ON THE EMERSON ENHANCEMENT EFFECT IN THE FERRICYANIDE HILL REACTION IN CHLOROPLAST FRAGMENTS*†

GOVINDJEE and MAARIB BAZZAZ

Department of Botany, University of Illinois, Urbana, Illinois, U.S.A.

Abstract—The Emerson effect is demonstrated in the ferricyanide Hill reaction when the rates of steady-state oxygen evolution are measured in spinach chloroplast fragments exposed to red (650 nm) and far-red (700 nm) light of high but not saturating intensity. However, at very low light intensity, the Emerson effect could not be observed. These experiments suggest that ferricyanide can be reduced at two sites. At low light intensity, the rate at one site predominates and at this site one photochemical system is active. At high light intensity, however, the action at a site that is dependent on the cooperation of two photochemical systems predominates.

The action spectra of the ferricyanide Hill reaction measured in the presence of an excess of 650 nm or in the excess of 700 nm light show two peaks: one at 650 nm due to chlorophyll *b* and the other around 675 nm due to chlorophyll *a*. The ratio of chlorophyll *a* to chlorophyll *b* peaks is about 1·4 when 650 nm background light is used; the same ratio is about 0·7 with 700 nm background light. The two pigment systems seem to contain both chlorophyll *a* and chlorophyll *b* but in different proportions.

INTRODUCTION

DURING the past 6 years, several workers have suggested that two light-induced steps are involved in various Hill reactions. These suggestions were based mainly on the observations of the Emerson enhancement effect. This Emerson effect was observed in the photoreduction of quinone by whole Chlorella cells[1–3] and by pokeweed chloroplasts,[1] and in the photoreduction of NADP+ by spinach chloroplast.[4–6] By the use of monochromatic light flashes, Whittingham and Bishop[7–9] observed an enhancement effect in the reduction of ferricyanide by chloroplasts. On the other hand, Mayne and Brown[10] failed to observe the enhancement phenomenon in the steady-state photoreduction of both quinones and ferricyanide. Gibbs et al.[11] found no enhancement, using NADP+ as the oxidant. In view of these controversial results, we have reinvestigated this problem using ferricyanide as the oxidant.

Fork[12] measured the action spectrum of the oxygen spike in chloroplasts without added oxidants and found a peak at 650 nm and a shoulder at 670 nm but the action spectrum with ferricyanide had a peak at 678 nm and a shoulder at 650 nm. Horio and San Pietro,[13] however, reported a peak at 660 nm and a shoulder at 680 nm for ferricyanide reduction.

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†*Abbreviations used in this paper*: DCPIP—2,6 dichloro phenol indophenol; NADP+—nicotinamide adenine dinucleotide phosphate; ATP—adenosine triphosphate.

R. Govindjee et al.[2,3,5] showed that the quantum yield of O_2 evolution, using quinone or NADP$^+$ as oxidant, declines beyond 680 nm. A similar "red drop" in the quantum yield of ferricyanide reduction was reported by Biggins and Sauer[14] and more recently by Sauer and Park.[15] The "red drop" seems to begin at $\sim$650 nm (ref. 14) or at about 680 nm (ref. 15). In order to characterize our system and compare it with those of other investigators, we have measured the quantum yield of O_2 evolution as a function of wavelength of light, using ferricyanide as the oxidant. Since there were differences in the results obtained by various workers, we have tabulated all the data with respect to the different methods and conditions used in those experiments.

MATERIALS AND METHODS

(1) Preparation of broken chloroplasts

Fresh young spinach leaves obtained from the greenhouse were chopped and ground with white sand and 0·35 M NaCl solution and filtered through eight layers of cheesecloth. The debris was discarded and the filtrate was centrifuged for 1 min at 200 $\times$ g to remove the sand particles. The supernatant was centrifuged for 10 min at 1200 $\times$ g. The pellet was suspended in 0·035 M NaCl and was recentrifuged for 10 min at 1200 $\times$ g. The washings and centrifugations were repeated five times. Finally, the chloroplasts were suspended in the reaction mixture (cf. refs. 16, 17).

(2) Reaction mixture

The 7-ml reaction mixture contained 95 μM Tris–Acetate buffer, pH 7·8, 3 μM (or 35 μM) of freshly prepared ferricyanide, 10 μM NaCl, and broken chloroplasts containing either 0·2 mg (or 2·3 mg) chlorophyll.

(3) Determination of oxygen evolution

Oxygen evolution was measured by "closed" manometers of Emerson and Chalmers[18] (see description in references 19–21).

The reaction mixture minus the chloroplasts was added to the respective vessels. Then, the chloroplasts were added in the dark and immediately the vessels were attached to the manometer and gassed with 99·99% Argon for 5 min. The manometer assembly was placed in the water bath for equilibration for 15–20 min. The temperature of the water bath was 10°C.

The pressure changes due to oxygen evolution were observed with a precision cathetometer at 1 min intervals. The gas exchange in the dark was checked and the light was turned on at the end of the 20 min equilibration period. Readings for 8 min were taken but the first three were always discarded because of the possible transients and the lag effects. The rate of the O_2-exchange was constant during this 5-min interval.

The light beam obtained from a ribbon filament tungsten lamp (operated at 31 A and 7·5 V) was filtered through the Emerson–Lewis monochromator. Another beam of light was obtained from a 1000 W tungsten lamp, and was filtered by means of Farrand interference filters (half band widths 10 nm or better).

(4) Measurements of light absorption and energy

The incident energies, in terms of quanta (or einsteins), were measured by a large surface bolometer previously calibrated by a standard lamp from the U.S. Bureau of

Standards.   For almost all the measurements on the quantum yields and the action spectra, the light intensity ranged from 4 to 10 μeinstein/hr/reaction vessel (or 0·1 to 0·2 nEinsteins $cm^{-2}\ sec^{-1}$).   The fractional absorption was determined in an integrating sphere spectrophotometer built by Cederstrand[22] or with a Bausch and Lomb (Spectronic 505) spectrophotometer equipped with an integrating sphere.

RESULTS AND DISCUSSION

(1) *The problem of the linearity of the rate–intensity curve*

The rate of the Hill-reaction (R) as a function of the light intensity (I) is fairly linear at very low light intensities but the curve $R = f(I)$ progressively decreases (Holt *et al.*[23]). Rieske *et al.*[24] and Lumry and Rieske[25] have made a thorough analysis of the shape of the $R = f(I)$ curve for ferricyanide reduction by isolated chloroplasts; they found this curve to be a rectangular hyperbola within narrow statistical limits.   As has been pointed out earlier,[6] this could mask the true enhancement effect, and the action spectra of the Hill reaction measured without regard to this problem may be unreliable.   Park and Sauer[15] have shown that the quantum yield of ferricyanide reduction is fairly linear with light intensity in the presence of ammonium chloride, an uncoupler of phosphorylation.   Sodium chloride also uncouples photophosphorylation.   The sodium chloride treated chloroplast fragments used in the present study also showed a linear rate versus
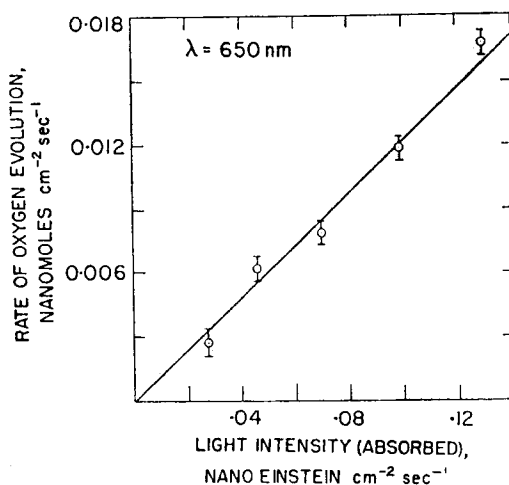

FIG. 1.   Rate of O_2 evolution at 10°C as a function of 650 nm light in chloroplast fragments from spinach with ferricyanide as the electron acceptor.   The bar on each point in this and the following figures is the standard deviation ($n = 5$).

intensity curve, at least, in the low light intensity range (cf. Fig. 1).   All the measurements on the quantum yield of the Hill reaction at different wavelengths of light were made in this linear portion of the R vs. I curve.   However, when the Emerson enhancement studies were made a second beam was added.   The intensity of the combined beams used to get the maximum enhancement effect, was often high enough that the non-linear (decreasing slope) part of the $R = f(I)$ curve was reached.   Under these conditions the protocol as

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suggested in ref. 6 was used to demonstrate the presence or the absence of the enhancement effect.

Because of the non-linearity of the light-curve at the intensities used for the Emerson effect studies, we calculate the enhancement on the basis of the following equation (see ref. 6):

$$E = \frac{R(700 + 650) - \{R(650 + 650') - R(650')\}}{R(700)} \tag{1}$$

where E stands for the Emerson enhancement factor and R for the rate of O_2 evolution; the numbers in parentheses refer to the wavelength of light, λ, in nm. The rate of O_2 evolution from the second 650 nm beam (650') and the 700 nm beam is adjusted to be the same.

(2) *The action spectra and the maximum quantum yield of the ferricyanide Hill reaction as a function of the wavelength of light*

Action spectra. The action spectra of the ferricyanide Hill reaction, as reported by Fork[12] and Horio and San Pietro,[13] show peaks at 678 nm and 660 nm, respectively. Table 1 summarizes the experimental conditions used by the two groups of investigators.

TABLE 1. COMPARISON OF EXPERIMENTAL CONDITIONS FOR ACTION SPECTRA MEASUREMENTS BY FORK AND HORIO AND SAN PIETRO

Conditions	Fork[12]	Horio and San Pietro[13]
Organism	*Beta vulgaris*	*Spinacea oleracea*
Reaction medium	0·4 M Sucrose 0·1 M NaCl 0·05 M K_2HPO_4—KH_2PO_4	0·05 M Tris–HCl 0·035 M NaCl 0·005 M $MgCl_2$ 0·0025 M ADP 0·0025 M Pi
pH	6·9	8·0
Temperature	20°C	15°C
Method of measurement of the Hill reaction	Rate of O_2 evolution by platinum electrode (covered by teflon); continuous measurement	Rate of reduction of ferricyanide by spectroscopic method (change in optical density at 420 nm); 3 min reaction.
Aerobic/Anaerobic	Nitrogen (anaerobic)	Anaerobic (?)
Concentration of ferricyanide	$4·3 \times 10^{-3}$ M	2×10^{-3} M
Intensity of incident light (ergs cm^{-2} sec^{-1}	$3·65 \times 10^2$	1×10^4
Absorption data	Not available; thin paste; path length: not available.	O.D. at Chl *a* peak = 0·6 for 12·5 µg Chl/ml; concentration in the reaction mixture = 20 µg Chl/ml. Path length = 1 cm.
Results from action spectra 　　　　Peaks 　　　　Shoulders	 678 nm 650 nm	 660 nm 680 nm
Ratio of activity at 650 nm to that at 680 nm	 0·9	 1·2

There are several differences including the choice of exciting light intensity; Fork used 3.65×10^2 ergs cm^{-2} sec^{-1} but Horio and San Pietro used 10^4 ergs cm^{-2} sec^{-1}. It is likely that the spectrum published by Fork is more reliable because of the use of low intensity excitation and the use of thin paste.

Quantum yield. The Maximum quantum yield Φ (No. molecules of O_2 evolved per absorbed quantum) of the Hill reaction was reported by French and Ribideau.[26] Their values ranged from 0.06 to 0.08 in spinach chloroplasts for the ferricyanide Hill reaction. Ehrmantraut and Rabinowitch[27] obtained consistently a value of 0.09 for the ferricyanide Hill reaction in the pokeweed chloroplasts. Wayrynen[28] reported a value of 0.12 when the yield was extrapolated to zero light intensity. Our experiments on the ferricyanide Hill reaction with 670 nm light confirm the maximum value of 0.12 at low light intensities.

Ehrmantraut and Rabinowitch[27] reported a value of 0.10 for the quinone Hill reaction in whole Chlorella cells with 669 nm light. They made one experiment on the quantum yield of the Hill reaction at four different wavelengths of light (633, 653, 669, and 698 nm). They found a high (0.10) value at 633, 653 and 669 nm and a very low value at 698 nm (the "red drop"). R. Govindjee *et al.*[2,3] reported the quantum yield, Φ, of the quinone Hill reaction as a function of the wavelength, λ, of light in whole Chlorella cells; the yield was almost constant (0.09) up to 680 nm and declined to half the maximal value around 695 nm. A similar result was obtained for the NADP Hill reaction.[4,5] For the ferricyanide reduction in isolated chloroplasts, the quantum yield as a function of the wavelength of light $\Phi = f(\lambda)$, has been recently reported by Biggins and Sauer[14] and Park and Sauer.[15] They also showed a decline in the $\Phi = f(\lambda)$ curve for the ferricyanide Hill reaction. Biggins and Sauer obtained very low values for the quantum yield of ferricyanide reduction (0.05 moles ferricyanide reduced/einsteins absorbed) and the "red drop" began at ~650 nm. Park and Sauer,[15] however, compensated for the shape of the velocity versus intensity curve and made other improvements in technique and obtained a constant quantum yield of 0.083 (0.33 electron per absorbed quantum) up to 680 nm; the "red drop" began at about 680 nm.

Emerson and Lewis[29] had shown a clear dip at 660 nm in the curve for the quantum yield of photosynthesis as a function of wavelength; no such dip was noted by Park and Sauer[15] in the ferricyanide Hill reaction.

In order to characterize our system, we remeasured the quantum yield of the ferricyanide Hill reaction as a function of the wavelength of light (Fig. 2). Like photosynthesis, a decline (the red drop[29,30]) beyond 680 nm and a dip around 660 nm is observed in the $\Phi = f(\lambda)$ curve for the O_2 evolution in the ferricyanide Hill reaction. The quantum yield is 50 per cent of the maximum at about 695 nm. The maximum quantum yield is 0.12; this corresponds to 8.3 quanta per O_2 evolved. In general, our results agree with those of Park and Sauer[15] but there are some differences: (1) there seems to be structure in the quantum yield versus wavelength curve; there is a dip at 660 nm, (2) the quantum yield values obtained in our experiments are higher than those of Park and Sauer. Table 2 summarizes the experimental conditions used by Biggins and Sauer, Park and Sauer and the present authors. We do not, as yet, know the exact reason for the differences.

(3) *The Emerson enhancement effect*

The Hill reaction in broken chloroplasts from spinach, as measured by O_2 evolution and using ferricyanide as the electron acceptor, shows the presence or the absence of the Emerson effect depending upon the light intensity. Experiments made with low intensities
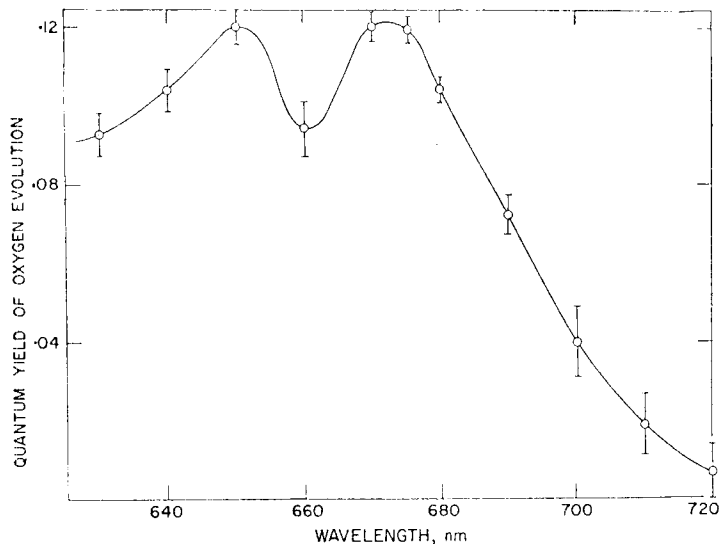
Fig. 2. Quantum yield of O_2 evolution at 10°C as a function of wavelength in chloroplast fragments with ferricyanide as the electron acceptor.

of light which give low rates of O_2 evolution show an absence of the Emerson effect as reported by Mayne and Brown.[10] However, a positive Emerson effect is demonstrated when two monochromatic light beams of 650 and 700 nm of high but not saturating intensities are used. Under these conditions, the intensity of the combined beams was high and the Emerson effect was calculated according to equation (1) given in section (1). On this basis, an enhancement factor of 1·7 ± 0·2 was obtained (Table 3).

The existence of the enhancement effect is usually interpreted as evidence for the two light reactions. However, the occurrence of two light reactions for the reduction of ferricyanide was unexpected because the oxidation-reduction potential of the ferricyanide (Fe^{+3})/ferrocyanide (Fe^{+2}) system $(E'_0 = + 0·4)$ is close to that of the P700, as measured by Kok.[31] Ferricyanide is known to bleach P700. As a working hypothesis, one may suggest that ferricyanide is reduced at two sites. At low light intensity, the rate at one site predominates and at this site one photochemical system is active but at high light intensities, the action at a site that is dependent on the cooperation of two photochemical systems predominates. Trebst[32] has earlier suggested two sites for the ferricyanide reduction on the basis of biochemical analysis.

(4) *The action spectra of the two pigment systems*

Duysens and Amesz[33] have suggested that an action spectrum of photosynthesis measured with high (but not saturating) intensity system II background light would yield the action spectrum of system I whereas an action spectrum measured with background of intense system I background light would yield the action spectrum of system II. French et al.[34] had earlier measured the action spectra of photosynthesis under similar conditions. These results suggest that the pigment system II is composed of a large part of chlorophyll *b* and more than 50 per cent of the short-wave form of chlorophyll *a* (often referred to as Chl *a* 672), whereas pigment system I is composed mainly of the long-wave form of chlorophyll *a* (Chl *a* 694), and a large proportion of Chl *a* 683, and less than 50 per cent of the

TABLE 2. COMPARISON OF EXPERIMENTAL CONDITIONS USED BY VARIOUS INVESTIGATORS FOR MEASUREMENTS OF THE QUANTUM YIELD OF FERRICYANIDE HILL REACTION AS A FUNCTION OF WAVELENGTH OF LIGHT (*Spinacea oleracea*)

	Biggins and Sauer[14]	Sauer and Park[15]	Govindjee and Bazzaz, (this paper)
Reaction mixture	μ mole/ml: 2 ml used	μ mole/ml: 2 ml used	μ mole/ml: 7 ml used
	50 Tris–HCl buffer 0·525 $K_3Fe(CN)_6$	35 K_2HPO_4 350 Sucrose 0·26 $K_3Fe(CN)_6$ 0·26 $K_4Fe(CN)_6\cdot3H_2O$ 10 $CH_3NH_2\cdot HCl$ (or NaCl)	14 Tris–Acetate 0·3 $K_3Fe(CN)_6$ 10 NaCl
pH	8·0	7·45	7·8
Temperature	Room temperature at Berkeley laboratory	Room temperature at Berkeley laboratory	10°C
Method of measurement	Reduction of ferricyanide (optical density at 420 nm); separation of chloroplasts by centrifugation	Reduction of ferricyanide (optical density at 420 nm)	Rate of O_2 evolution by manometry
Aerobic/Anaerobic	Anaerobic (?)	Anaerobic (?)	gassed with Argon (99·99%)
Incident intensity	9–14 nEinsteins cm^{-2} sec^{-1}	Results extrapolated to zero light intensity	0·1 to 0·2 nEinsteins cm^{-2} sec^{-1}
Optical path length	3 cm	3 mm	1 cm
Time of exposure	20 min	Measured continuously	Measured with dark light dark sequence of 8 min; corrections made for change in activity, if any
Absorption data (optical density)	0·225 at 678 nm for 1-cm path length (?)	0·3 to 0·7 at 678 nm for 1-cm path length	0·3 at 678 nm for 1-cm path
Light passed through:	Veril-200 interference filters	Bausch and Lomb monochromator	Emerson–Lewis monochromator
Half-band width	10 nm	7 nm	5–10 nm
Maximum quantum yield	0·05 mole ferricyanide per einstein absorbed; equivalent to 0·012 molecules of O_2 per absorbed quantum	0·33 electron transfer/ quantum absorbed; equivalent to 0·083 molecules of O_2 per absorbed quantum	0·12 molecule sof O_2 evolved per absorbed quantum (670 nm)
Beginning of decline in yield	650 nm	680 nm	680 nm

TABLE 3. EMERSON ENHANCEMENT IN O_2 EVOLUTION WITH FERRICYANIDE AS HILL OXIDANT

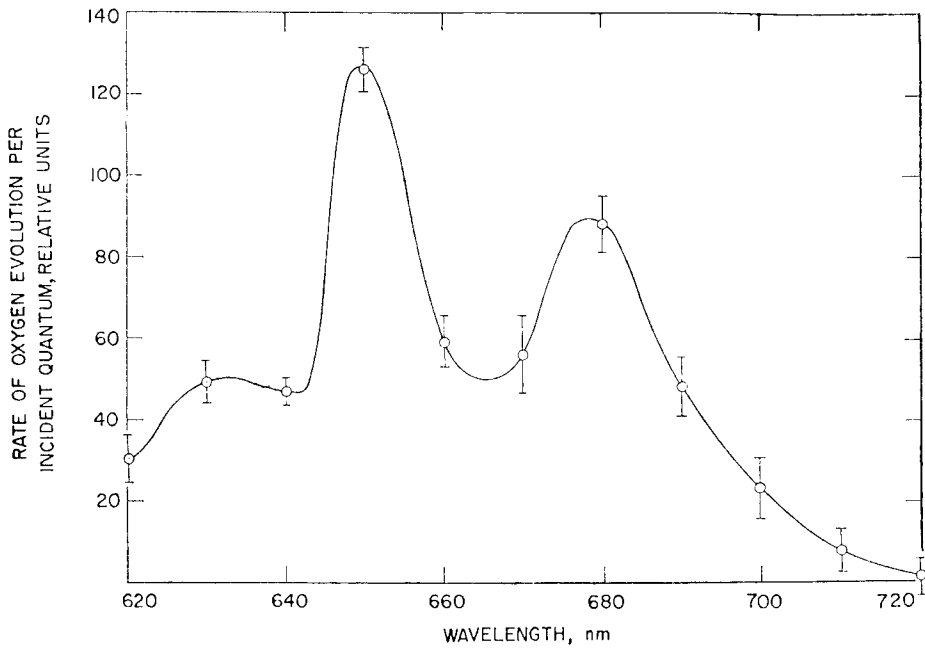|	Rate of O_2 evolution in relative units					Enhancement defined as $\dfrac{E-(D\text{-}B)}{C}$
	A	B	C	D	E	
Expt.	650 nm	650' nm	700 nm	(650+650')	(650+700)	
1	59	98	67	134	151	1·7
2	110	130	130	200	280	1·6
3	100	56	45	146	165	1·7

FIG. 3. Rate of O_2 evolution (corrected for incident quanta) as a function of wavelength of light; using 700 nm background light; chloroplast fragments with ferricyanide as the electron acceptor.
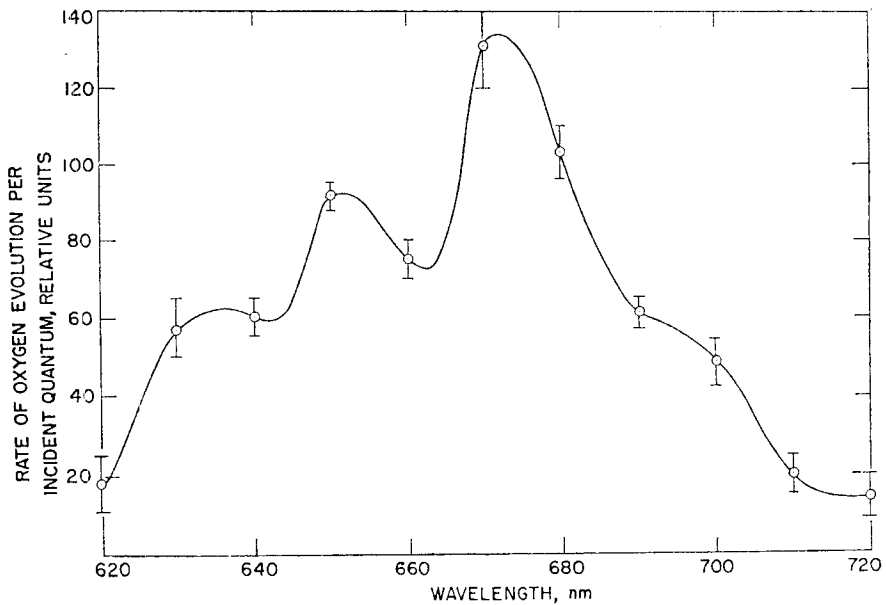
FIG. 4. Rate of O_2 evolution (corrected for incident quanta) as a function of wavelength of light; using 650 nm background light; chloroplast fragments with ferricyanide as the electron acceptor.

short-wave form of chlorophyll *a* (Chl *a* 672). (Also see solid curves for system I and II in Fig. 7 in ref. 35).

If two light reactions are involved in at least *one* route of the ferricyanide reduction, then we must have the two pigment systems performing the two postulated light reactions. In order to characterize these systems we have measured the action spectra of the Hill reaction in the presence of an excess of 700 nm (Fig. 3) and of 650 nm light (Fig. 4). These spectra may be somewhat distorted because of the non-linearity of the light curve at these intensities. The action spectrum measured in the presence of 700 nm light (system II spectrum) shows a dominant peak due to chlorophyll *b* and a smaller one due to chlorophyll *a* (one band envelope around 675 nm). The action spectrum in the presence of 650 nm light (system I spectrum) shows peaks due to chlorophyll *b* (about 70 per cent of that of Chl *a*), chlorophyll *a* (band envelope around 675 nm) and an additional shoulder around 700 nm (due to a long-wave form of chlorophyll *a*). Qualitatively, this confirms the earlier findings on photosynthesis.

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