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SALT-INDUCED ABSORBANCE CHANGES OF *P*-515 IN BROKEN CHLOROPLASTS

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SUMMARY

Absorbance changes, caused by adding KCl to a suspension of broken chloroplasts in the presence of a low concentration of $MgCl_2$, have been measured in the wavelength region 460–540 nm. The magnitude of the KCl-induced absorbance changes is shown to be proportional to the logarithm of the KCl concentration gradient initially induced across the thylakoid membrane. The difference spectrum of these absorbance changes is shown to be identical with the spectrum of the light-induced absorbance changes, which has been attributed to an electrochromic shift of *P*-515. This is interpreted as evidence that under these conditions salt-induced absorbance changes of *P*-515 occur in response to a membrane diffusion potential. The results indicate that the electrogenic potential across the thylakoid membrane, generated by a single turnover light flash, is in the range between 15 and 35 mV.

INTRODUCTION

Ample evidence has accumulated that the light-induced absorbance change of *P*-515, denoted as the 515 nm absorbance change and characterized by a difference spectrum with a minimum at about 480 nm and a maximum around 515 nm, is due to an electrochromic shift of the absorption bands of native pigments embedded in the membrane [1–3]. The extent of the 515 nm absorbance change has been considered to be a linear measure of the magnitude of the transmembrane potential associated with the electric field across the membrane [1–3]. Voltage calibration of the 515 nm absorbance change usually is made with reference to the absorbance change in response to a single turnover saturating light flash which, according to approximations about the number of charges loading the membrane capacitance, would generate a transmembrane potential of approx. 50 mV [1, 4]. Potential measurements with microcapillary glass electrodes inserted in a single granum stack of chloroplasts of *Peperomia metallica* have indicated that a single turnover light flash generates a potential across the thylakoid membrane in the range between 10 and 50 mV [5–8].

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

According to the results of recent studies on the effect of the voltage-dependent ionophore alamecithin on the kinetics of the 515 nm absorbance change, it has been concluded that the potential generated by a single turnover flash is of the order of 100 mV [9]. This value is at variance with the potential concluded from the former approaches. The discrepancy emphasizes the need for another independent voltage calibration method of the electrogenic charging of the thylakoid membrane.

Attempts have been made to measure electrochromic band shifts in isolated chloroplasts brought about by a transmembrane (diffusion) potential generated by salt additions [10, 11]. These measurements have successfully been done with respect to the carotenoid band shifts in bacterial chromatophores [12, 13]. They have been used as an elegant calibration method for determining the magnitude of the transmembrane potential associated with photosynthetic energy conservation in bacteria. However, this method could not be applied to chloroplast (thylakoid) membranes because of the fact that substantial changes in light scattering occur upon salt additions, which prevented accurate measurements of the 515 nm band shift [10, 11].

This communication reports on experiments in which KCl-induced absorbance changes were measured in the 460–540 nm wavelength region under proper conditions at which scattering changes were minimized. The results indicate that the magnitude of the 515 nm absorbance change per mV across the thylakoid membrane is approx. $1.1 \cdot 10^{-4}$ and that the electrogenic potential generated by a single turnover saturating light flash is in the range between 15 and 35 mV.

MATERIALS AND METHODS

Chloroplasts were isolated from fresh leaves of spinach, grown in the laboratory glass-house. Washed leaves were homogenized in an isolation medium containing 0.33 M sorbitol and 2 mM HEPES, adjusted to pH 7.1 with NaOH. The homogenate was filtered through four layers of perlon net (pore diameter 50 μm), and centrifuged for 2 min at 3000 rev./min. The sedimented chloroplasts were subjected to an osmotic shock by resuspending them in 10 ml distilled water. After 1 min, 10 ml of double strength isolation medium was added to the suspension. Chlorophyll content was determined spectrophotometrically [14]. The stock suspension of broken chloroplasts was diluted 10–15-fold in isolation medium giving a final concentration, equivalent to 15–25 μg chlorophyll/ml. Chloroplasts were used within 1 h after preparation.

Salt-induced absorbance changes were measured in an Aminco-Chance absorption difference spectrophotometer. The split-beam mode (monochromatic beam of measuring wavelength alternating between sample and reference compartments of the cuvette) was used for measuring the effects of subsequent additions of different salts at characteristic wavelengths in the region between 460 and 700 nm. The double beam mode (monochromatic beams of measuring and reference wavelength alternatively passing through single compartment of cuvette) was used to measure accurately the absorbance difference spectrum of KCl-induced changes in the presence of 3 μM valinomycin and 2 mM $MgCl_2$. The samples were continuously stirred at a high speed in the compartments of specially constructed perspex cuvettes (1 cm optical path, volume 2.5 ml) for both operation modes of the spectrophotometer. The mixing time of added salts under our experimental conditions was less than 1 s. Salts were added as concentrated solutions (1 M). Measurements were performed at 5 °C.

Absorbance changes induced by single turnover light flashes (half lifetime 8 μs) were measured in a single beam absorption-difference apparatus as described elsewhere [15]. The actinic flash light was of wavelengths above 665 nm. The absorbance changes were measured at various wavelengths, selected by interference filters, in the region 460–540 nm. A number of flashes were fired at a dark interval of 3 s. Usually the signals of 64 flashes were sampled and averaged (DL 102 A signal averager).

RESULTS

The characteristics of the absorbance changes at 515, 540, 460 and 475 nm upon subsequent additions of 10 mM KCl and 2 mM $MgCl_2$, or vice versa, to a suspension of chloroplasts are shown in Figs. 1a–1d. Addition of 10 mM KCl causes an absorbance decrease at these wavelengths. At 515 and 540 nm the decrease is preceded by a transient increase. It appears that the decrease in absorbance is composed of a fast and a slow component. The relative magnitude of these components is different at the wavelengths used. Subsequent addition of 2 mM $MgCl_2$ causes an

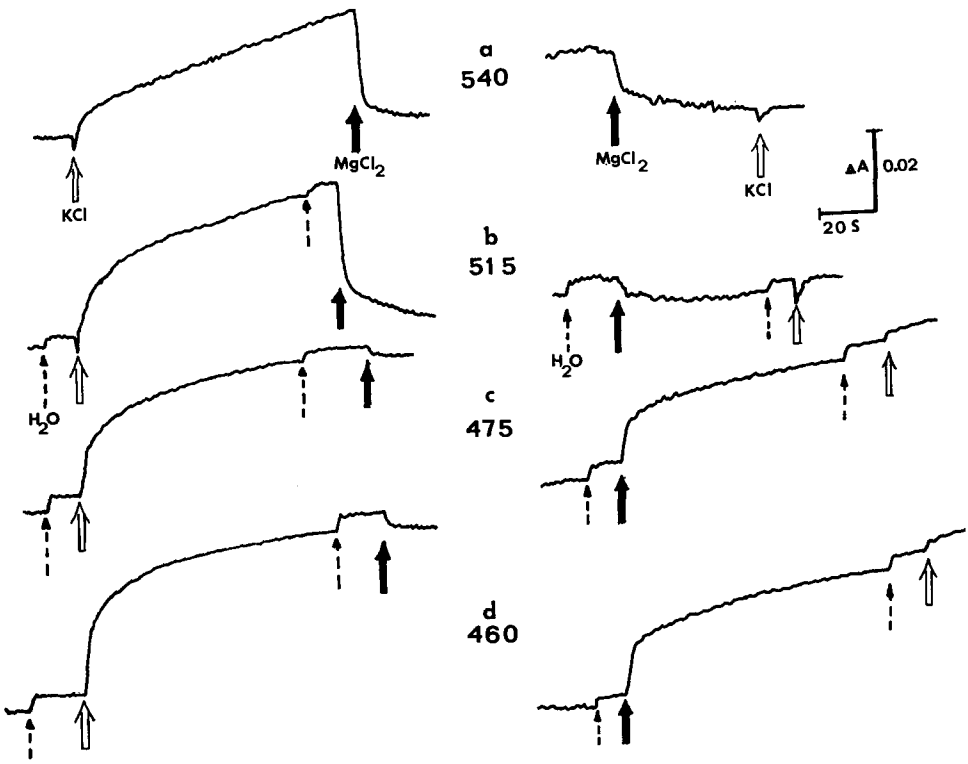

Fig. 1. Kinetics of salt-induced absorbance changes at 540 (a), 515 (b), 475 (c) and 460 nm (c) in a cation-free suspension of broken chloroplasts (15 μg chlorophyll/ml). Final concentrations of KCl and $MgCl_2$ were 10 and 2 mM, respectively. Addition of $MgCl_2$, KCl and H_2O are indicated by closed, open and dotted upward pointing arrows, respectively. The response upon additions of H_2O at the same amount as the salt solution indicates the dilution effect. The spectrophotometer was operating in the split-beam mode. An upward deflection means a decrease in absorbance.

increase in absorbance at 515 and 540 nm, which approximately equals the preceding decrease caused by the addition of KCl (Figs. 1a and 1b). As can be seen the kinetics of the $MgCl_2$–induced absorbance increase are multiphasic. The increase at 475 and 460 (Figs. 1c and 1d) caused by $MgCl_2$ in the presence of KCl is relatively small as compared to the increase at 515 and 540 nm. Addition of 2 mM $MgCl_2$ to a suspension of chloroplasts in the absence of KCl causes an increase in absorbance at 515 and 540 nm and a decrease at 475 and 460 nm. The absorbance changes caused by $MgCl_2$ at these wavelengths appear to be composed of a slow and a fast component. Subsequent addition of 10 mM KCl causes a small and reversible absorbance increase at 515 and 540 nm. Absorbance changes of this kind at 475 and 460 nm could not be resolved with sufficient accuracy from the response caused by the dilution effect. The absorbance changes brought about by $MgCl_2$ addition in the absence and presence of KCl were found to be saturated at a $MgCl_2$ concentration of 1–2 mM.

The characteristics of the absorbance changes upon KCl addition in the presence of $MgCl_2$, as observed at 515 (and 540 nm) (Figs. 1a and 1b), were studied in more detail. The accuracy of the measurements was improved by performing them in the double beam operation mode of the spectrophotometer. In order to suppress disturbances caused by the dilution effect, measurements were done with reference wavelengths in the 570–680 nm wavelength region, at which the absorbance of the chloroplast suspension was equal to the one at the measuring wavelengths in the 460–540 nm region. For example 570 and 642 nm were used as reference wavelengths for measurements at 540 and 515 nm, respectively. It was verified that the absorbance changes at the reference wavelengths upon addition of KCl in the presence of $MgCl_2$ and valinomycin, if occurring at all, were negligibly small as compared to the one at the measuring wavelength.

Fig. 2b shows the KCl-induced absorbance change at 515 nm in the presence of 2 mM $MgCl_2$ and 3 μM valinomycin. A fast increase is followed by a slow decrease to the initial level. A second addition of 10 mM KCl causes a similar but smaller absorbance change. A small effect is also observed upon a first addition of 10 mM NaCl

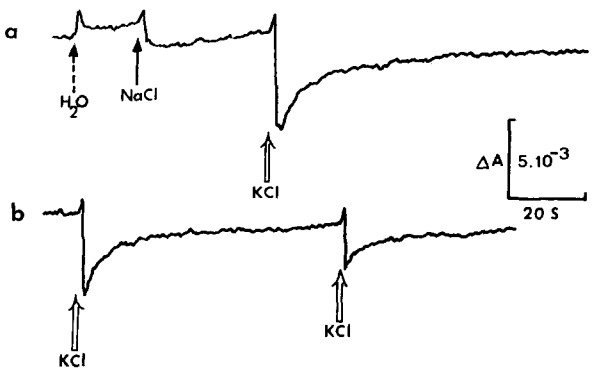

Fig. 2. Absorbance changes at 515 nm upon two subsequent additions of 10 mM KCl (b) or of 10 mM NaCl and 10 mM KCl (a) to a chloroplast suspension (25 μg chlorophyll/ml) in the presence of 2 mM $MgCl_2$ and 3 μM valinomycin. An upward deflection means a decrease in absorbance. The spectrophotometer was operating in the dual-wavelength mode. The reference wavelength was 642 nm. The small initial transient signal was caused by the addition of the salt solution.

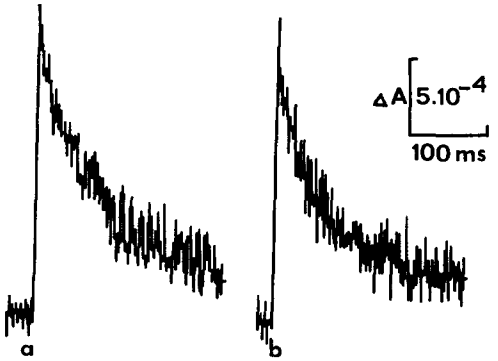

Fig. 3. Absorbance change at 515 nm upon a single turnover saturating light flash in the absence (a) and the presence (b) of 2 mM $MgCl_2$, respectively. Chlorophyll concentration, 23 μg/ml. Other conditions were as described in the text.

(Fig. 2a). A subsequent addition of 10 mM KCl in this case gives rise to an absorbance change, of which the magnitude is about equal to the one observed in the absence of NaCl and KCl in the suspending medium.

The time course of the light-induced absorbance change at 515 nm in the presence and absence of $MgCl_2$ is shown in Fig. 3. The magnitude of the fast initial increase in absorbance caused by a single turnover light flash, is independent on the presence of 2 mM $MgCl_2$ in the suspending medium. The decay towards the dark steady state is accelerated in the presence of $MgCl_2$.

In Fig. 4 the absorbance change at 515 nm is plotted as a function of the KCl added to the chloroplast suspension, containing 2 mM $MgCl_2$ and valinomycin. It

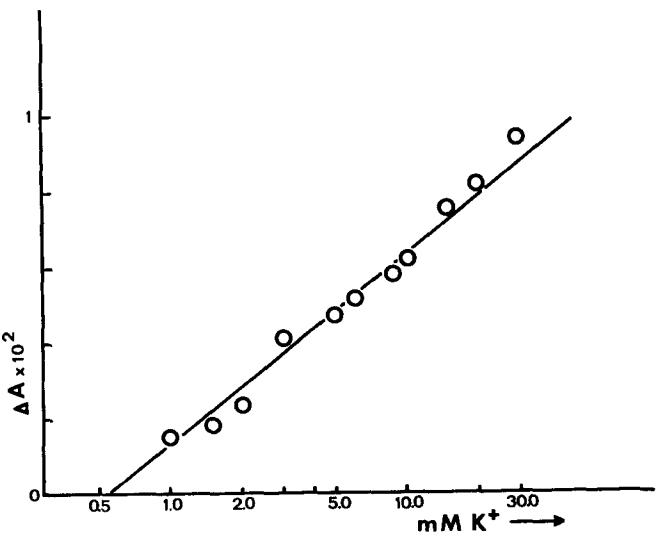

Fig. 4. The magnitude of the KCl-induced absorbance changes at 515 nm, measured in the presence of 2 mM $MgCl_2$ and 3 μM valinomycin, plotted as a function of the KCl concentration. Chlorophyll concentration was 25 μg/ml. Other conditions were as described in the text.

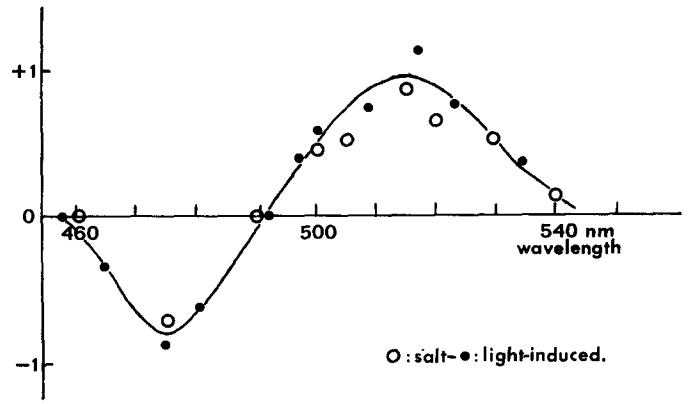

Fig. 5. Spectrum of absorbance changes induced by addition of 10 mM KCl (open circles), or by a single turnover light flash (closed circles), measured in the presence of 2 mM $MgCl_2$ and 3 μM valinomycin. The spectrum has been plotted in relative absorbance units.

appears that the increase in absorbance is proportional to the logarithm of the KCl concentration gradient initially induced across the thylakoid membrane, at least for a concentration below 50 mM. At a KCl concentration above 50 mM the absorbance increase routinely was observed to be higher than would be predicted from the relationship at the lower concentrations.

The spectrum of the reversible absorbance change caused by addition of 10 mM KCl in the presence of $MgCl_2$ and valinomycin and of the changes caused by a single turnover saturating light flash is shown in Fig. 5. There appears to be close correspondence between both.

DISCUSSION

The spectrum of the reversible absorbance change in the 460–540 nm wavelength region upon addition of KCl to a chloroplast suspension containing $MgCl_2$ and valinomycin (Fig. 5) suggests that the change is closely associated with a reaction of *P*-515. Light-induced absorbance changes attributed to *P*-515 have been argued to be due to an electrochromic bandshift of native pigments embedded in the membrane occurring in response to an electric field (potential) across the membrane [1, 4]. The spectrum of the absorbance change caused by a single turnover saturating light flash is identical with the difference spectrum of the salt-induced absorbance change (Fig. 5). A similar correspondence has been demonstrated for bacterial chromatophores with respect to the carotenoid band shifts [12, 13]. In strict analogy with these experiments, the data of Figs. 2 and 4 nicely confirm that the absorbance changes induced by salt addition in the presence of $MgCl_2$ occur in response to a membrane (diffusion) potential. (i) The absorbance change upon addition of 10 mM KCl in the presence of valinomycin is much higher than upon addition of 10 mM NaCl, as would be expected in the presence of this ionophore at which the membrane permeability of K^+ is high as compared to that of NaCl. (ii) The presence of NaCl in the suspending medium does not influence the extent of the absorbance change upon KCl addition, whereas the response upon KCl addition in the presence of KCl is significantly smaller. (iii) The

extent of the 515 nm absorbance change is proportional to the logarithm of the concentration of added KCl (Fig. 4). Moreover, the decay kinetics of the absorbance changes closely resemble the kinetics of the KCl-induced changes in delayed light emission in broken chloroplasts [16, 17]. These changes have been interpreted in terms of the decay of the membrane diffusion potential, due to the redistribution of K^+. The rate of this redistribution has been evidenced to be controlled by the rate of entry of Cl^- across the membrane into the thylakoid inner space [17]. It was found that the magnitude of the 515 nm absorbance change (Fig. 2) upon addition of a fixed amount of KCl (in the presence of $MgCl_2$ and valinomycin) is proportional to the chlorophyll concentration in the chloroplast suspension, for concentrations lower than 30 μg chlorophyll/ml. The same was found to be true for the magnitude of the light-induced absorbance change.

Fig. 4 shows that a 10-fold increase in K^+ concentration corresponds with a 515 nm absorbance change of approx. $4.9 \cdot 10^{-3}$. In the absence of permeating ions this increase would have corresponded with a change in the membrane diffusion potential of 58.5 mV (at 20 °C), according to the Nernst equation. Correcting for the presence of Cl^- in the medium (2 mM $MgCl_2$) and substituting an estimated [17] permeability ratio between Cl^- and K^+ of 0.04 in the presence of valinomycin, the Goldman constant field equation yields that, at the concentrations used, a 10-fold increase in K^+ concentration corresponds with a change in the membrane diffusion potential of about 35 mV. Thus the proportionality factor between 515 nm absorbance change and membrane potential is about $1.4 \cdot 10^{-4}$ per mV. The data of Fig. 4 may need a correction factor for a small absorbance change that could have occurred at the reference wavelength (642 nm) used. According to spectral data of Reich et al. [18] this correction factor is about 0.8. This would mean that the absorbance change at 515 nm has been $1.1 \cdot 10^{-4}$ per mV at a chlorophyll concentration of 25 μg/ml. At the same concentration a single turnover saturating light flash (e.g. Fig. 3) would cause an absorbance change of $2.2 \cdot 10^{-3}$, which corresponds with a membrane potential of 20 mV. Similar experiments done with various samples have indicated that a single turnover saturating light flash causes the generation of a transmembrane potential E_e in the range between 15 and 35 mV with an average value of 25 mV. This value is of the same order of magnitude as the approximated value of E_e associated with the charging of the membrane capacitance, due to one charge separation in all reaction centers oriented in the thylakoid membrane [1, 4, 8], and falls within the range of flash-generated potentials measured with micro-electrodes in chloroplasts of *P. metallica* [7, 8]. Values of about 100 mV have recently been concluded from analyses of the 515 nm absorption changes in chloroplasts in the presence of the voltage-dependent ionophore alamecithin [9]. The reason for the apparent discrepancy is unknown as yet. It might be that, contrary to the assumptions made [9], the proportionality factor between concentration and the characteristic potential of the ionophore is different in artificial lipid bilayers and thylakoid membranes. The present calibration method has the important advantage that the light- and the salt-induced 515 nm absorbance changes are measured in identical samples under equal conditions.

As can be concluded from the data of Fig. 1, the approach of setting potassium diffusion potentials across the thylakoid membrane in order to measure its associated electrochromic shifts could only be applied if low concentrations of $MgCl_2$, or high concentrations of NaCl (not shown), were present in the suspending medium. In

accordance with the results of others [11], the time course of the irreversible absorbance changes in the 450–550 nm wavelength region upon addition of KCl in the absence of $MgCl_2$ or KCl, respectively (Fig. 1), indicates a change in light scattering, probably due to structural changes in the membrane associated with cation binding at fixed negative charges. Subsequent addition of $MgCl_2$ results in an absorbance increase at wavelengths above 500 nm. This effect has been noticed by others [19]. It probably reflects the substitution of monovalent cations by divalent cations at the fixed charges, caused by changes of ionic concentrations near the electrical double layer [20]. In this wavelength region (above 500 nm) an absorption increase occurs upon $MgCl_2$ addition in the absence of KCl (Figs. 1a and 1b). This increase was found not to be accompanied by an increase in light scattering (Vredenberg, W. J. and Schapendonk, A. H. C. M., unpublished results) and is presumed to reflect a structural change in the membrane, which facilitates light absorption by pigments with absorption bands in this wavelength region. This salt effect, which needs further investigation, is different from that causing the absorbance changes in the 460–500 nm region (Figs. 1c and 1d). These have been suggested to be due to changes in mutual shading of pigments, which occurs in association with the shrinkage [11, 19] upon KCl or $MgCl_2$ addition. The apparent lack of any antagonistic effect between $MgCl_2$ and KCl upon the absorbance changes in the 460–500 nm region (Figs. 1c and 1d), is conclusive with this suggestion.

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REFERENCES

1 Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254
2 Emrich, H. M., Junge, W. and Witt, H. T. (1969) Z. Naturforsch. 24b, 144–146
3 Witt, H. T. and Zickler, A. (1973) FEBS Lett. 37, 307–310
4 Witt, H. T. (1971) Q. Rev. Biophys. 4, 365–477
5 Bulychev, A. A., Andrianov, V. K., Kurella, G. A. and Litvin, F. F. (1972) Nature 236, 175–176
6 Vredenberg, W. J., Homann, P. H. and Tonk, W. J. M. (1973) Biochim. Biophys. Acta 314, 261–265
7 Vredenberg, W. J. and Tonk, W. J. M. (1975) Biochim. Biophys. Acta 387, 580–587
8 Vredenberg, W. J. (1976) in The Intact Chloroplast (Barber, J., ed.), pp. 53–88, Elsevier, Amsterdam
9 Zickler, A., Witt, H. T. and Boheim, G. (1976) FEBS Lett. 66, 142–148
10 Strichartz, G. R. and Chance, B. (1972) Biochim. Biophys. Acta 256, 71–84
11 Gross, E. L. and Libbey, J. W. (1972) Arch. Biochem. Biophys. 153, 457–467
12 Crofts, A. R., Jackson, J. B., Evans, E. H. and Cogdell, R. J. (1972) in Proceedings of the 2nd International Congress of Photosynthesis Research (Forti, G., Avron, M. and Melandri, A., eds.), Vol. 2 pp. 873–902, Junk, The Hague
13 Jackson, J. B. and Crofts, A. R. (1969) FEBS Lett. 4, 185–189
14 Bruinsma, J. (1961) Biochim. Biophys. Acta 52, 576–578
15 Vredenberg, W. J. (1974) in Membrane Transport in Plants (Zimmermann, U. and Dainty, J., eds.), pp. 126–130, Springer Verlag, Heidelberg

16 Barber, J. (1972) FEBS Lett. 20, 251–254
17 Barber, J. (1972) Biochim. Biophys. Acta 275, 105–116
18 Reich, R., Scheerer, R., Sewe, K. U. and Witt, H. T. (1976) Biochim. Biophys. Acta 449, 285–294
19 Gross, E. L. and Prasher, S. H. (1974) Arch. Biochem. Biophys. 164, 460–468
20 Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174–181