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FLUOROMETRIC MEASUREMENT OF THE PHOTOREDUCTION OF FLAVIN BY ILLUMINATED CHLOROPLASTS

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SUMMARY

A fluorometric method is described for measuring the reduction and reoxidation of flavins in the presence of suspensions of chloroplasts or grana. The method is based on the fact that oxidized flavins have a strong fluorescence band in the green, whereas reduced flavins have no visible fluorescence. The fluorometric procedure has been used to show that illumination of chloroplasts with FMN in argon leads first to reduction of flavin, and that the reduced flavin is then reoxidized in a dark reaction which lags behind the reduction sufficiently to be directly demonstrable.

INTRODUCTION

As WARBURG[1] has previously noted, the blue-green fluorescence band of alloxazin disappears on reduction, and the fluorescence may therefore be used to measure the reduction and reoxidation of flavins. Here we shall describe a method for the fluorometric measurement of the photoreduction and dark reoxidation of flavins in the presence of chloroplasts or grana. The procedure is more sensitive than the direct spectrophotometric observations of VERNON and his associates[2,3], and permits observations over a wider range of flavin and chloroplast concentrations.

EXPERIMENTAL

The essential steps in the procedure and the arrangement of apparatus are shown in Figs. 1 and 2. It was necessary to remove O_2 from the reaction mixture without foaming, and this was accomplished as shown in Fig. 1. Furthermore, it was decisive that the reaction mixture could be stirred during illumination, and that the shift from illumination light to fluorescence-exciting light could be made rapidly. The arrangement of the apparatus is shown in Fig. 2.

Preparation of chloroplasts

Unwashed chloroplasts from spinach leaves (variety Matador) were used in all of the experiments described in the protocols. The leaves were ground in a mortar with sand in 0.35 M NaCl at 0°. The brei was strained through cloth and the intact

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chloroplasts were separated by differential centrifugation. Chlorophyll was determined by extraction into methanol and measurement of light transmission at 546 mμ (ref. 4).

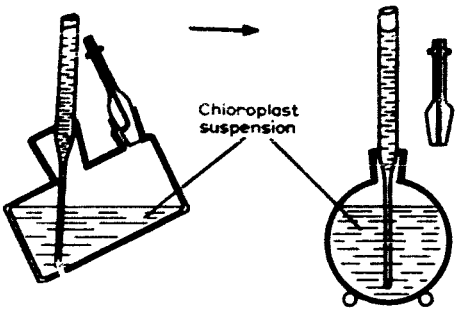

Fig. 1. Preparation of chloroplast suspension and filling of cuvettes. Chloroplast suspensions with all additions were equilibrated with argon by shaking in manometer vessels. The vessels were then removed from the manometer and the suspension was pipetted out without interrupting the flow of argon gas from the side arm (left). The cuvettes were filled to overflowing and immediately closed with a capillary stopper (right). Sufficient suspension was prepared to fill 2 matching cuvettes, one of which was used as a standard and was provided with an air bubble to keep the FMN completely oxidized. Except for the insertion of the air bubble, standard and experimental cuvettes were treated identically.

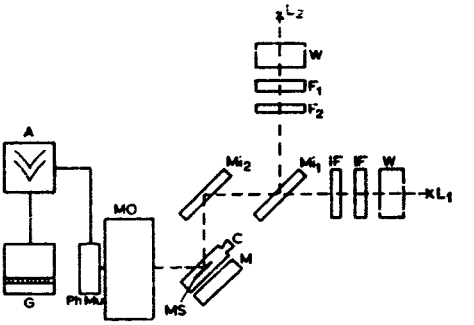

Fig. 2. Arrangement of apparatus for illumination and fluorescence measurements. L_1 = HBO 200 (Osram mercury high-pressure lamp); L_2 = 12V8A metal filament lamp (Osram); W = 10-cm water cuvette; IF = interference filter, λ = 366 mμ (Schott and Gen., Mainz, Type UV-PIL); F_1 = 2 cm 2% $CuSO_4$; F_2 = 2 mm RGI filter (Schott and Gen., Mainz), removed if white light desired; Mi_1 = silver mirror, only for illumination, removed for fluorescence measurement; Mi_2 = aluminium surface mirror; C = measurement cuvette (quartz); M = stirring motor; MS = magnetic stirrer; MO = monochromator (Zeiss, Type M4Q); λ_{max} FMN fluorescence = 530 mμ; PhMu = photomultiplier (RCA Type 1P28); A = amplifier (Zeiss); G = galvanometer (Bruno Lange, Type MFG2). (1) In general, the intensity of the ultraviolet light (L_1) was low (< 5%) relative to the intensity of the red or white light (L_2). (2) Stirring of the contents of the cuvettes was essential. (3) Both mirrors are totally reflecting, not partially transmitting. (4) The cuvette holder (not shown) contained slots for 2 cuvettes. The standard was used to adjust the galvanometer setting to 100 before each reading. The scale was calibrated by measuring the galvanometer response with varying amounts of FMN added to the same amount of chloroplasts.

RESULTS AND DISCUSSION

Reduction and reoxidation of FMN

The fluorometric procedure permits simple direct demonstration of the photoreduction of FMN. If the illumination is not too long and intense, the $FMNH_2$ formed in the light is rapidly reoxidized after the light is turned off. With properly chosen conditions, reduction and reoxidation can be repeated many times.

Fig. 3 shows the photoreduction of FMN, and the following reoxidation in the dark, repeated in sequence 7 times. The reactions may be represented by the overall Eqns. 1 and 2.

$$2\ FMN + 2\ H_2O \longrightarrow 2\ FMNH_2 + O_2 \qquad (1)$$

$$2\ FMNH_2 + O_2 \longrightarrow 2\ FMN + 2\ H_2O \qquad (2)$$

In the light, the rate of Reaction 1 exceeds the rate of Reaction 2, but one must of course visualize a state of affairs in which the FMN is cycling rapidly between reduced and oxidized forms.

The experiment shown in Fig. 3 illustrates another phenomenon—the gradual establishment of anaerobiosis in the light. Though O_2 was almost completely eliminated from the reaction mixture prior to the first illumination, the system can, in the light, generate O_2 almost equivalent to the amount of FMN added, and cannot therefore be regarded as anaerobic in the light. With continued illumination, a more

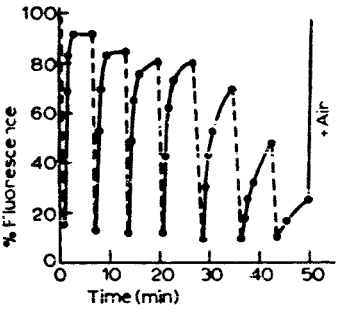

Fig. 3. The light reduction and dark reoxidation of FMN. The reaction mixture contained 75 μmoles of sodium phosphate buffer (pH 6.8), 20 μmoles of KCl, 25 μmoles of NaCl, 0.0375 μmole of FMN, and spinach chloroplasts containing 0.23 μmole of chlorophyll in 1.5 ml H_2O, equilibrated with argon. The dashed lines represent illumination with white light of about 600 μl quanta/min. The first three illuminations were for 0.5 min each, the fourth illumination was for 1 min, and the remaining illuminations were for 2 min each. The solid lines represent dark intervals between fluorescence readings. An air bubble was introduced into the cuvette at the end of the experiment.

complete anaerobiosis is gradually established, however, as shown by the fact that the reoxidation of $FMNH_2$ becomes less complete after each successive illumination period. The O_2 formed is not quite equivalent to the $FMNH_2$ formed, so that, as the system cycles, there is an accumulation of $FMNH_2$ without accumulation of an equivalent amount of O_2. This must occur at the expense of some reductant provided by the chloroplasts. Eventually, if enough light is used almost all of the FMN may be converted to $FMNH_2$ which is now relatively stable in the dark, *i.e.*, reoxidized only very slowly. This $FMNH_2$ is rapidly reoxidized on admission of a little O_2 to the system, which shows that the FMN has been reduced rather than destroyed, and that O_2 has really disappeared.

This gradual establishment of anaerobiosis in the light provides an explanation for the effect of anaerobiosis on phosphorylation (see Section *Effect of HCN and correlation with phosphorylation*).

The net proportion of FMN reduced and reoxidized under particular circumstances depends on the relative amounts of chlorophyll and FMN employed. Thus, the ratio of chlorophyll to FMN, on a molar basis, should be greater than 5 in order to demonstrate almost complete dark reoxidation of $FMNH_2$ following almost complete reduction of FMN in the light as shown in Fig. 3. With lower ratios of chlorophyll to FMN, more light is required in order to give almost complete FMN reduction, and the subsequent dark reoxidation becomes more incomplete because there has been a larger net consumption of oxidant during the illumination. Quite low ratios of chlorophyll to FMN are required for sufficient sensitivity when FMN reduction is measured by direct spectrophotometry[2,3]. The FMN reduction observed under such circumstances reflects mainly the net amount of oxidant consumed.

Quantum requirement for FMN reduction

At the beginning of illumination, the reoxidation rate of $FMNH_2$ is slow, since little O_2 is present. But as more $FMNH_2$ is formed with increasing time of illumination, the rate of the back reaction (reoxidation of $FMNH_2$) increases. The measure-

ments in Table I showed that in a particular case, when 0.421 μl quanta were absorbed in 4 sec, 0.0562 μl FMN was reduced, giving an apparent quantum requirement of 7.5 quanta/molecule FMN, but when 2·0.421 μl quanta were absorbed in 8 sec, the apparent quantum requirement had increased to 11.2 quanta/molecule of FMN reduced, because of the back reaction. An extrapolation of such data to zero time should give the quantum requirement of the photoreduction reaction alone, unaffected by the back reaction. Extrapolation of the results in Table I gives the value of about 4 quanta per molecule FMN reduced or 8 quanta/molecule O_2 evolved, without back reaction of the O_2, whereas in photosynthesis, 1 light quantum gives 1 molecule of O_2 without the back reaction[4,6-8]. This shows that the chlorophyll of the particular chloroplasts used for the measurements of Table I had one-eighth of the activity of the chlorophyll in living Chlorella under optimal conditions.

TABLE I

QUANTUM REQUIREMENT FOR PHOTOREDUCTION OF FMN

The reaction mixtures were equilibrated with argon, and contained, in a cuvette volume of 1.39 ml: 40 μmoles of phosphate (pH 6.2 or 7.5), 25 μmoles of NaCl, 0.0131 μmole of FMN, and spinach chloroplasts containing 0.124 μmole of chlorophyll. The intensity of the incident red light was 8.55 μl quanta/min (measured bolorimetrically), and 74% of this light was absorbed, as determined with the ULBRICHT sphere[5]. The cuvettes were cooled in an ice-bath in the dark for a minimum of 5 min between each illumination. Higher efficiencies were observed with this procedure than when measurements were made at room temperature. The dark period between illuminations was sufficient to permit at least 90% reoxidation of the $FMNH_2$ in all cases. The observed efficiency of the light was relatively low in the first illuminations, but increased with succeeding illumination periods to reach, eventually, constant values. Only these constant values were recorded and used in the calculations. The samples were stable for many hours, so that a given measurement could be repeated almost any desired number of times with the same sample.

Time of illumination (sec)	*μl quanta absorbed*	*f**	*No. of measurements*	*% FMN reduced*		*μl FMN reduced*	*$1/\Phi$**
				range	*average*		
4	0.421	7.5	5	(17.5–20)	19.1	0.0562	7.5
		6.2	5	(18.5–22)	20.4	0.0600	7.05
6	0.632	7.5	2	(23)	23	0.0676	9.3
		6.2	2	(24.5–26)	25.2	0.0740	8.55
8	0.842	7.5	7	(24–27)	25.7	0.0755	11.2
		6.2	7	(26–29.5)	28.1	0.0835	10.1

* Quantum requirement, $1/\Phi$ = μl quanta absorbed per μl FMN reduced.

For the measurements recorded in Table I, low concentrations of FMN were used to obtain high sensitivity and to keep the rate of the back reaction low relative to the rate of reduction of FMN. The results are typical of the better efficiencies we have observed to date, but there is no claim made that higher efficiencies cannot be obtained. Added ADP did not affect these efficiency measurements.

The reoxidation of $FMNH_2$

It is impossible to demonstrate any O_2 evolution manometrically when flavin is photoreduced by chloroplast suspensions, even in argon with large amounts of FMN. This is perhaps not surprising, since the fluorometric measurements showed that the

net amount of oxidant (O_2) accumulated in solution even in a bright light was only equivalent to about 15–20 % of the amount of chlorophyll present (see for example Fig. 4). If O_2 is present in the gas phase, there is a negative rather than a positive pressure change observed at the onset of illumination of chloroplast suspensions with FMN. O_2 is consumed, not given off. If 0.01 M HCN is added, there is a greatly enhanced O_2 consumption in the light, and H_2O_2 accumulates because catalase is inhibited[9,10]. The overall reaction sequence may be described by the equation

$$2\ FMN + 2\ H_2O \longrightarrow 2\ FMNH_2 + O_2$$
$$2\ FMNH_2 + 2\ O_2 \longrightarrow 2\ FMN + 2\ H_2O_2$$
$$\textit{Net:}\quad O_2 + 2\ H_2O \longrightarrow 2\ H_2O_2$$

The quantum requirement for the H_2O_2 production under aerobic conditions was measured and found to be about the same, within experimental error, with similarly prepared chloroplasts, as the quantum requirement for FMN reduction determined fluorometrically and extrapolated to zero time. This agreement suggests that the reactions have been formulated correctly. FMN functions with chloroplasts as a normal Hill reagent; its reduction can be observed directly; it is the very rapid re-oxidation of $FMNH_2$ which makes the O_2 evolution impossible to demonstrate.

Effect of HCN and correlation with phosphorylation

The light reaction is not directly affected by HCN; this reagent does not change the quantum requirement for FMN reduction when the initial reduction rate is determined by extrapolation. But HCN can nevertheless cause a striking enhancement of FMN reduction under some circumstances. An experiment to illustrate this effect is shown in Fig. 4. Here two reaction mixtures were compared—one without, and the other with 0.01 M HCN. The HCN enhanced the net reduction of FMN by inhibiting the dark reoxidation of $FMNH_2$. In fact, HCN causes a more rapid establishment of anaerobiosis with accumulation of $FMNH_2$ (with HCN, one can demonstrate FMN reduction in the light even when air is not removed from the reaction mixture by initial equilibration with argon).

The effects of HCN, described above, can account for the effect of this reagent

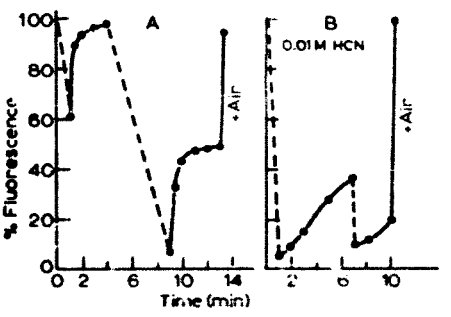

Fig. 4. Effect of HCN on flavin reactions. The reaction mixtures contained 40 μmoles of sodium phosphate (pH 6.8), 20 μmoles of KCl, 150 μmoles of NaCl, 0.086 μmole of FMN, and spinach chloroplasts containing 0.20 μmole of chlorophyll in 1.5 ml H_2O equilibrated with argon; 0.01 M HCN was added from the gas phase[11] to the reaction mixture of Expt. B. The dashed lines represent illumination with white light of about 600 μl quanta/min. Conditions were selected to show the apparent enhancement of photoreduction by HCN in the first illumination period of 1 min, and the inhibition of the dark reoxidation by HCN. The second illumination period with HCN was for 0.25 min, in contrast to the 5 min used for the sample without HCN. Such prolonged illumination was necessary to obtain almost complete reduction of FMN in the absence of HCN with the chlorophyll/FMN ratio employed in these experiments. Introduction of an air bubble at the end of the experiment led to rapid reoxidation of the accumulated reduced FMN both in the presence and in the absence of HCN.

on the phosphorylation which occurs when chloroplasts are illuminated with FMN[9,12–14]. With the fluorometric procedure one can show that the phosphorylation only occurs when oxidized FMN is reduced. When the reduction of FMN has become almost complete, and the reoxidation of $FMNH_2$ has become very slow, phosphorylation stops. One can convince oneself of this by observation of the FMN fluorescence of a phosphorylating system. Equilibration with argon to remove air leads to

TABLE II

EFFECT OF O_2, FMN CONCENTRATION AND HCN ON PHOSPHORYLATION

Experiments were done in manometric vessels illuminated while shaking, with 800 μl quanta white light per min at 10° for 20 min*. HCN was added in the gas phase[11]. Inorganic phosphate was determined on perchloric acid filtrates by the method of SUMNER[15], and the ΔP_i was calculated as the difference between the phosphate content of the illuminated sample and of a control incubated in the dark under identical conditions. Reaction mixtures contained 300 μmoles of $NaHCO_3$, 30 μmoles phosphate, 20 μmoles ADP, 20 μmoles AMP, 10 μmoles $MgSO_4$, 70 μmoles NaCl, with FMN and spinach chloroplasts as indicated, all in a volume of 3 ml. Qualitatively similar results were obtained if bicarbonate–CO_2 buffer was omitted and the initial pH of the reaction mixture was adjusted to 6.8–6.9. The bicarbonate–CO_2 buffer has the advantage that the course of the phosphorylation in argon may be followed by measuring the negative pressure changes, as CO_2 is taken up to compensate for H^+ consumed when ATP is synthesized. Thus, in Expt. 2, the pressure changes in the two vessels started at approximately equal rates, but after about 10 min the reaction in the presence of HCN ended abruptly. It was shown that the phosphorylation began again in such an experiment when more oxidized FMN was added from the side-arm. The termination of phosphorylation coincided with complete and permanent reduction of FMN.

Expt. No.	*FMN (μmoles)*	*Chlorophyll (μmoles)*	*Gas: 5% CO_2 in*	*0.01 M HCN*	*ΔP_i (μmoles)*
1	0.02	1.15	Air	–	−10.3
	0.02	1.15	Argon	–	0.3
2	1.0	1.20	Argon	–	−12.5
	1.0	1.20	Argon	+	− 5.7
3	1.0	1.01	Air	–	−10.8
	1.0	1.01	Air	+	−10.8

* If incubation is prolonged, 0.01 M HCN inhibits phosphorylation both aerobically and anaerobically. This effect requires time to develop, and is associated with a gradual destruction of the capacity of the chloroplasts to perform the Hill reaction.

inhibition of phosphorylation with small amounts of added FMN, but not with larger amounts; because complete conversion of the FMN to $FMNH_2$ stable in the dark occurs sooner with little FMN. In the presence of HCN, still larger amounts of FMN must be added to prevent the inhibition brought about by equilibration with argon, because HCN causes a more rapid complete conversion of FMN to $FMNH_2$ stable in the dark. Representative experiments to illustrate some of the above points are described in Table II.

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