THE MECHANISM OF THE PHOTOCHEMICAL ACTIVITY OF ISOLATED
CHLOROPLASTS. III.DEPENDENCE OF VELOCITY ON LIGHT INTENSITY 1,2

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Chloroplast fragments when illuminated in suspension media containing an oxidant such as \( \beta \)-benzoquinone, potassium ferricyanide or TPN support the photoproduction of oxygen from water (1). In previous papers we have shown that the actual free-energy requirement of this type of Hill reaction is approximately independent of oxidant despite the fact that the formal requirement varies from oxidant to oxidant and is in general less than that of the complete photosynthetic process (2, 3). As a consequence, it may be concluded from free energy considerations that such Hill-reaction systems retain the remarkable efficiency of the natural process of photosynthesis. Since we have also shown that such systems yield reproducible quantitative results in rate measurements, reactions with inhibitors and the like (4), they provide a relatively simple and attractive point of attack on the photosynthetic process. Recent work, particularly by Jagendorf and his co-workers (5), has intensified interest in the Hill reaction through demonstrations of the relationship between this reaction and phosphorylation. The later work has been carried out on chloroplast preparations which are undoubtedly more complex in chemical constitution and perhaps in structural interactions than fragments. The distinction between whole chloroplasts and fragments has not always been made in the past and, indeed, preparations have not usually been carefully characterized as to the presence or absence of intact chloroplasts. Nevertheless, it appears that chloroplast fragments, combined with necessary water-soluble cofactors and reactants, do carry out photophosphorylation just as well as intact chloroplasts. Thus, fragment preparations also provide a direct and much simplified system for investigation of this important reaction (6).

This paper confines itself to the Hill reaction of well-washed chloroplast fragments, our intention being to provide further fundamental information leading to a detailed characterization of the process. Eventually, the collection of biochemical, chemical and physical facts about photosynthesis will have to be organized on a framework of chemical kinetics if the process is to be understood in any really fundamental sense. It seems obvious that this undertaking will be very much facilitated if attention is first given to the Hill reaction, which has been shown consistently to yield simple kinetics entirely reminiscent of results obtained with chemical and enzymic systems. As will be shown in this paper, not the least fortunate appearance of simplicity is in the rate law relating velocity and light intensity. Under a very wide range of conditions, this rate law assumes a single, simple form in contradistinction to the behavior of the rate law of complete photosynthesis, which is complicated by many dependencies on extrinsic and intrinsic variables, some of which are difficult, if not impossible, to control (6). In this paper we shall establish the rate law for light intensity and provide quantitative data relative to temperature and wave length dependencies of the reaction parameters. This will be followed in the subsequent paper by a consideration of the consequences of the remarkably simple form of this law. In our treatment, we shall assume that the Hill reaction should be studied as an independent entity quite free of implications derived from observations on the total photosynthetic process in intact cells. Thus, we shall work to explain photosynthesis in terms of the Hill reaction, rather than the reverse.

1 Received March 23, 1959.
2 This work was supported by grants from the U.S. Atomic Energy Commission, the National Science Foundation (Grant G.1274) and the University of Utah Research Fund.
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LITERATURE CITED

Experimental

Experimental Materials: Washed chloroplast fragment preparations were obtained by standard methods from Swiss chard leaves (Fordhook giant) or from rhubarb chard leaves. The leaves were grown in such a way as to have high intrinsic Hill-reaction activity so that as high rates of reaction as possible could be secured. It was thus generally possible to work at temperatures near zero where the loss of intrinsic activity during an experiment is usually so small as to be negligible with respect to our other errors. Careful control of blending and centrifugation procedures produced material essentially free of intact chloroplasts (3, 8). Sufficient fragments were prepared at one time for each block of statistical experiments and were stored at \(-35^\circ\) C prior to use. Samples were thawed in exactly the same way before each experiment.

Velocity Determination: Reaction velocities were determined with conventional potentiometric or manometric techniques (3, 8, 9) using reaction mixtures which contained, in addition to chloroplast material, 0.05 to 0.10 M phosphate (total) buffer at pH 7.0, 0.167 M sucrose and the oxidant. It was previously established (4) that there are three ranges of oxidant effects on the Hill reaction of fragments: at very low concentrations (below \(10^{-4}\) M) the rate falls off with oxidant concentration and is probably diffusion controlled; above \(10^{-4}\) M and below \(7 \times 10^{-4}\) M, the velocity is very nearly independent of oxidant concentration; from \(10^{-4}\) M on up, there is a complex of activating and inhibiting reactions which now appears to be associated with the decoupling of photosynthesis by oxidant (10). Independence of rate on oxidant concentration in the middle region was reaffirmed and most experiments were carried out in this region using initial oxidant concentrations of \(2.5 \times 10^{-4}\) M potassium ferricyanide. The uppermost region of concentrations was also investigated with \(3 \times 10^{-4}\) M ferricyanide, but with the use of the less precise Warburg manometric method which is more convenient for this region.

The reaction vessel was of plastic (1.57 mm along the light path and 25.4 by 12.7 mm in rectangular cross-section across it) held in a thermostated, gas-tight metal box. A vibrator-driven metal rod provided a high rate of stirring.

Previous to this study, it was the custom in our laboratory when using the potentiometric method to evaluate the amount of ferrocyanide ion produced in the reaction by calculation from potentiometric curves using the apparent half-cell potential of the ferricyanide-ferrocyanide couple provided by each curve. It was discovered in this work that such a choice of half-cell potential leads to fictitious straightening of the product-time curves thus calculated and in so doing hides thermal inactivation. Choice of the true half-cell potential appropriate to a given reaction mixture is necessary if the experiments are of such long duration that inactivation occurs. In such a case, the correct rate can be obtained by extrapolation of the slope of the product-time curve, thus correctly calculated from potentials, to zero time. The true half-cell potential can be determined using known ratios of ferricyanide to ferrocyanide ion in each kind of non-illuminated reaction mixture. The total concentrations of the ions should be at least \(10^{-3}\) M to minimize dark reduction by the fragments. The validity of the method of correction was established by measuring reaction velocities over very short times, such that no inactivation occurred, and over long times, during which inactivation becomes so large that the rate law for inactivation can be calculated. In addition to the increase in precision obtained in this way, a further increase was secured by starting with half-reduced oxidant, for the reliability of the potential-concentration relationship is greatest in this region. Using these improvements and the fact that inactivation was usually slight, it was possible to obtain with little difficulty a standard deviation of velocity measurements of 2% or smaller, even at low velocities.

Light-intensity Considerations: With the potentiometric technique, light from an incandescent lamp was passed through an interference filter (width at half-maximum passage was 50 m\(\mu\) ) and an infra-red filter. Intensity was regulated by voltage variation and the interpositioning of neutral density filters calibrated to better than 1%. The intensity was maintained constant by manual regulation of the voltage and monitored continuously using a thermocouple or thermopile detector placed to the rear of the reaction cell. Absolute intensities were accurate to at least 10%, as established by conventional methods using a National Bureau of Standards carbon-filament lamp as secondary standard, and were maintained constant within 1%.

Because of the appreciable light absorption, even in the thinnest chloroplast suspensions, the intensity was measured as an average which can be expressed by:

\[
\bar{I} = \frac{I_0 - I}{I_0} = \frac{I_A}{\ln I} < a,
\]

where \(\bar{I}\) = average light intensity in the suspension;

\(I_A\) = absorbed light;

\(I_0\) = light intensity passed with water in the reaction cell;

\(I\) = light intensity passed with chloroplast suspension in the cell;

\(<\) = optical density due to light absorption per unit length of chloroplast suspension;

and

\(a\) = cell depth.

The total light attenuation factor \(<_T\) is obtained from measurements in an instrument such as a Beckmann DU spectrophotometer. It contains contributions from light absorption and light scattering. To a first approximation, scattering will not attenuate the local light field, so that an attenuation factor \(<\) must be defined which includes only the effect of light absorption. It is this factor determined by the methods
of this section which is used throughout this paper.

Since the sensitive elements of the thermopile or thermocouple only intercepted a small fraction of light beam transmitted through the reaction cell, it was necessary to correct the incident and transmitted light-intensity readings for divergence and scattering using a modification of the procedure of Wayrinen et al. (3). A correction for the light reflected from the plastic surfaces was also necessary. The light beam divergence correction factor, \( f_D \), was determined by placing the photo-detector in position such that it corresponded to the position of the reaction cell. With the light intensity kept constant, the galvanometer deflection was taken in this position and again with the detector in its normal position behind the cell space. The ratio of the galvanometer readings represented the divergence factor. The correction factor, \( f_r \), for reflection losses was determined from the following expressions:

\[
f_r = \frac{1}{1 - a} \text{ (gal. def. with water-filled cell)}
\]

\[
1 \text{ (gal. def. without cell)}
\]

The correction for the light lost due to scattering in the chloroplast suspension was usually calculated from galvanometer deflection readings taken with a selenium barrier layer photocell under the following conditions: 1) the cell filled with water; 2) the cell filled with the chloroplast fragment suspension; and 3) the above readings repeated with a mask over the face of the photocell in which an aperture of the same size and position of the receivers of the thermopile was cut. The scattering factor, \( f_s \), is then

\[
f_s = \frac{\text{W}_{(w/o)}}{\text{W}_{(w)}} \cdot \frac{\text{Chl}_{(w)}}{\text{Chl}_{(w/o)}}
\]

where \( \text{W}_{(w)} \) = galvanometer deflection with water in the cell and mask in front of the photocell;

\( \text{W}_{(w/o)} \) = deflection with water in the cell and no mask in front of photocell;

\( \text{Chl}_{(w)} \) = deflection with fragment suspension in the cell and mask in front of the photocell; and

\( \text{Chl}_{(w/o)} \) = deflection with fragment suspension in the cell and without the mask in front of the photocell.

The above procedure is based on the assumption that both the transmitted and the scattered light is picked up by the large surface of the photocell, whereas with the limited area of the aperture, the photocell picks up only the light normally received by the thermopile or thermocouple. This assumption is in keeping with the geometry of our cells, in which the depth was very much the smallest dimension. The scattering correction factor was found to vary with fragment concentration, as well as with the wave length of light, therefore it was necessary to determine the factor for every chloroplast preparation and every different light color used. Special scattering measurements are discussed in the Results section.

The reflection factor for single plastic surfaces, \( f_R \), was found to be 1.05, whereas the divergence factor, \( f_D \), normally varied between 1.05 and 1.10. When employing the thermocouple as the photosensitive receiver, the scattering factor, \( f_s \), varied between 1.10 and 1.48, depending on the fragment concentration and the light color. The corrections for reflection and divergence were applied to the incident light intensity readings, whereas the divergence and scattering factors were applied to the transmitted intensity reading. It was assumed that reflected light in the fragment-filled cell was absorbed in the reaction suspension. The totally corrected average light intensity reading should thus be given by:

\[
\bar{I} = \frac{f_D \cdot f_R \cdot I' - f_D \cdot f_s \cdot I'}{f_D I_0 \cdot f_s I'}
\]

where \( I' \) and \( I' \) represent the uncorrected light intensity readings with water and reaction suspension, respectively, in the reaction cell.

In manometric experiments, the light entered from below to fall on the flat bottom of the reaction vessels. Intensity was measured with a calibrated thermocouple contained in a simulated reaction vessel and shaken through the usual orbit. Only relative intensities were needed since only the form of the rate law was required. Precision in light and velocity measurements was of the order of 2 to 5 %, depending on the magnitudes of these quantities.

**Results**

**Macroscopic Rate Law**: The equation relating average Hill-reaction velocity, \( v \), and average light intensity, as obtained directly from the potentiometric time curve in a typical experimental situation, was indicated in an earlier work (9) to be of the form

\[
v = \frac{p k I}{I + K}
\]

in which \( p \) is determined by the fragment concentration and \( k \) and \( K \) are scale and form parameters, respectively. As a more severe test of this equation, 8 experimental velocities were determined at each of 8 light intensities. Statistical fitting to equation 3 can be carried out by an iterative least-squares method but the labor of such a treatment is justified only if the experimental errors are considerably less than those of the present investigation. It was easier and quite satisfactory to use an inverted form of equation 3 for statistical analysis:

\[
\frac{\bar{I}}{v} = \frac{K}{k p} + \frac{I}{v}
\]

Any such inverted form is suitable if proper weighting is applied. Since the standard deviation of \( \bar{I}/v \) determination was found to be proportional to \( \bar{I}/v \) (see table 1), equation 4 with weight of \( (v \cdot I)^2 \) was the obvious choice for least-squares treatment. The standard deviation of \( \bar{I} \) was found to be considerably less at all intensities than that of \( \bar{I}/v \), as is required for the simple least-squares method, which assumes
negligible error in the independent variable. The results, presented in table I, establish a high level of confidence in the validity of equation 4 and thus of equation 3. Calculated minus observed differences in velocity are all much smaller than the standard deviations in velocity and in most instances of the same order or less than the standard errors.

The reliability of the mean intercept (K/kp) and the mean slope (1/kp) of the regression plots according to equation 4 are measured by the standard errors (table I). Both means were found to have errors considerably less than 1%.

**Corrections for Suspension Absorbency:** The results of the previous section demonstrate that the rectangular-hyperbolic rate law of equation 3 provides a highly satisfactory fit for intensity-velocity data in the macroscopic experimental situation. It is apparent, however, that unless such data can be related to the actual intensity-velocity relationship existing in any small region of constant light intensity such as might contain a few hundred chlorophyll molecules, they will not be useful for the calculation of the fundamental reaction parameters of the microscopic reaction systems. The problems associated with heterogeneous light fields such as exist in chloroplast solutions have been well discussed by Rabinowitch (11). In principle, one may extrapolate through repeated dilutions of fragment suspension to a condition of zero light absorption. In practice, it is easier to vary the cell depth along the light path, holding fragment concentration constant. Using the latter procedure, one must assume a functional form for the microscopic rate law and average this velocity expression through the reaction cell by integration. Our final choice for the microscopic law was

\[ v = \frac{kpI}{I + K}, \tag{5} \]

in which \( v \) is the velocity in a local microscopic region in which the light intensity has a constant value I. A priori it might be expected that the average velocity expression derived from equation 5 by velocity averaging over the depth of the cell,

\[ V_v = \frac{kp}{I + K} \ln \left( \frac{K(e^{ka} - 1)}{K(e^{ka} - 1) + I_a} \right), \tag{6} \]

in which \( a \) and \( I_a \) are defined as in equation 1, would provide the experimental function of velocity in terms of light intensity and optical density due to light absorption if the microscopic rate law was chosen correctly. However, as will be discussed, it was found necessary to consider also the intensity-averaged form of the integrated rate law based on the same choice of microscopic rate law,

\[ V_t = \frac{kpI}{K + I} = \frac{kp(I_a/a)}{K + (I_a/a)} \tag{7} \]

Equation 6 yields a true rectangular hyperbola at vanishingly small values of \( a \), whereas equation 7 is always a rectangular hyperbola. The data of table I have associated too large an error to allow a distinction between these equations, but at higher optical intensities the distinction becomes marked, as shown in table II, in which it can be seen that equation 7 provides a highly superior fit to the experimental data at high, as well as low, optical densities. It should be noted that the only variable used in calculating the velocities is \( a \); \( K \) and \( k \) were the constants. Mayne has recently shown that similar verification of equations 7 and 5 results if \( a \) is held constant and \( < \) is changed (12). These tests are not only adequate to distinguish between the types of averaging necessary to obtain the macroscopic rate law, but since equation 7 is satisfactory at both high and low optical densities, the microscopic rate law, equation 5, on which it is based must be a satisfactory approximation to the natural rate law to the quite high accuracy of the present data.

**Table I**

**Linear Fit of Potentiometric Data**

Eight velocity determinations at each light intensity

<p>| pH = 7.0, Temp. 25° C, Ferricyanide conc = 2.5 × 10⁻⁴ M, ( \lambda = 675 \text{ m} \mu )* |
|---|---|
| <strong>Intensity</strong> | ( 10^{-2} ) (ergs cm⁻² sec⁻¹) |
|  | moles ferri |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>mole chlorophyll</th>
<th>min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.10</td>
<td>0.0705</td>
<td>0.126</td>
<td>0.189</td>
</tr>
<tr>
<td>2.36</td>
<td>0.0013</td>
<td>0.0033</td>
<td>0.0032</td>
</tr>
<tr>
<td>4.37</td>
<td>0.0005</td>
<td>0.0012</td>
<td>0.0011</td>
</tr>
<tr>
<td>7.08</td>
<td>1.566</td>
<td>1.873</td>
<td>2.312</td>
</tr>
<tr>
<td>9.20</td>
<td>0.0296</td>
<td>0.0452</td>
<td>0.0379</td>
</tr>
<tr>
<td>23.5</td>
<td>0.0105</td>
<td>0.0160</td>
<td>0.0134</td>
</tr>
<tr>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
<td>52.0</td>
</tr>
<tr>
<td>92.0</td>
<td>92.0</td>
<td>92.0</td>
<td>92.0</td>
</tr>
</tbody>
</table>

| **Least-squares** |
|---|---|
| \( I \times 10^{-2} \) | \( K \) |
| 2.112 | 1351.5 |
| \( K \) | 24.38 |
| Standard deviation | 8.620 |
| Standard error | \( 0.0085 \) |
| Calculated \( v \) | 0.00697 | 0.127 | 0.192 | 0.249 | 0.279 | 0.373 | 0.422 | 0.433 |
| Calculated-observed difference | 0.0008 | +0.001 | +0.003 | -0.004 | -0.001 | -0.001 | +0.001 | +0.002 |
| Ratio for \( v \) | 0.16 | +0.8 | +2.7 | -1.3 | -0.3 | -0.6 | +0.5 | +0.9 |

* Interference filter peak.
** The standard deviation for light intensity measurements was 0.4%.
TABLE II
Comparison of \( V_r \) and \( V_i \) with the Experimental Rates at Different Light Intensities and at Two Different Suspension Thicknesses

\[
\begin{array}{c|ccc|ccc}
\text{I}_a \times 10^{-3} & V_r & V_i & V_{\text{exp}} \\
\text{I}_{a*} \times 10^{-3} & V_r & V_i & V_{\text{exp}} \\
12.97 & .442 & .443 & .441 & 15.80 & .422 & .430 \\
7.33 & .420 & .422 & .421 & 8.94 & .385 & .401 \\
3.32 & .379 & .373 & .374 & 4.05 & .316 & .338 \\
1.297 & .278 & .279 & .280 & 1.58 & .217 & .234 \\
0.998 & .249 & .249 & .253 & 1.215 & .189 & .203 \\
0.616 & .195 & .192 & .189 & 0.750 & .142 & .149 \\
0.332 & .132 & .127 & .126 & 0.375 & .069 & .070 \\
0.156 & & & & & \\
\end{array}
\]

Experimental conditions as in table I.

* \( I_a \) = Absorbed light in ergs/cm\(^2\) - sec.
K for \( V_r \) = 590.
K for \( V_i \) = 640.

Validity of the Rectangular-Hyperbolic Rate Law for Light Intensity: The rectangular-hyperbolic rate law has quite generally been found to fit our velocity-light intensity data from Hill-reaction studies. Data published by Gorham and Clendenning (13) for the Hill reaction are fitted by this rate law and Burk and co-workers (14) have suggested that it is also satisfactory for photosynthesis data under some conditions (see also the discussion in Rabinowitch (11)). However, it is not possible with data of less than perfect accuracy to completely establish the validity of such an empirical analysis. We may, however, test a very great many alternative possibilities by the use of series expansions of \( \frac{1}{V} \) in powers of \( I \) since many more complicated rate laws may be approximated in this way. (For examples of photosynthesis rate laws treatable in this way see (6)). Two such expansions are sufficient:

\[
\frac{1}{V} = b + cI + dI^2 + eI^3 + \ldots \tag{8}
\]

\[
\frac{1}{V} = b + cI + dI^{-1} + eI^{-2} + \ldots \tag{9}
\]

With the use of weighted least-squares procedures, we have compared the fit of such equations to the data of table I with that of equation 7, retaining only one term beyond the linear one in these higher-order polynomials. The values of \( d \) so calculated (table III) are not only negative, but entirely insignificant by t-test procedures. Thus it is quite satisfactory to assume the validity of equations 5 and 7 for Hill-reaction data obtained at no higher precision than those of this investigation.

Temperature Dependence of the Rate Parameters: As a consequence of the above discussion, it is possible to evaluate the rate parameters, \( k_D \) and \( K \), of the microscopic rate law from "macroscopic" velocity measurements. In earlier studies, Bishop and co-workers (8) suggested that \( k_P/K \) was temperature-independent, from which they concluded that independent parameters \( k/K = k_{1D} \) and \( k = k_{0p} \) could be defined (see also (3)). \( k_L \) controls the rate at vanishingly small light intensities and is thus related to the light steps of the Hill reaction and to the minimum quantum requirement for this process. \( k_0 \) controls the velocity at \( I > K = k_0/k_{L} \). Bishop also estimated the activation energy for the temperature-dependent parameter as 10 kcal. His results were based on velocity measurements at high light intensity, but he was unable to extrapolate velocities to infinite intensity. It was thus necessary to improve Bishop's results by establishing the temperature-dependence of the 2 rate parameters. Typical temperature data are plotted in figure 1 according to the inverted linear form of equation 7. Values of \( k_P \) and \( k_L \) calculated from this data by least squares are given in table IV. It is apparent that \( k_P \) has an activation energy of zero with a high degree of confidence. We may thus conclude, following the arguments of Bishop et al, that \( k_P \) has significance as a separate parameter, a result which might be expected if this parameter is dominated by temperature-independent processes such as light absorption, migration of electronic quanta and quanta-trapping through temperature-independent processes in which systems change from one electronic state to another. The activation energy for \( k_D \) obtained by least-squares fitting of the Arrhenius plot of table IV data is 13.0 ± 0.3 kcal. As would be expected, the present value is significantly higher than that reported by Bishop et al and higher than nearly all reported values, most of which were obtained without extrapolation to infinite light intensity (see (8) and (6)).

TABLE III
Fit to Other Trial Equations

<table>
<thead>
<tr>
<th>Trial equation</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>( I ) = b + cI</td>
<td>14.690</td>
<td>2.112</td>
<td>\ldots</td>
</tr>
<tr>
<td>( I ) = b + cI + dI²</td>
<td>14.50</td>
<td>2.16</td>
<td>(-4.43 \times 10^{-4})</td>
</tr>
<tr>
<td>( I ) = b + cI + d/I</td>
<td>14.79</td>
<td>2.12</td>
<td>(-0.291)</td>
</tr>
</tbody>
</table>

Data from table I.

TABLE IV
Effect of Temperature upon the Hill Reaction-rate Parameters \( k_D \) and \( k_L \)

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>( k_D )</th>
<th>( k_L \times 10^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3</td>
<td>0.531</td>
<td>0.228</td>
</tr>
<tr>
<td>11.0</td>
<td>0.754</td>
<td>0.209</td>
</tr>
<tr>
<td>16.0</td>
<td>1.11</td>
<td>0.212</td>
</tr>
<tr>
<td>21.0</td>
<td>1.67</td>
<td>0.210</td>
</tr>
</tbody>
</table>

Experimental Conditions as in table I.
for summary). The one exception is the value 22 kcal reported by Holt and French (15). Although reliable values of the activation entropy for \( k_D \), \( \Delta S_D^P \), can not, of course, be calculated at the present time, \( \Delta S_D^P \) may be roughly estimated on the assumption that the rate is controlled by a fixed constituent of the fragments present in concentrations of 1 molecule per some average number of chlorophyll molecules which deliver their excitation energy to this "trapping" molecule. If we take the mean number of chlorophyll molecules to be 200, the value thus calculated is ~10.9 e.u. This number is the entropy of activation corresponding to the intrinsic dark-reaction rate of 1 mole of trapping centers. It is called \( k_D^0 \) (see (7) for complete description). There is no reason to assume at this time that the frequency factor is other than unity.

**Experiments at High Oxidant Concentrations:** In order to establish the form of the rate law at high (0.003 M) oxidant concentrations as well as at low, 13 carefully controlled manometric determinations of the rate law were made at the higher concentration and analyzed statistically. Although the errors inherent in the method considerably decrease the precision of these results as compared with potentiometrically determined velocities, the rate law was found to be identical in form in the 2 kinds of experiments, a confirmation of many previous observations from our laboratory. This conclusion is important since recent studies by Jagendorf and co-workers (10) suggest very strongly that the unusual behavior of the Hill reaction at high oxidant concentrations (4) is due to a decoupling by the oxidant of the photosynthetic-phosphorylation mechanism from the main sequence of Hill-reaction steps. If this indeed proves to be the case, it will be possible to conclude that the presence or absence of phosphorylation influences the values of the rate parameters but not the form of the rate law.

**Wave Length Dependence of \( k_D \) and \( k_L \):** Lumry et al (3) obtained values for the quantum requirement of the Hill reaction using equation 4 to evaluate \( k_L \), which, on conversion to absolute absorbed light intensities, measures the minimum requirement; i.e., that which occurs at zero light intensity. Those results indicated a rather surprising increase in the minimum requirement in the region of minimum extinction coefficient for chlorophyll (560 \( m \mu \)). Experience subsequent to that work suggested that the correction for scattered light may have been inadequate, particularly in the region of the spectrum in which light absorption is at a minimum. As a test of this possibility, the absolute quantum requirement was determined at 675,560 and 457.5 \( m \mu \) (peak values of interference filters with a 50 \( m \mu \) width at half-maximum transmission). The data are given in table V and figure 2. The earlier scattering correction was confirmed within error for blue and red illumination but not for the 560 region, where we found it necessary to use a special technique to determine the large correction. The method, due to Haxo and Blinks (16), consists in placing the chloroplast-fragment suspension against the face of a selenium barrier-
layer photcell and covering the suspension with a magnesia-coated hemisphere. A small slit in the hemisphere admits the light directly onto the suspension. Since any scattered or reflected light from the suspension will be reflected back to the photocell surface, the ratio of the photocell output with water in place of the suspension to that with the suspension in place will be the ratio of incident intensity to transmitted intensity. Comparison of this ratio with that obtained when the calibrated thermocouple was placed in the normal experimental arrangement, provided a simple calculation of the scattering factor. This factor at 560 mμ was found to be 1.48, as compared with 1.38 determined using the method described in the Experimental section. The value is considerably greater than the former value for a suspension of approximately the same fragment concentration (3).

The quantum requirements given in table V are the minimum values obtained by the extrapolation of the straight lines of figure 2 to zero light intensity and applying the necessary geometry and absorptivity factors to the kₚ values thus obtained. No corrections of kₛ for scattering, divergence, and reflection light losses were needed since these corrections were made on the individual light intensity readings as previously described. It will be noticed in this figure and table V that kₚ (measured by slope) is essentially independent of wave length but that kₛ does depend on wave length, as would be expected. It is perhaps worthy of mention that in photosynthesis, as in most other cases of biological photochemistry, the velocity of reaction at a fixed finite light intensity contains contributions from at least 2 rate processes—a light-dependent and a light-independent process—so that in the usual situation, in which the processes depend in different ways on the light intensity or on the light-wave length, a simple velocity measurement gives little, if any, useful information about the wave length dependence of this process. Thus, if it is to be useful, the concept of "action spectrum" must be more rigorously defined so that it is based on velocities extrapolated to zero light intensity. A useful action spectrum for the Hill reaction would then be a plot of properly corrected values of kₛ versus wave length.

Although the quantum requirements of table V are not strictly comparable with earlier results (3), which were obtained at pH 6.3, the pH of maximum efficiency, rather than 7.0 and were thus considerably smaller than present values, the newer results do serve to correct the formerly low value at 560 mμ and to emphasize the observation that the quantum requirement at 458 mμ (and now at 560 mμ) is about 35% higher than at 675 mμ. This latter fact may be a consequence of inefficient light absorption by accessory plant substances.

**Discussion**

**Relationship between Macroscopic and Microscopic Rate Laws:** The rather surprising fact that the macroscopic rate law must be obtained from the microscopic law by intensity rather than velocity averaging through the reaction cell (equation 6 versus equation 7) requires explanation. Failure of the Beer-Lambert Law in our suspensions, or the existence of a time lag between exposure of the fragments to light and the appearance of product which is long with respect to the time a local fragment region requires to move from one light field to another of appreciably different intensity, would explain the observation. Since <c₄ <l₄ was found experimentally to be a linear function of fragment concentration to considerably better than 90% light absorption, the second explanation may be assumed tentatively to be correct. In the second case, each local reaction unit in the course of its perambulations through the rapidly stirred reaction mixture must average the light intensity. The total time between the production of product molecules, presumably oxygen, must be sufficiently long so that each unit sees approximately the same time-average light field. Gilmour (18) and Kok (19) have found that this time may be an appreciable fraction of a second but even the time of Emerson and Arnold (20), which was about 0.01 sec, is probably sufficiently long for a fragment in a rapidly stirred suspension to make a large translation in our cells. Apparently the several quanta necessary to produce 1 product molecule arrive with rates corresponding to different light intensities and thus approximately average the effect of intensity. Slow stirring (< 30 cycles per sec) produces appreciably different rates of reaction than fast stirring, though this observation must be due in part to the time for diffusional equilibration of the oxidant concentration in the neighborhood of the electrodes. Normal rates of stirring were maintained at levels (> 80 cycles per sec) such that significant changes in these rates had no effect on reaction velocities. It might be suggested that even in the absence of stirring, simple diffusional rotation of the large, optically-dense fragments would provide

**Table V**

<table>
<thead>
<tr>
<th>Table V</th>
<th>Effects of the Light Wave Length upon the Hill Reaction Parameters kₚ and kₛ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wave length (mμ)</td>
<td>kₚ</td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>675</td>
<td>0.838</td>
</tr>
<tr>
<td>560</td>
<td>0.831</td>
</tr>
<tr>
<td>457.5</td>
<td>0.852</td>
</tr>
</tbody>
</table>

Experimental conditions except light intensity as in table I.

*<c₄/kₛ (0.436 × 10⁴), quanta per oxygen produced.
some averaging of the type under discussion. However, the extreme difference in light field on 2 sides of our small fragments (about 0.2 μ in diameter) would appear to be inadequate to produce the total observed averaging.

Our experiments do suggest the validity of the second explanation based on stirring-produced translational motion and thus indicate the presence of light-intermittency effects due to stirring. Hence, most so-called steady state velocity measurements on systems of chloroplasts, chloroplast fragments, algae and photosynthetic bacteria are probably to a greater or lesser degree flashing-light experiments. Nevertheless, when such experiments are carried out in such a way that reliable extrapolation to zero and to infinite light intensity is possible, the rate parameters evaluated at these limits should exclude such effects. In the case of the reactions of chloroplast fragments when experiments which have been carried out at both very low and very high light intensities are found to fit the straight line of equation 4, we may assume that the values of \( k_p \) and \( k_n \) are very close approximations to the true steady state parameters of the microscopic as well as the macroscopic rate laws.

**SUMMARY**

1. The experimental steady state, velocity-light intensity relationship for the Hill reaction of chloroplast fragments is shown to be a rectangular hyperbolic function within narrow statistical limits.
2. The rate law is analyzed in terms of the microscopic intensity-velocity relationship applying in small regions of the fragments to provide a means for evaluating fundamental rate parameters.
3. The temperature dependence of the experimental parameters is analyzed in terms of a light-step reaction parameter and a dark-step reaction parameter. The activation energy for the latter is 13.0 kcal.
4. The quantum requirement has been determined at 3 wave lengths to show that red light can be used 35 % more efficiently than green or blue light.
5. Apparent steady state studies of the Hill reaction are interpreted as flashing-light studies.

*Note added in proof:* In relating the rate law for the Hill reaction to the fluorescence yield-light relation, Lumry, Mayne and Spikes (Faraday Society Discussion, No. 27: Energy Transfer with Special Reference to Biological Systems, 1959) have observed that the fluorescence yield and thus presumably the local velocity at a "photosynthetic unit" for a given light intensity is independent of whether the reaction mixture is stirred or not. They conclude that the effects of stirring on measured velocity are due to the rate of equilibration of oxidant concentration at the electrode and that there is enough averaging of light intensity as a consequence of translational and rotational Brownian motion alone to explain the observed dependence of experimental velocity on average light intensity.

The paper by the above authors in the reference cited should be consulted for velocity expressions applicable to experimental situations in which the optical density is not large.

**LITERATURE CITED**