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MEASUREMENT OF TRANSMEMBRANE POTENTIALS IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES WITH AN OXACARBOCYANINE DYE

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SUMMARY

The fluorescent dye 3,3-dipentyloxacarbocyanine (OCC) can be used as a fluorescence probe to measure transmembrane potentials across *Rhodospirillum rubrum* chromatophore membranes. A reversible fluorescence increase is observed in the light which is sensitive to inhibitors, permeable ions and uncouplers.

Partial interchangeability between the electrical potential and the proton concentration gradient has been demonstrated by measurement of the fluorescence increase with OCC and the fluorescence quenching with 9-aminoacridine.

OCC fluorescence changes can be induced also in the dark by injection of permeable salts and by rapid pH changes presumably indicating diffusion potentials. Using salt-induced diffusion potentials for calibrating the light signals and with several assumptions, the light-induced potentials were estimated as 170 mV for the maximal signal and 90–110 mV at the steady state.

OCC has been shown to apparently increase the electrical conductivity of the chromatophore membrane, a fact which may be relevant to the mechanism of action of this probe.

A red shift in the OCC absorption spectrum occurs when mixed with chromatophores, with a difference spectrum maximum at 495 nm. The absorption changes at 495 nm taking place in the light are similar in kinetics to the fluorescence changes. The absorbance spectrum of OCC in organic solvents is red shifted and the extent of the shift depends on the hydrophobicity of the medium. The difference spectrum compared to water in *sec*-butyl acetate/*n*-hexane (3 : 1, v/v) with a dipole moment of 5 was nearly identical to that of chromatophore-associated dye.

The uncoupling properties of OCC at high concentrations and some difficulties in calibration limit the usefulness of this probe for quantitative measurements of transmembrane potentials.

Abbreviations: OCC, 3,3 dipentyloxacarbocyanine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; PMS, *N*-methylphenazonium methosulfate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCPIP, dichlorophenolindophenol; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; SF-6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; ANS, 1-anilino-8-napthalene sulfonate.

INTRODUCTION

Photophosphorylation in *Rhodospirillum rubrum* chromatophores is only partly inhibited by the presence of permeable ions like SCN^- and K^+ with valinomycin, or by amines and nigericin-type antibiotics alone [1, 2], but is synergintically uncoupled in the presence of both [3]. This suggested that in *R. rubrum* chromatophores, unlike chloroplasts [15], a transmembrane potential as well as a proton concentration gradient contribute to the proton motive force driving the phosphorylation of ADP, and further that some degree of interchangeability between the two parameters is possible. Several probes have been used to estimate transmembrane potentials in *R. rubrum* chromatophores, including carotenoid absorption shifts, the increase in 1-anilino-8-naphthalene sulfonate (ANS) fluorescence, the distribution of the permeable $S^{14}CN^-$ [7], and the uptake of phenyldicarbaundecarborane [8]. The estimated light-induced potentials vary from 90 [7] to nearly 250 mV [5] depending on the method used.

It was reported that cyanines, a synthetic group of fluorescent dyes can be used in different systems to measure transmembrane potentials [9–12]. This communication demonstrates that 3,3-dipentyloxacarbocyanine (OCC) can be used as a probe for measuring transmembrane potentials in *R. rubrum* chromatophores. Changes in OCC fluorescence can be observed either upon illumination or in response to diffusion potentials induced by salt injection or pH changes.

MATERIALS AND METHODS

Bacterial chromatophores from *R. rubrum* were prepared and stored as previously described [13]. Before use, chromatophores containing 1 mg bacteriochlorophyll were washed by suspension in 12 ml containing: 100 mM choline chloride, 5 mM $MgCl_2$, and 15 mM sodium tricine, pH 7.5, at 4 °C for 10 min. The chromatophores were then sedimented at 108 000 $\times g$ for 90 min and resuspended in a minimal volume of washing medium.

Cyanine fluorescence and 9-aminoacridine fluorescence were measured in an Eppendorf fluorimeter. The exciting light was filtered through a 405–436 filter. Emission was measured through a Corning CS4-96 filter, a Strand Electric Co. cinemoid filter No. 62 and a Wratten No. 58 filter. Actinic light was provided by a 24 V halogen lamp, filtered through a Schott RG 715 filter and 9 cm of water.

Absorption changes were measured in a Cary 16 spectrophotometer. The difference spectrum for OCC in the presence and absence of *R. rubrum* chromatophores was measured with partitioned cuvettes: in the reference cuvette, OCC and chromatophores were put separately into the different cell compartments and in the sample cuvette the dye and chromatophores were mixed.

Proton uptake, ATP formation and ATPase activity were measured with a Radiometer GK 2321C pH electrode connected to a Radiometer PHM64 pH meter.

OCC was a generous gift from Professor A. Waggoner, Amherst College, Massachusetts.

RESULTS

Illumination of *R. rubrum* chromatophores in the presence of OCC leads to an increase in cyanine fluorescence which is reversible in the dark (Fig. 1). The direction

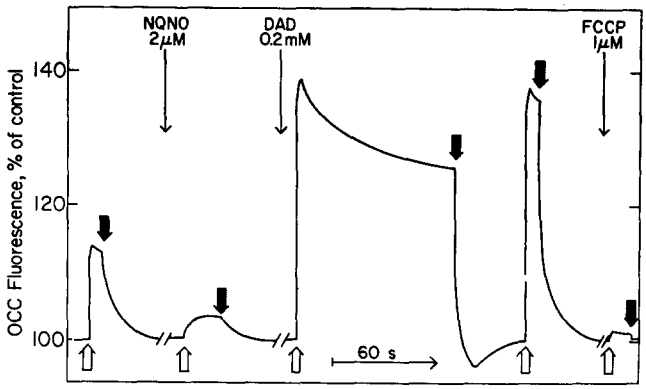

Fig. 1. Light-induced fluorescence changes of OCC. The reaction mixture contained in 3 ml: 25 mM choline chloride, 15 mM sodium tricine, 5 mM $MgCl_2$, chromatophores containing 22 μg bacteriochlorophyll and 3.3 μM OCC, pH 7.5. The indicated additions were administered in a small volume on a glass rod. Light arrow, light on; dark arrow, light off.

of the change indicates that the inside becomes more positive in the light and is in keeping with an inwardly directed electrogenic proton pump. As was previously shown in erythrocytes, liposomes and squid axon equilibrated with cyanines, the creation of a transmembrane potential negative inside leads to quenching of cyanine fluorescence whereas a potential difference positive inside enhances the fluorescence [9, 10].

Addition of the electron transport inhibitor (NQNO) inhibits the light-induced fluorescence enhancement. Further addition of diaminodiurene (DAD), an electron carrier that by-passes the NQNO site of inhibition and accelerates photophosphorylation, restores and markedly increases the signal. The uncoupler FCCP completely inhibits the fluorescence signal. Two phases can be clearly distinguished; a fast increase in fluorescence followed by a slower decrease towards a steady state of about 50–70 % of the initial peak. In the dark, the fluorescence level rapidly decreases to the initial level. This cycle can be repeated for at least five times with no significant change.

Fig. 2 illustrates the kinetics of light-induced fluorescence changes in a chromatophore suspension with two probes: OCC, presumably indicating a transmembrane potential (see below), and 9-aminoacridine, which has been previously reported to provide a quantitative measure of the proton concentration gradients (ΔpH) across liposomes [14], chloroplasts [15] and chromatophore membranes [16]. The kinetics of 9-aminoacridine quenching can be seen to resemble the kinetics of the decrease in OCC fluorescence towards the steady state in the light. This transition may therefore represent the interchange between a decreasing membrane potential and an increasing ΔpH. A similar interchange has been described earlier [5]. KCl in the presence of valinomycin decreased both the rate of the "on" and "off" response of OCC and its steady-state level. Simultaneously, it accelerated the kinetics of 9-aminoacridine responses and increased the quenching of 9-aminoacridine. These effects may be expected if we assume that KCl and valinomycin permit a rapid H^+/K^+ exchange to take place and so converts $\Delta\psi$ into ΔpH. KCl alone caused similar changes, but was less effective than KCl plus valinomycin.

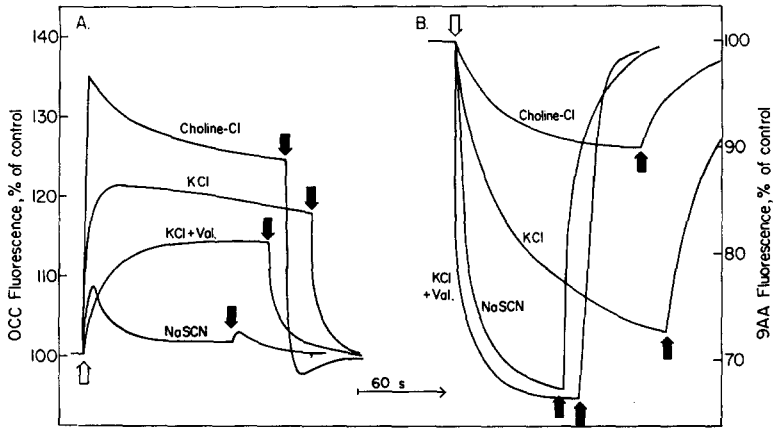

Fig. 2. The effect of permeable ions on the light-induced fluorescence signals with OCC and 9-aminoacridine. The reaction mixtures contained in 3 ml: 15 mM sodium tricine, pH 7.5, chromatophores containing 20 μg bacteriochlorophyll, and where indicated the following: 100 mM choline chloride, 100 mM KCl, 80 mM choline chloride, 20 mM NaSCN, 1 μM valinomycin. In A, 3.3 μM OCC, and in B, 1 μM 9-aminoacridine were added.

Similar overall effects were also observed by addition of the permeable SCN^-. However, the kinetics of the OCC change were markedly different and the steady-state level was very low indicating a poorer compensation between ΔpH and $\Delta\psi$.

The "off" responses often showed positive or negative overshoots (Figs. 1 and 2). These may indicate temporary hyperpolarization due to a diffusion potential induced by fast efflux of protons or thiocyanate that accumulated inside in the light.

Table I summarizes the effect of electron transport carriers, electron transport inhibitors and uncouplers on OCC and on 9-aminoacridine signals. The electron carriers DAD, PMS, TMPD or ascorbate plus DCPIP similarly stimulate the transmembrane potential and the ΔpH. The electron transport inhibitors, antimycin A, *o*-phenantroline, DBMIB and NQNO, inhibit both the endogenous transmembrane potential and ΔpH, but, as expected (see Fig. 1), the DAD- and PMS-stimulated signals are insensitive to NQNO and antimycin A.

The various uncoupling agents can be divided according to their mechanism of uncoupling into three groups: amines and nigericin-type antibiotics, which act primarily by catalyzing H^+/cation exchanges, inhibit primarily the ΔpH and at low concentrations did not markedly affect the transmembrane potential. Permeable ions, SCN^- and K^+ with valinomycin, inhibit the transmembrane potential and stimulate the ΔpH; and FCCP, gramicidin or combination of agents from the first two groups inhibit both the transmembrane potential and ΔpH. The latter are also the most efficient inhibitors of photophosphorylation in chromatophores.

The light-induced OCC fluorescence enhancement is nearly independent of pH in the range of 6.0–9.0, and of the osmolarity of the medium (from 30 to 500 mosM). Optimal signals are obtained at pH 7.0 and 60 mosmolar total salt concentrations.

The response of OCC to diffusion potentials generated across chromatophore membranes in the dark by injection of salts is illustrated in Fig. 3. Injection of KCl caused a rapid increase in fluorescence followed by a slower decay to nearly the initial

TABLE I

EFFECTS OF ELECTRON CARRIERS, ELECTRON TRANSPORT INHIBITORS AND UNCOUPLERS ON LIGHT-INDUCED OCC AND 9-AMINOACRIDINE FLUORESCENT CHANGES

The reaction medium was identical to that described in Fig. 1 except for the indicated additions. With all the uncouplers (III) DAD served as the electron carrier.

Additions	Salt present at 100 mM	OCC fluorescence		9-Aminoacridine fluorescence	
		Enhancement (%)	Control signal* (%)	Quenching (%)	Control signal* (%)
(I) Electron carriers					
None	Choline-chloride	4	14	6.1	43
DAD (0.2 mM)	Choline-chloride	25	86	17	122
PMS (66 μM)+succinate (0.5 mM)	Choline-chloride	29	(100)	14	(100)
TMPD (66 μM)	Choline-chloride	23	80	–	–
DCPIP (160 μM)+ ascorbate (1 mM)	Choline-chloride	13	45	9	65
(II) Electron transport inhibitors					
NQNO (2 μM)	Choline-chloride	0	0	0	0
Antimycin A (3 μM)	Choline-chloride	0	0	0	0
DBMIB (5 μM)	Choline-chloride	1	3	2	12
O-phenantroline (1 mM)	Choline-chloride	1	3	–	–
NQNO+DAD	Choline-chloride	30	104	17	100
NQNO + PMS + succinate	Choline-chloride	29	(100)	17	(100)
(III) Uncouplers	Choline-chloride				
–	or, NaCl	29	(100)	22	(100)
–	KCl	15	52	33	150
Valinomycin (1 μM)	KCl	0	0	35	160
NaSCN (20 mM)	Choline-chloride	6	21	32	146
NH_4Cl (0.5 mM)	Choline-chloride	26	90	5	23
NH_4Cl (5.0 mM)	Choline-chloride	14	48	0	0
Nigericin (1.7 μM)	NaCl	28	96	1	4.5
Nigericin (10 μM)	NaCl	15	52	0	0
FCCP (1 μM)	Choline-chloride	6	21	2	9
Gramicidin (1.7 μM)	NaCl	3	10	6	27
Valinomycin (1 μM)+ nigericin (1.7 μM)	KCl	0	0	0	0

* Percent of signal compared to the control: PMS+succinate (I and II) or DAD (III).

fluorescence level. This signal is in agreement with a transmembrane potential positive inside due to a higher permeability to K^+ than to Cl^-. For $NaNO_3$, where NO_3^- is the more permeable ion, a transmembrane potential negative inside is expected and the OCC signal is accordingly in the reverse direction. The fast decay with KNO_3 compared to KCl indicates that NO_3^- is more permeable than Cl^-, but K^+ is more permeable than either. NaCl causes only a slight signal indicating that both ionic species are relatively impermeable.

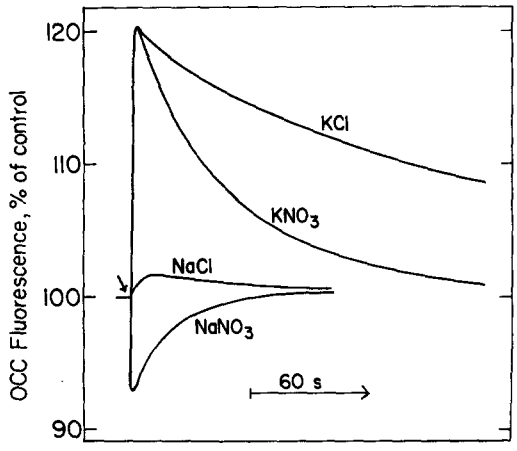

Fig. 3. Diffusion potentials induced by salt injection. The reaction mixture contained in 2.7 ml: 100 mM sorbitol, 2 mM $MgCl_2$, 10 mM Tris/succinate, pH 7.7, 3.3 μM OCC, and chromatophores containing 25 μg bacteriochlorophyll. At the point indicated by the arrow, salt was injected with a syringe in 0.3 ml to give a final concentration of 100 mM.

Table II summarizes the extents and half-times of decay for a variety of salts. Among the cations, rubidium appears to be the most permeable alkali cation, NH_4^+ is as permeable as K^+, whereas choline appears to be least permeable. Valinomycin increases K^+ permeability and monactin markedly increases Na^+ permeability as expected. The apparent high permeability of calcium may be an artifact due to specific

TABLE II

SALT-INDUCED DIFFUSION POTENTIALS

The preincubation medium contained in 2.7 ml: 10 mM $MgCl_2$, 3 mM sodium tricine, pH 7.5, 200 mM sorbitol, chromatophores equivalent to 25 μg bacteriochlorophyll and 3.3 μM OCC. Salt was injected in 0.3 ml.

Salt injected	Concentration (mM)	Addition to preincubation medium	OCC fluorescence (%) change	Decay half-time (s)
Choline chloride	100	–	0	–
LiCl	100	–	+ 4	–
NaCl	100	–	+ 4	–
NH_4Cl	100	–	+19	160
KCl	100	–	+20	150
CsCl	100	–	+23	–
RbCl	100	–	+25	–
KCl	100	valinomycin (1.7 μM)	+32	120
KCl	100	oligomycin (15 μg)	+20.5	25
NaCl	100	monactin (33 μg)	+24	60
$CaCl_2$	100	–	+ 6.5	30
NaN_3	100		0	–
$NaNO_3$	100	–	− 6.5	15
NaSCN	50		−10	–
NaI	50		−11	10

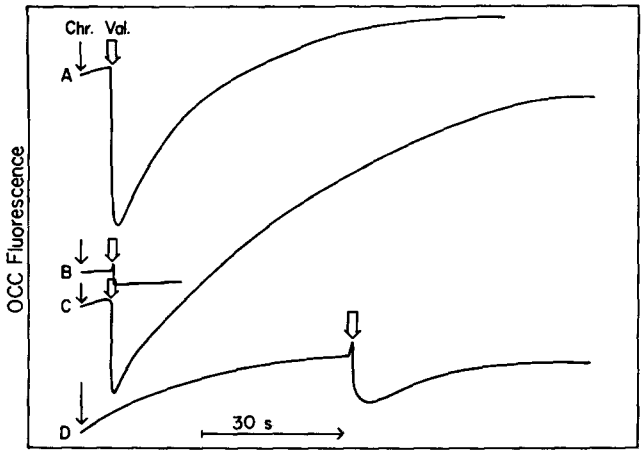

Fig. 4. KCl and valinomycin-induced diffusion potentials in "KCl-loaded" chromatophores. "KCl loaded" chromatophores were preincubated 15 min at 4 °C in a medium containing 100 mM KCl, 5 mM $MgCl_2$, and 15 mM sodium tricine. The chromatophores were then sedimented at 104 000 × *g* for 90 min and the pellet resuspended in 0.5 ml of a medium identical to the washing medium. The reaction medium contained in 2.7 ml; 25 mM choline chloride, 5 mM $MgCl_2$, 15 mM sodium tricine, pH 7.5, 3.3 μM OCC and chromatophores containing 19 μg bacteriochlorophyll. 100 mM KCl was included in B and 100 mM NaCl was included in C. Valinomycin was injected in 0.3 ml to a final concentration of 1.7 μM at the points indicated by the arrows.

interactions at the membrane level since $CaCl_2$ injection is accompanied by a large shift of the base-line fluorescence (20 % enhancement) and the decay is too fast to represent Cl^- influx. Among the anions CNS^-, I^- and to a lesser extent NO_3^- are the most permeable anions tested. Other permeable anions like ClO_4^-, dinitrophenol and tetraphenylboron were found to directly quench OCC fluorescence. Oligomycin was reported to increase permeability of mitochondria for Cl^- [17]. The acceleration of the decay of the diffusion potential with KCl is in agreement with this conclusion.

If the direction of the K^+ concentration gradient is experimentally reversed, quenching instead of enhancement of OCC fluorescence results (Fig. 4). Injection of valinomycin to chromatophores preloaded with KCl, and incubated in a K^+-free medium leads to a transient fluorescence quenching indicating a transmembrane potential negative inside (curve A). Addition of equimolar K^+ to the medium (curve B) but not Na^+ (curve C) eliminates the signal. If valinomycin injection is delayed 1 min after the addition of the chromatophores (curve D), the signal is markedly reduced, probably due to the rapid K^+ efflux from chromatophores even in the absence of valinomycin (notice the shift in baseline prior to valinomycin addition).

Rapid pH increase induced by injection of Tris base to chromatophores equilibrated at pH 5.6 in the presence of an internal buffer (succinate) also creates a transient fluorescence quenching (Fig. 5) and a reverse pH change due to the injection of HCl increases OCC fluorescence. These fluorescence changes are compatible with proton-diffusion potentials negative and positive inside, respectively. This interpretation is supported by the increase in the extent of these signals induced by FCCP, which increases proton permeability.

Injection of KCl with HCl to chromatophores at basic pH leads to an additive

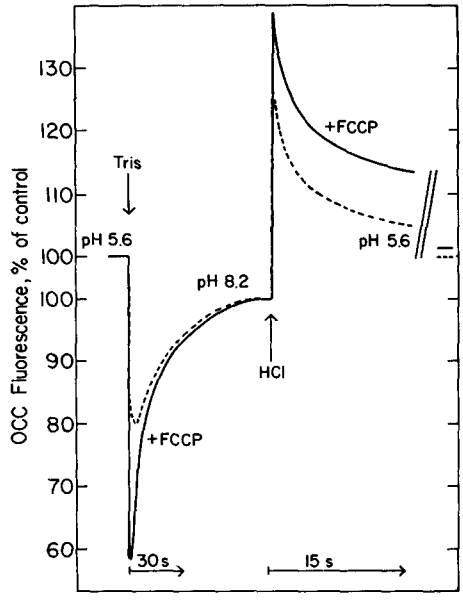

Fig. 5. Diffusion potentials induced by pH transitions. The reaction mixture contained in 2.7 ml: 40 mM Tris/succinate, 5 mM $MgCl_2$, 3.3 μM OCC, and chromatophores containing 25 μg bacteriochlorophyll. The pH was adjusted to 5.6. 0.3 ml 0.2 M Tris, pH 10, or 0.3 ml 0.2 M HCl, were injected where indicated. The pH changed to 8.2 and back to 5.6. 1 μM FCCP was included where indicated.

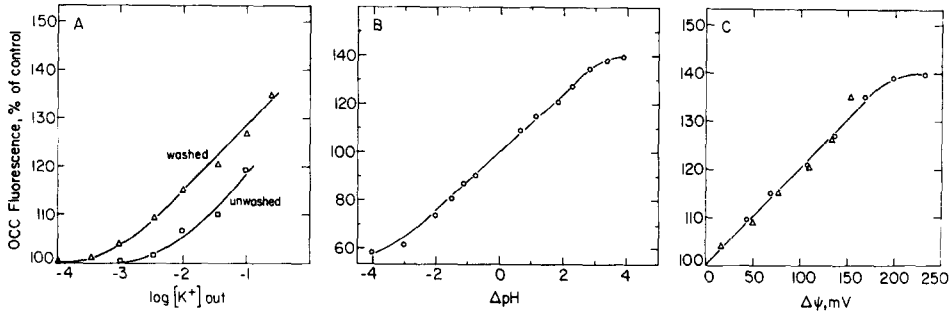

Fig. 6. Titration curves of potassium and proton gradient-induced diffusion potentials. (A) Washed ("KCl depleted") chromatophores (△–△) or unwashed chromatophores (□–□) were prepared in the following manner: chromatophores containing 0.5 mg bacteriochlorophyll were incubated in 12 ml containing: 100 mM choline chloride, 15 mM $MgCl_2$, 15 mM sodium-tricine, pH 7.5, with or without 5 μM valinomycin for 10 min at room temperature, cooled to 4 °C and sedimented as described under Materials and Methods. Chromatophores containing 25 μg bacteriochlorophyll from the pellet were resuspended in 27 ml containing; 25 mM choline chloride, 5 mM $MgCl_2$, 15 mM sodium-tricine, pH 7.5, 3.3 μM OCC, and 1 μM valinomycin. Isomolaric mixtures of choline chloride and KCl were injected in 0.3 ml to reach the final K^+ concentration indicated in the abscissa. (B) pH transitions were induced by injecting Tris, pH 10, or HCl, pH 1, at different concentrations in 0.3 ml to chromatophores at pH 5.3 as described under Fig. 5. The ΔpH was calculated by subtracting the initial pH from the final pH and plotted against the maximal fluorescence change. (C) The fluorescence change is plotted as a function of the maximal theoretical diffusion potential, calculated from the Nernst equation, for K^+ plus valinomycin (from A) and for H^+ plus FCCP (from B positive side).

increase in the fluorescence enhancement signal with biphasic decay kinetics (not shown).

Injection of KCl and Tris to an acidic chromatophore suspension induces an increase in fluorescence (not shown), but with a faster decay than the KCl induced signal.

Fig. 6 summarizes the dependence of the extent of fluorescence changes on the concentration of KCl injected (A), and on the extent of pH transition (B) which may be used as calibration curves for the light-induced signals. In Fig. 6A, K^+-induced diffusion potentials were followed by injecting KCl at different concentrations to chromatophores equilibrated with valinomycin and OCC. Chromatophores that have been washed free of K^+ showed higher fluorescence signals than unwashed preparations indicating a contribution of internal K^+ in the unwashed chromatophores to the magnitude of the observed potential.

In Fig. 6C the maximal theoretical diffusion potentials were calculated from the Nernst equation assuming that in the presence of FCCP or valinomycin the transmembrane potential is determined solely by H^+ or K^+, respectively, that the initial pH gradient is the difference between the preincubation and final pH measured, and

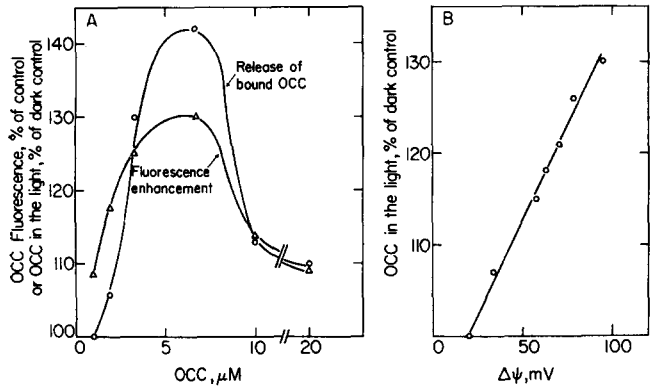

Fig. 7. (A) Correlation between OCC binding, fluorescence change, and membrane potential. The reaction mixture contained: 25 mM choline chloride, 5 mM $MgCl_2$, 15 mM sodium-tricine, pH 7.5, 0.2 mM DAD, 0.4 mg protamine sulphate, and chromatophores containing 25 μg chlorophyll and OCC at the concentration indicated. For the binding studies, the mixtures were distributed in 0.4 ml plastic tubes, placed in a Beckman 152 microcentrifuge, illuminated through 10 cm water (white light) for 30 s and centrifuged with illumination continued through the first 90 s of centrifugation. Centrifugation time was 4 min. The supernatant was collected and the OCC concentration was determined spectrophotometrically (at 436 nm). Dark controls were run for each OCC concentration. The results are expressed as percentages of cyanine present in the supernatant of the illuminated samples relative to the dark controls. The fluorescence enhancement experiments were performed as described under Fig. 1 using the same reaction mixtures. (B) Correlation between $S^{14}CN^-$ distribution and OCC binding. The reaction mixture contained in 2 ml: 100 mM NaCl, 7 mM sodium tricine, pH 7.5, 5 mM $MgCl_2$, 0.2 mM DAD, 0.4 mg protamine sulphate, chromatophores containing 100 μg bacteriochlorophyll and the uncoupler SF-6487 at concentrations 0.01–1 μM. For measurements of SCN^- distribution, $KS^{14}CN$, ^3H_2O, and 10 μM carrier KSCN were added. For OCC binding, 10 μM OCC were present, which was optimal at this chromatophore concentration. The two sets in triplicate were illuminated and sedimented under identical conditions as described under A. Samples from the supernatants and slices from the pellets were taken to determine the isotopic ratios. The transmembrane potential $\Delta\psi$ was calculated as described in ref. 7, assuming an osmotic volume of 20 μl per mg bacteriochlorophyll [7].

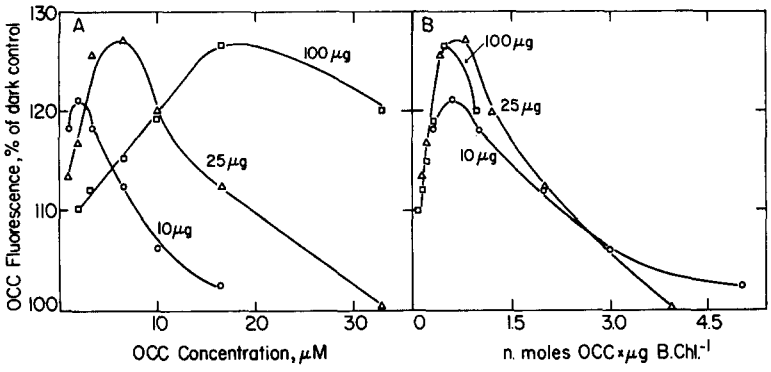

Fig. 8. Dependence of the light-induced OCC fluorescence change on the concentration of OCC and of the chromatophores. The reaction medium included in 3 ml: 100 mM choline chloride, 15 mM sodium tricine, pH 7.5, 5 mM $MgCl_2$, 0.2 mM DAD, and OCC and chromatophores at the concentrations indicated in the figure. The maximal initial light-induced changes are plotted.

that the internal K^+ concentration in washed chromatophores was equivalent to $5 \cdot 10^{-4}$ M.

The response of cyanine dyes to potentials was previously shown [10] to be correlated with its binding to the erythrocyte membrane. Fig. 7A demonstrates that a similar binding occurs in bacterial chromatophores. Thus, the extent of fluorescent enhancement in the light is similar to the percent free cyanine released from the chromatophores upon illumination.

In Fig. 7B, different concentrations of the uncoupler SF-6847 were added in the light, in order to decrease the transmembrane potential gradually. The potential calculated from the distribution of $S^{14}CN^-$ was then compared with cyanine binding. Clearly, the two parameters are linearly dependent. The fact that $\Delta\psi$ estimated from thiocyanate distribution does not extrapolate to zero may be a result of some thiocyanate binding or of the insensitivity of the method at the low concentrations of chromatophores which had to be used for adequate comparison with OCC binding.

The degree of enhancement of OCC fluorescence in the light, or due to a diffusion potential, is very sensitive to both the chromatophore and the OCC concentration. Fig. 8A shows the dependence of the maximal fluorescence enhancement obtained in the light on OCC concentrations at three different chromatophore concentrations. In Fig. 8B, the signal is plotted as a function of the ratio cyanine/chromatophores. The overlap suggests a partition of the dye between the membrane and the aqueous medium, as previously suggested [12]. Since the probe seems to bind to the membrane, it is important to check whether it has any effect by itself on the energization of the chromatophores. Fig. 9 shows that OCC indeed affects proton uptake and the rate of photophosphorylation. The extent of proton uptake in the light increases with OCC concentration up to 10 μM and decreases at higher concentrations. Also, the initial rate of H^+ uptake and release are markedly accelerated by OCC even at 1 μM concentration (not shown). Thus, it behaves in a manner analogous to permeable ions SCN^- or K^+ and valinomycin [3]. This suggests that OCC is either equilibrating across the membrane itself, or increases the permeability to other ions present in the medium.

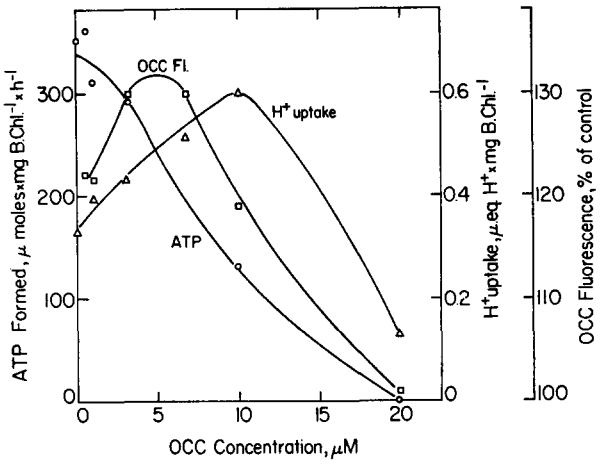

Fig. 9. The effect of OCC concentration on light-induced proton uptake and photophosphorylation. The reaction medium included in 3 ml: 50 mM choline chloride, 5 mM $MgCl_2$, 66 μM PMS, 0.5 mM sodium-succinate, 1 mM inorganic phosphate, OCC at the indicated concentrations and chromatophores containing 20 μg bacteriochlorophyll. The pH was 7.4. When photophosphorylation was followed, 1 mM ADP was also added. Proton uptake and photophosphorylation were measured with a pH electrode as described under Materials and Methods.

At the concentration normally employed (3.3 μM), photophosphorylation was inhibited by about 10 % and the extent of H^+ uptake increased by about 30 %. If the driving force for photophosphorylation is just the sum of a proton concentration gradient and a transmembrane potential, then clearly the transmembrane potential at this probe concentration is decreased by the probe itself, indicating that the real potential is underestimated, and the ΔpH overestimated. A direct interaction with the ATP-synthesizing enzyme may also be suggested. However, this possibility is shown to be unlikely by a comparison of the effect of OCC on photophosphorylation and on ATPase activities. Progressive inhibition of photophosphorylation in the light was observed in correlation with a progressive stimulation of the ATPase activity. For example, 10 μM OCC inhibited photophosphorylation to 40 % and stimulated ATPase to 240 % of the control rates. This would be expected from uncoupling but not from a direct interaction with the enzyme.

It has been noted earlier that the association of cyanine dyes with membrane particles is accompanied by a red shift in its absorption spectrum [10]. Similarly, the absorption peak in organic solvents is shifted to the red compared to water [10]. Fig. 10 shows that with chromatophores a red shift also occurs upon mixing of cyanine with the membranes (curves A and B) manifested by a difference spectrum with a sharp peak at 495 nm (curve C). Table III shows that the lower the polarity of the solvent, the greater is the red shift in the absorption peak in the difference spectrum. The difference spectrum between OCC in a mixture of *sec*-butyl acetate/*n*-hexane (3 : 1, v/v) and in water (Fig. 10D) can be seen to closely resemble the difference spectrum between chromatophore-bound and free OCC (Fig. 10C). It is interesting to note that this mixture of *sec*-butyl acetate/*n*-hexane (3 : 1, v/v) was also found to be most similar to chloroplast membranes when the distribution of 9-aminoacridine between two water phases having different pH values and separated by an organic phase was studied [18].

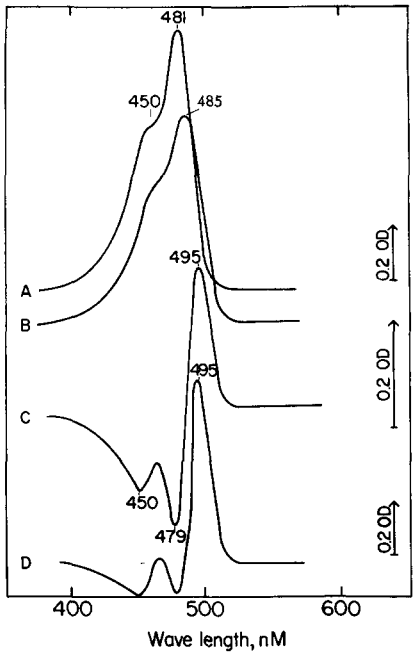

Fig. 10. Comparison of the absorption difference spectra for OCC interaction with chromatophores and with an organic solvent. (A) A spectrum of 3.3 μM OCC in a reaction medium identical to that described in Fig. 1. (B) A similar spectrum, but in the presence of 23 μg chromatophores in 3 ml versus a reference cell contain ng chromatophores without OCC. (C) Difference spectrum of 3.3 μM OCC mixed with chromatophores vs. a reference cell containing OCC and chromatophores separated by a partition. (D) The difference spectrum of 5 μM OCC in *sec*-butyl acetate/*n*-hexane (3 : 1, v/v) vs. a reference cell containing OCC in water.

TABLE III

PEAK ABSORPTION OF DIFFERENCE SPECTRA BETWEEN OCC IN ORGANIC SOLVENTS AND IN WATER

The difference spectra of OCC, 3.3 μM in the organic solvent relative to the same concentration in pure water were run in a spectrophotometer and the wavelength of maximal change was recorded. The partition coefficient was determined by mixing 5 ml organic solvent with 5 ml water containing 3.3 μM OCC. After phase separation, the concentration of dye in each phase was determined spectroscopically by comparison of the absorption of each phase with the absorption of 3.3 μM cyanine in the corresponding pure solvent at the maximal absorption wavelength. $\varepsilon_{20°C}$ refers to dipole moments.

Solvent	λ_{max} absorption difference (nm)	Partition coefficient solvent/water	$\varepsilon_{20°C}$ of solvent
Water	–	1	78
iso-Butanol	490	200	15.8
Sec-Butyl acetate/*n*-hexane (3 : 1, v/v)	495	3.4	4.3
Sec-Butyl acetate/*n*-hexane (1 : 9, v/v)	498	–	–
n-Hexane	502	0.075	2

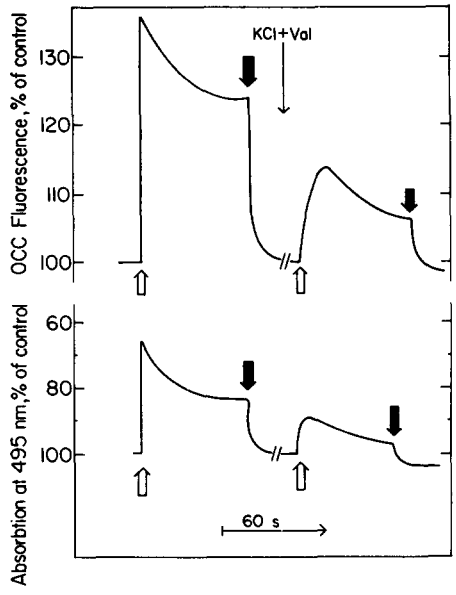

Fig. 11. Comparison of light-induced OCC fluorescence changes and absorption changes. The reaction medium contained in 3 ml: 50 mM choline chloride, 5 mM $MgCl_2$, 15 mM sodium-tricine, pH 7.5, 3.3 μM OCC, and chromatophores containing 25 μg bacteriochlorophyll. Where indicated 50 mM KCl and 1 μM valinomycin were added.

The absorption changes at 495 nm can also be used to monitor potential changes. A similar kinetic response of the fluorescence signal and the absorption difference signal is shown in Fig. 11.

DISCUSSION

The mechanism underlying the variation of cyanine fluorescence obtained upon induction of a diffusion potential has been previously correlated with the extent of binding to the membrane [10]. No definite conclusion was provided regarding the location of the probe, is it adsorbed to the membranes or concentrated in the intracellular phase? The quenching in the erythrocyte case was suggested to be due to the formation of non-fluorescent aggregates, which are at least to some extent associated with the membrane as indicated by the red shift. According to one of the models suggested by Sims et al. [10] the cyanine responds to the transmembrane potential by re-equilibration across the membrane which affects also the fraction of dye associated with the membrane. The basic assumption of this model is that the cyanine molecule can move electrophoretically across the membrane due to high delocalization of its positive charge.

A different mechanism based on absorption changes of membrane-associated cyanines was put forward by Chance et al. [19] who suggested isomerization of the probe at the membrane interface, in response to the applied field. Both neutral and negatively charged dyes have been used in this case to eliminate a possible contribution of electrophoretic movements of the dyes across the membrane.

Our results favor the former mechanism in the following respects: firstly, the quantitative correlation between cyanine binding and fluorescence changes was verified (Fig. 7A) for this system. Secondly, the stimulation of H^+ uptake (Fig. 10) suggests that the probe increases the electrical conductivity of the membrane which is in agreement with the suggestion that part of the cyanine located inside the chromatophores is released in response to the membrane potential resulting in OCC/H^+ exchange. For 30 % fluorescence stimulation in a mixture containing 3 μM OCC and 10 μg bacteriochlorophyll per ml this would imply release of 0.1 μequiv. OCC per mg bacteriochlorophyll, in keeping with the extent of stimulation of proton uptake by OCC (Fig. 9). Thirdly, the absorption shift that accompanies the interaction of OCC with chromatophores resembles solubilization in an organic solvent. The low partition coefficient of cyanine between *sec*-butyl acetate/*n*-hexane (3 : 1, v/v) and water would seem to rule out a simple partition between membrane and the aqueous phase. However, this type of probe could possibly orient itself at the medium-membrane interface with the dye strongly associated with the hydrophobic part of the membrane. And lastly, the optimal signal was found to depend on the cyanine/chromatophore concentration ratio favoring a mechanism of partition or binding to the membranes. The fact that the extent of the light-induced signal is independent of medium osmolarity indicates that the concentration of osmotically active dye trapped inside the chromatophores is negligible and the signal reflects mainly changes in association with the membrane.

We have only indirect evidence that the kinetics of the fluorescence changes in response to "on" or "off" light signals and to diffusion potentials are not secondary effects representing some complex reequilibration of the cyanine with the membrane in response to local potential differences at the membrane interface and really represent build up and decay of the transmembrane potential. Nevertheless, the effects of permeable ions and the fact that similar kinetic behavior was observed following the carotenoid absoption shifts [5] strengthens this suggestion.

An absolute calibration of the signal in terms of a membrane potential seems very difficult for the following reasons: (1) as indicated above, the probe itself affects the extent of the transmembrane potential which will lead to underestimation of the real value. (2) The extent of fluorescence change induced by KCl injection depends on the oxidation state of the membranal electron carriers and also on the pH of the medium. The presence of strong reducing agents (dithionate, hydroquinone and ascorbate) or oxidants ($Fe(CN)_3$) does not change the baseline fluorescence level but increases the KCl-induced dark change (data not shown). Since in the light oxidation-reduction reactions are induced in membrane-bound carriers and the internal pH is significantly lowered an absolute calibration of the light signal with the dark KCl-induced potential seems questionable. (3) The fact that the percent change of OCC fluorescence in Fig. 6A is not linear with log [K^+] out, indicates either that a non-linearity of the fluorescence with the transmembrane potential is indeed real and intrinsic to the system or that at low K^+ concentrations the transmembrane potential is not determined by K^+ alone but depends also on other ions (Na^+, Cl^-, Mg^{2+}). The internal K^+ concentration which needs to be known for calculation of the KCl-induced diffusion potential, is difficult to estimate and liable to serious errors.

pH transients in the presence of internal buffers and FCCP can also be used to calibrate the light-induced signals, assuming that in this case the proton concentration

gradient determines the magnitude of the membrane potential. In this case, one assumes independence of the signal on the external pH or on the internal pH, which may not be justified.

Bearing all these precautions in mind, the observations that calibration curves with pH transients, with KCl/valinomycin and with NaCl/monactin (not shown) give similar slopes and are linear over at least a certain concentration range, seem to indicate that they may be used for at least an approximate calibration of the light signal.

The value of the transmembrane potential may be estimated by taking into account the inhibition of the signal produced by the probe itself. The extent of this inhibition can be roughly estimated by comparing the stimulation of proton uptake induced by a permeable salt which equals the stimulation produced by the probe. This turns out to correspond to about 20 mV. Using the calibration curves of Fig. 6 and this correction, the maximal initial transmembrane potential has been estimated to be about 170 mV, the steady-state level about 110 mV, and in the presence of valinomycin and KCl about 50 mV. These values are considerably lower than those estimated from carotenoid absorption shifts [5] but similar in magnitude to the potential calculated from thiocyanate distribution [7].

It was reported recently [20] that in *R. rubrum* dark acid-base phosphorylation is very low but can be considerably increased if valinomycin plus KCl are injected together with the base. This would be in agreement with the following observations: our measurements indicate that injection of Tris to an acidic chromatophore suspension, creates a diffusion potential negative inside ranging up to 100 mV. This would detract from the effectiveness of the proton concentration gradient which drives protons in the opposite direction. When KCl plus valinomycin are included in the base injection the net effect recorded is a K^+-induced diffusion potential, positive inside, which ranges to about 100 mV and provides an additive driving force for H^+ efflux and so promotes phosphorylation. This may be particularly significant if the dependence of phosphorylation on the magnitude of the proton motive force is non-linear, and similar to that described for chloroplasts [21].

OCC may not be the ideal probe for quantitative measurements of transmembrane potential in *R. rubrum* but it compares favorably with any of the techniques employed hitherto. It may be worth reemphasizing here that the calibration of the light-induced signals for all probes from salt-induced diffusion potentials needs to be reevaluated for the reasons discussed earlier before the values obtained can be used with any assurance. A cyanine analog other than OCC which is a poorer uncoupler may well prove to be more useful in this system. It would also seem desirable to compare the responses of an anionic analog which, if the proposed model is correct, should give fluorescence changes in the light opposite in direction to those of OCC

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