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SALT-INDUCED LIGHT EMISSION FROM CHLOROPLASTS

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SUMMARY

1. The addition of alkali metal salts to preilluminated chloroplast suspensions buffered at neutral pH gave rise to enhanced light emission.

2. The magnitude of the signals obtained with the various salts tested suggested that this luminescence is governed by a membrane which shows a higher passive permeability to cations than anions.

3. It is argued that the diffusion potential, which would be expected to develop across this selective membrane on the initial addition of salts, triggers light emission from some metastable state produced during preillumination.

4. Comparison of the time-courses of the salt-induced signals with those obtained with acid–base shifts indicated that this triggering could be due to the efflux of protons from the chloroplasts brought about by the electrical gradient.

5. Treatment of the isolated chloroplasts with membrane-modifying agents, including valinomycin, supported this and suggested further that the kinetics of the salt-induced signals were controlled by the rate of cation influx rather than proton efflux.

6. From an estimate of the maximum possible electrical gradient created in these experiments it was concluded that both the pH- and salt-induced luminescence are independent of phosphorylation.

INTRODUCTION

JAGENDORF AND URIBE[1] have clearly demonstrated that dark-treated chloroplasts were able to produce ATP when first acidified and then brought to a more basic pH. This observation was particularly pertinent to the chemiosmotic hypothesis, presented by MITCHELL[2], which suggests that both oxidative and photosynthetic phosphorylation may arise from the energy available in a proton gradient. In addition MAYNE AND CLAYTON[3] have found that chloroplasts treated in a similar way as in the above experiments were capable of emitting light when the pH was suddenly increased. However, one difference was a requirement for preillumination of the chloroplasts prior to the acid–base transition[4]. Nevertheless, both these observations seem to suggest, as required for the MITCHELL scheme, that the grana membranes have a low passive permeability to H^+ such that the exit of this cation from the chloroplast interiors is restricted to specific sites.

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The above experiments demonstrated the effect of altering the pH to drive protons out of the chloroplasts, the usual change being from pH 4.0 to 8.0. However, since H^+ is electrically charged there is another way to expel it from the inner compartment. This would be to change the membrane potential Such a change could be brought about by suddenly increasing the external concentration of a salt which has a rapidly diffusing cation but a slower moving anion such that a cation–proton exchange can occur. More precisely, a diffusion potential would initially arise across the membrane which would act in such a way as to drive H^+ from the interior.

With this picture in mind we investigated the ability of spinach chloroplasts, suspended in sucrose buffered at neutral pH, to show luminescence upon addition of various alkali salts. Unknown to us at this time MILES AND JAGENDORF were also engaged in a similar line of research. Their results have recently been reported[5] and have shown, as we had also found, that the addition of salts can in fact induce luminescence from preilluminated chloroplasts. As with the pH-induced luminescence[3] they found that the light emission due to salt addition has a spectrum similar to that of chlorophyll fluorescence. They also found that changing the type of salt did induce varying amounts of luminescence but they did not attach any particular significance to this.

In this paper we are able to give some support to the above ideas involving the development of a membrane potential to induce luminescence both by presenting our own data and also by referring to the work of MILES AND JAGENDORF.

MATERIALS AND METHODS

The chloroplasts were isolated from market spinach by conventional methods. Essentially the leaves were washed with distilled water and macerated in cold 0.40 M sucrose solution buffered with 0.02 M Tris–HCl buffer to pH 7.2. The chloroplasts were separated from the homogenate by filtering through two layers of nylon gauze followed by centrifugation, once at 300 $\times$ *g* for 2 min to remove cell debris and then 1000 $\times$ *g* for 10 min to obtain the chloroplast pellet. This was then resuspended in the same buffered sucrose solution as above to give an absorbance using opal glass of about 0.4–0.7 per mm at 670 mμ measured with a Zeiss PMQ II spectrophotometer. The isolation procedure was carried out under very low illumination and the final suspension stored in a darkened vessel on ice until used.

The luminescence measurements were made with a laboratory constructed apparatus. 1 ml of chloroplast suspension was initially exposed to red light, transmitted by a filter combination consisting of Calflex C/Balzer B40 645 mμ/Schott RG630, for a fixed time period, usually 20 sec The suspension was then quickly pumped from the preillumination chamber, by means of air pressure, into a cylinder of an injection syringe together with the same volume of additional buffer or acid solution. A second injection syringe having exactly the same capacity (2 ml) contained the base or salt solutions. After a total dark period of 10 sec, except where stated, the two syringes were rapidly emptied by means of a piston driven simultaneously by compressed air. The liquids from the two syringes were thoroughly mixed in a common delivery tube leading to the reaction cuvette. The procedure therefore resulted in a four times dilution of the original chloroplast suspension. The filling time of the cuvette was about 50 msec with a total mixing time of approx. 70 msec. The resulting lu-

minescence was detected by means of an EMI photomultiplier type 9558(S-20) equipped with Schott RG 645 filter and the signal followed on a Siemens Oscillomink P or Hewlett-Packard Moosley (Model 7100 BM) chart recorder. An electronic integrator was used for determining the total light output produced over a fixed time interval. All the experiments were carried out at room temperature being about 20°.

The acid–base transition was accomplished by the addition of 0.02 M succinic acid and 0.1 M Tris which caused a pH shift from approx. 4.0 to 8.6. The chloroplasts were treated for 8 sec with acid during the total 10 sec dark period before the base was added. The salt solutions were either made up in distilled water or in the above buffer depending on the concentration used and did not cause any change of pH when mixed with the chloroplast suspension.

RESULTS

Luminescence induced by various K^+ salts

Preliminary experiments showed that K^+ salts could give rise to luminescence when mixed rapidly with chloroplast suspensions. As MILES AND JAGENDORF[5] have reported this salt-induced phenomenon, like the acid–base luminescence, required that the chloroplasts were preilluminated. MAYNE[4] has measured the action spectrum for this pretreatment and suggests that the pH-induced luminescence requires the activation of System II. To allow for this the experiments reported in this communication were conducted on chloroplasts which had been preilluminated with red light at a saturating intensity as outlined in MATERIALS AND METHODS.

TABLE I

LUMINESCENCE INDUCED BY ADDITION OF VARIOUS POTASSIUM SALTS

The luminescence induced by mixing preilluminated chloroplast suspensions buffered at pH 7.3 with various potassium salts to give a final external concentration of 0.3 M. The preillumination was for 20 sec using a saturating intensity of 645-mμ light. For comparison the table includes the magnitude of the luminescence due to the addition of only sucrose buffer (control) and also that resulting from an acid–base shift from pH 4.1 to 8.7. There was a dark time of 10 sec between ending the preillumination period and the addition of the salt solutions. For the pH transitions the chloroplasts were subjected to 8 sec of acid treatment during the dark period before adding the base. The data are expressed in relative units and are the mean of 3–5 experiments.

Experiment	*Initial signal height*	*Total light emission over the first 5 sec*
Control	1.0	1.0
Potassium salts		
Chloride	2.3	2.4
Bromide	2.3	1.8
Iodide	2.2	1.2
Nitrate	2.7	1.8
Sulphate	5.0	3.6
Citrate	6.2	4.4
Acetate	8.0	4.0
Benzoate	44.0	9.4
Acid–base shift	143.0	6.6

Table I shows the luminescence resulting from rapidly mixing chloroplast suspensions with various K^+ salts to give a final external concentration of 0.3 M K^+. The table also includes the luminescence resulting from an acid–base transition using the same chloroplast suspension. There was some problem in deciding exactly how to analyse the luminescence signals. We have chosen to take the initial signal height, as did MILES AND JAGENDORF[5], and also the total light emitted during the first 5 sec. The latter of these parameters is probably not so meaningful. As will be discussed later, there is some indication that the total light produced by any of these treatments is approximately the same when measured over a much longer time interval. In this case it would seem that the initial signal height is the most suitable parameter to analyse this type of chemiluminescence since it apparently represents the rate at which the energy stored during the preillumination is released. The control signal obtained upon addition of buffer represents the 'normal' delayed light emission resulting from preillumination[6]. Of the salts tested it seemed that the magnitude of the induced luminescence was dependent on the anion used. In particular the organic anions gave the largest signals with benzoate being very effective.

To some extent the magnitude of the signals produced either by salt addition or acid–base transitions were dependent on the condition of the chloroplasts. For example, ageing or hypo-osmotic treatment caused a reduction in the size of the luminescence and in this case the Cl^--induced signals could not always be detected. If the chloroplasts were gently heated to about 50° for a few minutes the salt and pH effects were totally destroyed.

The kinetics of the signals

In Fig. 1 it can be seen that the signal induced by potassium benzoate has a different time-course to that due to an acid–base transition. The rise time of these signals required the use of a fast responding recorder (Siemens Oscillomink P) or a storage oscilloscope and were limited by the filling time of the cuvette, which was about 50 msec. Although the peak height of the benzoate signal was lower than that due to the acid–base shift its decay was much slower, resulting in about the same light output for both treatments.

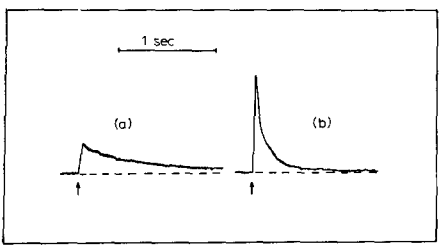

Fig. 1. A comparison of the kinetics of the luminescence signals induced either by the addition of potassium benzoate to give a final concentration of 0.3 M (Signal a) or by an acid–base transition from pH 4.1 to 8.7 (Signal b). The arrows mark the time of mixing and transfer of the suspension into the luminescence cuvette. The experimental procedure and conditions were the same as given in the legend of Table I.

Effect of various alkali metal cations

With freshly isolated chloroplasts a specificity to different alkali metal chlorides could be detected. In Table II it can be seen that NaCl and to a lesser extent LiCl

were more effective in producing luminescence than the other chlorides tested. However, when the anion was benzoate no clear difference could be detected between the Na^+ and K^+ salts using the same chloroplast suspension.

Variation of external salt concentration

The magnitude of the signals obtained at various external concentrations of potassium benzoate is shown in Fig. 2. The curve obtained shows that the luminescence is a linear function of the external K^+ concentration over the range tested. The plot also shows that there was no detectable threshold concentration since the addition of very small quantities of salt resulted in signals greater than the control.

TABLE II

LUMINESCENCE INDUCED BY THE ADDITION OF VARIOUS ALKALI METAL CHLORIDES

The alkali metal chlorides were added to preilluminated chloroplasts buffered at pH 7 3 The final salt concentration after mixing was 0.3 M and the control represents the light emission detected when only sucrose buffer (pH 7.3) was added to the chloroplasts The other experimental conditions were identical to those given in the legend of Table I The data are expressed in arbitrary chart units and are the mean of 4–5 experiments

Experiment	*Initial signal height*	*Total light emission over the first 5 sec*
Control	2 3	1.8
Salts (0 3 M)		
LiCl	8 6	5 4
NaCl	21 0	7 2
KCl	8 6	4 5
RbCl	8 6	4 4

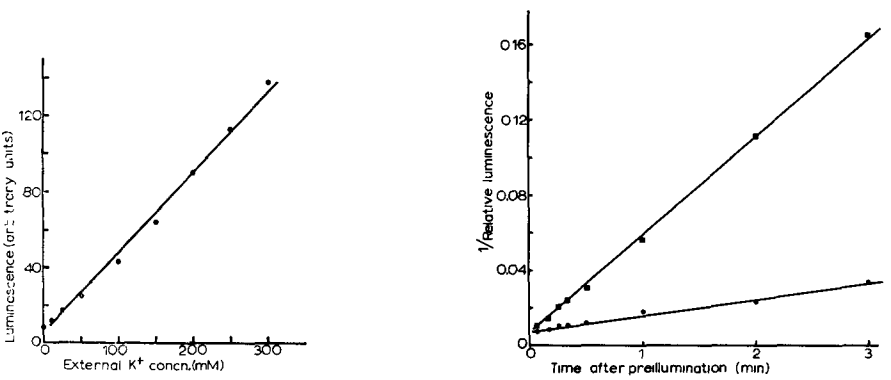

Fig 2. The luminescence induced by the addition of various concentrations of potassium benzoates to preilluminated chloroplast suspensions buffered at pH 7.8. The chloroplasts were preilluminated with saturating 645-mμ light for 5 sec followed by a 10-sec dark period before adding the benzoate solutions The luminescence values are expressed in arbitrary chart units

Fig. 3. Decay of the salt-induced luminescence capacity with increasing dark periods following the 20-sec preillumination. The light emission was brought about by the addition of potassium benzoate giving a final external concentration of 0.3 M to chloroplasts suspended in sucrose buffer at pH 7.3. The luminescence is expressed either as the initial signal height (■) or as the total light output over the initial 5 sec after salt addition (●) and is plotted as a reciprocal against time.

In order to eliminate any possible errors due to changes in osmolarity appropriate quantities of sucrose were added to the various salt solutions used in this experiment.

Variation of the dark times

The dark time between the end of the preillumination period and the addition of salt were varied. Even after 3–5 min some luminescence could be induced by the addition of potassium benzoate or by subjecting the suspension to acid–base shifts. A plot of the reciprocal of either the signal height or total light output over the initial 5 sec produced by the addition of potassium benzoate against dark time yields straight lines as shown in Fig. 3. This seems to suggest that a second-order decay is occurring of some metastable state produced by the preillumination and is consistent with similar measurements reported earlier[4] for acid–base-induced luminescence and delayed light emission.

The effect of gramicidin and carbonyl cyanide m-chlorophenylhydrazone

When the chloroplasts were incubated for a few minutes with low concentrations of these compounds the acid–base signals were slightly decreased while the salt-induced luminescence was stimulated. Table III shows the action of gramicidin at several concentrations on the KCl and NaCl-induced signals. With 0.1 mg/l of this antibiotic the light emission due to the addition of these alkali metal chlorides

TABLE III

THE EFFECT OF GRAMICIDIN ON THE LUMINESCENCE INDUCED BY THE ADDITION OF KCl AND NaCl AND BY pH SHIFTS

The results are given as the initial height of the signals in arbitrary chart units (mV) and each is the mean of three experiments carried out on the same chloroplast preparation suspended in sucrose buffer at pH 7.2. The chlorides were added to give a final external concentration of 0.3 M. The pH shift was brought about in the usual way as given in the legend of Table I. The gramicidin originated from Sigma but its exact type was not known. The chloroplasts were pretreated for about 20 min with this antibiotic before commencement of the experiment. The other experimental conditions were the same as those given for the data in Table I.

Experiment	*Gramicidin conc. (mg/l)* · 0.0	0.1	0.5	1.0
Control	2.1	2.0	2.0	2.2
KCl	6.8	17.8	13.2	6.4
NaCl	16.7	25.5	16.4	7.4
pH shift	206.0	160.0	78.0	58.5

was stimulated while the acid–base luminescence was reduced. The same stimulation occurred with luminescence brought about by the addition of potassium or sodium benzoates. The kinetics of the signals due to the addition of potassium benzoate before and after treatment with low concentrations of gramicidin are shown in Fig. 4 together with the corresponding time-course of the acid–base luminescence. The stimulated potassium benzoate signal in this figure can now be seen to have a profile more like that found with the acid–base-induced luminescence. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone at about 0.1 μM also stimulated the salt-induced signals in a similar way while inhibiting those due to an acid–base transition.

At higher concentrations both these compounds considerably inhibited the acid–base and to some extent the salt-induced signals (see Table III).

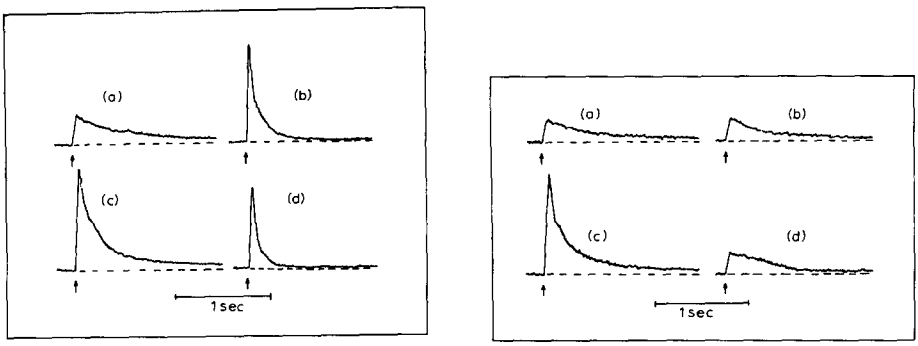

Fig 4 The effect of gramicidin on the kinetics of the salt- and pH-induced luminescence Signal a is the time-course of the luminescence resulting from mixing a preilluminated chloroplast suspension of pH 7 4 with potassium benzoate solution to give an external concentration of 0 3 M Curve b is the signal obtained from the same preparation when it was subjected to a shift of pH from 4 1 to 8 8 Signals c and d are the same experiment as a and b respectively, but after treating the chloroplasts for about 20 min with 0 1 mg/l gramicidin before the preillumination The arrows mark the initial onset of mixing and transfer to the luminescence cuvette The other conditions were the same as given in the legend of Table I

Fig 5 Time-courses of the luminescence induced from preilluminated chloroplasts buffered at pH 7 2 by the addition of potassium benzoate (Signal a) and sodium benzoate (Signal b) to give an external concentration of 0 3 M Curves c and d respectively giving the corresponding signals but after pretreatment of the chloroplast suspension with 3 3 μM valinomycin for about 20 min The other conditions were the same as given for Fig 5

The action of valinomycin

In contrast to the results with the above uncouplers, valinomycin did not inhibit the acid–base signals or stimulate the Na^+-induced luminescence. However, it can be seen in Table IV that with K^+ salts a stimulation of the luminescence occurred in a manner similar to that observed with low concentrations of gramicidin.

TABLE IV

THE EFFECT OF VALINOMYCIN ON THE LUMINESCENCE FROM PREILLUMINATED CHLOROPLASTS INDUCED BY THE ADDITION OF K^+ AND Na^+ SALTS AND BY pH SHIFTS

The results are given as the initial height of the signals in arbitrary units (mV) and each is the mean of three experiments carried out on the same chloroplast suspension buffered at pH 7 2 After the addition of the salts the final external concentration was 0 3 M The pH shifts, the pretreatment with valinomycin, and other experimental procedures were the same as those given in the legend of Table III

Experiment	*Valinomycin concn (μM)*	*0*	*0 06*	*0 33*	*0 60*	*3.3*
Control		0 7	0 6	0 7	0 8	0 8
KCl		1 2	3 5	4 0	4 5	4 6
NaCl		2 1	2 0	1 9	2.0	2 4
Potassium benzoate		35 0	45 0	66 0	90 0	135 0
Sodium benzoate		35 0	35 0	34 0	34 0	31 0
pH shift		110 0	105 0	108 0	104 0	106 0

Typical signals obtained with valinomycin-treated chloroplasts are shown in Fig. 5. The kinetics of the signals due to sodium and potassium benzoates were about the same in the controls but after treatment with 3 3 μM of this ringed polypeptide the K^+ signal looked more like that due to an acid–base shift while the Na^+ signal was virtually unaffected.

DISCUSSION

In many respects these observations are in agreement with the suggestion that it is the electrical potential developed across a membrane by the salt gradient which gives rise to the triggering of luminescence at neutral pH. The magnitude of the predicted diffusion potential will not only depend on the concentration difference but also on the relative permeabilities of the cation and its anion through the membrane. For the addition of a single salt C^+A^- the electrical potential developed across a thin biological membrane may be expressed approximately by[7]:

$$E = \frac{RT}{F} \ln \frac{P_C(C^+)_0 + P_A(A^-)_1}{P_C(C^+)_1 + P_A(A^-)_0} \qquad (1)$$

where P_C and P_A are the permeability coefficients, $(\ )_0$ and $(\ )_1$ denote outside and inside activities respectively and the other symbols have their usual meanings. The permeability coefficients contain the product of the mobility of the ions in the membrane and their partition coefficients between membrane and solution. Clearly if the anion cannot penetrate the membrane, $P_A = 0$, then the maximum potential is developed and given by $E = RT/F \ln (C^+)_0/(C^+)_1$. If, however, the anion and cation permeabilities are equal then no electrical potential is generated.

Interpreting our results in this way would imply that the chloroplasts have rate-limiting membranes which show a higher passive permeability to cations than to anions. The increasing size of the luminescence signals with various anions tested would therefore give an indication of the decrease in their relative permeabilities. For example, benzoate, an anion which would possibly be expected to show very low membrane permeability, gave the largest signals. The reduction of the salt effect by ageing or after osmotic treatment may be due to an overall increase in anion permeability or to a partial breakdown in structure of the relevant membranes. It was interesting that the addition of various alkali metal chlorides indicated that the rate-limiting membranes may show a higher passive permeability to Na^+ than K^+, a situation opposite to that found for the plasma membranes of both plant and animal cells. As would be expected this difference was not seen when the co-ion was benzoate where the relative cation and anion permeabilities could be large (*i. e.*, $P_C \gg P_A$). MILES AND JAGENDORF[5] also obtained various amounts of luminescence by the addition of different salts Essentially they detected a similar anion specificity as given in this communication when the external concentration was 0.3 M but this was apparently lost to some extent as the salt levels were increased to 0.8 M. When they varied the cation keeping Cl^- as the common anion, they found little difference in the ability of K^+ and Na^+ to induce luminescence. However, further evidence that this salt-induced light emission may be controlled by the rate of cation diffusion through a membrane came from their studies with the chlorides of Mg^{2+} and Ca^{2+}. They report, as might be expected, that both these cations,

and incidentally choline also, are relatively ineffective at producing luminescence.

Measurement of the signal height at various external potassium benzoate concentrations followed a linear relationship over the range tested. If the mechanism involves the production of a diffusion potential of a magnitude given by Eqn. 1, then the rate of triggering luminescence would seem to be proportional to the exponential of the electrical gradient created. Also it would be expected that a threshold concentration, given by the value of the internal K^+ activity, should be reached before detecting any increase of luminescence above the control level. In fact enhanced signals could be detected at salt concentrations below 50 mM suggesting perhaps that the internal activity of this cation may be very low in these aqueously isolated spinach chloroplasts.

The comparison of the normal acid–base signals with that obtained with salts before and after treatment with gramicidin or carbonyl cyanide *m*-chlorophenylhydrazone seems to be consistent with the above arguments. These uncouplers are believed to increase the permeability of lipid membranes to cations[8,9]. At low concentrations of these compounds the salt-induced signals were stimulated perhaps by the increased rate of cation entry. With the benzoates the signals obtained after treatment with the uncoupler had a similar time-course to those resulting from an acid–base transition. These observations may suggest that the kinetics of the salt-induced luminescence is controlled by the rate of cation penetration rather than proton efflux. On the other hand, if low concentrations of these uncouplers allow an increase in the membrane permeability with a higher specificity to protons it would be difficult to reconcile the stimulated salt signals with the development of a diffusion potential. The possibility of a collapse of the electrical gradient in this sort of way certainly seems probable when using higher concentrations of these compounds. Under such conditions it could be argued that the number of cation channels induced in the chloroplast membranes were large enough to allow a proton leak by-passing the luminescence site, even during the sudden influx of the added cation. With valinomycin the signal could only be stimulated by the addition of K^+ salts This agrees with the reports that this compound is able to increase membrane permeability specifically for K^+ (ref. 9).

The possibility of an electrical gradient driving protons from an inner chloroplast compartment is of importance to the Mitchell hypothesis and GLYNN[10] has already used a similar argument to interpret some experiments of COCKRELL and co-workers[11]. Presumably the H^+ which are thought to be expelled would normally be associated with fixed negative charges within the chloroplasts[12].

The salt gradient, as MAYNE[4] has suggested for the pH gradient, seems to trigger the release of light resulting from some metastable state within the chloroplast produced during the preillumination. The energy for the triggering does not seem to require a threshold value as high as would be expected for a pH-induced phosphorylation reaction. Variation of salts and of external cation concentrations were found to produce signals of various magnitudes. In any case it would be unlikely that a membrane potential greater than 100 mV is developed by the salt additions employed in these experiments. To generate such a potential would require in the absence of a net inward diffusion of anion ($P_A = 0$) that the internal activity of the particular cation is only 3 mequiv/l. Nevertheless, the addition of benzoates, especially after treatment with membrane modifying agents, resulted in signals

which were comparable with those obtained from acid–base transitions. This then also suggests that the activation energy associated with an acid–base-induced luminescence is lower than that required for acid–base-induced phosphorylation since the latter probably requires an electrical potential gradient greater than 100 mV (ref. 2). From these considerations it would indicate that the salt- and pH-induced luminescence is independent of phosphorylation

Although MILES AND JAGENDORF[5] did not interpret their results in terms of the production of electrical gradients they did come to the same conclusion that these ionic treatments trigger luminescence by some means independent of phosphorylation. Indeed, they were unable to detect ATP formation or ATPase activation when chloroplasts were subjected to a sudden increase in external salt concentration.

So far the discussion has been based on the hypothesis that the efflux of protons driven either by a concentration or electrical gradient results in the triggering of luminescence. Essentially we have visualized the effluxing protons as electron carriers able, for example, to increase the rate of recombination of electrons and positive holes in a membrane located site. Although this model fits well with the requirements of the Mitchell scheme[2] there are some disturbing observations which may place doubt on this exact mechanism. MILES AND JAGENDORF[5] found that they could induce acid–base signals using glutamate, a non-penetrating acid, indicating that the luminescence induced this way does not require the presence of a large internal reservoir of protons Coupled to this is their observation that the addition of acid alone can cause considerable light emission from preilluminated chloroplasts especially when the final pH is reduced below 3

In summary it seems that ionic treatments either with salts or by pH shifts can bring about luminescence from preilluminated chloroplasts. The magnitude and kinetics of the signals strongly suggest that they are controlled by a rate-determining membrane which shows a difference in cation and anion permeability. Whether the mechanism is to induce a proton efflux through a luminescence site is not certain. An alternative possibility is that a diffusion potential developed across the relevant membrane directly reduces the energy barrier for triggering luminescence. As MILES AND JAGENDORF[5] have pointed out this triggering of luminescence is almost certainly related in a very intimate way to 'normal' delayed light emission[6] as represented by the controls in our experiments. Thus the initial signal heights give an approximate measure of the rate at which the metastable states produced during the preillumination are able to donate electrons to chlorophyll such as to induce fluorescence. Because of this it might be expected, for identical preillumination conditions, that the total light emitted would be approximately the same however it is produced bearing in mind that there is also likely to be some time-dependent non-radiational loss of the stored energy. Essentially we did find this to be the case for the treatments presented above as long as the total light output was measured over a reasonably long time interval of about a minute or so. Clearly the luminescence parameter which should be measured for any particular treatment is the initial rate of rise of the signal $d(h\nu)/dt$ but unfortunately this has not yet been experimentally possible.

Finally it should be mentioned that CLAYTON[13] has also observed 'chemiluminescence' induced by another mechanism involving a change in fluorescence yield of chlorophyll associated with System II. This possibility, however, does not seem

to totally explain the luminescence induced by salt additions[5,13] even when an organic anion like benzoate is used (G. P. B. KRAAN, unpublished observation).

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