ON THE CALIBRATION OF THE CAROTENOID BAND SHIFT WITH DIFFUSION POTENTIALS

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1. Introduction

Carotenoid absorption changes have been used widely as indicators of membrane potential in chloroplasts and photosynthetic bacteria [1–5]. In their classical paper Jackson and Crofts [6] introduced the method of calibrating these absorption changes by diffusion potentials of K^+ in the presence of valinomycin. However, considerable discrepancies exist between values obtained with this method and those obtained by other indicators of membrane potential [7–10], the former giving much larger values than any other method used.

In this work we investigated the effect of various parameters such as pH, concentration of valinomycin and initial concentration of permeable ion on the ΔA induced by a diffusion potential and tried to optimize the experimental conditions.

2. Methods

Rhodopseudomonas capsulata wild-type was grown anaerobically in the medium described [11] and harvested after 2 days. Chromatophores were prepared as in [12] except that they were sonicated twice for 30 s separated by a 5 min period.

Abbreviations: ΔA, absorption change; DMSO, dimethylsulfoxide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TEA, tetraethylammonium; MOPS, 3-(*N*-morpholino) propane sulphonic acid; MES, 2-(*N*-morpholino) ethane sulphonic acid

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The chromatophores were resuspended in a minimal volume of the washing medium (which was 10 mM, half the molarity of the preparation medium, which was 20 mM) to which 10% DMSO was added, and stored under liquid nitrogen. The buffers used in the preparation medium were the same as those composing the different measuring media, as stated in the text. Valinomycin concentration, except for the experiments stated in table 2, was 0.5 μM.

The measurements were carried out as in [12] with a Cary-17 spectrophotometer. The suspension was allowed to equilibrate for ~20 min, in the presence of valinomycin. Different solutions of the ions to be added were prepared at 3–0.015 M. In this way the same volume could be administered for each pulse. Corrections were made for dilution artefacts. A magnetic stirrer was used. The mixing time was <0.5 s. The experiments were carried out at room temperature.

Light-induced $\Delta\Psi$ was calculated by comparing carotenoid ΔA changes in the light with the ΔA induced by a 10-fold change in concentration of the permeable ion, which corresponds to 59 mV (see eq. (1)).

3. Results and discussion

3.1. *Kinetics of the diffusion potential*

Figure 1 shows the decay kinetics of Rb^+ diffusion potentials at different concentrations of RbCl. The diffusion potential-induced ΔA reached its maximum in <2 s after administration of Rb^+ or K^+. The decay of the change was predominantly biphasic; the relative extent of the phases depended on the valinomycin

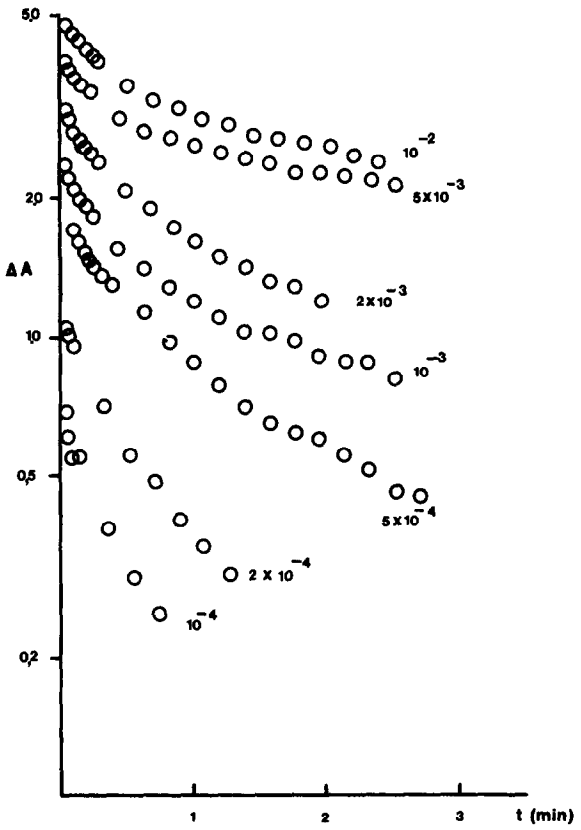

Fig.1. Kinetics of diffusion potentials, given by ΔA_{530}, for different final concentrations of RbCl (in M). Chromatophores from *Rps. capsulata* wild-type were suspended to 10 μg/ml bacteriochlorophyll in a medium containing 3.7 mM MES, 1.3 mM succinic acid and 3.5 mM NaCl. pH was 5.4. ΔA is in arbitrary units.

concentration, the half times on the size of the diffusion potential. Some variation could be observed from preparation to preparation, but, in general, for diffusion potentials in the order of 120 mV the half decay times were 10 s and 6 min for the fast and the slow phases, respectively. For diffusion potentials of 50 mV, half decay times of 8 s and 2 min were observed. The relative extent, however, remained constant over this region.

We believe that the fast phase reflected the concomitant outflow of H^+ and inflow of Rb^+ (or K^+) and that thereafter both the H^+ and the Rb^+ (or K^+) gradients were in equilibrium with the transmembrane potential, which decayed very slowly, due to the flow of other ions (the slow phase). This assumption was supported by the observation that CCCP did not affect the slow phase at all. Low concentrations of CCCP accelerated the fast phase only, but at 1 μM the extent of the fast phase was also inhibited.

In order to calculate the extent of the ΔA induced by a diffusion potential we extrapolated the changes to time zero. In this way, errors of the estimation of the diffusion potentials, due to the fast decay (which, moreover, depended on the size of the change) could be avoided.

3.2. *The optimal valinomycin concentration*

Even in the absence of valinomycin, diffusion potentials were obtained which were a mere 10–20% smaller than those obtained in the presence of an optimal concentration of valinomycin (see table 1). From the table it is also obvious that the optimal valinomycin concentration is different for Rb^+ as compared to K^+. This concentration difference cannot be explained by the affinity difference of

Table 1
Diffusion potentials given by increase in A_{530} after addition of 10 mM KCl or RbCl

Valinomycin (μM) :	0	0.1	0.5	1.0	2.0
K^+ diffusion potential	1.55	1.57	1.81	1.64	1.59
Rb^+ diffusion potential	1.97	2.00	1.91	1.78	1.68
Extent slow phase in % of total Rb^+ diffusion potential	74	82	66	53	42

ΔA are indicated in % of A_{510} (maximum of the band). Measuring medium: MOPS, 5 mM; Na^+ 3.5 mM (pH 7.0). Chromatophore 10 μg/ml bacteriochlorophyll

valinomycin for Rb^+ and K^+, which is no more than a few percent [13,14].

Probably, even in the presence of the ionophore, the intrinsic permeability of the chromatophore membrane can contribute to the total ionic permeability. However, since it has been suggested that complexation of valinomycin with the cation occurs rather close to the hydrocarbon core of the membrane [15], even in the case of 'pure ionophoric' cation transport the intrinsic membrane permeability can be a factor which has to be taken into account.

The optimal valinomycin concentration difference between Rb^+ and K^+ gradients could explain the observation that the slope of the calibration curve for a K^+-diffusion potential was ~25% smaller as compared to the case of Rb^+ [16]. When NaCl was used to correct for the ionic strength changes (see below) the discrepancy between the K^+ and Rb^+ values became <10%, which was also the case when TEA, an agent known to block K^+-channels in nerve membranes [17], was added.

The permeability difference between Rb^+ and K^+ led us to the conclusion that diffusion potentials measured with Rb^+ concentration gradients are more likely to approach the ideal value of 59 mV for a 10-fold concentration difference. Furthermore, we observed a variability in intrinsic membrane permeability for different batches of chromatophores, so that it seems to be desirable to determine optimal values of the valinomycin concentration for each preparation.

3.3. *Effect of surface potential*

As shown in [20] carotenoid ΔA can be observed upon alteration of the surface potential. Since by administration of a solution of RbCl to the sample, the ionic strength, and concomitantly the surface potential, is altered, one expects the carotenoid ΔA produced by a diffusion potential to consist of two parts:

(i) An absorption increase (at the given measuring wavelength, which in the case of *Rhodopseudomonas capsulata* wild-type is 530 nm) due to the generation of the transmembrane potential itself;

(ii) A decrease, due to the alteration of the initially negative surface potential [18,19].

The contribution of the surface potential diminishes the slope of the calibration curve. It is possible to correct for this error, by taking care that the added solutions always have the same ionic strength, as shown in fig. 2. The ion used to compensate for Rb^+ or K^+ was Na^+, which does not permeate through the chromatophore membrane. The influence of the correction for the surface potential changes on the slope of the calibration curve is a function of pH as shown in table 2, pH 5 being about isoelectric. This is comparable with the observations in [19,20]. It follows that at high pH, where surface charges are less screened, the correction is very important.

In order to calculate the correct intercept with the concentration axis, one has to estimate the ΔA caused by the alteration of the ionic strength and add this value to each point. Since for the concentration range of interest the effect is linear with the logarithm of the concentration [21], this correction is easily applied. As is apparent from table 2, even in plots were we did correct for surface potential

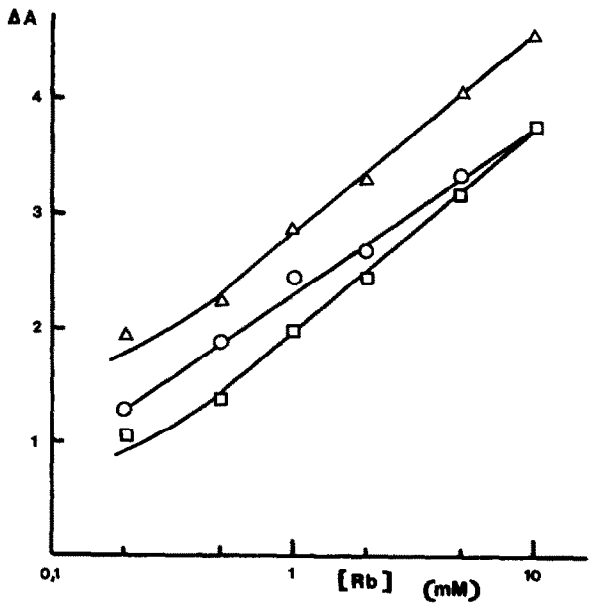

Fig.2. ΔA_{530} induced by Rb^+ diffusion potentials. Circles: chromatophores from *Rps. capsulata* wild type, suspended in 5 mM MES and 3.5 mM Na^+ (pH 6). Squares: same as circles but with the ionic strength of the added solution kept constant with NaCl. Triangles: calibration curve corrected for the surface potential changes (i.e., same as squares but shifted by the amount due to the surface potential change). Other conditions as in table 1.

Table 2
Diffusion potentials as given by the slope of the calibration curve in arbitrary units

pH values:	8	6	5.4	5
NaCl corrected Rb^+ diffusion potentials	1.97	1.82	1.37	1.10
Uncorrected Rb^+ diffusion potentials	1.35	1.41	1.27	1.04

Chromatophores of *Rps. capsulata* wild-type were prepared in 20 mM MOPS (pH 6.9). Measuring media contained for: pH 8, Tricine, 5 mM; pH 6, MES, 5 mM; pH 5.4, 3.4 mM MES and 1.6 mM succinic acid; pH 5, 2.5 mM MES and 2.5 mM succinic acid. Total Na^+ conc. was 4.5 mM for all the measuring media. Other conditions as in table 1

changes a variation of the slope with pH was observed. This could have been caused by the existence of a H^+-diffusion potential created by taking up the chromatophores (which all were sonicated at pH 6.9) in the respective suspension media. Furthermore, we believe that the calibration value for pH 5 was less reliable because the intercept with the concentration axis of the curve deviated considerably from those measured at other pH values.

3.4. *Internal concentration*

As shown in fig. 3, the calibration curve can deviate considerably from linearity at small diffusion potentials. This effect has been analysed [22], where it was described by the following equation:

$$\Delta\Psi = \frac{RT}{F} \ln \frac{[Rb]_o + [Rb]_{add}}{[Rb]_o + \frac{C\,\Delta\Psi}{F\,V_i}} \qquad (1)$$

where $\Delta\Psi$ is the membrane potential difference, $[Rb]_o$ the initial concentration of Rb^+, which is assumed to be equal inside and outside the chromato-

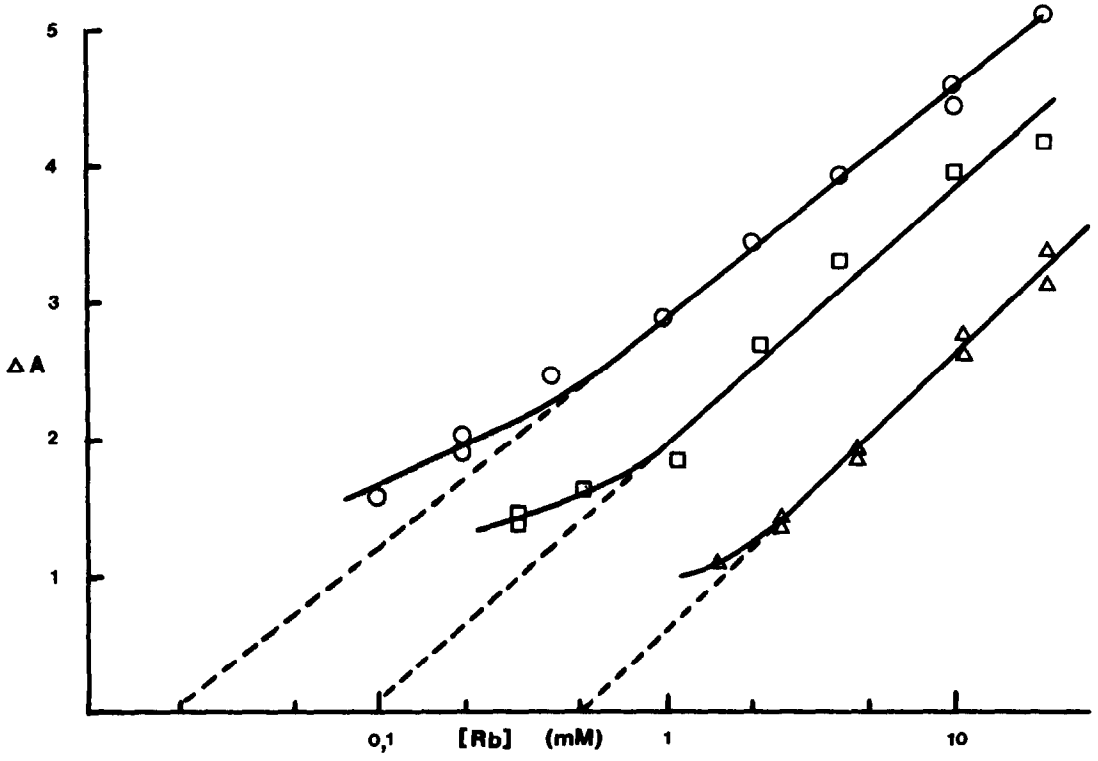

Fig.3. ΔA_{550} induced by diffusion potentials at varying initial concentrations of Rb^+. Triangles: 0.5 mM RbCl. Squares: 0.1 mM RbCl. Circles: no added RbCl. Chromatophores from *Rps. capsulata* were washed and prepared with Rb^+ and K^+ free buffer. The suspension medium contained in addition 2.5 mM MOPS and 2.5 mM MES (pH 6.2). Other conditions as in table 1.

phores and $[Rb]_{add}$ the concentration of Rb^+ added during the pulse. V_i is the inner volume of a chromatophore, C the capacitance of the chromatophore membrane, and F, R and T are the Faraday and gas constant and absolute temperature, respectively.

Some additional conclusions can be drawn from the solution of this equation: for small initial concentrations of Rb^+ linearity is only obtained for relatively high diffusion potentials and furthermore, a feature which is more dramatic, the slope of the calibration curve in the pseudolinear region only approaches the ideal value of 59 mV to within 20%. For initial concentrations of Rb^+ of 1 mM the situation is much better and the calibration value has an error of less than a few percent. These features are clearly shown in fig. 3. Linearity is obtained for diffusion potentials at >40 mV for initial concentrations of 0.5 mM Rb^+ and for potentials >80 mV when no RbCl was added. The slope of the former is ~15% larger than is that of the latter.

4. Conclusion

As one can verify, all these corrections are additive so that it is not amazing that values seen in the literature for the light-induced membrane potential as calibrated with diffusion potential-induced carotenoid ΔA, corrected in the proper way, approach those obtained with other methods.

If we assume, for example, that the ΔA are continuously monitored during the application of the diffusion potential, and that the initial concentration of valinomycin is not chosen optimally, a supis <50 μM (i.e., the chromatophores have been prepared in the absence of K^+ or Rb^+) a correction of 15% is needed (see section 3). If the concentration of valinomycin is not choosen optimally, a supplementary correction of 10% is needed. Even if measurements are carried out at pH 7, without keeping the changes in ionic strength equal for all the points measured, the diffusion potentials are underestimated by 30%. In total this gives way to an overestimation of the electrical potential generated in the light by a factor 2. Peak values of 400 mV, currently reported in the literature would turn out to be only 200 mV after correction. In our hands, for *Rhodopseudomonas capsulata* wild-type chromatophores peak values of 210 mV were measured, yielding steady state potentials (after 7 min continuous illumination) of 70 mV. Both the initial and steady state potentials have errors of ~15 mV.

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