THE ACTION SPECTRUM OF THE HILL REACTION IN WHOLE ALGAL CELLS AND CHLOROPLAST SUSPENSIONS. (RED DROP, SECOND EMERSON EFFECT AND INHIBITION BY EXTREME RED LIGHT).

BY

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THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY RAJNI VARMA GOVINDJEE

ENTITLED THE ACTION SPECTRUM OF THE HILL REACTION IN WHOLE ALGAL CELLS AND CHLOROPLAST SUSPENSIONS. (RED DROP, SECOND EMERSON EFFECT AND INHIBITION BY EXTREME RED LIGHT).

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

In Charge of Thesis

Head of Department

Recommendation concurred in†

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† Required for doctor's degree but not for master's

D517

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CHAPTER I

INTRODUCTION

It has been believed until recently, that chlorophyll a is the primary sensitizer of photosynthesis, because of its universal occurrence in all true photosynthetic organisms, and also because the excitation energy taken up by other pigments often is re-emitted as fluorescence of chlorophyll a (55,56,9,24,10,44,45,43). However, the discovery of Emerson and Lewis in 1943 (13; also cf. 29,49,34 and 16) that the quantum efficiency of photosynthesis declines well within the long-wave absorption band of chlorophyll a, posed the question -- not recognized as such until years later -- whether excitation of chlorophyll a by itself is sufficient to give a full yield of photosynthesis. There was no experimental evidence as to the cause of the "red drop" and the factors controlling its occurrence, until Emerson and coworkers (14,15,16) discovered that, if light of shorter wavelengths (< 680 mμ) is simultaneously provided, the red drop disappears. This enhancement has been called the "second Emerson effect",* (abbreviated to "Emerson effect" in further discussion). The question arose that whether the enhancement was due simply to the supply of higher energy quanta, or to the need to excite an accessory pigment together with chlorophyll a. Emerson et. al (15,18) studied the action spectrum of the enhancement effect, and found that in Chlorella, it follows the curve showing the fraction of absorbed light absorbed by chlorophyll b, with peaks at 480 mμ, 560 mμ, 600 mμ and 650 mμ. In Navicula, it follows the curve for the participation of fucoxanthol in the absorption,

*It has been called the second Emerson effect, to distinguish it from the CO_2 burst at the beginning of the light period -- also known as the Emerson effect.

with a peak at 540 mμ; in Anacystis, that of phycocyanin absorption with a peak at about 600 mμ; and in Porphyridium, that of phycoerythrin absorption with a peak at 540 mμ. These findings suggested a more specific role for the accessory pigments than mere absorption of light energy in spectral regions where chlorophyll a absorption is poor, and transfer of the excitation to chlorophyll a -- the only function that had been ascribed, until lately, to the accessory pigments. The new findings suggested that for photosynthesis to take place with full yield, at least one accessory pigment must be excited simultaneously with chlorophyll a; failure to do so causes the red drop.

Franck (23) suggested a somewhat different interpretation, by assuming two forms of chlorophyll a; the red drop occurs when light is preferentially absorbed by one form of chlorophyll a only. Simultaneous excitation of another form (directly, by absorption of light < 680 mμ, or indirectly, by energy transfer from accessory pigments) is required for efficient photosynthesis. At that time, no spectroscopic evidence for the existence of two forms of chlorophyll a existed. Myers and French (42) confirmed the findings of Emerson and found additional evidence of specific participation of chlorophyll b in certain induction phenomena in photosynthesis.

Hlinks (2) observed that transient changes in the rate of photosynthesis occur upon changes in the spectral composition of incident light. Myers and French (42) found that the action spectrum of this effect is similar to that of the Emerson effect, indicating specific participation of chlorophyll b.

Laverel (39), from his studies of the absorption spectra of fluorescein and chlorophyll a in solution, suggested that variable amounts of chlorophyll a may exist in a dimeric form in living cells. Krasnovsky

and co-workers (37,38) also suggested that the red drop is due to an "aggregate" form of chlorophyll a. They thought that the photochemically active monomeric form absorbs around 670 mμ, whereas the form absorbing at about 680 mμ is an inactive "aggregate".

Albers and Knorr (1) reported that they could observe, in the spectra of chloroplasts, in addition to the main absorption peak at 682 mμ, another one at 670 mμ, and several minor maxima. Because of the asymmetric and broad nature of the chlorophyll a red absorption band *in vivo*, Duysens (11) assumed that there may be more than one form of chlorophyll a, a situation similar to that known to exist in bacteriochlorophyll *in vivo*. French and co-workers (5,25) using differential spectroscopy, confirmed the existence of several peaks of chlorophyll a *in vivo*, at about 670 mμ, 680 mμ and 690 mμ. Complexity of the red absorption band of chlorophyll a was noted also by Halldal (33) in *Anacystis nidulans*, by Thomas and Marsman (50) in *Porphyra perforata*, and by Cederstrand (7) and Thomas and Govindjee (52) in *Porphyridium cruentum*.

The findings of Govindjee and Rabinowitch (29) and Rabinowitch and Govindjee (46) in addition to confirming that preferential light absorption by fucoxanthol in *Navicula*, phycocyanin in *Anacystis*, phycoerythrin in *Porphyridium* and chlorophyll b in *Chlorella*, accounts for most peaks in the action spectra of the Emerson effect, showed that these action spectra also contain peaks due not to accessory pigments, but to a special form of chlorophyll a (Chl a 670)* (cf. 26). This gave direct confirmation not only of the *existence* of different forms of chlorophyll a but, what is more interesting, of their *different photochemical function*. The peak at 670 mμ was most

*Chl a 670 refers to the form of chlorophyll a absorbing around 670 mμ. The other forms of chlorophyll a, with absorption peaks at about 680 mμ and 690 mμ, will be referred to as Chl a 680 and Chl a 690.

pronounced in those of Anacystis and Porphyridium. Beside the peak at 670 mμ, in the action spectrum of the Emerson effect, Govindjee (27) also noted peaks in the blue region, which too, could be attributed to Chl a 670 -- a peak at 420 mμ and one at 440 mμ.

These findings of Govindjee and Rabinowitch (26,29) lend support to Franck's (23) assumption of the existence of two forms of chlorophyll a with different functions in photosynthesis.

The present investigation deals chiefly with the red drop, the Emerson effect and more generally with the effect of combining two light beams of different wavelengths, on the rate of the Hill reaction, in whole Chlorella cells and in chloroplast suspensions from Phytolacca americana. Even though in the work on photosynthesis a very carefully estimated respiration correction was made, there was no direct evidence that the Emerson effect was not due, at least in part, to an inhibition of respiration by light (rather than to an enhancement of photosynthesis), because the techniques employed -- manometry (4,21,22) and polarography (42) -- could not distinguish between positive changes in the rate of photosynthesis and negative changes in the rate of respiration. There was also no evidence as to the locus of the effect -- in the oxygen-evolving or in the carbon-dioxide reducing phase of photosynthesis. The Hill reaction (photochemical reduction of quinone and other oxidants, and liberation of oxygen, sensitized by plant cells or chloroplast suspensions) lacks the intricate enzymatic reactions of photosynthesis, but retains its photochemical mechanism. It was thought that studies of the Hill reaction could answer the above questions.

To see whether accessory pigments (such as phycocyanin) can participate in the observed effects, a blue-green alga, Anacystis, was studied in addition to Chlorella and chloroplast suspensions.

In Emerson's action spectrum of the enhancement effect, the curve dipped below the 100 line at some wavelengths, showing photosynthesis to be inhibited rather than enhanced by the combination of the two beams. Govindjee and Rabinowitch (29) noticed a similar "negative" effect in photosynthesis. They also noted that the negative effect could be converted into a positive effect, if the light action of the far red light alone was reduced. Furthermore, it was observed by Mcleod (41), and by Govindjee and Rabinowitch (29) that photosynthesis sensitized by 700 mμ light had a very low saturation level; it is not impossible that this low saturation is, at least partly, responsible for the apparent inhibition. The negative Emerson effects will be discussed in chapter 2.

Another interesting phenomenon was found by Thomas and Govindjee (51,52). They noted that white light filtered through a dense phycobilin filter produced no photosynthesis at all in Porphyridium cruentum, although the far red light transmitted by the phycobilin filter was strong enough to cause some excitation of chlorophyll a. Later Rabinowitch, Govindjee and Thomas (47) and Govindjee, Rabinowitch and Thomas (28) showed that extreme red light $>$ 720 mμ (contained in the phycobilin-filtered white light) was inhibiting photosynthesis produced by far red light (680-720 mμ). From the study of the action spectrum of this inhibition they found that its maximum lies around 740-760 mμ.

A similar study revealed an inhibition of the Hill reaction by extreme red light ($>$ 720 mμ); these results will be discussed in chapter 4.

CHAPTER II

THE SECOND EMERSON EFFECT IN THE HILL REACTION IN ALGAL CELLS

A. MATERIALS AND METHODS:

I. Description of the Algae and Preparation of the Sample for Manometry:

Two species of algae (Chlorella pyrenoidosa, Emerson's strain 3; and Anacystis nidulans) were used. These are unicellular organisms; as such, they offer fewer difficulties for manometric measurements than multicellular plants, because of a more efficient gas exchange with the medium. Following is a brief summary of the properties of the two algae.

	Chlorella pyrenoidosa (Emerson's Strain 3) a green alga	Anacystis nidulans a blue-green alga
Description	unicellular	unicellular in turbulent cultures; sometimes forming two to four celled filaments.
Size	average diameter = 5 mμ	ca. 1.6 x 2.2 μ
Pigments	chlorophyll a, chlorophyll b and carotenoids	chlorophyll a, phycocyanins and carotenoids (traces of phycoerythrin?)

These algae were grown in inorganic culture media, as indicated in tables 1 and 2. Table 3 summarizes the growing conditions.

The algal suspensions were centrifuged at 1600 x g in the case of Chlorella, five minutes and in that of Anacystis ten minutes were enough to spin the cells down. The supernatant was removed and the cells were resuspended in .15M phosphate buffer (pH 6.8). After washing the cells twice

in the buffer, the suspension was finally diluted until absorption at the peak of chlorophyll a absorption (680 mμ) was about 60% for Chlorella and about 90% for Anacystis, when measured in 1 cm. Beckman cuvettes. Pure nitrogen (99.99%) was conducted through the algal suspension for about 15 minutes. 6.8 ml of the cell suspension were then transferred into the rectangular manometric vessel. A solution of 0.25% recrystallized para-benzoquinone (purified by sublimation) in 0.01 N sulphuric acid was prepared and 0.2 ml of it added to the 6.8 ml of the algal suspension, thus bringing the total fluid volume in the manometric vessel to 7 ml (the vessel constant k was calculated for this volume). Phosphate buffer was used in the Hill reaction studies (in contrast to carbonate-bicarbonate buffers used for photosynthesis measurements), because the para-benzoquinone is unstable at the high pH of the carbonate buffers (cf. Erhmantraut and Rabinowitch 12). The compensating vessel, used in the double manometer, received 7.0 ml of the phosphate buffer alone. Nitrogen was passed for another 10 minutes through the manometer vessels after they had been attached to the manometer. No carbon dioxide was added to the gas phase, but no attempt was made to remove traces of this gas [see Warburg (57), Brown (3) and Stern and Vennesland (48) concerning a possible need for traces of carbon dioxide in the Hill reaction]. After the addition of quinone to the cell suspension, care was taken to keep and handle them in the dark, since exposure to light causes a destruction of quinone. The quinone solution was always stored in darkness and in the cold, and a fresh solution was prepared every week.

Evolution of oxygen due to the reduction of quinone in light was measured by a differential manometer [Emerson and Chalmers (17)] at 10°C. Measurements were made at intervals of one minute, with a precision of 0.01 mm, by using a double cathetometer. The two telescopes of the cathetometer were focussed on the menisci of the manometric fluid, which were illuminated by

Table 1

CULTURE MEDIA

Main constituents are described in this table,
for the micronutrients see Table 2

	CHLORELLA	ANACYSTIS
	g/l	g/l
$MgSO_4 \cdot 7H_2O$	0.25	0.25
KH_2PO_4	1.875	--
Na_2HPO_4	0.025	--
K_2HPO_4	--	1.00
KNO_3	1.25	1.00
$CaCO_3$	0.016	--
$Ca(NO_3)_2 \cdot 4H_2O$	--	0.025
KCl	0.025	--
Sodium citrate	--	0.165
$FeSO_4 \cdot 7H_2O$	0.0057	0.0057
Micronutrients		
*A_2	1 ml/l	--
***A_5	--	1 ml/l
##ABC	0.05 ml/l	--

Table 2

MICRONUTRIENTS

*A_2	$MnCl_2 \cdot 4H_2O$	1.81 g/l
	H_3BO_3	2.86 g/l
**A_3	$ZnSO_4 \cdot 7H_2O$	0.22 g/l
	$CuSO_4 \cdot 5H_2O$	0.079 g/l
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.20 g/l
***A_5	$A_2 + A_3$ (with slight modifications taken from Hoagland, 35)	
#B_9	(after Brody and Emerson, 4, with slight modifications)	
		mg/l
	$Al_2(SO_4)_3Na_2SO_4 \cdot 24H_2O$	.786
	KBr	.119
	KI	.083
	$Cd(NO_3)_2 \cdot 4H_2O$	.154
	$Co(NO_3)_2 \cdot 6H_2O$	.145
	$Cr(NO_3)_3 \cdot 9H_2O$	.040
	$NiSO_4 \cdot 6H_2O$	.131
	$Na_3VO_4 \cdot 16H_2O$	.041
	$Na_2WO_4 \cdot 2H_2O$	.033

Table 2 continued

		mg/l
C_{10}	As_2O_3	6.61
	$SrSO_4$	10.49
	$HgCl_2$	6.77
	$PbCl_2$	6.71
	LiCl	30.55
	RbCl	14.20
	$K_2TiF_6 \cdot H_2O$	5.00
	$NaSeO_4$	11.96
	$Be(NO_3)_2 \cdot 3H_2O$	103.7
	Uranyl nitrate	10.0
## ABC	5 ml A_3 + 5 ml B_9 + 5 ml C_{10} made up to 100 ml	

Table 3

SUMMARY OF GROWING PROCEDURES

Algae Isolated by	*Chlorella pyrenoidosa* Emerson	*Anacystis nidulans* Kratz and Allen
Inoculum in µl/200 ml	15-20	15-20
Culture medium described by	Emerson and Chalmers	Kratz and Myers
Illumination	four tungsten lamps and one white fluorescent ring	four white fluorescent rods and one tungsten bulb
Wattage	60 watt tungsten lamp and 32 watt white fluorescent ring	40 watt white fluorescent rod and 100 watt tungsten bulb
Distance from the bottom of flask	6" (tungsten lamps) 4" (fluorescent ring)	7 1/2"
Axis to axis distance	3" (tungsten lamps)	3 1/16" (fluorescent rods)
No. of days algal cultures were on line	2-3	2-3
Temperature of culture bath	20°C 2°C	27°C 2°C
Gas mixture	5% CO_2 in air	Filtered room air or 5% CO_2 in air
Growth (x fold)	40 fold	40 fold

" means inches

two lights, to permit the taking of readings while the manometer was being shaken, and thereby saving time and eliminating errors involved in the stopping of the shaker.

The manometer was mounted on a shaking panel with the vessels submerged in a water bath. The temperature of the water was maintained constant by means of a thermoregulator, a heater and a refrigeration unit. The manometer was shaken at a speed of 200 oscillations per minute, with an amplitude of 18 mm. Before starting the experiment, the cells were allowed to equilibrate in the Warburg tank for 30 minutes.

II. Optical Arrangement:

Monochromatic "supplementary" light was obtained from the Emerson-Lewis grating monochromator (13) with a General Electric 225 watt tungsten ribbon filament lamp. The lamp was run at 7.5 volts and 30 amperes on an A.C. power supply* connected to 110 volts, 60 cycles power line. The monochromator was calibrated with a cadmium lamp. The band widths used in the experiments were 10 mμ in the 600-700 mμ region and 20-30 mμ in the 400-600 mμ region. The light from the monochromator was focussed, by means of a lens, on an adjustable mirror, situated under the thermostated tank. The mirror reflected the beam, through a glass window, into the tank, and onto the bottom of the manometric vessel.

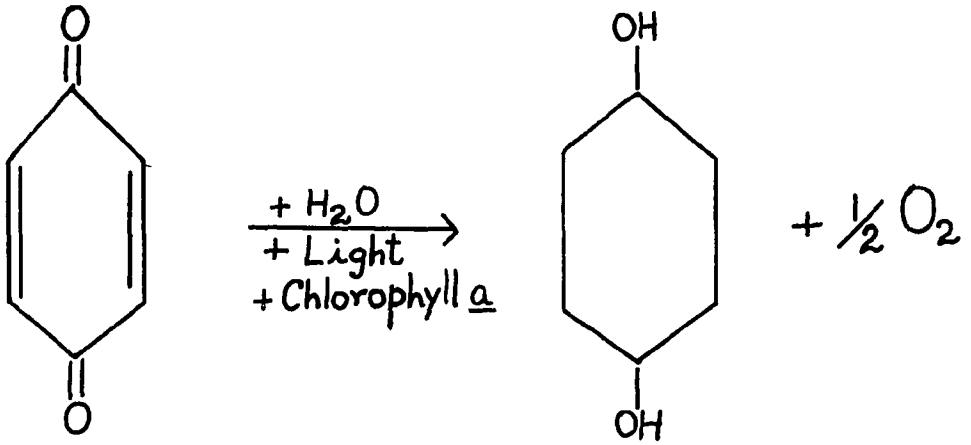
Far red light was provided by another assembly, consisting of a 1000 watt tungsten lamp, infra-red absorbing glass filter, three red cut off filters Schott RG8, RG5, and a Corning filter CS#2-64, a 45° reflecting prism, a lucite light pipe (to prevent the scattering of light by the strongly agitated water in the tank), and a stainless-steel mirror which reflected

*The power supply used was built by Mr. Carl N. Cederstrand.

this second light beam, too, ente the bottom of the manometric vessel. Fig. 1 curve A shows the transmission curve of the filter system used. In some later experiments, the Schott and Corning filters were replaced by a single interference filter (Farrand 109556) with maximum transmission at 700 mμ; its transmission spectrum is shown by curve B in fig. 1. Fig. 2 shows the optical system used.

III. Measurement of the Hill Activity:

Hill reaction in whole Chlorella cells was measured by following the pressure changes due to the evolution of oxygen in light in the presence of quinone (which is being reduced to hydroquinone).



Quinone Hydro-quinone

Quinone inhibits respiration and the enzymatic dark reactions of photosynthesis, so that there is no CO_2 exchange to interfere with the measurements of oxygen evolution. In most of the experiments, there was no dark reaction leading to observable pressure changes. In the few cases when small pressure changes were noticed in the dark, a correction was made by adding a corresponding term to the observed pressure changes in light. Whenever there was such a dark

Figure 1. A: Transmission curve of the combination of Schott glass filters RG5 and RG8, with Corning filter CS#2-64, used to obtain light $>$ 680 mμ.

B: Transmission curve of a Farrand interference filter (#109556), used to obtain light of $\sim$ 700 mμ.

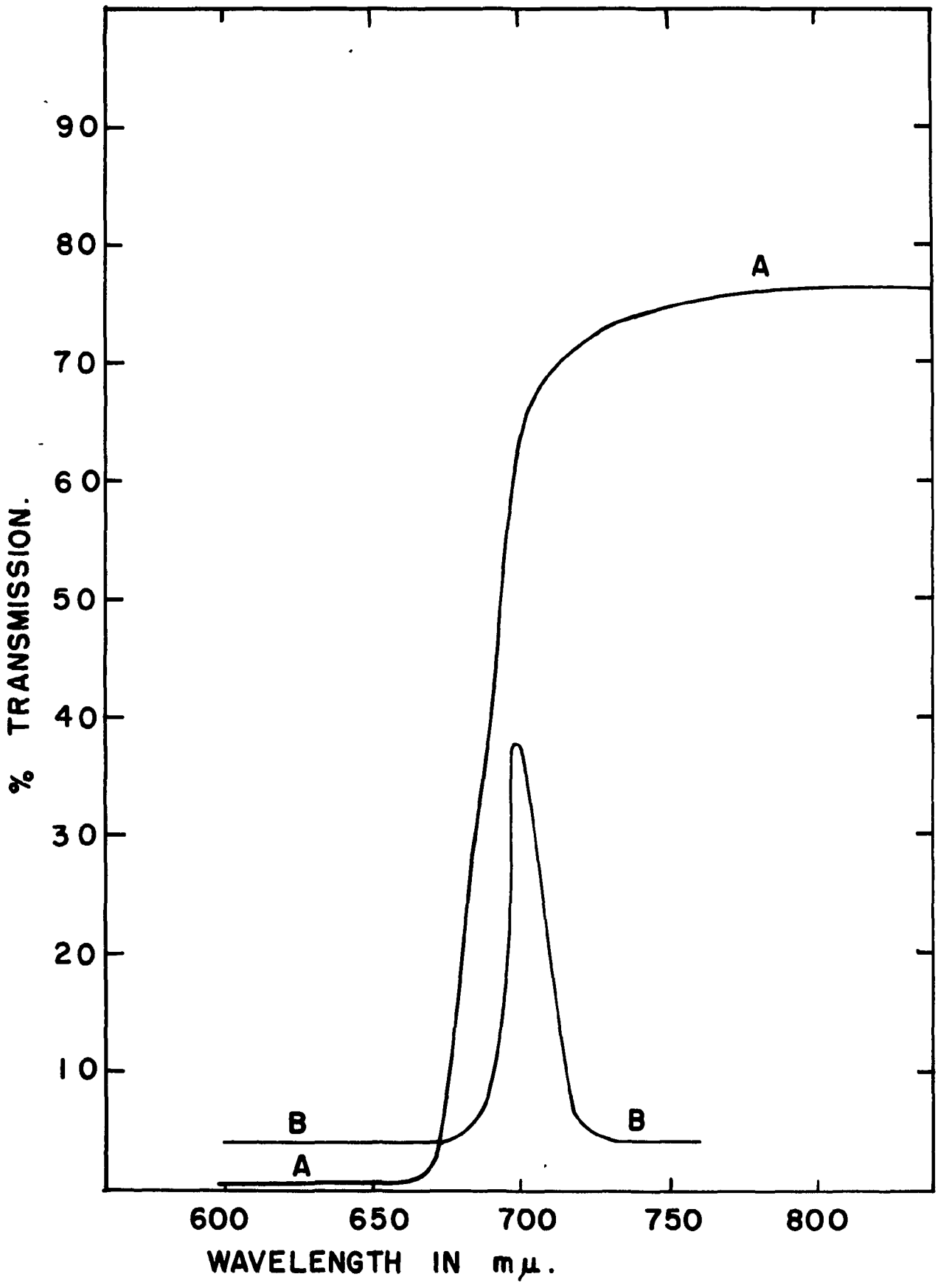
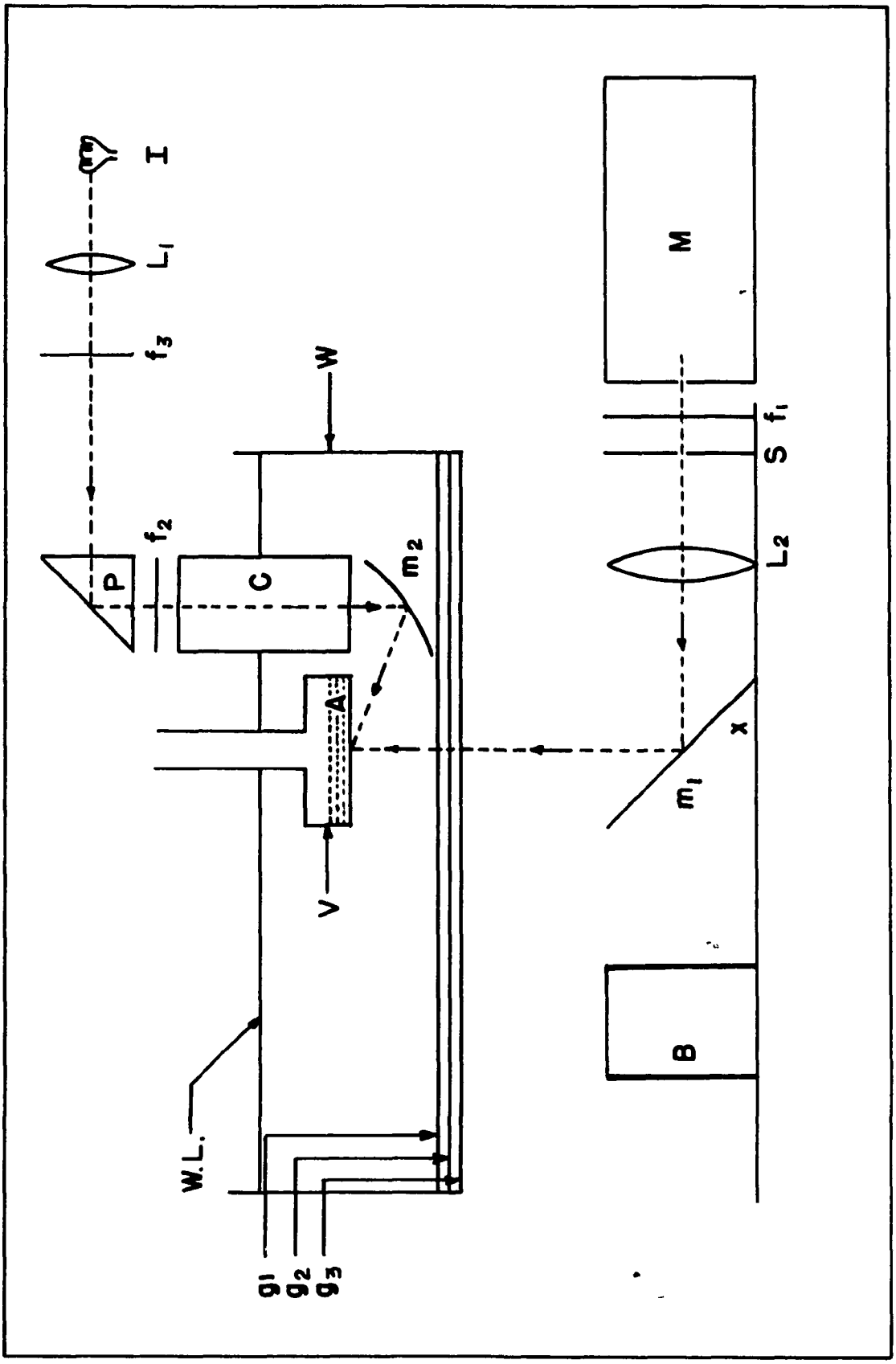

90
80
70
60
50
40
30
20
10
% TRANSMISSION.
A
B
B
A
600
650
700
750
800
WAVELENGTH IN mμ.

Figure 2: Optical arrangement used to illuminate cells with two beams of light. M: Emerson-Lewis monochromator; f_1: glass filter used to cut off overlapping higher orders in the far-red and extreme-red regions; f_2: interference filter used to isolate 700 mμ light; (or a combination of Schott glass filters RG5 and RG8 and a Corning filter CS#2-64 giving light $>$ 680 mμ); f_3: infrared-absorbing American Optical Co. filter; L_1 and L_2: lenses; I: Lighthouse 1000 Watt tungsten lamp; P: 45 degree total reflection prism; C: lucite column ("light pipe"; V: manometer vessel; A: algal suspension; m_1: mirror movable along axis X; m_2: stainless steel concave mirror; W: water bath; W.L.: water line; B: Bolometer; S: Exit slit of monochromator; g_1, g_2 and g_3: glass plates at the bottom of the water bath. Note that the diagram has not been drawn to scale (diagram after Thomas and Govindjee (22)).


I
L1
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S f1
f2
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C
m2
L2
A
x
m1
V
B
W.L.
g1
g2
g3

reaction, its speed was repeatedly checked during the experiment, in order to make a more accurate correction.

With the concentration of the quinone used, and under the conditions specified above, the Hill activity lasted 4-5 hours and remained fairly constant throughout the duration of measurements needed to cover the intended spectral range. This constancy was checked by repeating at the end of the series the measurement at the wavelength, at which the experiment was begun. If a decline was observed, an appropriate correction was made by plotting the measured light action at the reference wave length against time and interpolating linearly to determine the correction terms for the intermediate measurements.

Quantum yield measurements were made in the "red drop" region by using thin suspensions of algae, and making a correction for the incompleteness of absorption. For this purpose the true absorption curve of the suspension was measured by placing the algal suspension in the manometer vessel in an integrating spectrophotometer.* The quantum yield was calculated according to the following equation:

$$\Phi = \frac{L \times K_{O_2}}{22{\cdot}4 \times M \times K \times A}$$

where Φ is the quantum yield,

A is the fraction of light absorbed by the algae

L is the light action (in mm pressure change per hour),

K_{O_2} is the vessel constant for oxygen at the temperature of the experiment (10°C),

22.4 is the conversion factor to convert from microliters to micromoles,

M is the bolometer reading in microvolts

K is the factor to convert the microvolts read by the bolometer

*This instrument was designed and built by Mr. C. Cederstrand.

into the number of microeinsteins incident on the algal suspension per hour (for details of the evaluation of this factor see appendix 2 and figure 33 in the thesis of Govindjee (27)).

Measurements of the action spectrum of the Emerson effect did not involve determination of absolute quantum yields. The light action of the far red light of constant intensity was measured, in the presence and in the absence of the supplementary light of shorter wavelengths, as a function of this wavelength. The following sequence of readings (each lasting for a period of 5 minutes) was used: Dark, Far red, Dark, Supplementary, Supplementary + Far red, Supplementary, Dark, etc. The intensity of the supplementary light was varied from wavelength to wavelength, so as to get about the same rate of oxygen production at all wavelengths. The light action due to the far red light alone was designated as 100. The action of the far red light in the presence of supplementary light was calculated by subtracting the light action of the supplementary light alone from the light action of the combined supplementary and far red light. The Emerson effect was given, in per cent, by the expression:

$$E = \frac{\text{light action of the far red light (in presence of supplementary light)}}{\text{light action of the far red light (in absence of supplementary light)}} \times 100$$

The values of E were plotted against the wavelength of the supplementary light; the plot is the action spectrum of the Emerson effect. E values higher than 100 meant a positive Emerson effect; those below 100 a negative effect.

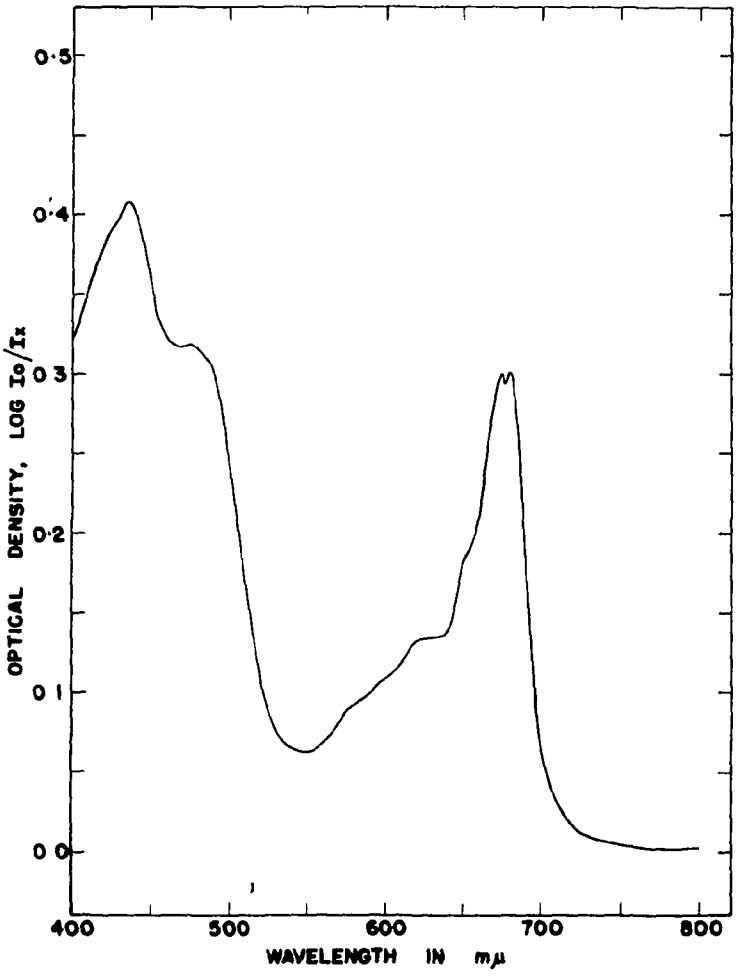

Figure 3: <u>Doublet structure of the red chlorophyll a absorption band of Chlorella pyrenoidosa.</u> Measured by Beckman DU spectrophotometer using the wet filter paper technique (52) Note peaks at 673 mμ and 680 mμ.

IV. Absorption Measurements:

The doublet nature of the red absorption band of chlorophyll a is shown in figure 3. It was measured in a Beckman DU spectrophotometer by the wet filter paper technique of Thomas and Govindjee (52). In this method the algal cell suspension is soaked onto a (Whatman #1 filter) paper strip, which is placed in a Beckman cuvette adhering to the side nearest to the photo-cell. Another strip is soaked in the culture medium or the buffer solution used as the blank. The differences in scattering due to variations in the scattering power of the filter strips were eliminated by measuring the optical density at 800 mμ, where the algae are transparent.

V. Measurement of Light Energy:

For the measurement of absolute quantum yield, it was necessary to know accurately the amount of light energy absorbed by the cells. For this purpose a bolometer was used. The advantages of using a bolometer are i) It has a large surface permitting to include in the measurement the whole beam incident on the manometric vessel; ii) its response is the same to equal energies at all wavelengths and iii) it measures light energy in absolute units. The bolometer circuit consists of 4 sets of very thin black platinum strips, two sets of these strips are in the dark all the time, whereas the other two are illuminated with the light beam whose energy is to be measured. The black surface on the strips absorbs light energy, heating the strips. A mirror galvanometer is put across one set of strips, and a resistance across the other set in the dark; the galvanometer is adjusted to zero. Now the strips are illuminated, by lowering the mirror in figure 2 out of the beam, and the potential required to bring the galvanometer needle back to zero is measured. The reading is converted into the number of einsteins falling on the manometric vessel per second, by multiplying it with a factor taken from a calibration graph (see Appendix 2, thesis of Govindjee (27),

for details of the calculation of this factor). It takes into account the differences in the optical path of the light beam incident upon the manometric vessel and upon the bolometer and the reflection losses at the several surfaces. The bolometer was calibrated by Emerson in 1950; and rechecked by Emerson and co-workers in 1958 (19), against a standard light source from the U.S. Bureau of Standards.

B. THE RED DROP

Ehrmantraut and Rabinowitch (12) noticed that the quantum yield of the Hill reaction (quinone reduction) in the green alga Chlorella declined between 669 mμ and 698 mμ; but they did not determine the complete action spectrum of this reaction. Therefore, a more systematic investigation was now made. Figure 4 shows a plot of the quantum yield of the Hill reaction with quinone as oxidant in Chlorella pyrenoidosa at 10°C, as a function of the wavelength, in the "red drop" region. Since the measurements were made with optically thin suspensions, a correction had to be made for the incompleteness of absorption; this was done by measuring the true absorption curve of the same suspension in Cederstrand's integrating spectrophotometer. Figure 4 shows that the maximum quantum yield of the Hill reaction, found in our cultures of Chlorella, was about 0.09. The decline in yield began at about 680 mμ -- the same wavelength at which the quantum yield of photosynthesis is known to show a decline. The quantum yield fell to half the maximum value at about 695 mμ. The existence of the "red drop" in the Hill reaction in Chlorella is clearly confirmed by this experiment. In a preliminary report (30) on this study, a curve was given which declined more steeply than the one presented here. This difference is due to the fact that accurate correction for incomplete absorption could not be made in the earlier experiments.

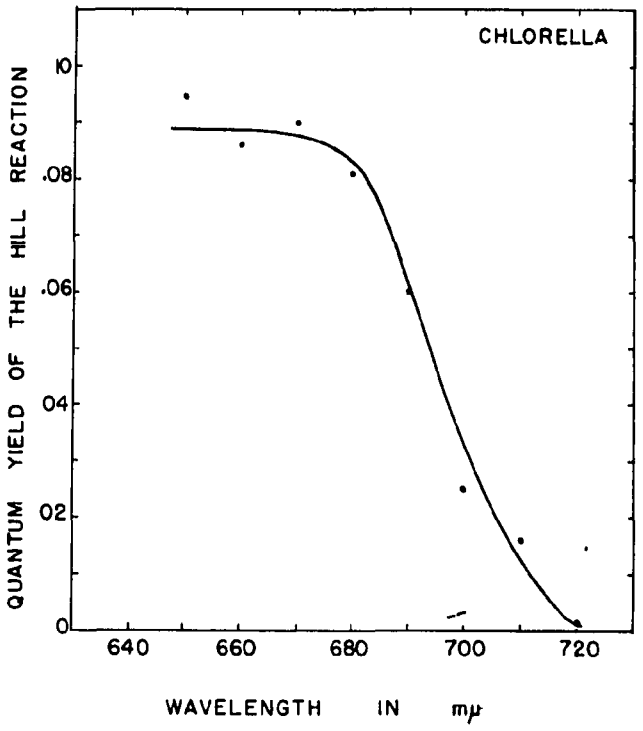

Figure 4: **"Red drop" in the action spectrum of the Hill reaction (quinone reduction) in Chlorella pyrenoidosa, at 10°C.**

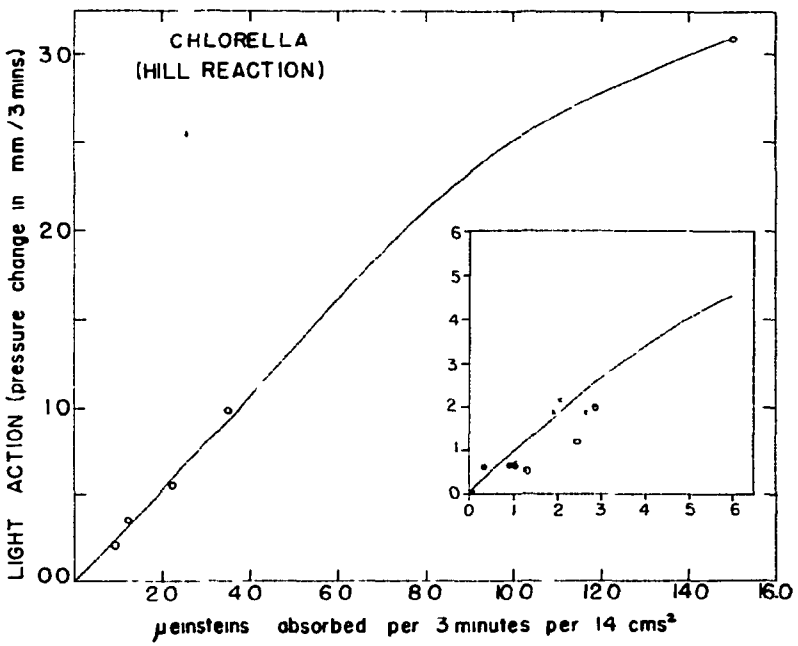

Figure 5: Light curves of the Hill reaction in <u>Chlorella pyrenoidosa</u> in White light. Insert shows the light curves for 680 mμ (solid line and crosses), and 700 mμ (dotted line and circles with dots).

The quantum yield measurements were carried out considerably below light saturation to ensure that they were restricted to the linear part of the "light curve" of the Hill reaction. Incidentally, determination of the light curves of the Hill reaction at different wavelengths (cf. figure 5) showed that the saturating light intensities may be quite different at different wavelengths -- again in analogy to recent observations on photosynthesis.

C. THE ACTION SPECTRUM:

Emerson and co-workers (15,18,20) showed that the quantum yield of photosynthesis in the far red light (in the region of the red drop i.e. $>$ 680 mμ) can be raised significantly by simultaneous exposure of the cells to light of shorter wavelengths. Upon studying the action spectrum of this enhancement, Emerson concluded that chlorophyll a by itself is relatively inefficient in photosynthesis and that it is necessary to excite some accessory pigment simultaneously with chlorophyll a in order to obtain a high quantum yield. The action spectrum of the enhancement effect follows in general the curve showing the fraction of total absorbed light absorbed by the accessory pigment. A more detailed study by Govindjee and Rabinowitch (29), showed however that there are two forms of chlorophyll a -- Chl a 670 and Chl a 690). A peak in the action spectrum of the Emerson effect appears at 670 mμ, suggesting that only one form of chlorophyll a -- Chl a 690 -- is "inactive"; when this form alone is excited, full yield of photosynthesis is not obtained. In order to obtain high yield, it is necessary to excite also the other form -- Chl a 670 -- (either directly or indirectly through transfer of excitation energy from the auxiliary pigments).

The finding of the Emerson effect in the Hill reaction (discussed below) proved convincingly that this effect is not due, at least in part, to light inhibition of respiration -- since respiration is completely suppressed

by quinone. Also, it proves that the locus of the effect is in the light phase -- often described (not quite correctly) as "photolysis of water" -- and not in the enzymatic phase of carbon dioxide reduction.

I. Role of the Chlorophylls in the Hill Reaction :

Figure 6 shows the action spectrum of the Emerson effect in the Hill reaction in Chlorella (a two-days-old culture) in the region of 640-700 mμ. Table 4 gives a summary of five experiments indicating the extent of differences between the behavior of different cultures. In all cultures there are two distinct peaks in the 640-700 mμ region. That at 650 mμ is due to chlorophyll b [cf. figure 7 in Emerson and Rabinowitch (18)] , while the peak at 670 mμ, found earlier in the action spectra of the Emerson effect in photosynthesis by Govindjee and Rabinowitch (29), must be attributed to a special form of chlorophyll a. The absorption spectrum of the Chlorella suspension (figure 3) shows peaks at 650 mμ, 670 mμ and 680 mμ in the red region. The peak at 650 mμ belongs to chlorophyll b. The peaks at 670 mμ and 680 mμ must belong to two distinct forms of chlorophyll a ("Chl a 670" and "Chl a 680").

Figure 7 is a continuation of the action spectrum of the Emerson effect in the Hill reaction of a two-day-old culture of Chlorella, into the region of shorter waves between 400 mμ and 640 mμ. The peaks at 480 mμ, 560 mμ, 580 mμ and 600 mμ, also present in Emerson's curve for the same effect in photosynthesis, can be ascribed to chlorophyll b. The peaks at 620 mμ and 520 mμ were noticeable neither in Emerson's curve (Figure 7, Emerson and Rabinowitch (18)), nor in that by Govindjee (27). However, since the 520 mμ peak did appear in Emerson's "fractional absorption curve" (18), its occurrence in the action spectrum was to be expected. This peak, too, must be due to chlorophyll b. The 620 mμ peak can be tentatively assigned to a vibrational sub-band of Chl a 670.

Table 4

THE SECOND EMERSON EFFECT IN THE HILL REACTION (QUINONE REDUCTION) IN CHLORELLA

Enhancement effect E (see text)

Wavelength, Supplementary light, mμ	Exp. # 1	Exp. # 2	Exp. # 3	Exp. # 4	Exp. # 5	Average
640	100	100	110	120	111	108
645	-	-	-	220	-	-
650	187	180	210	240	145	192.4
655	-	140	-	180	-	160
660	125	120	146	160	111	132.4
665	-	180	-	160	-	170
670	181	240	208	280	255	232.8
675	175	240	-	180	-	198.1
680	137.5	140	155	140	145	145.5
690	-	100	123	115	118	114
700	-	-	105	-	108	106

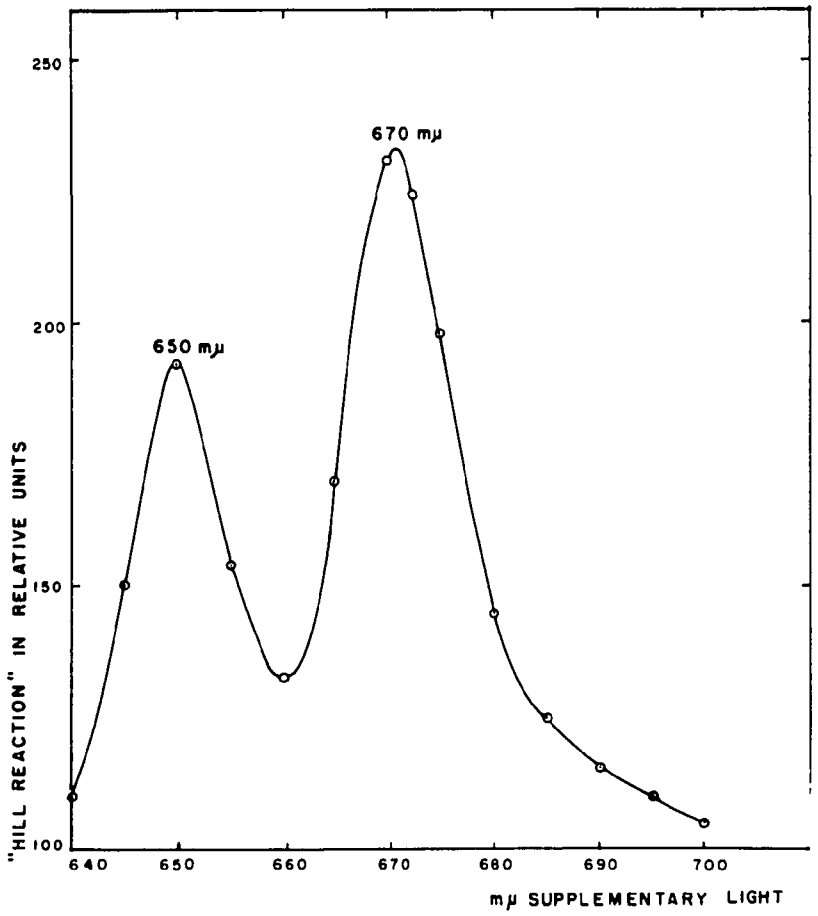

Figure 6: <u>The 670 mμ peak</u> (due to Chl <u>a</u> 670) <u>in the action spectrum of Emerson effect in the Hill reaction in Chlorella pyrenoidosa</u>. Action due to far red light (using Schott red cut-off filters RG8 and RG5 combined with a Corning filter CS#2-64; $>$ 680 mμ) was 3.0 $\mu l O_2$/hr/60 μl cells. Ratio of the light action of far red light alone to light action of supplementary light alone, 1:6. (This action spectrum was confirmed by using an interference filter, λ max = 700 mμ, to produce far red light.)

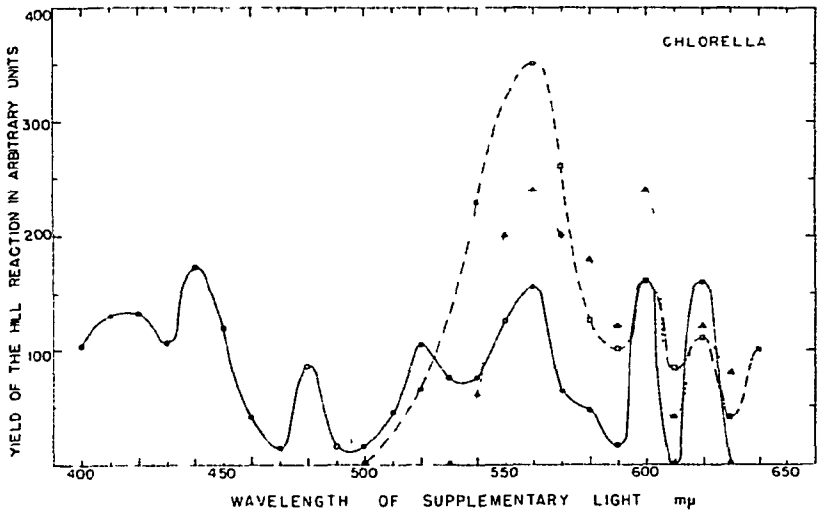

Figure 7: **Complete action spectra of the Emerson effect in the Hill reaction in Chlorella pyrenoidosa** (3 experiments). Peaks ascribed to chlorophyll b: 480 mμ, 520 mμ, 560 mμ and 600 mμ; peaks ascribed to Chl a 670: 410-420 mμ, 440 mμ and 620 mμ. Light action due to far red light alone (> 680 mμ) = 2.5 μl O_2/hr./60 μl cells. Ratio of the light action of far red light to supplementary light was about 1:1.5.

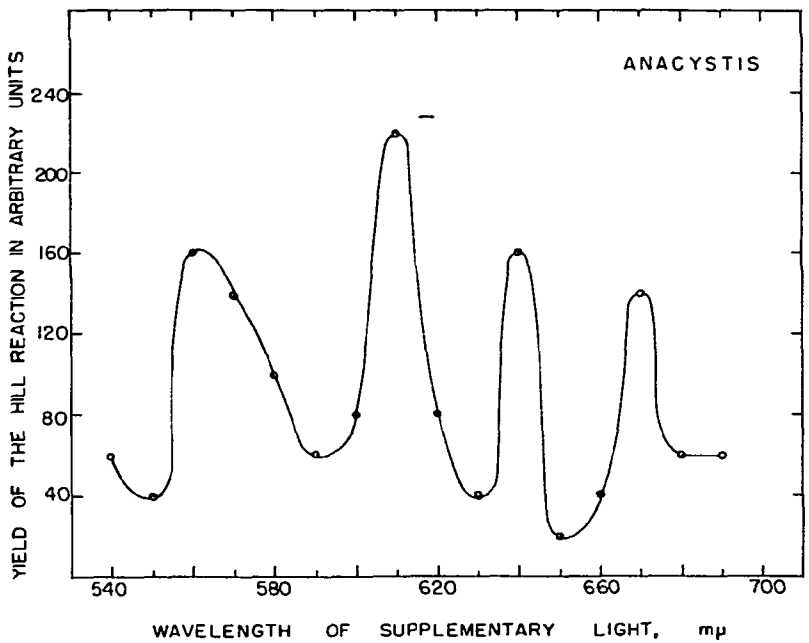

Figure 8: **Action spectrum of the Emerson Effect in the Hill reaction in Anacystis nidulans.** Peaks due to phycocyanin: 560 mμ, 610 mμ and 640 mμ. Peaks ascribed to Chl a 670 — 670 mμ. Light action of far red light = 1.2 $\mu l O_2$/hr/60 μl cells. Ratio of light action of far red light to that of supplementary light = 1:1.5.

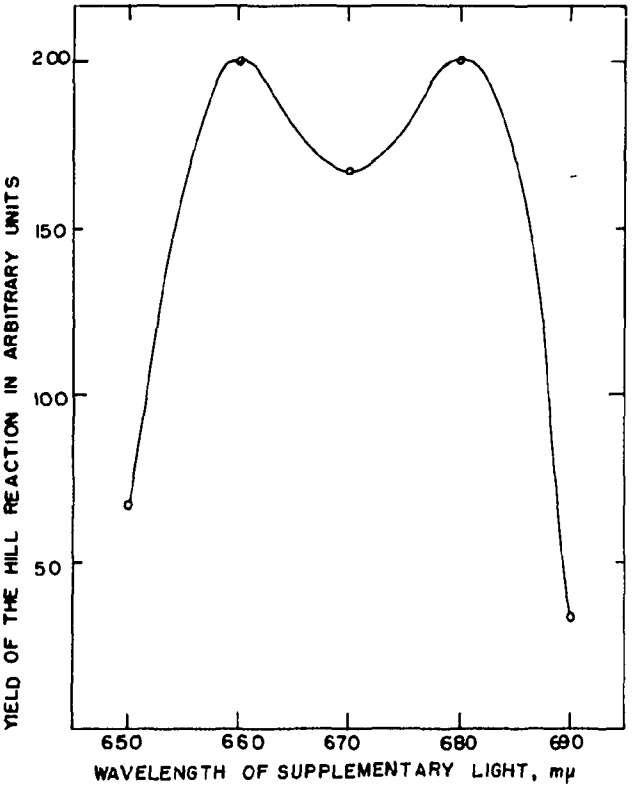

Figure 9: Action spectrum of the Emerson effect in the Hill reaction in 10-15 days old cultures of Chlorella at 10°C.

The precise locations of the action peaks at 410-420 mμ and 440 mμ could not be determined, because wide slits had to be used in this region, where the emission of the tungsten lamp is weak. These peaks can be tentatively assigned to the Soret band of Chl a 670, as suggested by Govindjee (27) in the case of photosynthesis. Cederstrand and Govindjee (8) have shown the existence of five different forms of chlorophyll a by the study of the Soret band of chlorophyll a.

Figure 8 shows the action spectrum of the Emerson effect in the Hill reaction in Anacystis. A peak at 670 mμ can be clearly noted in this spectrum, too.

Some experiments on the Emerson effect were carried out with Chlorella cells from 10-15 days old cultures. In these experiments, peaks were found not at 650 mμ and 670 mμ, but instead at 660 mμ and 680 mμ. Figure 9 shows the action spectrum of the Emerson effect for such "old" cells. The explanation for this behavior is not yet clear, but shifts may be due to a change in the relative amounts of the different forms of chlorophyll a.

II. Role of Phycocyanin in the Hill Reaction

Thomas and DeRover (53) showed that phycocyanin can serve as sensitizer for the Hill reaction in blue-green algae; but there has been no previous information on its involvement in the Emerson effect. Figure 8 shows an action spectrum of the latter. Beside a peak at 670 mμ (to be ascribed to Chl a 670), there are three other peaks, at 560 mμ, 610 mμ, and 640 mμ respectively. These peaks coincide approximately with peaks in the calculated curve showing the fraction of total absorbed light absorbed by phycocyanin [figure 6 in Emerson and Rabinowitch (18)]. Analogous results were found with the same alga Anacystis, also in the photosynthesis measurements by Govindjee and Rabinowitch (Figure 6 in (29)).

III. The Negative Emerson Effect in the Hill Reaction:

It was noted that the Emerson effect E, often falls below the 100 level, suggesting mutual inhibition of the two beams rather than enhancement. This is clearly shown by figures 7 and 8,9. Negative Emerson effects have been noticed previously also in photosynthesis (cf. Emerson and Rabinowitch (18) and Govindjee and Rabinowitch (29)). It has been suggested (29,41) that lower saturation level of photosynthesis in 700 mμ light may explain the negative Emerson effect, at least partly. For this, we must assume that when light of a wavelength at which the negative effect is observed (and which by itself, gives a normal saturation level) is added to light of 700 mμ, (whose saturation level is low), the saturation level of the combined beam is low. This assumption remains to be proved, and it is by no means certain whether it can provide a complete interpretation of all negative Emerson effects. Figure 5 shows that the Hill reaction, similarly to photosynthesis, is saturated at different levels in white light, at 680 mμ and at 700 mμ.

Experiments on the relation between the light intensity and the sign of the Emerson effect are illustrated by figures 10 A,B,C and D. The solid line curves show the Emerson effect at four different ratios between the "light action" of the far-red light and the "light action" of the supplementary light alone. Curves A correspond to 1:1 ratio, B to a 1:2 ratio, curves C to a 1:3 ratio and curves D to a 1:4 ratio. It is seen that a ratio 1:4 produces most extensive negative effects. However, the ratio of the two light intensities is not the only thing that matters. If the absolute intensity of far red light is lowered, the Emerson effects become positive, whatever the intensity of the supplementary light. If the intensity of the far red light is raised, negative effects appear at all ratios (cf. the solid and the dashed curve in Figures 10 B and C). This is in agreement with the above-suggested interpretation. No attempt has yet been made to study experimentally the light curves

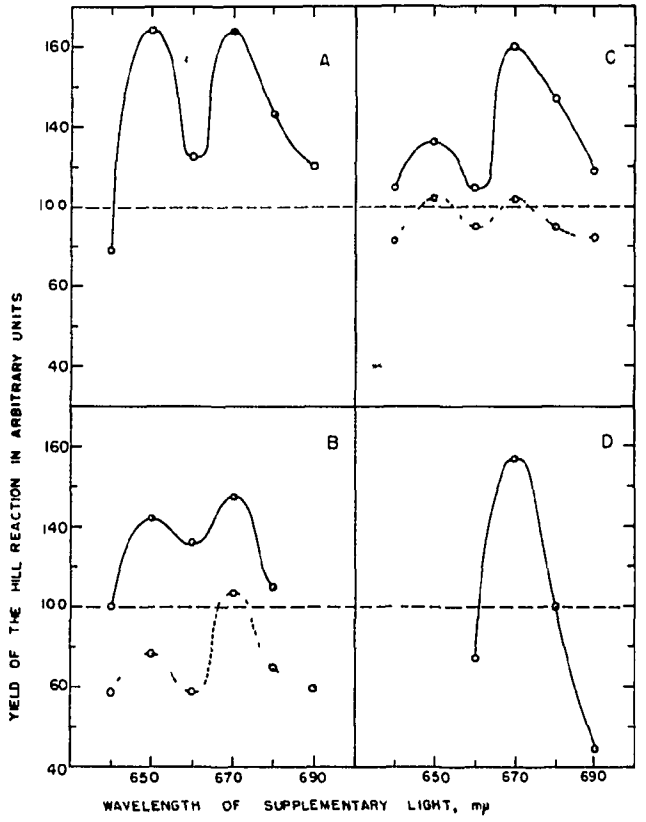

Figure 10: Effect of the absolute values and of the ratio of the light actions of the far red light and the supplementary light on the sign of the Emerson effect. Solid line curves in figure A, B, C and D.
Effect of increasing the absolute light actions, keeping the ratio of the action of far red light and supplementary light constant; see dotted lines in B and C
Effect of the rates: see solid lines in A, B, C and D.

A: Action of far red light = 2.0 μlO_2/hr. Ratio of the light action of far red and of supplementary light = 1:1.

B: Action of far red light -- solid line curve 2.4 μlO_2/hr; dashed curve 6.2 μlO_2/hr. Ratio of the light actions due to far red and supplementary light, 1:2.

C: Action due to far red light: solid curve, 2.4 μlO_2/hr; dashed curve 6.2 μlO_2/hr. Ratio of the light actions of far red and of supplementary light, 1:3.

D: Action of far red light = 4.0 μlO_2/hr. Ratio of the light actions of far red and supplementary, 1:4.

of the combined light, and compare them with those of the separate monochromatic beams.

The negative Emerson effect requires further investigation, before a definitive explanation can be proposed. The usual assumption is that the saturation of photosynthesis is due to a rate-limitation imposed by the available amount of a certain "limiting" enzyme. It obviously needs revision in the light of the finding that the saturation level in the 700 mμ region is different from that in shorter-wave light. This phenomenon must be understood before we will be able to predict the saturation level of mixed light.

CHAPTER III

SECOND EMERSON EFFECT IN THE HILL REACTION OF CELL-FREE LEAF EXTRACTS FROM POKEWEED (PHYTOLACCA AMERICANA)

A. MATERIALS AND METHODS:

Cell-free extracts were prepared according to Thomas (54). Young leaves (small leaves near the stem tips) of Phytolacca americana were freed from the petioles and the midribs and put in ice-cold phosphate buffer, pH 6.5, (7.62 gms. K H_2PO_4 + 2.37 gms. Na_2HPO_4 in 1 liter of glass-distilled water) containing 0.35 M NaCl. The wet fragments were macerated as gently as possible using a pre-cooled juice extractor (Drachenberg Products Manufactering Co., Detroit). Some buffer was added while the leaves were being macerated. The juice extractor was preferred to a Waring blendor because it has a milder action, leaving most of the chloroplasts whole; preparations obtained in a Waring blendor contain mostly chloroplast fragments, whose Hill activity is much shorter than that of whole chloroplasts. The brei obtained in the juice extractor was filtered through cotton, freeing it from unbroken, whole cells. The extract was collected in a glass tube placed in ice. All this was done in dim light. Fractions were prepared from this filtrate by repeated fractional centrifugation, as follows: The filtrate was first centrifuged at about 200 x g for three minutes, the sediment was discarded and the supernatant centrifuged at 1200 x g for 7 minutes; the supernatant was thrown away, and the sediment, consisting mainly of whole chloroplasts, was washed by suspending it in phosphate buffer containing 0.35 M NaCl. The suspension was again centrifuged at 1200 x g for 7 minutes, and the sediment re-suspended in phosphate buffer (pH 6.8). All this was done at 0°C.

Argon containing 5% CO_2 was bubbled through the solution for 15 minutes. Then 6.8 ml of this solution were pipetted out into a previously cooled rectangular manometric vessel and 0.2 ml of para-benzoquinone were added, bringing the total fluid volume in the vessel up to 7.0 ml. A stream of argon containing 5% CO_2 was conducted through the manometer vessel, after attaching it to the manometer, for about 15-20 minutes. While the vessel was being gassed with argon, the suspension in it was kept cool by surrounding the manometric vessel with ice; this stage, too, was carried out in the dark or in very dim light. Carbon dioxide was used in the gas phase in view of the reports which indicate that the Hill activity of chloroplasts is increased by the presence of CO_2 (cf. Warburg (57), Brown (3) and Stern and Vennesland (48)). (That carbon dioxide is indispensable for the Hill reaction, is denied by Brown (3) but asserted by Warburg (57)).

The oxygen evolution was measured with a differential manometer, as described in chapter 2 of this thesis. The experiments were carried out at 10°C. The activity of the preparations lasted for about three hours in the crude, cell-free leaf extracts, and for about two hours in purified chloroplast preparations. The suspensions absorbed approximately 90% of the incident light.

B. THE RED DROP:

Figure 11 is a plot of the quantum yield of the Hill reaction, in the extract from pokeweed leaves, against wavelength covering the red part of the spectrum. This curve represents an average of several experiments; table 5 gives a summary of these experiments, showing the variations in the quantum yield shown by the different samples. The average quantum yield values are similar to those that have been reported for photosynthesis (13,14,15,16) and the Hill reaction in whole Chlorella cells. The highest single yields obtained in these measurements were about 0.175 ($\sim$ 1/6) -- significantly higher than

Table 5

QUANTUM YIELD OF THE HILL REACTION IN LEAF EXTRACT PREPARATIONS FROM PHYTOLACCA AMERICANA

Wavelength, mμ	Exp.# 1	Exp.# 2	Exp.# 3	Exp.# 4	Exp.# 5	Exp.# 6	Exp.# 7	Average
650	.1680	.1430	.0742	.1090	.0577	.0468	.0715	.09574
660	.1750	.1140	.0935	.1750	.0602	.0473	.0728	.1054
670	.1040	.0995	.1040	.1250	.0635	.0411	.0616	.0855
680	.0842	.1060	.0590	.0990	.0340	.0478	.0686	.0712
690	.0660	.0540	.0374	.0652	.0209	.0326	.0275	.0434
700	.0213	.0286	.0327	.0485	.0103	.0212	.0266	.02702
710	-	.0087	.0225	-	.0286	-	.0099	.01743
720	-	.0000	.0115	-	.0000	-	-	.0038

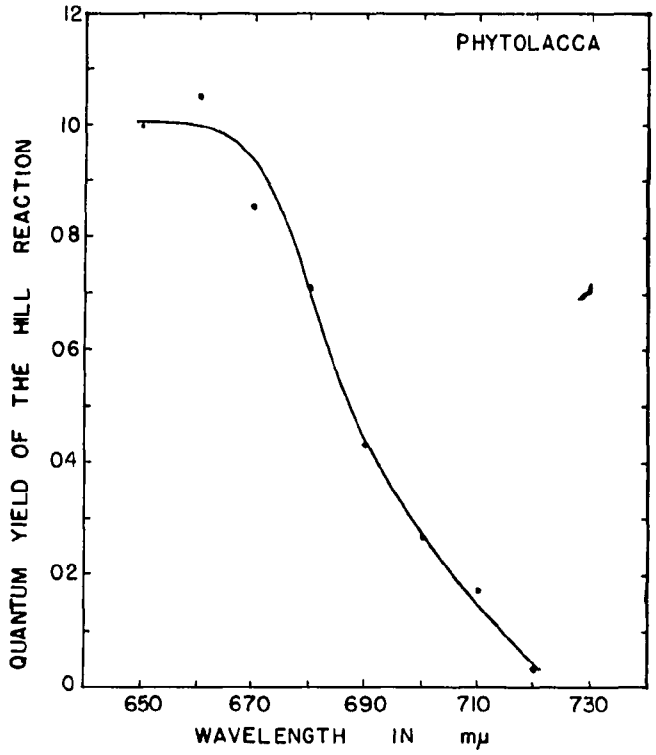

Figure 11: "Red drop" in the action spectrum of the Hill reaction in Phytolacca americana, at 10°C.

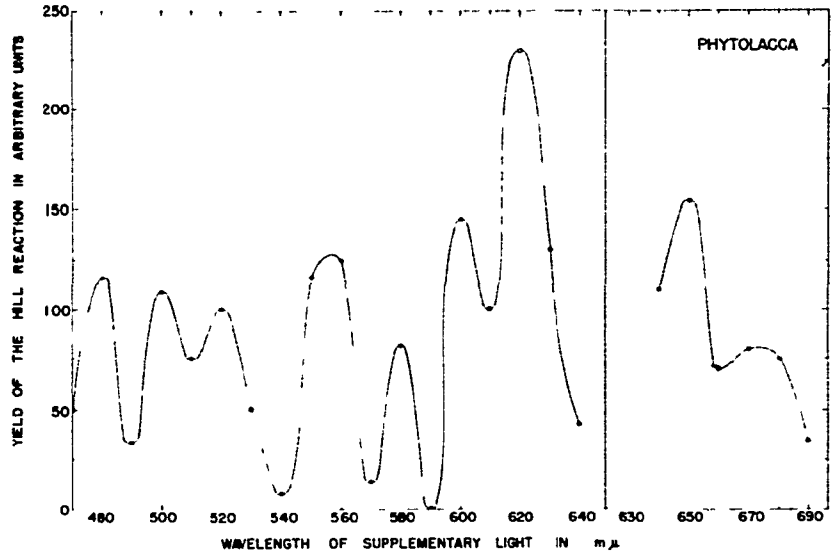

Figure 12: Action spectrum of the Emerson effect in the Hill reaction in chloroplasts from pokeweed. The peaks ascribed to chlorophyll b are 480 mμ, 520 mμ, 560 mμ, 600 mμ and 650 mμ; peaks ascribed to Chl a 670 are: 620 mμ and 670 mμ; the peaks at 500 mμ and 580 mμ may be due to carotenoids.

the quantum yields usually observed for photosynthesis; however, the average of all determinations was about 1/10 (table 5). In Chlorella, no similarly high quantum yields of the Hill reaction were observed. Whether the high yields found in some chloroplast experiments are truly significant, remains to be investigated.

It can be noted that the quantum yield begins to decline at 670 mμ -- 10 mμ earlier than observed in the Hill reaction of whole Chlorella cells, or in photosynthesis of the same algae. The quantum yield drops to one half of its maximum value at 688 mμ.

C. THE ACTION SPECTRUM:

Figure 12 is a plot of the action spectrum of the Emerson effect in the Hill reaction of cell-free leaf extracts from Phytolacca americana, with quinone as oxidant. The intensity of the far red light was constant throughout each experiment. The Emerson effect was determined as described in Chapter 2, part C of this thesis. Supplementary monochromatic light was obtained from the Emerson-Lewis grating monochromator; it was varied from 470 mμ to 690 mμ. The intensity of the supplementary light was adjusted so that it produced, by itself, about the same oxygen liberation at all wavelengths. The results can be interpreted by comparing the action spectrum of the Emerson effect (Figure 12) with the curve [fig. 7 in (18)] calculated by Emerson for the fraction of total absorbed light absorbed, at each given wavelength, by chlorophyll b. This comparison suggests the attribution of the peaks in the action spectrum at 650 mμ, 600 mμ, 560 mμ, 580 mμ, 520 mμ, 500 mμ, and 480 mμ to chlorophyll b. The peak at 670 mμ, seen in fig. 12, can be attributed to a special form of chlorophyll a (Chl a 670). That at 500 mμ and 580 mμ may be due in part to carotenoids (cf. Govindjee thesis (27), page 50), while the peak at 620 mμ may be attributed to Chl a 670.

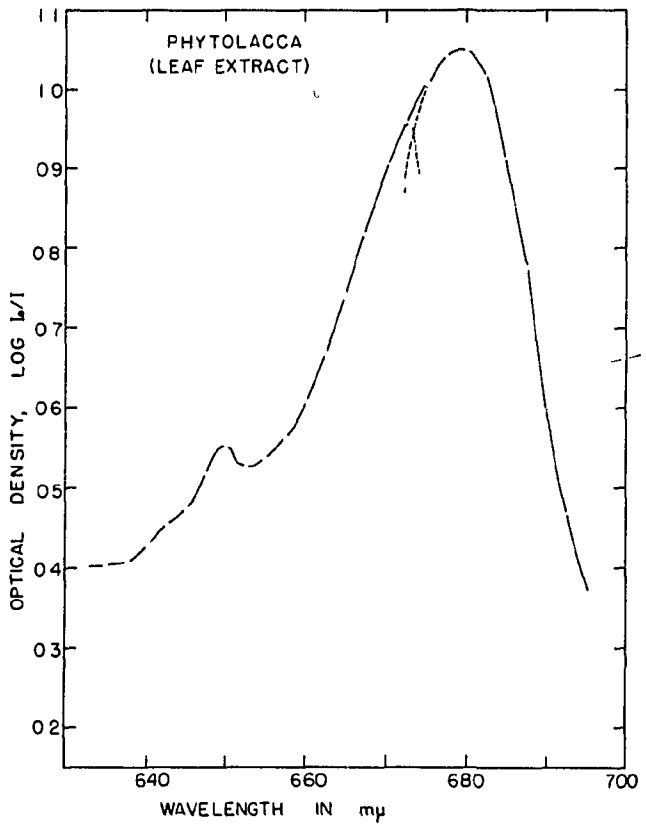

Figure 13: Absorption spectrum of a chloroplast suspension from pokeweed, in the red region. Dotted lines suggest the doublet nature of the peak with individual peaks at 670 mμ and 680 mμ.

It may be noted that a large portion of the action spectrum in figure 12 lies below the 100 lines, i.e. it suggests a "negative Emerson effect" -- mutual inhibition, instead of enhancement. It has been shown before (fig. 10) and also demonstrated by Govindjee and Rabinowitch (29) for photosynthesis, that, by reducing the intensity of the far red light, the whole action spectrum of the Emerson effect can be raised above the 100 line and the negative Emerson effects made to disappear. As explained in Chapter 2, part C III of this thesis, early light saturation of photosynthesis in one of the combined beams may explain this phenomenon, at least partially.

The above-described results are clearly analogous to those obtained in the study of the Emerson effect in the Hill reaction, and in the photosynthesis of Chlorella. These results provide additional evidence of the existence of (at least) two distinct forms of chlorophyll a in the higher plants, with different photochemical functions -- a fact previously demonstrated for photosynthesis in the algae Chlorella, Navicula, Anacystis and Porphyridium by Govindjee and Rabinowitch (29).

Figure 13, obtained by the wet filter paper technique (52), shows the doublet nature of the red absorption band of chlorophyll a in a chloroplast suspension from pokeweed.

A curious thing was noticed in the study of the Emerson effect in the Hill reaction in leaf extracts prepared from older leaves (big leaves away from the stem tips) in the same plant, Phytolacca americana. Figure 14 shows the curve of the Emerson effect in this material plotted against the wavelength of the supplementary light. In this leaf extract, peaks appear at 660 mμ and 680 mμ. The results resemble those found with the Emerson effect in old cultures of Chlorella (cf. figure 9 in Chapter 2). Figure 15 shows an absorption spectrum of a leaf-extract from old leaves; it is

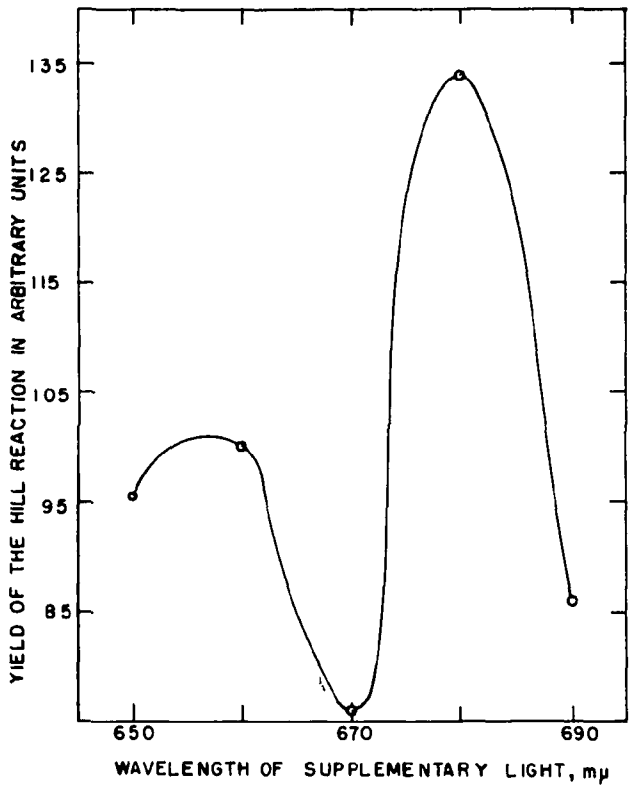

Figure 14: Action spectrum of the Emerson effect in chloroplasts from old leaves of pokeweed, showing peaks at 660 mμ and 680 mμ.

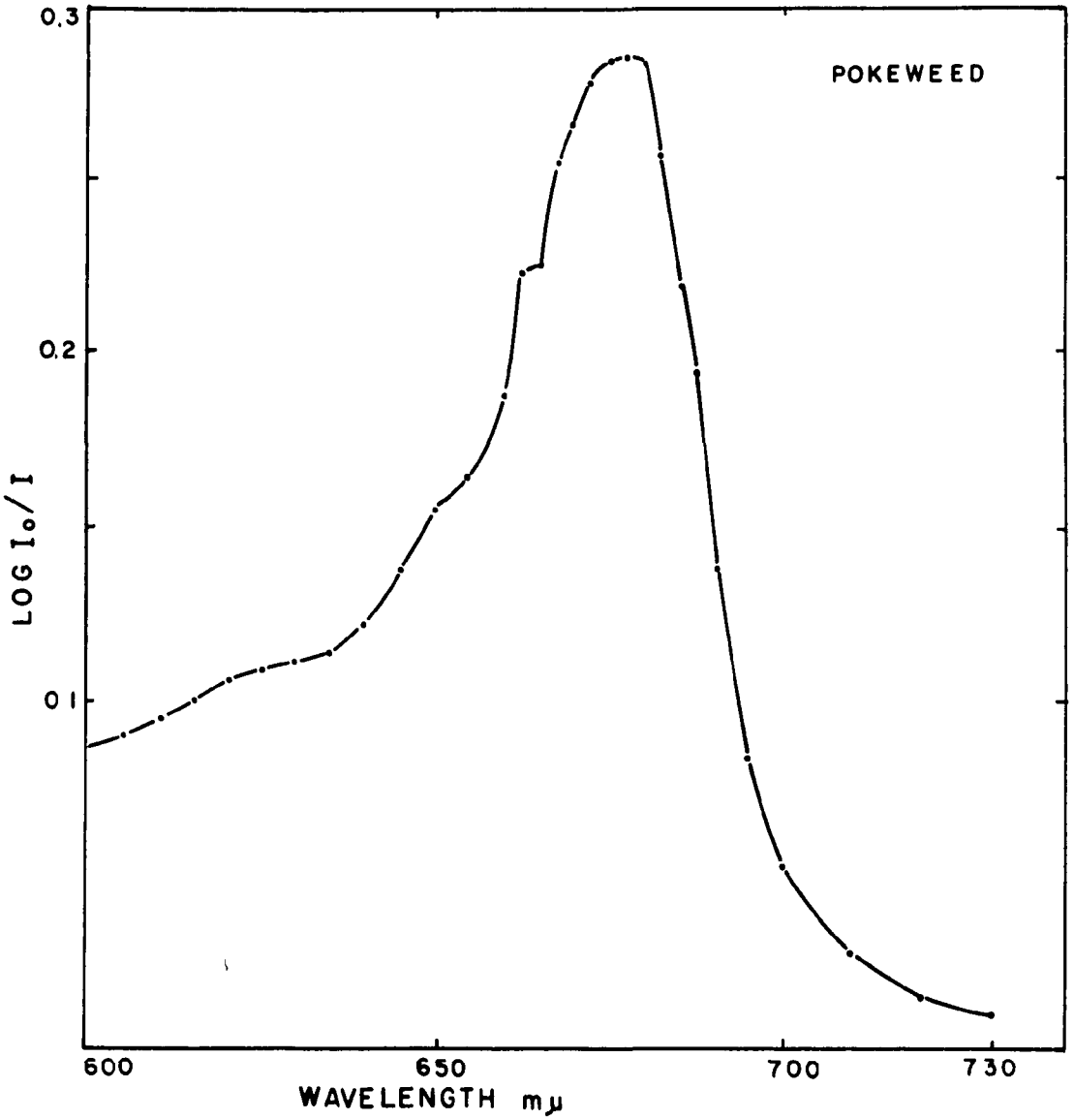

Figure 15: Absorption spectrum of a chloroplast suspension from Pokeweed; note the shoulder at 662 mμ.

characterized by a shoulder at about 662.5 mμ. The implications of these results are not yet clear.

CHAPTER IV

THE INHIBITION OF THE HILL REACTION BY EXTREME RED LIGHT ($>$ 720 mμ).

Rabinowitch, Govindjee and Thomas (47), and Govindjee, Rabinowitch and Thomas (28) discovered that "extreme red" light ($>$ 720 mμ) can inhibit photosynthesis of Porphyridium cruentum and Chlorella pyrenoidosa produced by "far red" light (680 to 720 mμ). The same effect was found to exist also in the photochemical quinone reduction by Chlorella suspensions, and by leaf extracts, from Phytolacca americana.

The Hill activity (quinone reduction) was measured as described in chapters 2 and 3 of this thesis. Two monochromatic light beams were thrown simultaneously on the suspension of algae, or of chloroplast fragments. One beam, $\lambda \cong 700$ mμ, was obtained by means of an interference filter (Farrand 1322; half band width about 20 mμ) in the path of a parallel beam of white light. The other monochromatic light band (half band width about 10 mμ) was obtained from the Emerson-Lewis grating monochromator, with a Schott RG-10 filter placed at the exit slit to cut off the overlapping higher orders. The rate of the Hill reaction was measured first in the far red light ($\sim$ 700 mμ) alone, then in the presence of supplementary "extreme red" light of varying wavelength (720 mμ to 780 mμ). The "extreme red" light alone caused no oxygen evolution at all. The results of these experiments are shown in figures 17 and 16.

Since a two mm thick RG-10 Schott glass filter was used at the exit slit of the monochromator, it is certain that visible light $<$ 720 mμ, could not have affected the results. Figure 17 agrees with figure 16 in that the inhibition effect has two maxima, one at about 740 mμ and another at about

760 mμ. Since the grating first used in our work was blazed for the visible region, these wave length determinations may not have been precise.

However, the results were later confirmed (at least for the inhibition of photosynthesis) with a new grating, blazed for the 750 mμ region (see figures 18 and 19). These figures (also obtained with a RG-10 Schott glass filter at the exit slit) again show two maxima of inhibition at approximately 730 mμ and 760 mμ. The danger of a contamination of the 760 mμ light with the 730-740 mμ light is absent with this grating. (This was tested by putting 720, 730 and 740 mμ interference filters at the exit slit of the monochromator, and measuring the energy transmitted at 760 mμ. The reading of the bolometer was always zero.) Therefore, it appears certain that the pigment (or pigments) responsible for inhibition have two absorption peaks in the "extreme red" region.

However, not <u>all</u> experiments showed these two peaks in exactly the same location [(cf. figures 1 and 2 in (28)]. Many more experiments will be needed to identify the cause of the variability in the location and number of the maxima of inhibition observed in different cultures.

Govindjee, Cederstrand and Rabinowitch (31) have reported the finding in the absorption spectrum of algal cells of two very weak bands in this region, where the action spectrum of inhibition has its maxima. Lippincott et. al (40) noted that the presence of methanol (or other water-miscible solvents causes a band at 740 mμ to develop in the absorption spectrum of several higher plants, as well as of <u>Chlorella</u>. They suggested that this treatment causes chlorophyll to crystallize <u>in vivo</u>. (the absorption band of crystalline chlorophyll a is located at 740 mμ).

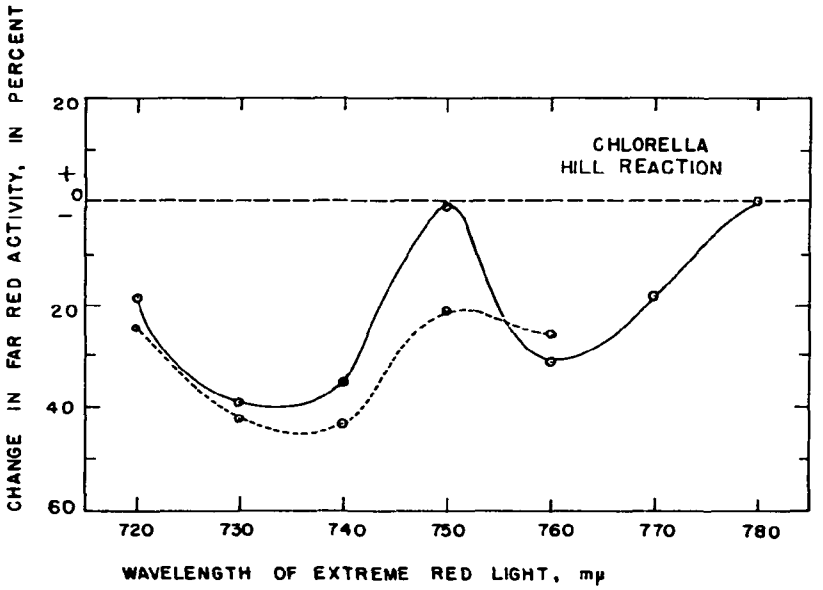

Figure 16: Doublet nature of the action spectrum of the inhibition of the Hill reaction in Chlorella pyrenoidosa, caused by the addition of monochromatic "extreme red" light (> 720 mμ) to "far red" light of about 700 mμ.

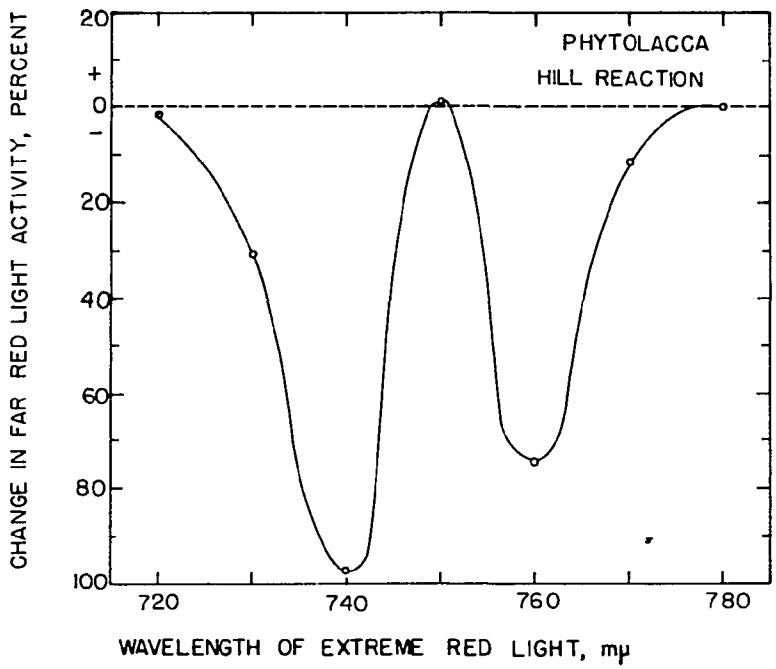

Figure 17: Action spectrum of the inhibition of the Hill reaction (produced by light ~ 700 mμ) in Pokeweed chloroplasts, caused by the addition of "extreme red" light (>720 mμ).

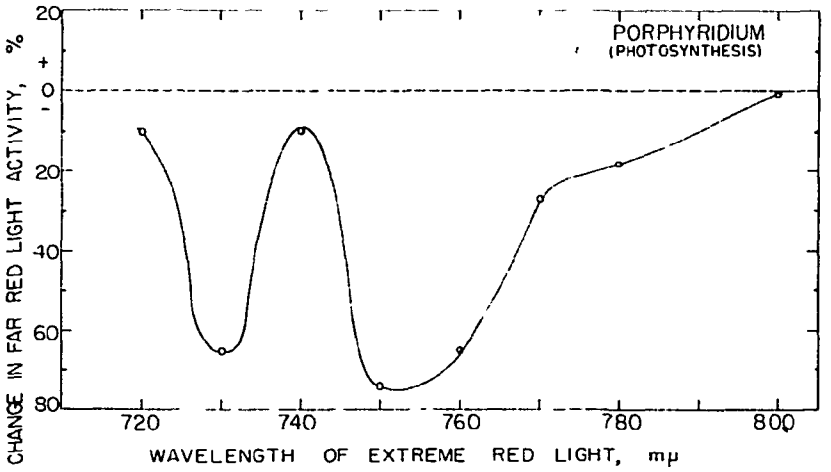

Figure 18: Doublet structure of the action spectrum of the inhibition of photosynthesis (produced by ~ 700 mμ light) in Porphyridium cruentum, caused by the addition of "extreme red" light (> 720 mμ).

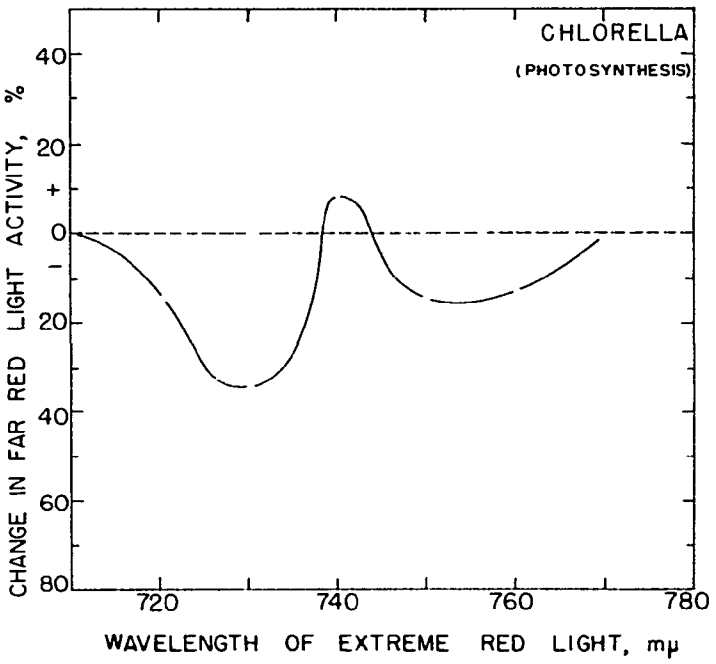

Figure 19: Action spectrum of the inhibition of photosynthesis (produced by ~ 700 mμ light) in Chlorella pyrenoidosa, caused by the "extreme red" light (> 720 mμ).

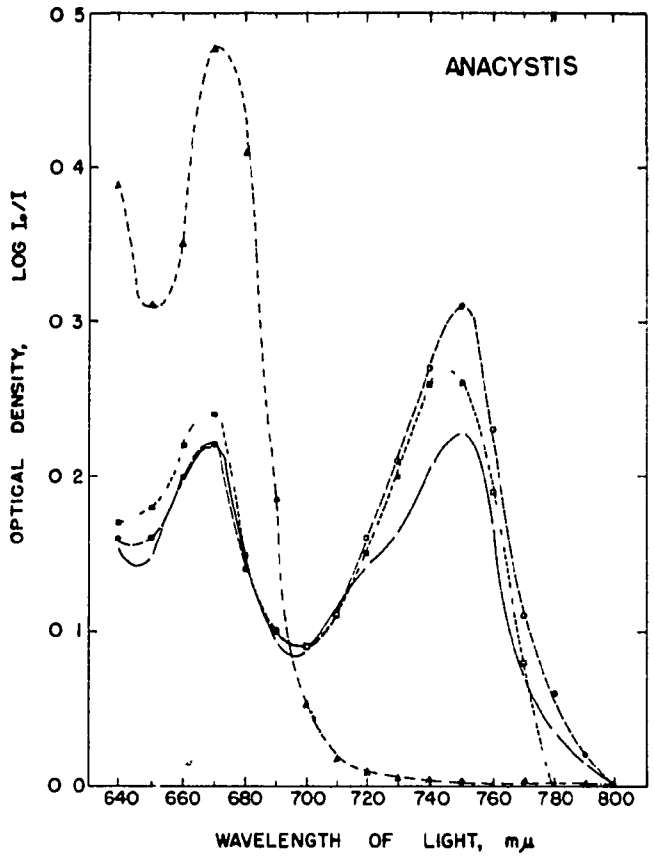

Figure 20: Appearance of the 750 mμ absorption peak in Anacystis nidulans after treatment with 65% methanol (V/V).

When 65% methanol (V/V) is added to a suspension of Anacystis cells, a very sharp absorption peak appears in the 740-750 mμ region (cf. figure 20).

In this blue-green alga, an absorption peak at about 750 mμ was noted earlier in our laboratory (32); in thick suspensions, it is visible even without methanol treatment. Upon treatment of Chlorella suspensions with 65% methanol (V/V) a small shoulder appeared in the absorption spectrum in the region of 750 mμ. A test was made, both in Anacystis and in Chlorella to see whether any easily crystallizable chlorophyllide was formed upon treatment with 65% methanol. The "HCl test" (58) was employed; an ether extract of the pigment was shaken with 22% HCl and allowed to stand; any chlorophyllide present must appear in the acid layer. This test applied to Anacystis cells treated with 65% methanol revealed the presence of some chlorophyllide. In Chlorella, too, a trace of chlorophyllide was found to be present. The relative concentration of the chlorophyllide in methanol treated Anacystis and Chlorella cells paralleled qualitatively the relative heights of the absorption peaks of the 740-750 mμ pigment formed by the methanol treatment. The question whether the 740-750 mμ absorption band, present in natural state in blue-green algae, could be due to crystalline chlorophyllide, the quantity of which increases when the algae are treated with methanol, is difficult to answer. An HCl test applied to Anacystis cells without methanol treatment suggested that some chlorophyllide may be present in them even in the natural state. These conclusions can be considered as certain only if the "HCl test" can be taken as absolutely certain. No attempt has been made by the author to test this point and to check the quantitative reliability of the "HCl test".

It is doubtful that Lippincott's pigment (crystalline chlorophyll or chlorophyllide) is responsible for the inhibition of photosynthesis and of the Hill reaction by far red light. Except for Anacystis, this pigment is formed

only after treatment with organic solvents, and in Anacystis, no inhibition of photosynthesis by "extreme red" light (28) could be so far observed at all.

Butler et al. (6) found a photolabile pigment in higher plants which exists in two mutually convertible states (730 mμ state and 660 mμ state), and is supposed to be responsible for photomorphogenic phenomena. Govindjee et al. (32) noted an increase in absorption at 745 mμ of Porphyridium, Anacystis and Chlorella after pre-illumination with 660 mμ light; but it is by no means certain that the pigment responsible for this increase is the same one that causes the inhibition of photosynthesis and of the Hill reaction.

CHAPTER V

SUMMARY AND GENERAL DISCUSSION

The results presented in chapters II and III have clearly demonstrated the existence of the 'red drop' and the Emerson effect in the Hill reaction, in Chlorella pyrenoidosa, Anacystis nidulans, and the leaf macerates of Phytolacca americana.

Peaks found in the action spectrum of the Emerson effect in the Hill reaction at 480 mμ, 520 mμ, 560 mμ, 600 mμ and 650 mμ can be ascribed to chlorophyll b, and peaks at 500 mμ and 580 mμ to the carotenoids. Those found in Anacystis at 560 mμ, 610 mμ and 640 mμ may be due to phycocyanin.

Absorption by at least one form of chlorophyll a (Chl a 670) can raise the quantum yield of the Hill reaction in the region of the red drop in all the three organisms studied.

Finding of the red drop and of the Emerson effect in the Hill reaction with quinone as oxidant means that this effect cannot be due to changes in the rate of respiration in light, since quinone stops effectively all respiratory activity. The carbon dioxide fixation and reduction do not occur in the Hill reaction, but the Emerson effect is there; this suggests that this effect is perhaps located in the oxygen-evolution phase (and not the carbon dioxide reducing phase) of photosynthesis.

The existence of a peak at 670 mμ in the action spectrum of the Emerson effect has been interpreted as meaning that simultaneous excitation of the two forms of chlorophyll a is necessary for photosynthesis as well as the oxygen evolution by chloroplast suspensions (cf. Franck, 23; Rabinowitch and Govindjee 46). It has been suggested that accessory pigments cause peaks in the action spectrum of the Emerson effect because they transfer their energy of excitation to Chl a 670 by resonance transfer.

It has been shown in chapter IV that there exists a pigment (or two pigments) absorbing in the extreme red region of the spectrum (740 mμ and 760 mμ), which, when excited simultaneously with the chlorophyll a form (or forms) absorbing at 680-700 mμ, causes an inhibition of the Hill reaction in Chlorella and in Phytolacca chloroplasts.

A negative Emerson effect was found to occur in the Hill reaction (chapter II); it may be due in part to early light saturation of oxygen production in 700 mμ light.

A. DIFFERENT PHOTOCHEMICAL ACTIVITIES OF DIFFERENT FORMS OF CHLOROPHYLL A IN VIVO

Several forms of chlorophyll a are now known to exist in plant cells. The question is: do they have different functions? Franck (23) suggested an interpretation of the Emerson effect based on the assumption that there are two forms of chlorophyll a. This was confirmed by the findings of Govindjee and Rabinowitch (29), who showed that simultaneous excitation of Chl a 670 and of a longer-wave absorbing form of chlorophyll a is necessary for a full yield of photosynthesis. A similar interpretation can be suggested for the observations on the kinetics of the Hill reaction, presented in this thesis.

It was not stated clearly in Govindjee's papers whether "Chl a 680" should be listed as an "efficient" form (together with Chl a 670) or as an "inefficient" form together with Chl a 690. The results obtained in the present study suggest that Chl a 680 belongs to the first active group. In the first place, the quantum yield of both photosynthesis and Hill reaction at 680 mμ is still high in green and brown algae. Furthermore, it has been found that certain "old" cultures of Chlorella and chloroplasts from "old" leaves of Phytolacca have a peak at 680 mμ in the action spectrum of the Emerson effect in the Hill reaction.

The action spectrum of the Emerson effect in the photosynthesis of Chlorella and Porphyridium (29) and in the Hill reaction of Chlorella show peaks also in the blue part of the spectrum, where chlorophyll a is the predominant absorber. This may mean that the Soret band of Chl a 670, which is probably located at about 440 mu, also contributes to the Emerson effect.

Any theory of photosynthesis will now have to include the concept of (at least) two photochemical reactions. (cf. Franck 23). A general scheme for photosynthesis, which is based on the ideas of Rabinowitch and Govindjee (personal communication) and on certain fragmentary results concerning the effect on the red drop of variations in the composition of the medium are presented below.

A. I Light reaction(s) (Accessory pigments)

$$X + H_2O + \text{Chl a } 670 \xrightarrow{h\nu_1} XH + \text{Chl a } 670 + \{OH\}$$

B. II Light Reaction(s)

$$ADP + i\ P + \text{Chl a } 690 \text{ (or Chl a } 700) \xrightarrow{h\nu_2} \text{Chl a } 690 + ATP$$

C. Dark Reaction(s) (oxygen evolution phase)

$$\{OH\} \text{ (+ ATP?)} \xrightarrow{\text{Enzymes}} O_2 + ADP$$

D. CO_2 fixation phase (Dark Reactions)

$$XH + ATP + \{R.CO_2\} \xrightarrow{\text{Enzymes}} \{R.COOH\} + X$$

In Hill reaction, reactions A, B and C are unchanged but D is replaced by

$$XH + Q \text{ (or } Fe^{+++}) + ATP\ (?) \longrightarrow H_2Q + X \quad \text{or} \quad (Fe^{++} + X)$$

where

X is some hydrogen acceptor

ADP is Adenosine diphosphate

iP is inorganic phosphate

ATP is Adenosine triphosphate

Q is Quinone

Fe^{+++} is Ferric compound

B. PARTICIPATION OF ACCESSORY PIGMENTS IN THE HILL REACTION

As described in chapters II and III, occurrence of peaks in the action spectrum of the Emerson effect in the Hill reaction which are ascribed to the accessory pigments (viz. chlorophyll b and phycobilins) could be suggested to mean that it is necessary to excite an accessory pigment as well as the "long-wave form" of chlorophyll a to achieve a full yield of the Hill reaction. In the above-given general scheme the accessory pigments would then perform the reactions A and chlorophyll a, the reaction B. It is more logical, however, to assume (following Franck and Rabinowitch) that the energy absorbed by the accessory pigments is transferred to Chl a 670 before it can contribute to photosynthesis or Hill reaction and that chlorophyll a (in the form of Chl a 670) can bring about the reactions A. (It is known from observation of sensitized fluorescence that light absorbed by the accessory pigments is effectively transferred to chlorophyll a (cf. Duysens 10).

Further experiments on the fluorescence of different forms of chlorophyll a and other pigments could help in clarifying this matter.

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VITA

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She is a coauthor of the following publications:

1. "Increased Formation of Asparagine in Carica-curl Virus Infected Leaves." Experientia XII/2, 1956.
2. "Second Emerson Effect in the Hill Reaction of Chlorella cells with Quinone as Oxidant." Science, 132, 1960.