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ABSORBANCE CHANGES AT 520 nm CAUSED BY SALT ADDITION TO CHLOROPLAST SUSPENSIONS IN THE DARK

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SUMMARY

1. Absorbance changes near 520 nm, similar to those resulting from illumination, are produced by adding alkali cation salts to a suspension of fragmented chloroplasts in the dark. These absorbance changes depend on the osmolarity of the suspending medium, the anion of the salt added, the intactness of chloroplast thylakoids, and are modified by divalent cations and by valinomycin. The absorbance changes are insensitive to pH, from pH 5 to 9, and to electron transport inhibitors. They are changed only slightly if the chloroplasts are illuminated during salt addition.

2. A model is presented to explain both salt and light-induced absorbance changes in terms of a hypothetical electric field within the membrane.

INTRODUCTION

A light-induced absorbance increase centered near 520 nm was first observed in Chlorella by DUYSENS[1]. Similar absorbance changes have been reported for a variety of green plants[2,3] and algae[4–6], but are notably absent in some species[7,8]. Although the the change at 520 nm (hereafter called "$A_{520\,nm}$") is one of the most prominent light-induced absorbance changes occuring in plants and seems to be intimately related to photosynthetic activity[1,2,9], no generally satisfactory explanation of its origin has been presented to date.

The prime candidates for pigments which actively change their absorbance at 520 nm and at 475 nm, the wavelength of an accompanying absorbance decrease, are carotene[10,11] and chlorophyll[12,13]. We have shown in a previous paper[14] that extraction of carotene from chloroplasts removes the 520 and 475 nm absorbance changes and that reconstitution with pure β-carotene restores these absorbance changes. Similarly, HILDRETH[11] has shown that the extent of laser flash-activated 520 nm absorbance changes in wild-type and mutant barley leaves is proportional to the β-carotene content of these tissues.

Abbreviations: MOPS, morpholinopropane sulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, (*N*-morpholino)ethane sulfonic acid.

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It has been suggested that the electric field associated with a transmembrane potential is responsible for the 520 nm change in green plants[12] and for the carotenoid absorbance changes observed in some photosynthetic bacteria[15]. In this paper we report an attempt to produce an electric potential difference across the chloroplast thylakoid membrane by addition of concentrated salt solutions to a chloroplast suspension in the dark. Such salt additions did cause absorbance changes similar to those activated by light. The dependence of these absorbance changes on the concentration of added salt, medium osmolarity, vesicle intactness, and to the presence of ionophorous antibiotics suggests that they are responses to a membrane potential.

MATERIALS AND METHODS

Preparation of chloroplasts

Chloroplasts were isolated from 60 g of deribbed leaves of spinach (*Spinacia oleracea*) or pokeweed (*Phytolacca americana*) by homogenizing in a blender in 200 ml of 0.4 sorbitol, 0.01 M NaCl, 0.02 M morpholinopropane sulfonic acid (MOPS), pH 7.8. The homogenate of ground leaves was filtered through four layers of cheesecloth and the filtrate centrifuged at 500 $\times$ *g* for 30 sec. The supernatant was then centrifuged at 1500 $\times$ *g* for 10 min and the resulting pellet resuspended in a minimal volume of homogenizing medium to give a final concentration of 2 to 4 mg chlorophyll/ml.

The reaction medium was usually 0.01 M MOPS adjusted to pH 7 with 10% trimethylammonium hydroxide to minimize the concentration of alkali metal cations.

Separation of chloroplast components

The various chloroplast components were separated using a method developed by Drs. W. D. Bonner and S. B. Wilson. Intact chloroplasts (Class I) were prepared by the method of COCKBURN *et al.*[16]. The final pellet of intact chloroplasts was suspended in cold 80% aqueous glycerol at a concentration of approximately 5 mg chlorophyll/ml. 5 ml of this suspension was then injected by a hypodermic syringe into 2l of 0.01 M MOPS, pH 7.5, effectively bursting the chloroplasts by osmotic shock. The suspended fragments were centrifuged at 600 $\times$ *g* for 15 min, and the pellet was resuspended in isolating medium and layered on a discontinuous sucrose gradient (Fig. 1). The gradient was centrifuged for 3 h at 75000 $\times$ *g* in a Beckman Model L2-65B ultracentrifuge. As a result of this centrifugation, three bands of chloroplast material were distributed as shown in Fig. 1. The bands were withdrawn from the gradient, diluted into four volumes of 0.01 M MOPS, pH 7.5, and centrifuged at 6000 $\times$ *g* for 15 min. The pellets, containing the separated material from the three green bands, were resuspended in a 0.3 M sorbitol solution and the contents identified by examination under the phase contrast microscope. Assignment of different chloroplast fragments to the various bands in the gradient is shown in Fig. 1.

Optical measurements

Salt-induced absorbance changes and light-dark difference spectra were measured in a dual-wavelength spectrophotometer similar to the one described by CHANCE[17]. Measurements of the rise time of rapid salt-induced absorbance changes were made using the flow apparatus designed and constructed at the Johnson Foundation. All measurements were performed in the stopped-flow mode.

Chemical assays

Chlorophyll was assayed by the method of NISHIMURA[18] derived from the formulae of MACKINNEY[19]. All chemicals used were of reagent grade. MOPS was purchased from Calbiochem, Los Angeles, Calif., and trimethylammonium hydroxide from Eastman Organic Chemicals, Rochester, N.Y. Valinomycin was also purchased from Calbiochem, and nigericin was the generous gift of Dr. B. C. Pressman.

Actinic illumination was provided by a tungsten filament lamp equipped with a (Corning Glass 2030) filter which transmitted light above 650 nm and with a 3-cm water filter. The photomultiplier was shielded against this illumination using a Corning 9782 glass filter.

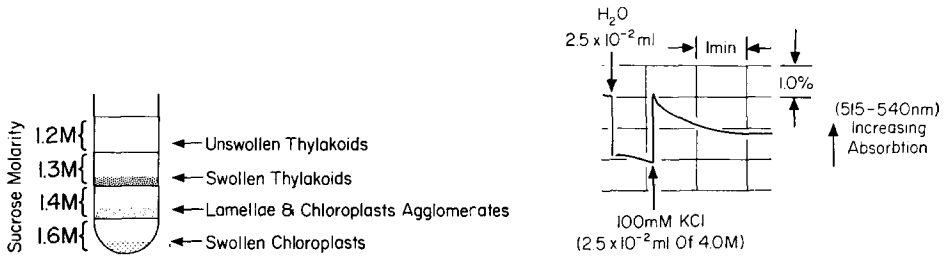


Fig. 1. Distribution of chloroplast fragments in a discontinuous sucrose gradient. The separation was produced by centrifugation at 75000 × *g* for 3 h. Details are given under MATERIALS AND METHODS.

Fig. 2. Time course of absorbance changes following KCl addition to a suspension of fragmented chloroplasts. Spinach chloroplasts (60 μg chlorophyll/ml) suspended in 0.01 M MOPS, pH 7.1. The response time of the detecting system is 0.1 sec.

EXPERIMENTAL RESULTS

Kinetic and spectral parameters of salt-induced absorbance changes

The addition of a solution of salt of an alkali metal cation to a suspension of fragmented chloroplasts produces rapid absorbance changes as shown in Fig. 2. Measurements at 520 nm show a rapid absorbance increase followed by a slower absorbance decrease. The fast absorbance increase may be followed by stopped-flow techniques; the kinetics are approximately exponential with a characteristic time of 600 $\pm$ 60 msec. The slow reversal is biphasic and has a fast exponential phase with a characteristic time ranging from 5 to 10 sec, and a slow exponential phase with a characteristic time between 50 and 100 sec. These kinetic parameters depend on the identity of both the cation and the anion of the salt which is added. The parameters are collected in Table I.

The two phases of the absorbance decrease have extents which vary between preparations. The slower phase is always evident, the faster phase is present to a variable degree.

The dilution artifact appearing in Fig. 2 is very reproducible. All subsequently measured absorbance changes have been corrected for dilution artifact.

Fig. 3 shows the wavelength dependence of the rapid absorbance increase which occurs upon KCl addition (solid line). It is very similar to "A_{520nm}" (produced by illumination of the same chloroplast suspension) in the region from 500 to 540 nm. At shorter wavelengths the two spectra differ considerably. The shape of the spectrum

TABLE I

KINETIC PARAMETERS OF SALT-INDUCED ABSORBANCE CHANGES IN FRAGMENTED CHLOROPLASTS

	Conditions	*Rise time (sec)*	*Characteristic time fast decay (sec)*	*Characteristic time slow decay (sec)*
I.	KCl addition			
	Control *	0.46 ± 0.02	15 ± 2	34 ± 2
	+ Valinomycin (0.1 μM)	0.09 ± 0.01	15 ± 2	22 ± 2
	Control **	—	8 ± 2	80 ± 4
	+ Nigericin (2.8 μM)	—	8 ± 2	55 ± 5
II.	NaCl addition **			
	Control	—	8 ± 2	109 ± 7
	+ Nigericin	—	7 ± 2	108 ± 7
	+ Valinomycin (both 2.8 μM)	—	7 ± 2	103 ± 7
III.	Potassium acetate addition **			
	Control	—	5 ± 2	86 ± 6

* and ** denote two different preparations, done on separate days.

near 520 nm resulting from KCl addition is independent of the concentration of KCl which is added, as shown by comparing Fig. 3 with the insets of Fig. 5, and is identical in chloroplasts from pokeweed or from Spinach.

Salt-induced absorbance changes at 520 nm are much diminished by extraction of β-carotene[14] from chloroplasts as shown in Fig. 4 (broken line). The extraction also reduces the extent of absorbance changes at wavelengths lower than 500 nm. The difference between salt-induced spectra in unextracted and extracted chloroplasts is similar to the light-induced change, but still has larger absorbance changes at 475 nm (dashed line in Fig. 4).

The spectrum of the fast reversal phase of the KCl-induced absorbance change is the inverse of the spectrum of the initial rapid absorbance increase; *i.e.* these spectra are symmetrical about the zero absorbance change axis. The slow reversal phase has no maxima or minima from 500 to 540 nm but decreases monotonically as the wavelength increases over this range.

The dependence on KCl concentration of the rapid absorbance change at 520 nm is shown in Fig. 5. The experimental curve is very similar for KCl, NaCl, $NaNO_3$, and potassium acetate. As shown by the dotted curves in Fig. 5, the experimental curve can be resolved into a straight line and a hyperbolic curve. The half-maximum for the hyperbola occurs at 5.5 ± 0.5 mM added salt concentration.

The extent of the salt-induced absorbance change depends on the tonicity of the medium. Large absorbance changes can only be observed for chloroplasts suspended in hypotonic medium (Fig. 6). In suspensions in isotonic medium (0.33 M) addition of salts causes little absorbance change above that of the dilution artifact.

Suspending intact chloroplasts in very hypotonic medium (0.01 M) causes the chloroplasts to swell rapidly, bursting the outer membrane. This treatment produces several types of chloroplast fragments which may be separated by the discontinuous sucrose gradient described earlier.

Both light-induced and salt-induced activities are localized in the unswollen

(intact) grana fraction (Table II) and, as with the usual mixture of chloroplast fragments, the salt-induced change can only be seen in hypotonic suspensions. The activity and the kinetics of the salt-induced changes in the isolated grana fraction are identical to the absorbance changes seen with unseparated chloroplast fragments. Hence, in

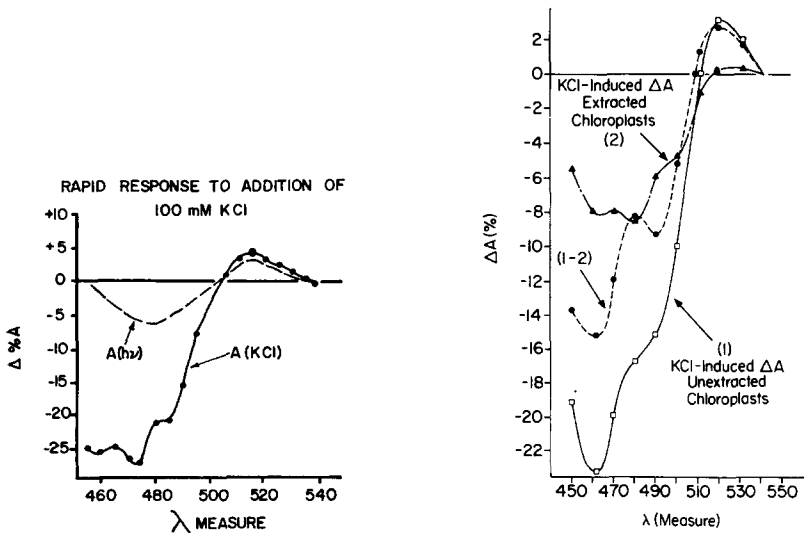


Fig. 3. Light-induced and salt-induced difference spectra in fragmented chloroplasts (60 μg chlorophyll/ml) suspended in 0.02 M MOPS, pH 7.0. Continuous actinic illumination by broad band red light at saturating intensity. Reference wavelengths are 540 nm for measurements including and greater than 500 nm, and 500 nm for measurements below this wavelength.

Fig. 4. KCl-induced absorbance changes in unextracted and extracted chloroplasts. The final KCl concentration is 0.028 M. Other conditions as in Fig. 3.

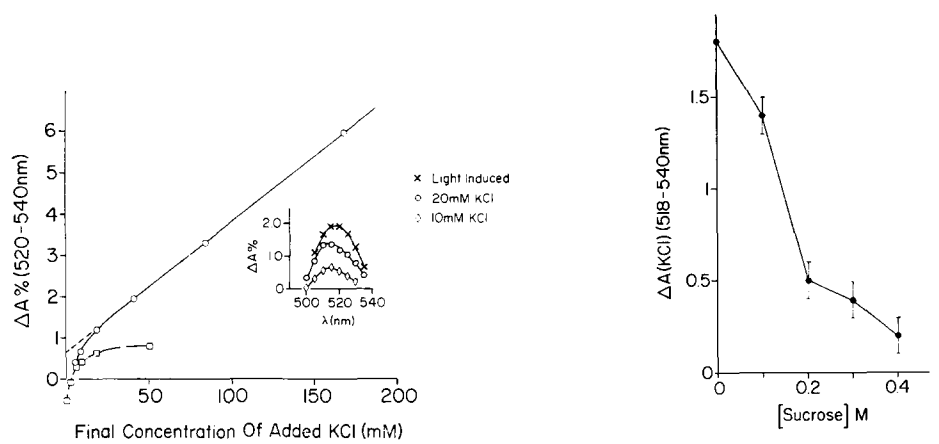


Fig. 5. Dependence of rapid absorbance changes at 520 nm on the concentration of KCl added. Chloroplast fragments at a concentration of 58 μg chlorophyll/ml in 0.01 M MOPS, pH 7.1. The insert shows the absorbance changes as a function of wavelength for two different KCl concentrations.

Fig. 6. Extent of rapid KCl-induced absorbance changes as a function of medium osmolarity. All suspensions buffered by 0.01 M MOPS, pH 7.1. Final KCl concentration, 0.04 M.

TABLE II

LOCALIZATION OF "$A_{520\ nm}$" AND KCl-INDUCED ABSORBANCE CHANGES IN CHLOROPLAST FRAGMENTS

The activities are in arbitrary units but are normalized for chlorophyll concentration. Actinic illumination is by broad band red light (Corning 2030 filter). The fractions correspond to the bands from centrifugation shown in Fig. 1.

Outer membrane chloroplast fragments		*Swollen grana*		*Unswollen grana*	
		Hypotonic	*Isotonic*	*Hypotonic*	*Isotonic*
Light-induced absorbance changes at 518 nm	0.27 ± 0.02	—	0.61	1.1	1.3
KCl-induced absorbance changes at 518 nm	—	0.25 ± 0.2	0.3	1.45	0.24

mixed suspensions, the observed absorbance changes are attributable only to the intact grana which are present.

It was possible that the absorbance changes caused by salt addition were responses to the changes in osmolarity. All of the electrolytes added are known to cause osmotic volume changes in chloroplast grana[20]. However, addition of sucrose or mannitol having the same osmotic activity as an aliquot of KCl results in only one fifth of the absorbance change which the KCl produces. Therefore, the greater part of the absorbance change does not appear to arise from an osmotic response.

The addition of divalent cations produces very different absorbance changes from those produced by monovalent cations, both spectrally and kinetically. The presence of divalent cations diminishes the rapid salt-induced absorbance changes as well as the light-induced change. In the second paper in this series we detail the responses of salt- and light-induced 520-nm absorbance changes to divalent cations.

Effect of pH on salt-induced absorbance changes

The curves in Fig. 7 demonstrate that although the light-induced absorbance change varies with pH the salt-induced absorbance changes are roughly constant in the range from pH 5 to 9. The pH profile for the light-induced "$A_{520\ nm}$" is quite similar to the pH profile for Hill activity[21]. Since one apparent "pK_a" of the chloroplast membrane is about 6.8[22], it appears that salt-induced absorbance changes are not responsive to the degree of protonation of this group on the thylakoid membrane.

Effect of ionophorous antibiotics on salt-induced absorbance changes

The ionophorous antibiotics, which catalyze the entry or exchange of ions across artificial and biological membranes modify the salt-induced absorbance changes. Valinomycin, a K^+-specific ionophore, enhances KCl-induced absorbance changes. Fig. 8 shows the time course of KCl-induced absorbance changes in the presence and absence of valinomycin. The enhancement by valinomycin is seen only with KCl addition, but not with NaCl additions. If nigericin, which catalyzes an electrically neutral exchange of K^+ and H^+, is substituted for valinomycin no enhancement of KCl-induced absorbance changes occurs.

Valinomycin also effects the kinetics of the KCl-induced response (Table I;

Fig. 9). The rate of the fast absorbance increase, as measured in the stopped-flow apparatus, is accelerated by the same range of valinomycin concentrations which enhance the extent of KCl-induced absorbance changes. The data imply that the increase in the absorbance change is a result of the enhancement of the rate of appearance of that change. The rate of the faster decay phase is unaffected by valinomycin or nigericin (Table I).

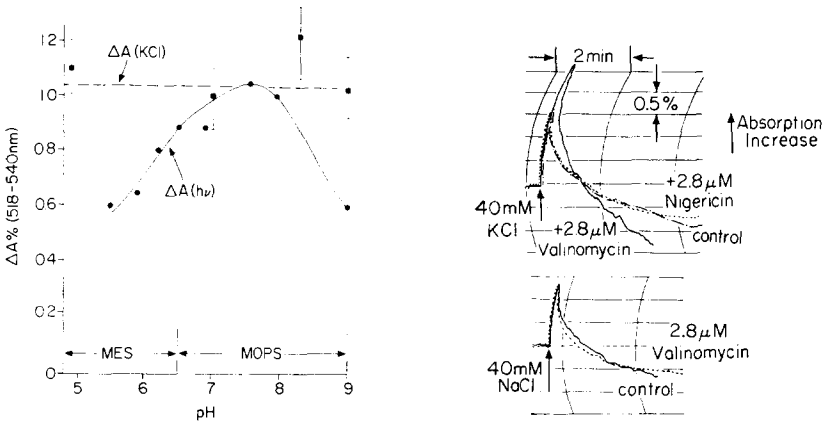


Fig. 7. The pH dependence of light- and salt-induced 520-nm absorbance changes. The cuvette contains 0.01 M MOPS or MES, as indicated, and chloroplasts at 51 μg chlorophyll/ml. For KCl-induced changes the final KCl concentration was 0.04 M.

Fig. 8. Enhancement of KCl-induced absorbance changes by valinomycin. Chloroplast fragments at 70 μg chlorophyll/ml suspended in 0.005 M glycylglycine, pH 6.5. The measuring wavelength pair is 518–540 nm.

Valinomycin also affects the light-induced absorbance changes at 520 nm. Even in the presence of only endogenous ions (K^+), increasing the concentration of valinomycin inhibits the appearance of "$A_{520\,nm}$" caused by continuous illumination. This is shown by the curve labeled $\Delta A(h\nu)$ in Fig. 9. The same concentrations of valinomycin which inhibit "$A_{520\,nm}$" activated by continuous illumination and accelerate the decay rate of laser-activated "$A_{520\,nm}$" enhance the KCl-induced absorbance changes[23].

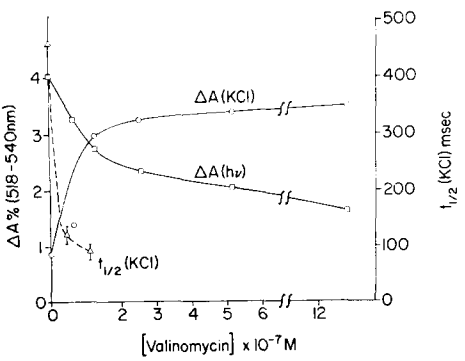
Valinomycin also modifies the dependence of absorbance changes on the concentration of KCl. Fig. 10 shows that in the presence of valinomycin absorbance changes vary linearly with the logarithm of the concentration of added KCl. The value of KCl which gives a zero absorbance change is 1.8 ± 0.4 mM KCl.

The ability of valinomycin to modify absorbance changes caused by KCl and those caused by illumination is not permanent, but disappears slowly, vanishing at about four hours after chloroplast isolation.

Effects of sonication of intact chloroplasts on 520-nm responses

Sonication of intact chloroplasts drastically changes the nature of 520-nm absorbance response. Both light-induced and KCl-induced absorbance changes are diminished, but the latter response shows a transient increase in samples sonicated for relatively short periods. The eventual inhibition is probably due to the destruction of

the thylakoid membrane. The transient increase in the salt-induced change indicates that the thylakoids, previously contained in the intact chloroplasts, are being exposed by a mild sonication which destroys the more fragile outer chloroplast membrane without seriously damaging the grana. Sonication for periods longer than 6 sec changes completely the direction and the kinetics of the monovalent cation response. Even so, some light-induced activity still persists, indicating that the processes driving light-induced absorbance changes at 520 nm can be differentiated from those which drive the salt-induced absorbance changes.



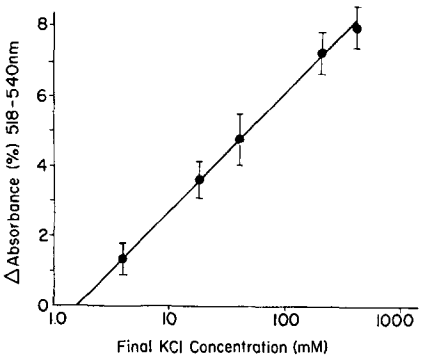


Fig. 9. Effect of increasing valinomycin concentration on the rate and extent of KCl-induced absorbance changes and on the extent of light-induced absorbance changes. Final KCl concentration is 0.02 M in a suspension of chloroplast fragments at 50–70 μg chlorophyll/ml in 0.01 M MOPS, pH 7.2.

Fig. 10. Concentration dependence of KCl-induced absorbance changes in the presence of valinomycin. Chloroplasts at 62 μg chlorophyll/ml in 0.01 M MOPS, pH 7.06. Valinomycin concentration was $8 \cdot 10^{-7}$ M.

Relationship between salt-induced and light-induced 520-nm changes

The absorbance changes caused by monovalent cation addition and those caused by light do not appear to compete with each other. If salt-induced absorbance changes are produced during illumination of the suspension, the decay rate of the change is faster than it would be in the dark. However, the extent of the salt-induced change is independent of the extent of the light-induced change upon which it is superimposed. Over a range of light intensities, from dark to saturating illumination, the extent of salt-induced absorbance changes is constant.

Finally, the salt-induced change is insensitive to electron transfer inhibitors, such as DCMU, at concentrations which are capable of abolishing 90 % of the light-induced absorbance changes.

DISCUSSION

The features of salt-induced absorbance changes and light-induced absorbance changes are collected and compared in Table III. Both DCMU and pH limit the electric field by inhibiting electron transfer[21] and the subsequent membrane potential associated with charge separation and ionic gradients. Hypotonicity causes a decrease in steady-state "$A_{520\,nm}$" by swelling the thylakoids, increasing ion permeability

TABLE III

A COMPARISON OF THE RESPONSES OF LIGHT AND SALT-INDUCED 520-nm ABSORBANCE CHANGES TO VARIOUS EFFECTS

+, increase of $\Delta A_{520\ nm}$; −, decrease of $\Delta A_{520\ nm}$; o, no effect on $\Delta A_{520\ nm}$.

Effect	*Salt*	*Light*
Electron transfer inhibitor (DCMU)	o	−
pH	o	−
Extraction	−	−
Hypotonicity	−	o
Ionophores		
Valinomycin	+ (KCl)	−
Nigericin	o (KCl)	o
Divalent cations	−	−
Localized in thylakoid	Yes	Yes

through the membrane, thus allowing faster discharge of the field. Ionophores act similarly, by catalyzing ion transport as noted below.

While extraction removes both light- and salt-induced absorbance changes at 518 nm it affects the latter only partially at 475 nm (Fig. 4). Even after removal of β-carotene there is a large absorbance change from 450 to 500 nm resulting from KCl addition. The shape of the remaining difference spectrum suggests that other carotenoids and/or chlorophyll contribute to the KCl-induced changes at shorter wavelengths (475 nm band) but make little contribution above 500 nm.

There are several phenomena capable of producing the salt-induced absorbance changes. Among these are osmotic swelling and shrinking, conformational changes resulting from an increase in the ionic strength of the medium or from binding of ions, and responses to an ion-induced electric field within the membrane phase. The last phenomenon is the most probable one based on the reported evidence that: (1) The absorbance changes require intact vesicles. (2) Addition of osmotically active solutes which are uncharged produces little absorbance change. (3) Salt-induced changes are enhanced by antibiotics which specifically increase the permeability of the cation being added. (4) Antibiotics which catalyze a neutral uptake (exchange) of cations have no effect on the absorbance changes.

If it is assumed that the absorbance at 520 nm responds to an electric field in the membrane, then the experimental results might be explained by either a dffusion potential model or one which considers the interaction between added electrolytes and fixed charges on the membrane. We shall discuss these two mechanisms in this order. Following that, we will argue the possibility that the 520-nm absorbance change is an electrochromic shift of carotene absorbance bands caused by the electric field.

First, we consider the probability of the formation of a diffusion potential. If they are permeant, the added ions will diffuse through the membrane producing a potential. Our data is consistent with a model in which the cations of the added salt are more permeant than the anions since valinomycin enhances absorbances changes. In this view the initial absorbance increase corresponds to a faster influx of cations, causing the potential inside to become more positive with respect to the outside. The slower permeation of anions tends to reverse the direction of the potential change and to decrease the absorbance change.

Furthermore, an increase in cation permeability affects only the rate of the fast aborbance decrease (Table I). The symmetry between the spectra of the absorbance increase and the fast absorbance decrease also imply that they reflect opposite changes of a common factor.

According to this diffusion potential model the voltage across the thylakoid membrane ($E_{in} - E_{out}$) first increases from the cation uptake and then decreases from the anion uptake. At the peak of the absorbance increase the rate of voltage change is zero and the net current density should be zero. Under these circumstances the membrane potential due to ion distribution is described by the constant field Eq. (1):

$$E_{\text{outside}-\text{inside}} = \frac{RT}{F} \ln \frac{\sum_{C} P_C[C]_i + \sum_{A} P_A[A]_0}{\sum_{C} P_C[C]_0 + \sum_{A} P_A[A]_i} \tag{1}$$

where P_C and P_A are the permeabilities of cation C and anion A, respectively, and $[\ ]_i$ and $[\ ]_0$ represent inside and outside electrochemical activities, and R, T, and F have their usual meanings[24,25]. If one of the ions is much more permeant than the others, a condition best approximated by KCl addition in the presence of valinomycin[26,27], Eq. (1) is approximated by the Nernst equation

$$E_{\text{outside}-\text{inside}} = \frac{RT}{F} \ln \frac{[C]_i}{[C]_0} \tag{2}$$

Indeed, in the presence of valinomycin the extent of the maximum absorbance change varies linearly with the logarithm of the concentration of added KCl (Fig. 10). If we assume that Eq. (2) is applicable under these conditions, then Fig. 10 may be considered as its graphical analogue, and the measured absorbance change can be calibrated on a membrane potential scale, in the manner of JACKSON AND CROFTS[15].

The maximum light-induced absorbance change, equal to approximately 0.008 absorbance unit, is reproduced by an addition of 6.5 mM KCl (Fig. 10). Taking the concentration of K^+ inside the thylakoid as 2 mM, 2 % absorption change corresponds to a membrane potential of about 30 mV.

In contrast to potential changes from ion diffusion, addition of an electrolyte can change the electric field within the membrane by screening fixed charges on the membrane surface. For example, increasing the electrolyte concentration, C, on one side of the membrane increases the space charge of cations around a fixed layer of anions. Although the net potential difference between bulk solutions on either side of the membrane will not change, the potential from one membrane surface to another, V_0, will decrease as C increases[28].

Approximate functions have been obtained for the relation between the transmembrane potential and concentrations of screening ions[29]. But calculations based on these equations are difficult with the data presented here since necessary parameters such as surface charge density have not been evaluated.

At best we can suggest a maximum value of ΔV_0 due to electrolyte screening of surface charges. Assuming a value for σ of one e^- per 50 $Å^2$, a figure derived from model phospholipid membranes studies[30], the maximum potential change is approximately 30 mV. Both the screening of surface charges and the diffusion of ions might cause changes in membrane potential. However, the latter phenomenon in the presence of

valinomycin is evidently the predominant affector and leads to more interpretable results.

How is an electric field able to change absorbances of intramembrane pigments? JUNGE AND WITT[12] have proposed that an electric field in the thylakoid membrane causes absorbance changes *via* an electrochromic, or Stark, shift. The first-order electrochromic shifts in optical transitions, Δ, are related to the difference in dipole moments between excited and ground states, $\vec{\mu}^* - \vec{\mu}$, and the local electric field strength, $\vec{\varepsilon}$, by the equation

$$\Delta = (\vec{\mu}^* - \vec{\mu}) \cdot \vec{\varepsilon} \tag{3}$$

Second order terms, proportional to ε^2, do occur but these are usually several orders of magnitude smaller than the first-order terms[31].

Previously we have shown that β-carotene is necessary for the 520-nm change[14] (also *cf.* ref. 11) and that the absorbance spectrum of this carotenoid in the chloroplast differs from its spectrum in solution (Fig. 11A).

Resolution of the spectrum in Fig. 11A on a Dupont curve analyzer shows that in the chloroplast the long wavelength peak is shifted by 20 $\pm$ 2 nm to the red and broadened by 5 nm, compared to its parameters in solution, whereas the other two peaks are shifted by 12 $\pm$ 2 nm to the red while their widths remain constant, within $\pm$ 2 nm. This red shift is analogous to the spectral change in retinal upon its incorporation into the rhodopsin complex[32]. The broadening and the relative increase of oscillator strength of the long wavelength β-carotene band may be accounted for by the same factors which cause the red shift of retinal[33–35]. We will assume that the

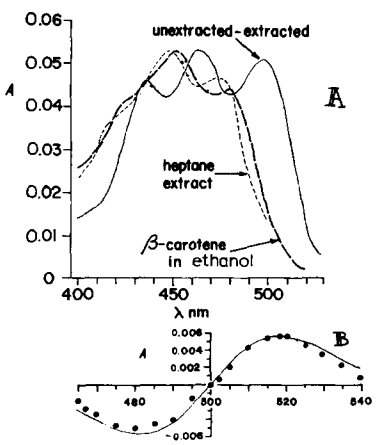


Fig. 11. A. Absolute spectra of heptane extract from chloroplasts and solution of β-carotene in ethanol compared to difference spectrum of unextracted–extracted chloroplasts. The spectra have been scaled to give the same absorbance at the maximum of the middle peak. B. The solid line is a theoretical difference spectrum calculated by shifting a Gaussian curve, which fits the far red peak of the difference spectrum in A, towards longer wavelengths by 8 Å and then subtracting the original, unshifted curve from it. The points are from one experimental light–dark difference spectrum in chloroplasts. The theoretical curve has been multiplied by the ratio of chlorophyll concentration (in the light–dark assay) to chlorophyll concentration (in the unextracted–extracted samples) (78/13 = 6.0).

long wavelength peak of β-carotene directly causes the 520-nm change. The agreement between results based on this assumption and experimental data is reinforcing.

The long wavelength absorbance band can be fitted by a Gaussian curve of the form:

$$A(\lambda) = \frac{I}{(2\pi\sigma^2)^{1/2}} \exp\left[-(\lambda - \lambda_0)^2/2\sigma^2\right] \tag{4}$$

where λ is the wavelength of the measured absorbance, A, λ_0 is the peak wavelength, and σ is a wavelength difference such that the area under a section of the curve taken σ on each side of λ_0 is 68 % of the total area; for this band $\sigma = 18 \pm 2$ nm. The change in A at a fixed wavelength, λ, resulting from a small shift of λ_0, $\Delta\lambda_0$, is greatest at a distance $\pm\sigma$ from λ_0, and is given by Eq. (5):

$$\Delta A(\lambda) = \frac{(\lambda - \lambda_0)(\Delta\lambda_0)}{\sigma^2} A(\lambda) \tag{5}$$

By shifting the undistorted long wavelength band 8 Å to the red, the light-induced absorbance changes are simulated (Fig. 11B). From Eq. 3 the calculated dipole difference to give an 8 Å electrochromic shift is 19 debyes in a field of 10^5 V/cm. This field strength is the order of magnitude corresponding to a potential difference of 100 mV across a unit biological membrane 50 to 100 Å wide. The transition dipole of 19 debyes is large for the usual $\pi \rightarrow \pi$ transitions which characterize conjugated hydrocarbons, but could be due to the delocalization of excited state electrons over as large a molecule as β-carotene[36].

Throughout these calculations we have assumed that the β-carotene moiety of the chloroplast responded as a homogeneous pool to a uniform electric field. Indeed, studies of maize chloroplasts have shown that 99 % of the β-carotene is extractable as a homogeneously bound pool[37]. AMESZ AND VREDENBERG[38] have calculated that carotenoid absorbance changes in *Rps. spheroides* can be accounted for by shifting 8–12 % of the pigment 10 nm to the red. While large shifts of small amounts of pigment can mimic peak absorbance changes, such shifts result in detectable changes of the difference spectrum isosbestic point[39]. Since isosbestic points appear constant for all intensities of carotenoid difference bands in bacteria as well as green plants (480-, 518-nm bands), large shifts of small amounts of pigment are not possible.

BARBER AND KRAAN[40] have observed an enhancement of fluorescence induced by salt addition to pre-illuminated chloroplast suspensions similar to the enhancement triggered by acid–base transitions. They propose, after MAYNE[42], that a salt-induced membrane potential triggers the release of light from some metastable state. Our results do not bear directly on these fluorescence studies, but they do support the concept of a light-driven membrane potential. Such potentials can affect a redistribution of the energy of the metastable state among reactions in the membrane phase by (1) directly contributing to the energetics of reactions, such as dismutations or dipole formation–dissipation reactions, and changing their equilibrium free energy; (2) directly affecting the rates of competing reactions by energizing or de-energizing transition states; or (3) indirectly influencing such reactions by influencing the immediate environment of the reaction. An example of the last case might be the potential-induced volume decrease of the membrane phase which contributes to the disposition

of reaction equilibria through pressure volume ($P\Delta V$) terms[43]. To determine which of these mechanisms is most influential is impossible in the absence of knowledge about the metastable state which activates delayed light.

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