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ENHANCEMENT STUDIES ON ALGAE AND ISOLATED CHLOROPLASTS

PART II. ENHANCEMENT OF OXYGEN EVOLUTION IN INTACT CHLOROPLASTS

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SUMMARY

Intact isolated chloroplasts were shown to exhibit a characteristic three-phase pattern of development of oxygen evolution activity. The first phase, Phase I, appeared to be an equilibration phase in which the isolated chloroplasts adapted to the conditions on the electrode surface. It was characterised by a rapidly increasing rate of oxygen evolution accompanied by decreasing enhancement signals. The second phase, Phase II, was an intermediate phase in which the rate of oxygen evolution was maximal and no enhancement was observed. In the last phase, Phase III, the rate of oxygen fell again, normal enhancement was still missing, but the samples appeared to undergo slow adaptive changes closely related to the State I-State II changes previously reported for whole cell systems.

The concentrations of Mg^{2+} within the chloroplast were shown to play an important role in the control of the development of both the oxygen evolution and enhancement signals. It was shown how these signals could be explained in terms of a model that was consistent with that developed in Part I of this investigation to account for the variability of enhancement of the alga *Chlorella pyrenoidosa*.

INTRODUCTION

In Part I of this study we reported the results of an investigation into the variability of enhancement in the alga *Chlorella pyrenoidosa* [1]. In this paper we report the results of a parallel study on the variability of enhancement in intact spinach chloroplasts.

All enhancement studies on isolated chloroplasts reported to date have suffered from the disadvantage that they have been performed on broken or badly damaged

Abbreviations: PPNR, photosynthetic pyridine nucleotide reductase; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulphonic acid.

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chloroplasts incapable of oxygen evolution in the absence of added electron acceptors [2–8]. The normal practice in such studies has been to add back either ferredoxin and NADP, or alternatively a non-physiological intermediate such as methyl viologen, to the chloroplasts to act as the terminal electron acceptor. The work of Sun and Sauer [6] and of Sinclair [7] on magnesium stimulation, and Sane and Park [8] on PPNR stimulation, of enhancement has clearly demonstrated the great variability of results that can be obtained in such studies depending on the presence or absence of co-factors of this type.

In an attempt to avoid these problems, we have chosen to use intact chloroplasts capable of fixing carbon dioxide and evolving oxygen in the absence of added co-factors. Despite difficulties arising from the heterogeneity and inherent lability of such samples, we were able to identify a characteristic pattern of oxygen evolution. In this paper, we describe the development of this pattern and the enhancement signals associated with it. The significance of these signals is then explained in terms of the model proposed in Part I of this study to account for the variability of enhancement in *Chlorella*.

MATERIALS AND METHODS

Isolation of Chloroplasts

Intact chloroplasts were isolated from spinach leaves (*Spinacea oleracea*) by the method of Stokes and Walker [9]. They were finally suspended in an assay medium consisting of 50mM HEPES (pH 7.6), 0.33 M sorbitol, 1mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. The same medium, supplemented with 0.3mM $KHCO_3$, was used as the circulating medium in the oxygen electrode.

Chloroplasts prepared by this method were 30–50 % intact as measured by the ferricyanide method [10]. The non-intact chloroplasts present in the samples did not appear to be significantly involved in the oxygen measurements since the oxygen uptake signals from the samples were negligible even after total removal of the outer chloroplast membranes by osmotic shock.

Algae Culture. Details of culturing procedure for *Chlorella pyrenoidosa* are described in Part I [1].

Oxygen Evolution Measurements. The a.c. oxygen electrode system was described in Part I of this investigation. All measurements unless specifically stated were made using a chopped 645 nm beam for Light II and an unchopped 705 nm beam for Light I. The light intensities for individual measurements are quoted in the relevant figure captions.

Enhancement was expressed in terms of the enahncement ratio, E, defined as:

$$E = \frac{\text{a.c. } O_2 \text{ evolution in Light II plus Light I}}{\text{a.c. } O_2 \text{ evolution in Light II alone}} \qquad (1)$$

Especial care was taken to check and re-adjust the phase shift control of the amplifier system at frequent intervals so as to minimise any effects associated with changes in the length of the oxygen diffusion path of the type described by Sinclair [7].

Materials. The divalent cation ionophore A23187 was a gift from Lilly Laboratories. All other chemicals were normal analytical grade materials.

RESULTS AND DISCUSSION

General pattern of development of oxygen evolution signals

The range of patterns of oxygen evolution and of enhancement signals observed for intact chloroplasts was much wider than that reported in Part I of this study for *Chlorella* [1]. This was probably in part due to the greater initial heterogeneity of the experimental material and in part due to the inherent lability of this material. Nevertheless, a general pattern in the development of the oxygen signal from the initial placing of the chloroplasts on the electrode until the cessation of their oxygen evolution activity (usually 1–2 h), could be readily discerned.

A typical trace of the development of such a signal is shown in Fig. 1. It can be conveniently divided into three phases.

In Phase I, the rate of oxygen evolution in response to weak chopped 645 nm light (Light II) increases with time over a period usually lasting 10–20 min. At the same time the enhancement ratio, observed on the superposition of unchopped 705 nm light (Light I), decreases. The ratio of the initial and final rates of oxygen evolution in Light II alone and the initial value of the enhancement ratio both varied from sample to sample. They appeared, however, to show a distinct inverse relationship: a low initial Light II oxygen evolution rate usually corresponded to a high initial enhancement ratio and a high initial rate to a low enhancement ratio.

In Phase II, the rate of oxygen evolution reaches a maximum and no enhancement is observed. In most cases this phase lasted 5–10 min and then Phase III commenced. This latter phase was not, however, always observed. In some cases Phase II persisted, with only a very slow diminution in the rate of oxygen evolution and no

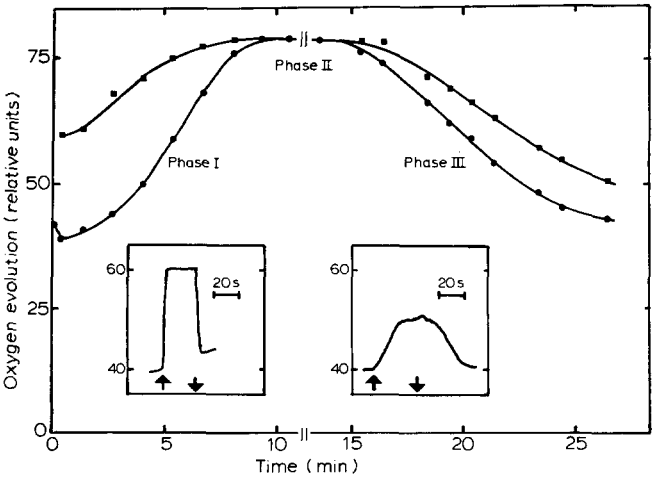

Fig. 1. Typical plot of the development of the oxygen evolution signal in intact chloroplasts showing Phase I, Phase II and Phase III. The rates of oxygen evolution are plotted both for Light II + Light I (■–■) and Light II alone (●–●). Typical enhancement signals for Phase I and Phase III - Signal I and Signal III, respectively, are shown in the insets. The enhancement signals show the changes in the rate of oxygen evolution in Light II (intensity 50 $\mu W \cdot cm^{-2}$) on the superposition of Light I (intensity 90 $\mu W \cdot cm^{-2}$). The points at which Light I is switched on and switched off are indicated by the vertical arrows.

restoration of enhancement. In a very few cases no Phase II was observed and the sample moved directly from Phase I to Phase III.

In Phase III, the rate of oxygen evolution in Light II alone drops again and enhancement returns. The kinetics of the enhancement signals (cf. insets in Fig. 1) were, however, very different from those observed in Phase I. Again, an inverse relationship appeared to exist between enhancement ratio and the rates of oxygen evolution in Light II alone. As the rate of oxygen evolution falls, E rises.

This three-phase pattern of oxygen evolution was only observed if measurements were commenced as soon as possbile after placing the sample on the electrode surface. If the samples were pre-equilibrated on the electrode surface for 10–20 min before measurement, no Phase I was observed.

Introduction of dark periods into Phase I had no effect on its development, suggesting that it was essentially a light independent equilibration of the chloroplasts on the electrode surface. Part of this equilibration can be attributed to mechanical settling effects leading to an increased efficiency in the diffusion of oxygen to the electrode surface. The change in enhancement ratio, however, cannot be accounted for in this way as E, being a ratio of rates, is independent of this effect. The major part of the equilibration process was thus clearly physiological in origin. Further support for this view was provided by measurements made on chloroplast samples diluted with assay medium and kept at room temperature for 15 min before application to the electrode. These pre-equilibrated samples showed a much smaller rise in oxygen evolution, (10–20 %), over the first fifteen minutes of measurement, and no enhancement activity.

The three-phase pattern of development of the oxygen evolution can be explained on the basis of the model put forward in Part I to account for the variability of enhancement in *Chlorella*. The variability of *Chlorella* enhancement was explained in terms of the varying photochemical efficiencies of PSI and PSII, termed ε_{I} and $\varepsilon_{\mathrm{II}}$, as a consequence of changes in the concentration of a control factor, probably Mg^{2+}. In the case of *Chlorella* these changes were attributed to a growth cycle linked variation (diurnal changes) or to a light driven variation (State I–State II changes).

The changes observed in chloroplasts can be explained by assuming that the chloroplasts as isolated contain an abnormally high concentration of the control factor which falls as the chloroplasts re-equilibrate with their surroundings. Assuming a similar concentration dependence to that postulated for *Chlorella* (Fig. 13, Part I), the high initial concentration would ensure that the initial rate of oxygen evolution, limited by PSI, would be low and E correspondingly high. As the concentration falls during Phase I, the efficiency of PSI increases, oxygen evolution rises and E falls. This continues until the efficiencies of the two photosystems are balanced, Phase II. Any further decrease in concentration then leads to PSII becoming rate-limiting and the commencement of Phase III.

If the concentration dependence of ε_{I} and $\varepsilon_{\mathrm{II}}$ were identical to those postulated for *Chlorella*, the rate of oxygen evolution in Light I+Light II during Phase I would be expected to remain at a constant value which should equal that observed in Phase II. In practice it does not; it always falls well below this value (Fig. 1). Part of this apparent deficit in oxygen evolution can be accounted for by the unavoidable artifacts arising from the settling of the chloroplasts on the electrode. A further factor that must be borne in mind is that the re-equilibration taking place during Phase I will

almost certainly involve changes in the pool sizes of a large number of intermediates, particularly those of the Calvin cycle, and that any one of these may be exerting an additional control on the rate of electron transport.

Shape of enhancement signals

The shape of the enhancement signal observed on the superposition of Light I upon Light II changed markedly during the course of the development of the oxygen evolution signal. The characterisation of the signal shapes in intact chloroplasts was much more difficult than for algae for a number of reasons. Firstly, the enhancement signals were very sensitive to heterogeneities within the sample. Secondly, the lability of the samples meant that intact chloroplasts were much more suspect to changes in morphology and ionic integrity than algae. Thirdly, in view of the continuous changes taking place in the sample it was not always possible to ensure that all signals were measured at optimum light intensities.

Despite these difficulties, we were able to identify a number of different types of enhancement signal. These signals could be classified into two basic signals, Signal I typical of Phase I and Signal III typical of Phase III, and a number of other commonly observed signal-types that were either variants or composities of these two basic signals.

For the present, we shall concentrate on the two basic types of enhancement

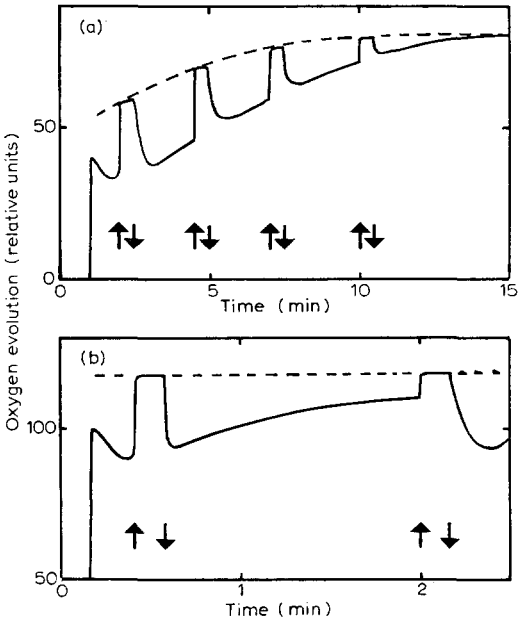

Fig. 2. Comparison of the enhancement changes (a) during Phase I for intact chloroplasts and (b) during equilibration for a Light I pre-illuminated *Chlorella* sample showing Type B responses. The vertical arrows indicate the times of the addition and removal of Light I. The measurements for the intact chloroplasts were made on a sample freshly placed on the electrode. The intensities of the background Light II and of Light I were 50 $\mu W \cdot cm^{-2}$ and 80 $\mu W \cdot cm^{-2}$, respectively. The *Chlorella* sample was pre-illuminated for 5 min in Light I, intensity 200 $\mu W \cdot cm^{-2}$; the enhancement measurements were made using Light II, intensity 65 $\mu W \cdot cm^{-2}$, and Light I, intensity 75 $\mu W \cdot cm^{-2}$.

signal, examples of which are shown in the insets to Fig. 1. Signal I is characterised by an initial rapid rise-time, typically of the order of 2–3s, and a slightly slower fall, 5–10s. The rate of these changes are directly comparable to those observed for the fast component of enhancement in algae. Owing to the rapidity of the changes in Phase I, the signals were normally measured over periods of the order of 20–30s, thus giving little time for the possible development of any slow component of enhancement. If the signals were measured over a longer period, the rate of oxygen evolution in Light II+Light I was observed to increase, but it was impossible to determine whether this was due to the normal light independent development of Phase I or to a specific adaptive response.

Signal III in contrast to Signal I is characterised by a very slow rise time, 20–40s, and a very slow decay, 40–60s. The decay of the signal was often complicated by the existence of a small burst in oxygen evolution directly following the removal of Light I. We have occasionally observed similar bursts in algal samples measured shortly after placing on the electrode. They appear to have a similar origin to the oxygen evolution gush observed on the initial illumination with Light II of samples of dark adapted or Light I pre-illuminated chloroplasts or algae [7].

The rapid rise-time and decay of Signal I is typical of a situation in which PSI is rate-limiting in Light II. The chloroplast signals are, in fact, strikingly similar to those observed for the State I–State II transition of *Chlorella* samples showing Type B responses (see Fig. 2). The rise in oxygen evolution and the decrease in E

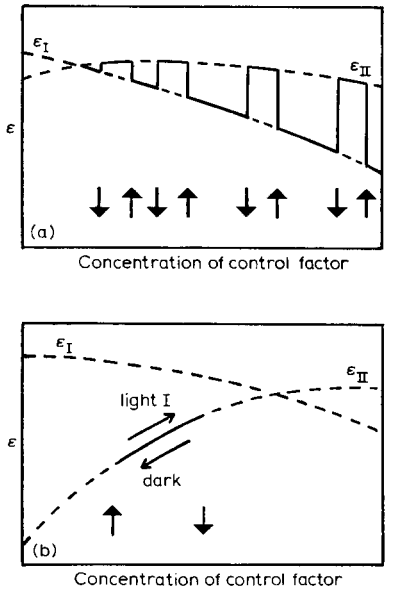

Fig. 3. Sketches showing how the enchancement signals characterising Phase I and Phase III can be explained in terms of the relative photochemical efficiencies of the two systems. The concentration dependencies of ε_I and ε_{II} are assumed to be of a similar form to that postulated for *Chlorella* (cf. Fig. 13, Part I). The arrows indicate the points of addition, or removal, of Light I. (a) The shape of Signal I is determined by changes in the relative values of ε_I and ε_{II} as the concentration of control factor falls. (b) The shape of Signal III is determined by the changes in ε_{II} as the concentration of control factor first rises in response to Light I adaptation and subsequently falls on its reversal.

can, according to our model, in both cases be attributed to a fall in concentration of the control factor leading to a narrowing of the gap between the efficiencies of the two photosystems (cf. Fig. 3a).

The slow rise-time and slow decay of Signal III, on the other hand, is consistent with a slow adaptive increase in the rate of PSII brought about by a Light I driven increase in the concentration of control factor and the slow reversal of this increase on removal of Light I (see Fig. 3b). Signal III is particularly interesting in that it appears to correspond to the fourth member of the series of adaptive responses, described in Part I. This signal, in contrast to the Type A, Type B and Type C responses described in that paper, takes place in a situation in which PSII is rate-limiting at all times, i.e. $\varepsilon_I > \varepsilon_{II}$. This means that the rate-limiting step in oxygen evolution does not change on removal of Light I and explains why this signal, unlike the others, is characterised by a slow decay.

Effect of Mg^{2+} on oxygen evolution and enhancement

The most likely candidate for the role of the central control factor postulated in our model is, as discussed in Part I, Mg^{2+}. With this in mind, the effect of added Mg^{2+} on the development of the oxygen evolution and enhancement signals was investigated in some detail. Three types of studies were performed; measurements of the effects of omitting Mg^{2+} from the isolation or assay media, of the effects on normally isolated chloroplasts of Mg^{2+} ions added in Phase I and of the effects on such chloroplasts of Mg^{2+} ions added in Phase III.

Effects of omission of Mg^{2+}

The assessment of the role of Mg^{2+} was complicated by the fact that both the isolation and normal assay media contained low concentrations of these ions. We therefore examined the effects both of isolating the chloroplasts in media lacking divalent ions, low salt media, and of prewashing the chloroplasts isolated in normal media in magnesium free assay medium. Chloroplasts prepared in low salt media were generally less active than the normal chloroplasts. They showed no Phase I behaviour other than a 20–30 % increase in the rate of oxygen evolution. This rise, as explained above, can be largely attributed to mechanical settling. Prewashing chloroplasts isolated in normal media did not appreciably change the overall activity of the samples but often led to a similar drastic reduction in Phase I. This loss of Phase I behaviour was irreversible; it was not restored on the re-addition of Mg^{2+}.

Effects of adding Mg^{2+} during Phase I

The cold concentrated chloroplast samples were either pre-equilibrated with equal volumes of buffered 20 mM $MgCl_2$ and the subsequent measurements carried out using a circulating medium that was 10mM with respect to Mg^{2+}, or the concentration of Mg^{2+} was changed during the course of the measurements by the addition of small volumes of concentrated $MgCl_2$ to the circulating medium.

The direct addition of Mg^{2+} during the course of Phase I appeared in all cases to have negligible effect on the development of the signal. Pre-equilibration with Mg^{2+} did, however, appear to have some effect on the subsequent development of Phase I, especially in those samples that showed low initial rates of oxygen evolution. These initial rates could often be further depressed and the initial values of E increased

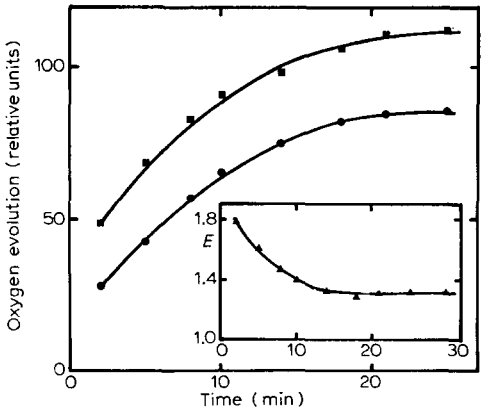

Fig. 4. Measurements of the oxygen evolution rate for a sample pretreated with Mg^{2+} and ionophore so as to yield a magnesium concentration of 20mM and an ionophore concentration of 5 $\mu g \cdot ml^{-1}$. The rates of oxygen evolution are plotted both for Light II + Light I (■–■) and Light II alone (●–●). The corresponding values of the enhancement ratio, E, are shown in the inset. The intensities of Light II and Light I were 50 $\mu W \cdot cm^{-2}$ and 80 $\mu W \cdot cm^{-2}$, respectively.

by the Mg^{2+}. Pre-equilibration, however, had little or no effect on those samples showing relatively high initial rates of oxygen evolution.

Pretreatment of the chloroplast samples with the divalent ionophore A23187 (at concentrations below the uncoupling level [11]) and Mg^{2+} again had little or no effect on the development of Phase I (see Fig. 4). Phase II and Phase III were, however, abolished by this treatment.

Effects of adding Mg^{2+} during Pase III

The addition of Mg^{2+} during Phase III, in contrast to their addition during Phase I, usually had a very marked effect both on the rate of oxygen evolution and on the shape of the enhancement signals. The nature of these latter changes depended, however, to some extent on whether or not the samples exhibited normal Phase III behaviour (reduced rate of oxygen evolution and Signal III) or a prolongation of Phase II (reduced oxygen rate but no enhancement signal).

Typical traces both for samples showing, and samples lacking, Signal III are given in Fig. 5. The initial response to the addition of Mg^{2+} in both cases is an increase in the rate of oxygen evolution in Light II alone. The extent of the increase varied from sample to sample. A few samples showed no increase but most samples showed increases of up to 30 %. The concentration of Mg^{2+} in the circulating medium required to give maximum response varied from sample to sample. The oxygen evolution rate could, however, be readily maximised in all cases by the addition of low concentrations of A23187, thus suggesting that the factor limiting the size of increase was normally Mg^{2+} permeability rather than magnesium concentration.

The changes in the rate of oxygen evolution in Light II were accompanied by characteristic changes in the enhancement signals. Samples showing no Signal III before the addition of Mg^{2+}, tended to develop an enhancement signal that was typified by a fast positive going spike of oxygen evolution immediately following the superposition of Light I and an equally rapid negative going spike immediately following its removal (cf. Fig. 5a). This signal grew in size and slowly changed to a square

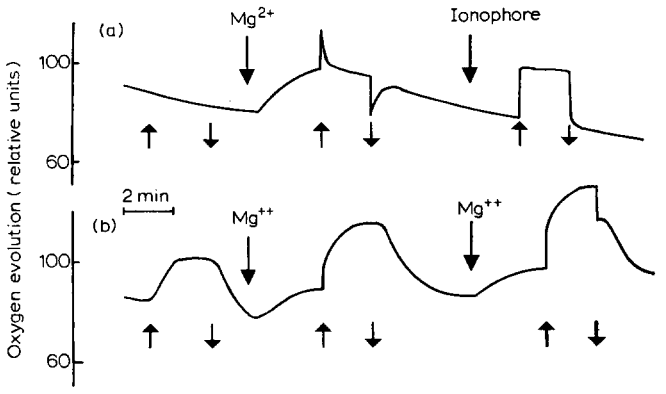

Fig. 5. Traces showing the effects on Light II stimulated oxygen evolution of adding concentrated buffered $MgCl_2$ to samples of intact chloroplasts during Phase III for (a) a sample showing no enhancement signal during Phase III and (b) a sample showing a Signal III type response. The times of switching on and off Light I are indicated by the small arrows in the normal way. The times of adding Mg^{2+} and ionophore are indicated by the large arrows. After the additions sample (a) was 10 mM with respect to Mg^{2+} and contained 5 $\mu g \cdot ml^{-1}$ of ionophore. Sample (b) was 10 mM with respect to Mg^{2+} after the first addition and 20 mM after the second. The intensities of Light II and Light I were the same (50 $\mu W \cdot cm^{-2}$ and 100 $\mu W \cdot cm^{-2}$, respectively), for the two sets of measurements.

pulse that closely resembled Signal I (with the exception that the basal level of oxygen evolution in Light II alone remained constant), as the sample aged or more Mg^{2+} was added. Addition of ionophore A23187 led to a very rapid conversion of the signal to the square pulse form.

The size and rates of decay of the spikes accompanying the addition and

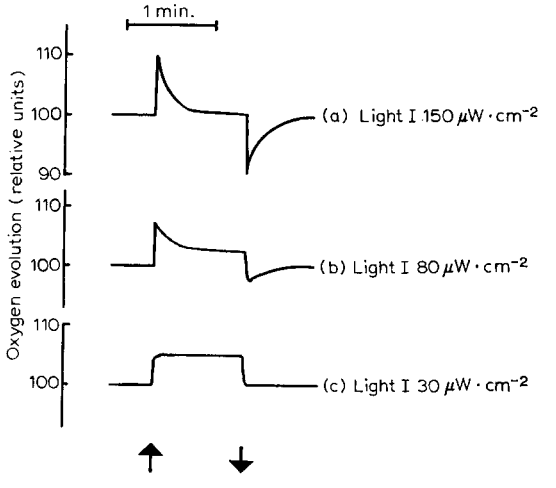

Fig. 6. The effect of changing intensities of Light I on the magnesium controlled enhancement signal observed in samples of the type shown in Fig. 5a. The magnesium concentration in the circulating medium was 10 mM. The intensity of Light II was 50 $\mu W \cdot cm^{-2}$ throughout. The vertical arrows indicate the times of the switching on and switching off of Light I.

removal of Light I in samples of this type were extremely sensitive to changes in the intensity of Light I (Fig. 6). This sensitivity decreased as the samples aged and the signals took on their characteristic square shape. It was completely abolished by the addition of the ionophore. The presence or absence of the spikes again appeared to be closely related to the magnesium permeability of the samples. The Light I intensity dependence of the spikes suggests that they might be associated with light driven magnesium movements within the chloroplast which could be "damped out" by increasing the magnesium permeability of the chloroplast membranes.

Samples with Signal III enhancement before the addition of Mg^{2+} showed a slightly different pattern of responses (Fig. 5b). The increase in rate of oxygen evolution was accompanied by the addition of a fast component to the enhancement signal yielding a signal which appeared to be a composite of the original slow changes and a square pulse type signal. Addition of more Mg^{2+}, ageing or the addition of ionophore A23187 (not shown) resulted as before in a change in the enhancement signal to a square pulse shape that resembled Signal I.

The relative lack of sensitivity of the rate of oxygen evolution in Phase I to Mg^{2+}, its much greater sensitivity in Phase III and the observation that Mg^{2+} can restore Signal I type enhancement (Fig. 5) are all consistent with the view that Mg^{2+} are the control factor referred to in our model.

Oxygen gush and enhancement

Sinclair [7] has reported a Mg^{2+} dependent linear relationship between E and the oxygen gush observed on the initial illumination of dark adapted, or PSI pre-illuminated, broken chloroplasts. He suggested that this relationship might be a consequence of the fact that both parameters were reflections of the relative photochemical efficiencies of the two photosystems.

In order to test this hypothesis measurements were made, using *Chlorella*, in which the balance between the two photosystems was deliberately altered by changing the wavelength of Light II. The resulting linear relationship between oxygen gush (taken to equal the ratio of the maximum rate of oxygen evolution during the gush to the rate immediately following the gush) and E (Fig. 7b), appears to confirm the hypothesis. The relationship between the two parameters was then measured for intact chloroplasts in which, according to our model, the relative efficiencies of the two photosystems change in response to changes in their internal Mg^{2+} concentrations. The relationship during Phase I, although non-linear, did suggest a marked correlation between the two parameters (see Fig. 8). The size of the oxygen gush in Phase III, however, appeared to be effectively independent of E, suggesting that the two phenomena are not related during this phase.

The non-linearity of the plot for Phase I is probably a reflection of the heterogeneity of the samples rather than a basic difference between the intact and broken chloroplast systems. It is significant that if the measurements for intact chloroplasts are repeated using chloroplasts pretreated with Mg^{2+} and low concentrations of ionophore A23187 to equilibrate their stromal Mg^{2+} levels (Fig. 7a), linear plots directly analogous to those observed for broken chloroplasts were obtained.

The existence of a linear relationship between oxygen gush and E for algae (Fig. 7b), for broken chloroplasts treated with Mg^{2+} [7] and intact chloroplasts in which the stromal Mg^{2+} levels have been equalised (Fig. 7a) all argue that the con-

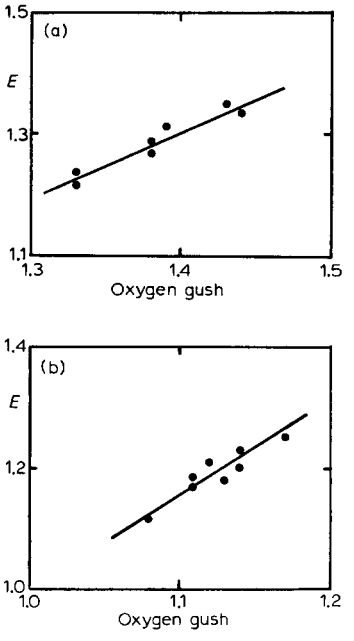

Fig. 7. Comparison of plots of the enhancement ratio, E, against the size of the oxygen gush for (a) a sample of intact chloroplasts pretreated with Mg^{2+} and ionophore to yield a magnesium concentration of 20mM and an ionophore concentration of 5 $\mu g \cdot ml^{-1}$ and (b) a *Chlorella* sample in which the balance between the two photosystems has been altered by changing the wavelength of Light II. The light intensities used in the chloroplast measurements were 50 $\mu W \cdot cm^{-2}$ and 80 $\mu W \cdot cm^{-2}$ for Light II and Light I, respectively. The *Chlorella* measurements were made using an initial Light II intensity of 80 $\mu W \cdot cm^{-2}$ at 645 nm and adjusting the intensity at the other wavelengths (in the range 600–677 nm) to yield the same rate of oxygen evolution. The intensity of Light I was kept at 100 $\mu W \cdot cm^{-2}$ throughout.

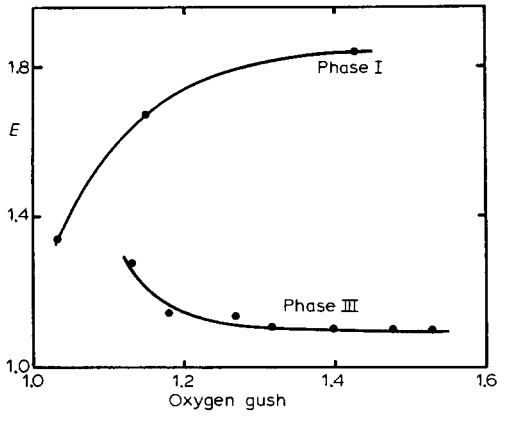

Fig. 8. Typical plots of the enhancement ratio, E, against the size of the oxygen gush following 2 minute dark periods during Phase I and Phase III. The intensities of Light II and Light I were 50 $\mu W \cdot cm^{-2}$ and 80 $\mu W \cdot cm^{-2}$, respectively.

trol factor referred to in the model outlined in Part I of this investigation is indeed Mg^{2+}. The point, or points, at which the Mg^{2+} acts is not, however, entirely clear. The sensitivity of enhancement in Phase III to Light I illumination under conditions of limited Mg^{2+} permeability (Fig. 6) and the loss of this sensitivity on addition of A23187 at concentrations below the uncoupling level, suggests that the level of Mg^{2+} in the stromal compartment is important. The fact that Phase I still persists in chloroplasts pretreated with low concentrations of this ionophore (Figs. 4 and 7a) suggests, on the other hand, that the Mg^{2+} level in the intra-thylakoid space might be the point of primary control.

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