). The chemical and kinetic behavior of this effect may prove extremely important in elucidating the Hill mechanism. It may be that an interaction between quinone itself or more probably a derivative such as hydroxyquinones (21), and 6T either natural or added, results in the inhibition, supporting the idea that 6T is a natural Hill intermediate. It is also of great interest to discover whether the quinone-saturation of rate is primarily the result of the inhibitory effect, as the saturation rates are important clues as to the mode of 6T stimulatory action.

Summary

1. Conditions have been defined under which 6-thioctic acid, 6T, increases the quantum efficienty of the Hill reaction in Scenedesmus. This increase occurs when the control rate is quinone-limited, under conditions of high light intensity, low quinone concentration, high temperature, and high algal density. When the control rate is inhibited by high concentrations of quinone or photolyzed quinone, 6T results in a further decrease in rate.

2. Fundamental characteristics of the Hill reaction suggest that 6T either increases the rate at which water is separated into reduced and oxidized fragments (quantum conversion agent) or decreases the rate at which these fragments later recombine (hydrogen carrier or quinone diffusion).

3. The variation of the effect with 6T concentration, incubation conditions, pH, quinone concentration, temperature, preillumination of quinone, dark contact time with quinone, light intensity, algal density, sulfhydryl poisons, plant species, and other reagents does not permit an unequivocal discrimination between the two possible mechanisms.

Footnotes

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(**) The following abbreviations will be used throughout this paper: 6T, 6,8-dithiooctanoic acid; 6DT, 6,8-dithioloctanoic acid; 6MO, 6,8-dithiooctanoic acid monoxide; 5T, 5,8-dithiooctanoic acid; 4T, 4,8-dithiooctanoic acid; 6MT, 8-methyl,6,8-dithiooctanoic acid; DPN, diphosphopyridine nucleotide.

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