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# A POLAROGRAPHIC METHOD FOR DETECTION OF OXYGEN PRODUCTION AND REDUCTION OF HILL REAGENT BY ISOLATED CHLOROPLASTS

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SUMMARY

In preceding papers[2,3] a new amperometric method was described for the detection of photosynthetic oxygen production by unicellular algae. The algae are illuminated by a modulated light and only the modulated component of the amperometric current is detected.

This apparatus has now been modified to allow the study of isolated chloroplasts in the presence of electron acceptors and donors. The time resolution of the method has been improved and reaches 5 msec under the best conditions. The method can be used to measure, in relative units, the rate of reduction of electro-active Hill reagents such as quinone, ferricyanide or methyl viologen. It is also possible to measure the relative amounts of oxygen evolved or Hill reagent reduced, by a single saturating flash of short duration. The latter determinations can be performed simultaneously with the modulated rate measurements.

## INTRODUCTION

In previous papers we described an amperometric method for the measurement of photosynthetic $O_2$ production using modulated illumination and phase- and frequency-sensitive signal detection[1-3]. In this paper we describe the following modifications and improvements of the technique: Firstly, its use has been extended so that, besides $O_2$ evolution, the photoreduction of Hill oxidants can be measured. Secondly, the time resolution has been increased to $\geqslant 5$ msec. Thirdly, we can measure, in relative units, the amount of $O_2$ evolved (or Hill reagent reduced) by a single brief flash.

## I. DESCRIPTION OF THE APPARATUS

As shown in Fig. 1 the apparatus consists of three compartments separated by dialysis membranes. The bottom of the lowest compartment is a bare platinum electrode (2 mm × 10 mm) upon which chloroplasts or algae are deposited. The platinum is about 0.1 mm recessed so that when the electrode assembly is pushed

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol.

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upwards against a taut dialysis membrane (0.001 inch, wall thickness) the total volume of the electrode chamber is approx. 2 $\mu$l. A second dialysis membrane is mounted about 0.5 mm above the first one. In the lucite spacer plate, which separates the two membranes, is an oblong opening with dimensions slightly exceeding those of the platinum electrode. Through grooves on each side of the cavity and holes in the spacer plate and the lower membrane, the cavity communicates with two channels in the lower lucite block (L), fitted with injection needles. One of these is connected to a reservoir containing a buffer solution *plus* 0.1 M KCl to which a Hill oxidant can be added. By gravity flow, the buffer solution continuously renews the $O_2$ and solute content of the middle compartment. The upper lucite block (U) contains the third (top) compartment (approx. 4 mm deep) which is sealed with a glass window; its side-wall is lined with thin silver foil which serves as the reference electrode. The horizontal dimensions of the top compartment are somewhat larger than those of the lower compartment to allow masking of the silver electrode against illumination which might cause spurious signals. Also, this compartment is provided with entrance and exit channels to allow a gravity-fed liquid flow, this time of pure buffer solution enriched with 0.1 M KCl.

The two main lucite parts, U and L, each containing influx and efflux parts, and the spacer between the membranes fit into a cylindrical metal holder and can be compressed tightly with a screw cap. To assemble the instrument, wetted discs of dialysis membrane are placed on the upper (U) and lower (L) parts, using nylon rings to stretch and hold them in place. The bottom part is placed in the metal mounting

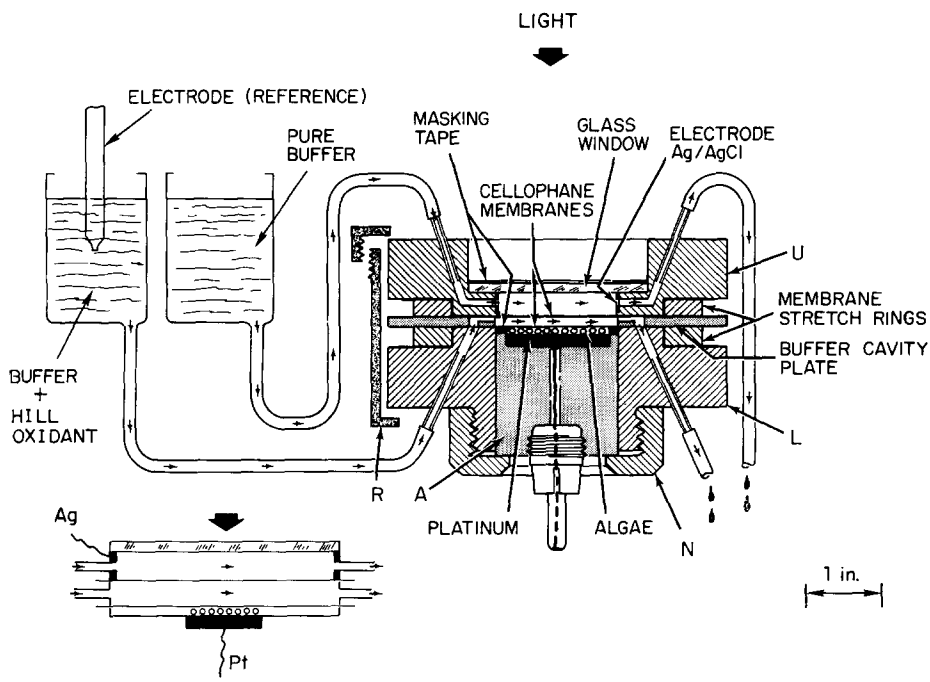



Fig. 1. Bottom left: simplified scheme of the apparatus shown in more detail on the right hand side. The detailed drawing is more or less to scale except for the algae-containing chamber. For explanation see text.

cylinder (R) (shown on left side only), and the walls are keyed to each other to ensure alignment of the three parts. The spacer plate is put on top and 2 holes are pierced in the membrane matching the feeding and draining channels, respectively. The top part is added, the screw cap tightened and the apparatus mounted in the proper optical arrangement. The Pt electrode is embedded with epoxy cement in a cylindrical piece of lucite (A) and connected to a shielded plug. The top face of this assembly is polished flat and provided with a disc of black electrician's tape, with a cut-out of the same dimensions as the Pt electrode to obtain an approx. 0.1-mm deep cavity for retaining the algae. This electrode assembly fits snugly into the lower lucite block and is easily removable.

After a drop of a chloroplast or algae suspension (approx. 0.3 mg chlorophyll per ml) is placed on the platinum surface, the electrode assembly is pushed upward and secured against the lower membrane with a nut (N). A slot and key arrangement aligns the assembly. During insertion, excess suspension is drained through two tiny grooves at the side of the electrode assembly: air bubbles on the electrode must be carefully avoided. Depending on the size of the particles, adequate settling, *i.e.*, a steady response was obtained 10 min after assembly.

The essence of this improved design is the middle compartment through which buffer medium flows, containing $O_2$ and Hill oxidant. These diffuse through the dialysis membrane into the electrode compartment and are available to the chloroplasts. Even a considerable dark rate of reduction or oxidation of such a reagent at the platinum surface does not interfere with the measurement, since it yields a d.c. current which is not detected. If the presence of a high molecular weight substrate or catalyst, such as ferredoxin, is required it has to be added in the chloroplast suspension itself because it will not diffuse through the membrane. The flow of pure buffer–KCl solution through the top compartment prevents chloroplast oxidants from reaching the silver electrode, an essential requirement for reliable operation. Low resistance of the electrode assembly favors high time resolution, one reason why especially the lower and middle compartments are thin. A secondary advantage of using thin layers is that light absorption by colored oxidants such as indophenol dye is restricted.

The flow rate of the two liquids proved not very critical (a few ml/min); the same batch (some 100 ml) of each medium could be used during several hours by returning the efflux into the feed reservoir.

To illuminate the sample, two beams were available, each of which could serve either as the modulated detecting light or provide a continuous background or preillumination. One was the output of a small grating monochromator (half band width, $>3$ m$\mu$), focussed on the platinum strip. The other was used when a higher intensity was required, its spectral composition and intensity could be varied with color filters or wire screens. Either of the beams could be modulated by inserting a four-sector rotating disc, driven by a small variable speed d.c. motor (Brion LeRoux Birotax, Model 2, rev./min proportional to voltage). The modulation frequency could be varied between 1 and 200 cycles/sec by varying the voltage which was regulated so that, at any setting, speed variations were within $\pm$ 1 %.

Finally, in a third optical beam a GE-FT 230 flash lamp was focussed on the sample *via* a lens system and partial transmitting mirror. This allowed the illumination of the sample with short (20 $\mu$sec) flashes of saturating intensity (20 J).

## II. ELECTRICAL CIRCUITS (Fig. 2)

### *1. Polarization voltage*

The platinum electrode can be polarized either positively or negatively with respect to the reference electrode. Since the potential of the reference electrode VE can vary, especially with high d.c. amperometric currents, a reference calomel electrode was used to monitor the potential between the platinum and the solution. This reference electrode was immersed in the buffer reservoir feeding the middle compartment and connected to the input of a servo amplifier. The potential of the silver electrode was regulated so as to maintain VE at the selected value. The time constant of this amplifier was about 1 sec to eliminate noise of higher frequency. VE could be varied over a range +1 to −1 V.

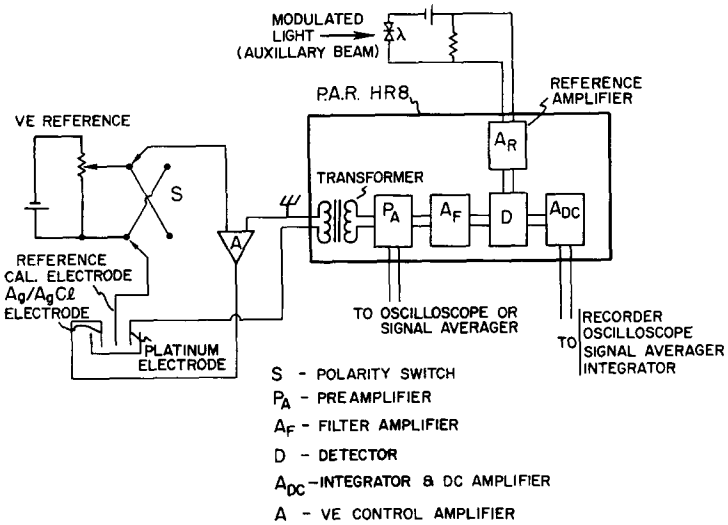



Fig. 2. Electrical circuit.

### *2. Signal detection*

The amperometric current is fed into a lock-in amplifier (PAR Model HR 8). The low impedance preamplifier was used, including a transformer with a turns ratio of 20, which yielded good impedance matching for modulation frequencies between 3 and 200 cycles/sec. The lock-in signal, needed to synchronize the detector with the light modulation, was obtained from a small photodiode (Texas Instrument, H-35) which was intermittently illuminated by a small pen light probe, interrupted by the same rotating disc which chopped the illuminating beam. The use of the lock-in amplifier which allows measurement of the phase of the signal in respect to the phase of the exciting beam was discussed in earlier papers[2,3]. In most experiments discussed here and in following reports, the phase angle between the modulated signal and the reference signal was adjusted for maximum response. Under some experimental conditions, however, the phase of the signal varies with the wavelength or intensity of the modulated exciting light. Such a variation generally indicates the simultaneous occurrence of two different modulated signals, for instance, one due to the reduction of $O_2$, the other to the reduction of quinone (see Fig. 6). In these in-

stances interpretation of the response can be quite difficult despite the possibility of obtaining considerable separation between two such signals by choosing an optimal setting of the phase angle.

The output of the HR 8 amplifier, which indicates the rate of the reaction, could be connected to a pen recorder (time response, 0.5 sec), to an oscilloscope for viewing faster phenomena, or to a signal averager to increase the signal to noise ratio of weak, repeatable phenomena.

If we wanted to determine the amount of $O_2$, *e.g.*, as evolved in the initial gush upon illumination, the signal was fed into an integrator and recorded with an oscilloscope or pen recorder. The sensitivity of the amplifier used to detect the modulated signals ranged between 2 $\mu$V and 5 mV, full scale.

*3. Time response*

Time response and selectivity of the amplifier can be adjusted by varying the integration time after demodulation. We generally used a time constant 10–20 times the period of modulation, which yielded excellent signal to noise ratio. Only if very weak, stationary rates had to be observed did we use higher time constants (*e.g.*, 30 sec).

Very rapid time response, of the same order of magnitude as the modulation period of the illuminating light, could be obtained by using the amplifier with time constant zero after demodulation and suppressing the selectivity of the first filter amplifier. In this case one must guard against saturation of the d.c. amplifier by noise or 60-cycle interference. For a modulation frequency of 200 cycles/sec the time response is about 5 msec. Without a decrease of time resolution the signal to noise ratio now can be improved, if necessary, by repeating the observation and averaging the signals (for this we used a Fabri Tek No. 1052 computer). Examples of the use of the apparatus in this way are given in Figs. 7 and 8.

## III. MEASUREMENT OF RELATIVE FLASH YIELDS

A quantitative interpretation of fast transitory signals observed with the classical rate electrode is nearly impossible because of the complex diffusion and electrical properties of the system. Still, the instrument allows a quantitative comparison of signals which have identical time courses[4].

We have used our apparatus to determine the relative amounts of $O_2$ evolved, or oxidant reduced, by light flashes of very short duration. For this measurement the output of the preamplifier is connected directly to the oscilloscope or the signal averager. Fig. 3 shows examples of the signal obtained with negative polarization ($O_2$ evolution) after a 20-$\mu$sec flash. To vary the size of the $O_2$ gush, three flashes were given after preillumination of the sample with three different intensities. These preilluminations varied the concentration of the photoreductant of System II and thus the amount of $O_2$ liberated by each flash. Despite the fact that, mainly due to the transformer circuit, the signal is strongly distorted, the time course is exactly identical in each case. This shows that the time course of $O_2$ evolution after a flash is independent of the amount of $O_2$ evolved and the amplitude of the signal is proportional to this amount. Fig. 4 illustrates similar experiments in which positive polarization was used to detect the amounts of methyl viologen reduced by single flashes.

Since the two types of observation do not significantly interfere with each other, the production by a flash can be measured during the observation of the rate induced by a modulated background beam.

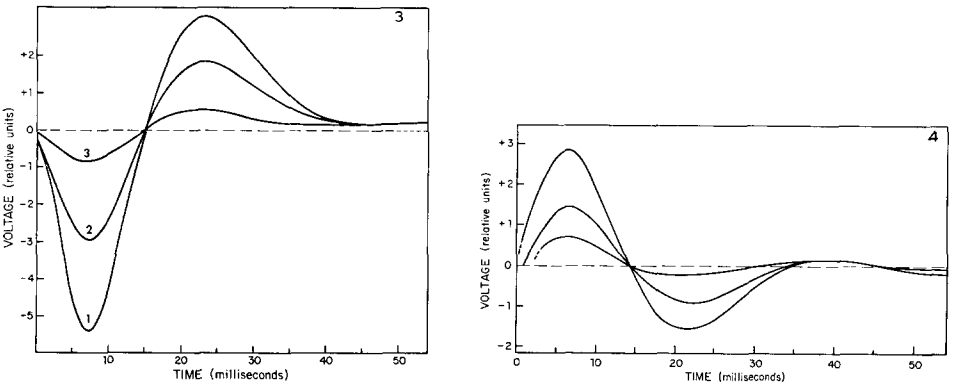



Fig. 3. Effect of brief saturating flashes. Negative polarization (−0.6), $O_2$ detection. Isolated chloroplasts suspended in phosphate buffer (pH 6.4) *plus* 0.1 M KCl; no acceptor added. Room temperature. Flash duration, approx. 20 $\mu$sec; flash energy, 31 J. The flashes are superimposed on a continuous back-ground of 650-m$\mu$ light, the intensity of which was increased from Flash 1 to Flash 3. The amount of $O_2$ evolved by the flash is proportional to the magnitude of the signal (for instance, the distance between the minimum and the maximum).

Fig. 4. Effect of brief saturating flashes. Positive polarization (+0.6). Acceptor methyl viologen $5 \cdot 10^{-5}$ M. Same chloroplasts and conditions as used for the experiment of Fig. 3. As in Fig. 3, the amount of methyl viologen reduced by the flash is proportional to the magnitude of the signal.

Checks in the absence of chloroplasts, and with chloroplasts inhibited by DCMU, revealed no artifacts due to photoelectric or thermal effects caused by the flash. A limitation of the method is that the $O_2$ production by flashes of different duration cannot be directly compared.

## IV. POLAROGRAM

The polarogram represents the dependence of the modulated signal upon the voltage VE applied to the platinum electrode. Since the phase of the signal varies with VE, it is necessary to tune the phase angle of detection for maximum response at each VE setting.

In the absence of added oxidants, the polarogram observed with negative polarization is similar to the one described in ref. 3.

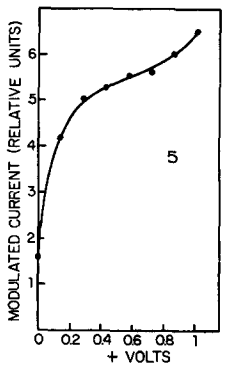
Fig. 5 shows a polarogram obtained with positive polarization in the presence of methyl viologen. Because of relatively slow equilibration after a change of VE, precise polarograms were difficult to obtain. The absence of well-defined plateaus in these polarograms necessitates careful stabilization of voltage VE.

## V. EXPERIMENTAL RESULTS

We will demonstrate the use and performance of the method by describing a few actual experiments; the biological significance of some of these will be discussed elsewhere in more detail.

*1. Experiments using negative polarization*

With whole algae or chloroplasts in the absence of electron acceptors the only response which can be detected at frequencies higher than 5 cycles/sec is due to the



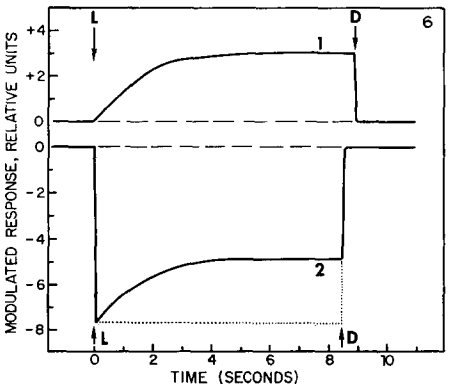




Fig. 5. Polarogram of the modulated response in the presence of $5 \cdot 10^{-5}$ M methyl viologen. Modulation frequency: 90 cycles/sec. Phosphate buffer (pH 7.9) *plus* 0.1 M KCl.

Fig. 6. Modulated response obtained in weak light —after a long dark period (3 min). Polarization, —0.6 V. Frequency of modulation: 27 cycles/sec. Chloroplasts suspended in phosphate buffer (pH 7.1) *plus* 0.1 M KCl. L = light; D = dark. Curve 1: No acceptor; Curve 2: Acceptor benzoquinone, $10^{-4}$ M.

modulated evolution of $O_2$. At lower frequency, a modulated $O_2$ uptake by chloroplasts, due to the autoxidation of the photoreductant (Mehler reaction), is simultaneously detected. Apparently the time constant of this autoxidation is quite slow (0.5 sec), so that the modulated signal corresponding to this uptake vanishes at higher frequencies. In this case discrimination between the two signals is relatively simple.

Fig. 6 compares the response to a modulated illumination in the presence or absence of $10^{-4}$ M benzoquinone. A very weak intensity was given after a long dark period. Curve 1 shows the slow increase of the rate to the final level due to the photoactivation of the $O_2$-evolving system described earlier[6]. In the presence of quinone (Curve 2) we observe the same slow activation kinetics, now, however, superimposed upon a constant negative signal which responds immediately to illumination and darkening (dotted line). This fast component indicates the modulated reduction of quinone which proceeds independently of the slowly activated $O_2$ evolution.

In experiments like this, one can often choose a frequency where the $O_2$ response and quinone response are about $\pm 90°$ out of phase. By choosing the correct phase angle of detection, it is possible to cancel either of the two signals. Still, interpretation of mixed signals necessitates quite careful experimentation. In studies of the $O_2$ evolution by chloroplasts, this difficulty can be largely avoided by using $NADP^+$ as a substrate in the presence of catalytic amounts of ferredoxin. These reagents do not react with the Pt electrode at the potential used for $O_2$ detection.

Figs. 7 and 8 illustrate the application of the method in studies of the gush of $O_2$ at the onset of illumination[5]. This measurement was made with a modulation frequency of 103 cycles/sec and a time response as fast as 10 msec, the signal being retrieved from the PAR without filtering or integration. In Fig. 7 each experiment

shows the response to a sudden admission of a modulated beam of 650 m$\mu$ (approx. $10^4$ erg·cm$^{-2}$·sec$^{-1}$). Curve was 1 observed after the chloroplasts had been in the dark for 15 min prior to illumination. The lag observed at the onset of illumination again shows the photoactivation which now, due to the high intensity, is completed within 100 msec. (In the experiment of Fig. 6, made with very dim modulated light, this activation took 5 sec.) Following the activation the rate remains fairly constant for a while and then declines to a low steady-state value. Curves 2 and 3 demonstrate that no activation lag is observed, if the chloroplasts have been pre-exposed to a very weak continuous light, or if, after a long dark period, a brief flash precedes the illumination. Actually in the latter case we observed an initially enhanced rate, which decreases in 0.1 sec to the plateau rate.

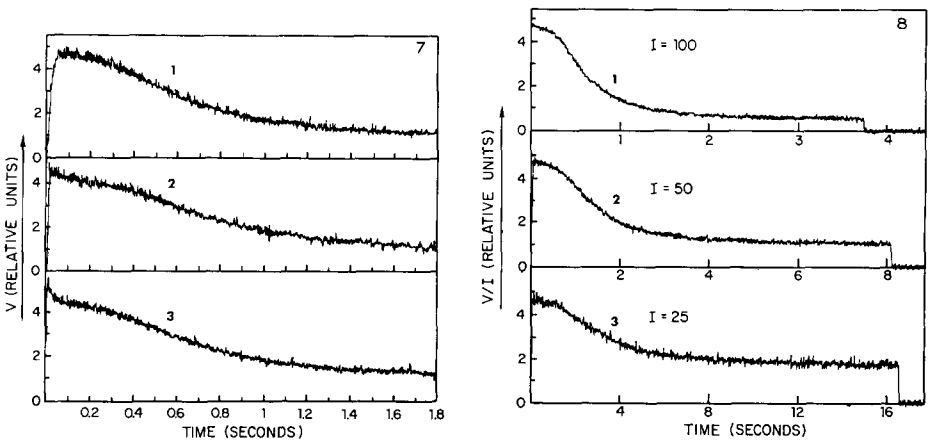



Fig. 7. $O_2$ gush at the onset of illumination. Modulated response; $V$ = rate of $O_2$ evolution. Frequency of modulation, 103 cycles/sec. Polarization —0.6 V. pH = 7.1. No acceptor. At zero time the sample was illuminated by a modulated 650-m$\mu$ light (about $10^4$ erg·cm$^{-2}$·sec$^{-1}$). Curve 1: After a dark period of 15 min. Curve 2: After preillumination with a weak, continuous 720-m$\mu$ beam during 1 min. Curve 3: After a dark period of 15 min. A 20-$\mu$sec saturating flash was given at the onset of the 650-m$\mu$ illumination.

Fig. 8. Oxygen gush at 3 different light intensities. Same conditions as Fig. 7. Preillumination during 3 min with a weak 720-m$\mu$ continuous beam. Ordinate shows the ratio between the rate of $O_2$ evolution, $V$, and the light intensity, $I$. Curve 1: $I$ = 100 relative units (approx. $10^4$ erg·cm$^{-2}$·sec$^{-1}$). Curve 2: $I$ = 50. Curve 3: $I$ = 25. The minimum rates measured at the end of the gush were 5.5/1 = 5.5 (Curve 1), 10.5/2 = 5.25 (Curve 2) and 18/4 = 4.5 (Curve 3). The equality of these 3 values indicate that a dark reaction limits the rate of the process.

The experiments of Fig. 8 demonstrate the strict proportionality between the rate of $O_2$ evolution and the amplitude of the modulated signal even during rapid rate transients. Each trace represents results of a duplicate experiment, averaged with the computer. Three different intensities of the same modulated beam, as used in the experiment reported in Fig. 7, were given to a chloroplast suspension which was preilluminated with weak (720 m$\mu$) light. Since the $O_2$ gush is due to the photochemical depletion of a fixed pool of reductant in the chloroplasts, the amount of $O_2$ evolved in the gush is independent of intensity. Thus, whereas the number of quanta ($I \cdot t$) required to deplete this pool should be independent of intensity, the rate ($V$) of depletion should be proportional to intensity. The plots of the rate, ex-

pressed as $V/I$ in dependence of $I \cdot t$ indeed reveal this clearly, especially for Curves 1 and 2. In Expt. 3 the intensity was actually too low to completely deplete the pool. In the final phase of the exposures the rate becomes limited by a thermal reaction and thus independent of intensity.

*2. Experiments using positive polarization*

With positive polarization, $O_2$ is not detected and the signal exclusively indicates the modulated reduction of added oxidant. With high potential oxidants such as DCIP, ferricyanide or quinone, we encountered some ambiguities, which can be ascribed to concomitant photoreduction by Photosystems I and II. Like $NADP^+$ in

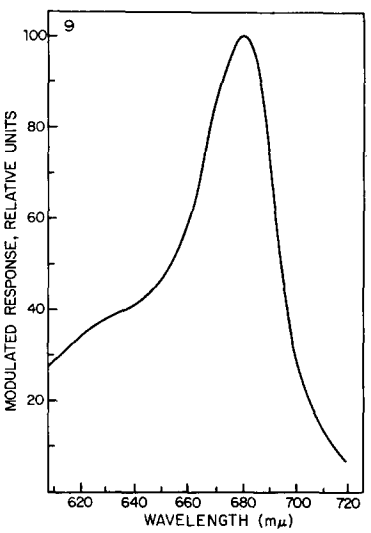



Fig. 9. Action spectrum of the reduction of methyl viologen by chloroplasts in the presence of DCMU and an electron donor system. Frequency of modulation, 90 cycles/sec. Polarization, +0.6 V. Isolated chloroplasts were suspended in phosphate KCl buffer (as in experiment, Fig. 4) containing (1) methyl viologen, $5 \cdot 10^{-5}$ M; (2) DCMU, $10^{-5}$ M; (3) diaminodural, $10^{-3}$ M; (4) and excess ascorbate. For each wavelength, the modulated signal was divided by the number of incident quanta.

conjunction with ferredoxin, a low potential oxidant such as methyl viologen is reduced by Photosystem I exclusively. Methyl viologen proved a very convenient electron acceptor, despite the fact that its autoxidation by molecular oxygen limits the lifetime of its reduced state. Excellent response could be obtained at frequencies higher than 30 cycles/sec which proves that the electrode reaction is faster than reoxidation by $O_2$. Fig. 9 shows the action spectrum of the photoreduction of methyl viologen by chloroplasts in the presence of DCMU, ascorbate and diaminodural[6]. Use of DCIP instead of diaminodural, yielded very similar results but with lower efficiency. The maximum activity at 681 mμ and the absence of a 650-mμ band, indicates that, as expected, a pure System I activity is observed.

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