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MEASUREMENT BY A FLOW DIALYSIS TECHNIQUE OF THE STEADY-STATE PROTON-MOTIVE FORCE IN CHROMATOPHORES FROM *RHODOSPIRILLUM RUBRUM*

COMPARISON WITH PHOSPHORYLATION POTENTIAL

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Summary

1. In the light a transmembrane electrical potential of 100 mV has been estimated to occur in chromatophores from *Rhodospirillum rubrum*. The potential was determined by measuring the steady-state distribution of the permeant SCN^- across the chromatophore membrane using a flow dialysis technique. The potential was not observed in the dark, nor in the presence of antimycin. It was dissipated on the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The potential was reduced by between 15 and 20 mV when ADP and P_i were added. Hydrolysis of ATP by the chromatophores generated a membrane potential of about 80 mV.

2. Using a flow dialysis technique light-dependent uptake of methylamine was observed only in the presence of concentrations of SCN^- that were 500-fold higher than were used to measure the membrane potential. It is concluded that the pH gradient across the illuminated chromatophore membrane is insignificant except in the presence of relatively high concentrations of a permeant anion like thiocyanate. Further evidence that a negligible pH gradient was generated by the chromatophores is that addition of K^+ and nigericin to illuminated chromatophores did not stimulate uptake of SCN^-.

3. In the light the chromatophores established and maintained a phosphorylation potential of up to 14 kcal/mol. If a phosphorylation potential of this magnitude is to be poised against a proton-motive force that comprises solely a

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, membrane potential; ΔpH, transmembrane pH gradient.

membrane potential of approx. 100 mV, then at least five protons must be translocated for each ATP synthesised via a chemiosmotic mechanism.

Introduction

Chromatophores from photosynthetic bacteria are a useful system for studying the mechanism of coupling between electron flow and ATP synthesis in biological membranes. Much experimental data has accrued to indicate that this coupling involves a transmembrane electrical potential difference ($\Delta\psi$), and a transmembrane pH gradient (ΔpH) [1—3] as the two components of the proton-motive force that is defined in the chemiosmotic hypothesis [4]. Indeed, chromatophores from *Rhodopseudomonas capsulata* are the only energy-transducing system for which evidence has been obtained [1] for a sufficiently large proton-motive force to account for the extent of ATP synthesis on the basis that two H^+ are translocated across the membrane for each ATP molecule synthesised, as originally suggested by Mitchell [4]. However, the methods used for measuring the membrane potential and the pH gradient, respectively, the extent of the carotenoid shift and the quenching of 9-amino acridine fluorescence, are not universally accepted as quantitative indicators of these two parameters [5—8].

The more direct, and more generally acceptable, method for determining the membrane potential across energy-transducing membranes is to measure the distribution of an appropriately charged permeant ion between the lumen of the membrane vesicle and the suspending medium, while the pH gradient is determined from the distribution of a weak acid or base across the membrane [7]. Schuldiner et al. [9] have already employed these methods for illuminated chromatophores from *Rhodospirillum rubrum*, using SCN^- as permeant ion and methylamine as a weak base, but were hampered by the difficulty of rapidly separating chromatophores from the suspending medium, for which precipitation of the chromatophores with protamine sulfate was necessary. In the present work we have overcome this difficulty by using the flow dialysis technique to monitor the concentration of either SCN^- or methylamine in reaction mixtures containing *R. rubrum* chromatophores. This technique allows the extent of uptake of these two species into the chromatophores to be followed without separating the chromatophores from the reaction medium. It was introduced by Colowick and Womack [10] and applied more recently to studies of bacterial membrane vesicles [11,12]. Our main aim was to compare the size of the proton-motive force measured using the flow dialysis technique with the magnitude of the phosphorylation potential that the chromatophores could generate.

Methods

Growth of organism and preparation of chromatophores. R. rubrum (strain S1, a gift from Dr. O.T.G. Jones, University of Bristol, U.K.) was grown anaerobically in the medium of Ormerod et al. [13] under continuous illumination from a 100 W tungsten bulb at about 23°C. Cells were harvested in late

logarithmic or early stationary phase, washed once in cold 0.2 M glycylglycine, pH 7.4, resuspended in the same buffer and stored at −20° C. Chromatophores were prepared by exposing cells (about 0.2 mM in bacteriochlorophyll) suspended in 0.2 M glycylglycine and 5 mM $MgCl_2$ at pH 7.4 to sonic oscillation at the maximum output of a Dawe Soniprobe for two periods of 1 min. The temperature of the cell suspension did not rise above 10° C during this treatment. Cell debris was removed by centrifugation at 17 000 rev./min (28 000 × *g*) for 15 min in the SS 34 rotor of a Sorvall RC2B centrifuge. Chromatophores were sedimented at 110 000 × *g* by centrifugation at 44 000 rev./min for 50 min in a Spinco 50 rotor, and then resuspended in 19 mM Tris/acetate (pH 8.0) containing 5 mM magnesium acetate. They were either used immediately or stored overnight under nitrogen at 4°C.

Flow dialysis. The uptake of $S^{14}CN^-$ and [^{14}C]methylamine was measured in a cylindrical flow dialysis cell (constructed in the workshop of the Botany School) which essentially followed the design of Colowick and Womack [10]. The volume of the lower chamber was 1.2 ml and the upper chamber had a maximum capacity of 2.5 ml. Visking dialysis tubing (Gallenkamp) of average pore diameter 24 Å was boiled for 1 h in 5 mM EDTA (sodium salt) and stored in distilled water at 4° C before being inserted between the two chambers with Parafilm gaskets to ensure water tightness. Water was pumped through the lower chamber at 2 ml/min by means of a Watson-Marlow MHRE peristaltic pump. 1-ml fractions of the outflow were collected in scintillation vial inserts containing 2 ml Triton-toluene scintillant [14], held in an LKB Ultrorac fraction collector. The dead volume between the flow dialysis cell and the fraction collector was 0.3 ml. Radioactivity was counted in a Tracerlab Corumatic 200 liquid scintillation counter. Chromatophores in the flow dialysis cell were illuminated by a 500 W tungsten bulb from which the light was filtered through a Kodak Cinemoid 5A deep orange filter and 5 cm of water. The light intensity behind the cell was approx. $2.8 \cdot 10^5$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} as measured with an EEL Lightmaster photometer. For dark controls, the cell was covered with aluminium foil. Details of the reaction mixtures are given in the legends to the figures. The flow dialysis experiments were done at room temperature (23—24° C).

Bacteriochlorophyll and protein determinations. Bacteriochlorophyll concentrations were estimated from the absorbance at 880 nm using the in vivo millimolar extinction coefficient ($E_{880}^{1cm} = 140$) given by Clayton [15]. Protein was determined by the Folin method [16] using bovine serum albumin (Cohn fraction V) as standard. The bacteriochlorophyll : protein ratio for three different preparations of chromatophores was 37 μg bacteriochlorophyll/mg protein (S.D. = 3.7).

Reagents. All radioisotopes were from the Radiochemical Centre, Amersham (U.K.). $KS^{14}CN$ and [^{14}C]methylamine hydrochloride were made up carrier-free to give stock solutions of 2.08 and 2.25 mM (60 and 55.5 Ci/mol), respectively. [U-^{14}C]Sucrose and ^3H_2O were, respectively, diluted to specific activities of 5 Ci/mol and 400 Ci/ml. FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was a generous gift from Dr. P.G. Heytler (E.I. Du Pont de Nemours and Co., Wilmington, U.S.A.), and nigericin was kindly provided by Dr. R.L. Hammill (Lilly Research Laboratories, Indianapolis, U.S.A.). All

enzymes were from Boehringer. Other chemicals and biochemicals were from Sigma, London, or B.D.H. Chemicals Ltd., Poole, U.K. and were of the highest grade commercially available.

Calculation of $\Delta\psi$ and ΔpH. $\Delta\psi$ was measured assuming that SCN^- passively equilibrates across the membrane so that there is no electrochemical potential difference for the SCN^- (which has a very low pK_a, −1.8 [33]) across the chromatophore membrane. In this case, from the Nernst equation,

$$\Delta\psi = \frac{RT}{zF} \frac{\ln[SCN^-]_{in}}{[SCN^-]_{out}} \quad (1)$$

The concentration terms, $[\text{thiocyanate}]_{in}$ and $[\text{thiocyanate}]_{out}$ are obtained from the amount of thiocyanate uptake and an estimate of the internal volume of the chromatophores. ΔpH was evaluated from the extent of methylamine uptake. As the pK_a of methylamine (10.47) is substantially greater than the pH on either side of the membrane then [7]

$$\Delta pH = \frac{\log[\text{methylamine}]_{in}}{[\text{methylamine}]_{out}} \quad (2)$$

Again the internal volume must be known. Details of this determination are given in Table I. Use of Eqns. 1 and 2 assumes equal activity coefficients for both methylamine and thiocyanate ions on each side of the membrane.

Results

Determination of the internal volume of the chromatophores

The internal volume of the chromatophores was estimated by a modification of the sucrose impermeable space method (e.g. ref. 17), which gave a value of 50 μl per mg bacteriochlorophyll (S.E. = 1.6) at the osmolarity of 65 mosM that was used in the present work (Table I). This volume is close to that obtained by others for chromatophores from *R. rubrum* [9,18,19].

It is evident from Eqns. 1 and 2 that an accurate determination of the internal volume of the chromatophores is critical for the quantitative estimation of $\Delta\psi$ and ΔpH across the membrane. For this reason it is instructive to compare our experimentally determined value with the internal volume obtained by direct calculation. If it is assumed that there are between 2000 and 4000 bacteriochlorophyll molecules per chromatophore [20—22] and that the average chromatophore radius is 30 nm, an estimate of between 40 and 20 μl per mg bacteriochlorophyll is obtained. Such calculated values are in good agreement with our experimentally determined value. In the present work we have assumed that the chromatophore volume remains constant upon illumination as there are no changes in light scattering by chromatophores under these conditions (Crofts, A.R., unpublished, cited in ref. 23). Fig. 1 shows how the potential calculated from Eqn. 1 depends upon the internal volume of the chromatophores at a given decrease in the external concentration of SCN^-. Underestimation by a factor of two of the internal volume will increase the value of $\Delta\psi$ calculated from Eqn. 1 by only 18 mV. Similar considerations

TABLE I

DETERMINATION OF THE INTERNAL VOLUME OF CHROMATOPHORES

Chromatophores were suspended in the reaction medium given in Fig. 2 with approx. 0.15 μCi [U-^{14}C]-sucrose and 2.5 μCi $^{3}H_2O$ to a final volume of 5 ml containing 0.82 (Expt. 1) or 2.52 (Expt. 2) mg bacteriochlorophyll. The mixtures were centrifuged at 100 000 × *g* in a Spinco 50 rotor at 4°C for 1 h. 1-ml aliquots of the supernatant were counted for radioactivity in 9 ml of Triton-toluene scintillant [14]. The pellets of the chromatophores were dried by removing as much as possible of the remaining supernatant by suction. Then the pellets were resuspended to a volume of 5 ml with homogenisation in the original medium lacking the radioactive components, and centrifuged as above. 1-ml aliquots of the supernatant were again counted for radioactivity. This protocol avoids counting radioactivity in the pellet from the first spin, and thus no correction for differential quenching of ^{3}H and ^{14}C counts is required. The channels on the liquid scintillation counter were set such that ^{3}H registered only in channel A in which ^{14}C was counted with an efficiency of about 93%. The efficiency of counting of ^{14}C in channel B was approx. 42%. A standard containing only ^{14}C was run so as to obtain the factor by which the ^{14}C counts in channel B had to be multiplied to obtain the ^{14}C counts in channel A. The ^{3}H counts in channel A were thus calculated by subtracting the ^{14}C counts in channel A (calculated from the ^{14}C counts in channel B) from the total counts in channel A. V_i, the specific internal volume of the chromatophores in $\mu l/mg^{-1}$ bacteriochlorophyll, is given by:

$$V_i = \frac{5000}{x}\left[\frac{\text{cpm } ^{3}H \text{ in 2nd supernatant}}{\text{cpm } ^{3}H \text{ in 1st supernatant}} - \frac{\text{cpm } ^{14}C \text{ in 2nd supernatant}}{\text{cpm } ^{14}C \text{ in 1st supernatant}}\right]$$

where x = mg bacteriochlorophyll in the 5 ml starting reaction mixture. The results from the two experiments given are typical. From a series of determinations the mean and S.D. for V_i were 49.8 and 4.3 μl/mg bacteriochlorophyll.

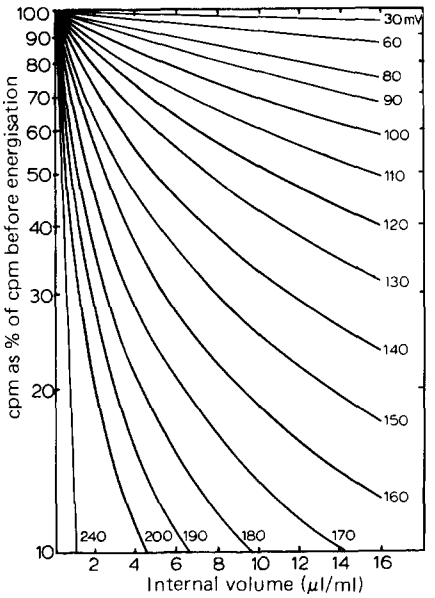
	cpm 1st supernatant	cpm 2nd supernatant	V_i (μl/mg bacteriochlorophyll)
Expt. 1			
^{14}C	50 469	1 713	49.9
^{3}H	270 215	11 388	
Expt. 2			
^{14}C	57 637	5 983	52.2
^{3}H	354 097	46 053	

apply to the determination of ΔpH from the uptake of methylamine into chromatophores whose inside is acidic relative to the outside.

Determination of $\Delta\psi$

Fig. 2 shows the SCN^- concentration, monitored by counting radioactivity, in the outflow from the flow dialysis cell. $KS^{14}CN$ was added to the upper compartment of the cell either in the presence or in the absence of chromatophores. Before the cell was illuminated the amount of SCN^- equilibrating present. Thus, in the dark, there appears to be neither a significant binding of SCN^- to chromatophores, nor a detectable Donnan potential across the chromatophore membranes. The flow rate through the lower chamber of the flow dialysis cells was 2 ml/min and thus it can be seen from Fig. 2 that equilibrium across the dialysis membrane was attained after about 3 min.

Illumination of the chromatophores resulted in a marked decrease in the SCN^- concentration in the outflow from the cell, and a new equilibrium of SCN^- across the dialysis membrane was reached (Fig. 2). This result indi-



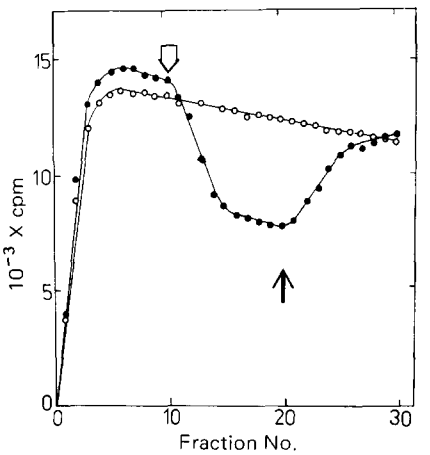


Fig. 1. Nomograph for calculating transmembrane ion gradients from flow dialysis measurements. The ordinate represents the external ligand concentration as a percentage of the concentration before energisation. The abscissa represents the enclosed volume of the membrane vesicles in μl/ml reaction mixture. The values of the lines of isopotential are in mV at 30°C.

Fig. 2. Uptake of $S^{14}CN^-$ by *R. rubrum* chromatophores. Flow dialysis was performed as described in Methods. The radioactivity measured in the outflow of the flow dialysis cell is plotted against the fraction number. Reaction mixtures contained in a final volume of 1 ml: 10 mM Tris/phosphate (pH 8.0), 5 mM magnesium acetate, 20 mM sucrose, 0.2 mM sodium succinate. At time zero, 10 μl of 2.08 mM $KS^{14}CN$ (60 Ci/mol) were added to the upper chamber and the flow started. After collecting fraction 10 of the outflow from the lower chamber, the light was turned on (open arrow), and after fraction 20 had been collected, 1 μl of 2.5 mM FCCP was added to the upper chamber (second arrow). ○———○, no chromatophores; ●———●, plus chromatophores (0.18 mg bacteriochlorophyll).

cates that SCN^- were taken up by the illuminated chromatophores, thus lowering the free external thiocyanate concentration in the upper chamber of the cell. The SCN^- was released from the chromatophores when FCCP was added (Fig. 2). From a series of eleven experiments an average light-dependent $\Delta\psi$ of 100 mV (S.D. = ±9 mV) was obtained. After addition of FCCP the radioactivity in the outflow corresponded to an extrapolation of the radioactivity found in the fractions of the outflow that were collected before the chromatophores were illuminated (Fig. 2). Hence the uptake of SCN^- into chromatophores is fully reversible.

Fig. 2 also shows that uptake of SCN^- into the chromatophores was complete within 5 min of illumination. It is noteworthy that very little thiocyanate was lost from the upper chamber during this period so that the rate of SCN^- uptake by the chromatophores is faster than the rate of dialysis.

The light-dependent $\Delta\psi$ was reduced by between 15 and 20 mV when ADP (0.2 mM) and P_i were present (plus 10 mM glucose and 5 units hexokinase) so as to permit continuous ATP synthesis. This observation indicates that SCN^-

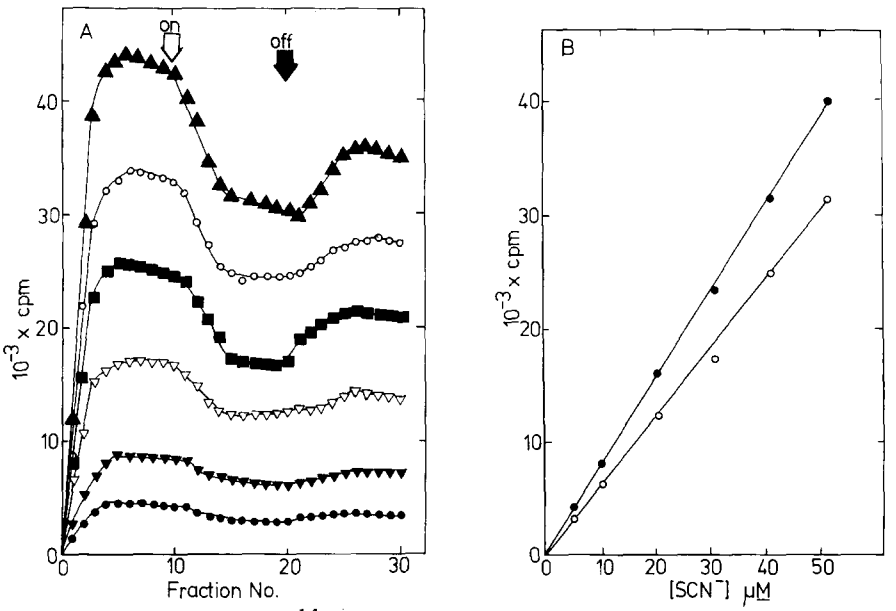
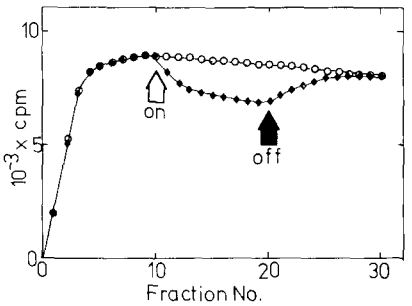


Fig. 3. Effect of varying the $S^{14}CN^-$ concentration on the proportion of thiocyanate taken up by illuminated chromatophores. The reaction conditions were as in the legend to Fig. 2, except that the concentration of radioactive thiocyanate used to start the reaction was varied. (A) The initial thiocyanate concentrations in the upper chamber (constant specific activity) were 5.2 μM (●———●), 10.4 μM (▼———▼), 20.8 μM (▽———▽), 31.2 μM (■———■), 41.6 μM (○———○) and 52.0 μM (▲———▲). Open arrow: light on. Closed arrow: light off. For clarity, the chromatophore-free controls have been omitted. (B) Data of A replotted to show that a constant fraction of the total thiocyanate added is taken up by the chromatophores, over the whole range of thiocyanate concentrations tested. ○———○, represents the radioactivity in fraction 16 as a function of the total added thiocyanate concentration. ●———●, represents the radioactivity that was found in fraction 16 of the chromatophore-free controls, or calculated by interpolation of the counts found in fractions 8 and 28 so as to obtain the value which would be expected for fraction 16 in the dark. As may be seen in Fig. 1, the constancy of the fraction taken up indicates that a constant potential is formed across the chromatophore membrane.

uptake is responsive to the magnitude of $\Delta\psi$, since ATP synthesis is known to lower the steady-state proton-motive force in several systems including chloroplasts [40] and mitochondria [7,41] as well as in *R. rubrum* chromatophores [19].

The extent of thiocyanate uptake was not increased by addition of oligomycin (30 μg/mg bacteriochlorophyll) to the chromatophores, in harmony with the results of Leiser and Gromet-Elhanan [19] who found that oligomycin did not cause any additional energy-linked enhancement of anilinonaphthalene sulphonate fluorescence with *R. rubrum* chromatophores.

Fig. 3 shows some of the results from an experiment in which the uptake of SCN^- into chromatophores was monitored over a 10-fold range of initial SCN^- concentration. The percentage of thiocyanate taken up in the light was similar at all thiocyanate concentrations tested. Therefore it appears that SCN^- is not binding to the membranes, which would be a saturable effect, but rather that it is taken up into the chromatophore lumen. Further evidence that SCN^- taken up is not bound was obtained by determining that the measured $\Delta\psi$ was independent of the chromatophore concentration over the range 0.05—0.25 mg



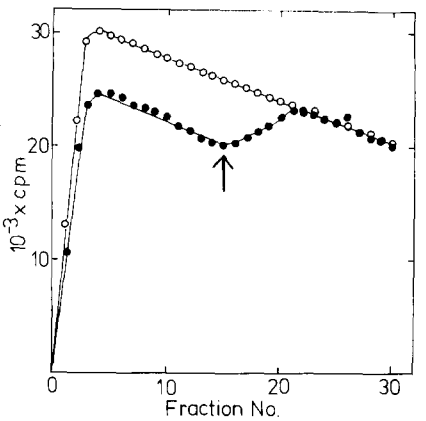


Fig. 4. Effect of antimycin on thio[^{14}C]cyanate uptake by chromatophores. The reaction mixture was as in the legend to Fig. 2 except that the chromatophore concentration was 0.15 mg bacteriochlorophyll/ml and antimycin (0.37 μg/μg bacteriochlorophyll) was present (○———○). ♦———♦, shows a similar experiment in which antimycin was present at the same concentration and 0.1 mM phenazine methosulphate was also added.

Fig. 5. ATP-driven uptake of thio[^{14}C]cyanate by *R. rubrum* chromatophores. Flow dialysis was performed as described in Methods except that light was excluded throughout. The reaction mixture contained in a final volume of 1 ml: 10 mM Tris-acetate (pH 8.0), 10 mM magnesium acetate and 10 mM ATP (sodium salt, pH 8.0). The flow rate was 1 ml/min, and the reaction was initiated by adding thiocyanate as described in the legend to Fig. 2. 2 μl of 2.5 mM FCCP were added where indicated by the arrow. ○———○, no chromatophores; ●———●, plus chromatophores (0.12 mg bacteriochlorophyll).

bacteriochlorophyll/ml at a constant SCN^- concentration. Whole cells of *R. rubrum* would presumably contain the same putative membrane binding sites as chromatophores for the permeant SCN^-. Illumination of cells of *R. rubrum* did not drive an uptake of SCN^-, thus confirming that SCN^- uptake occurs only into membrane preparations (chromatophores) that have an opposite polarity to whole cells, and that light-induced binding of SCN^- to the membrane is insignificant.

When antimycin (0.37 μg/μg bacteriochlorophyll) was present, no light-dependent uptake of SCN^- into chromatophores was observed unless the site of antimycin inhibition was bypassed through addition of 0.1 mM phenazine methosulphate (Fig. 4).

Hydrolysis of ATP by the chromatophores in the dark caused an uptake of SCN^- that corresponded to a $\Delta\psi$ of 80 mV. As shown in Fig. 5 this determination was made by adding ATP at the beginning of the experiment, and subsequently adding an uncoupler so that $\Delta\psi$ was determined from the SCN^- released. It was found that the addition of ATP (as its sodium salt) to the upper chamber of the flow dialysis cell altered the rate of flow of SCN^- across the dialysis membrane irrespective of whether chromatophores were also present.

The addition of NO_3^- (as 10 mM KNO_3) decreased the light dependent $\Delta\psi$ from 103 to 83 mV, estimated from the decrease in SCN^- uptake into chromatophores, a result consistent with previous work which has shown that NO_3^- permeates chromatophores and thus reduces the membrane potential [21,24].

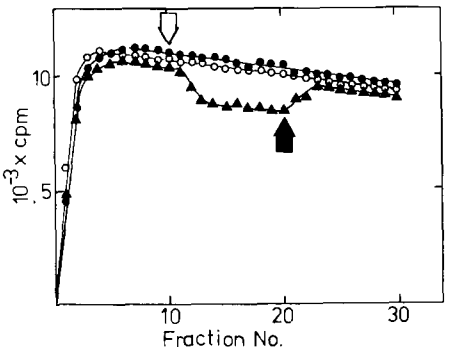


Fig. 6. Uptake of [^{14}C]methylamine by *R. rubrum* chromatophores. Conditions as in Fig. 2 except that the radioactive thiocyanate was replaced by 10μl of 2.25 mM [^{14}C]methylamine hydrochloride (55.5 Ci/mol). •———•, no chromatophores; ○———○, plus chromatophores (0.12 mg bacteriochlorophyll); ▲———▲, plus chromatophores (0.12 mg bacteriochlorophyll) plus 10 mM unlabelled KSCN. Open arrow: light on. Closed arrow: light off.

Determination of ΔpH

Under those conditions that were routinely employed to measure $\Delta\psi$ (20 μM KSCN present), there was no observable light-dependent methylamine uptake (Fig. 6). However, on addition of 10 mM KSCN (unlabelled), methylamine uptake into the chromatophores corresponding to a ΔpH of 1.6 units (acid inside) was observed (Fig. 6). Addition of SCN^- to chromatophores from *R. rubrum* is known to stimulate light-induced proton uptake [25], but the data of Fig. 6 indicate that only at relatively high initial concentrations of SCN^- is the proton uptake sufficient to produce a significant ΔpH. The appearance of this ΔpH of 1.6, which is energetically equivalent to a $\Delta\psi$ of 100 mV, coincided with a decrease in $\Delta\psi$ to practically zero in agreement with previous evidence that the $\Delta\psi$ and ΔpH components of the protonmotive force are interchangeable in chromatophores [25]. Further evidence for this interpretation of the methylamine uptake at high concentrations of SCN^- was that addition of nigericin (5 μg/mg bacteriochlorophyll) in the presence of 10 mM KSCN completely blocked methylamine uptake. Nigericin catalyses an electrically neutral K^+/H^+ exchange [26] and so should decrease any ΔpH that can be formed in the presence of 10 mM KSCN.

Fig. 1 shows that a small pH gradient will be reflected by only slight changes in the concentration of methylamine in the suspending medium in the upper chamber of the flow-dialysis cell. Therefore the question arises as to the magnitude of the smallest ΔpH that would have been detected in our experiments. In an experiment in which sufficient chromatophores where used to give 16 μl of internal volume per ml of reaction mixture, no methylamine uptake was detected upon illuminating the chromatophores. The experimental conditions were such that an uptake of 3% of the methylamine would have been detected. Fig. 1 shows that uptake of 3% of the methylamine corresponds to a pH gradient of less than 0.3 unit (20 mV) with 16 μl internal volume per ml reaction mixture.

As a further check against the possibility that the flow dialysis method was

failing to detect a small but significant ΔpH when $\Delta\psi$ was large, or a small $\Delta\psi$ when ΔpH was large, both $\Delta\psi$ and ΔpH were estimated at concentrations of KSCN at which $\Delta\psi$ and ΔpH were expected to be of comparable magnitude. These reaction conditions should allow uptake of both SCN^- and methylamine to be detected by the flow dialysis method. It was found that, with a preparation of chromatophores that generated a $\Delta\psi$ of 100 mV and an insignificant ΔpH under standard conditions, $\Delta\psi$ was decreased to 50 mV and 59 ΔpH increased to 40 mV in the presence of 2 mM KSCN, and with 5 mM KSCN $\Delta\psi$ was 35 mV and 59 ΔpH was 60 mV.

The conclusion from these experiments is that a $\Delta\psi$ of 100 mV and a ΔpH of virtually zero does reflect the proton-motive force under our reaction conditions. If the method of measurement had been failing to detect a ΔpH of as much as 30 mV, then at intermediate concentrations of KSCN, where both components of the proton-motive force are detectable, it might have been anticipated that the total measured proton-motive force would have been greater than the value obtained under standard conditions when only $\Delta\psi$ could be detected.

The quantitative conversion of $\Delta\psi$ to ΔpH upon addition of 10 mM KSCN indicates that the uptake of $S^{14}CN^-$ or [^{14}C]methylamine does reflect an equilibrium distribution of these solutes with $\Delta\psi$ and ΔpH, respectively.

Addition of potassium acetate (10 mM) and nigericin (5 μg/mg bacteriochlorophyll) to illuminated chromatophores did not stimulate an extra uptake of SCN^- (Fig. 7). The ΔpH component of a proton-motive force should, in the presence of K^+ and nigericin, be replaced by an energetically equivalent $\Delta\psi$ as a result of nigericin exchanging any accumulated H^+ for added K^+. Hence our failure to observe an increased uptake of SCN^- under these conditions suggests that a significant ΔpH is not normally formed. A light-dependent ΔpH was

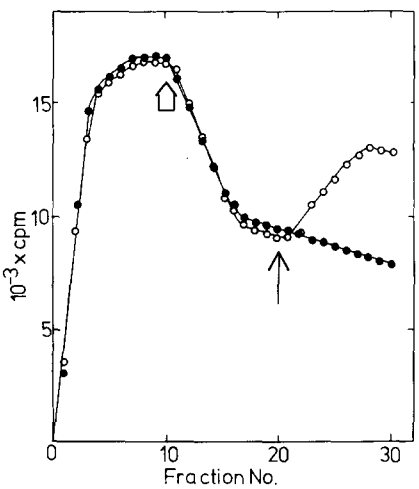


Fig. 7. Effect of nigericin and valinomycin on the uptake of thio[^{14}C]cyanate by *R. rubrum* chromatophores. Reaction conditions as in the legend to Fig. 2 except that 10 mM potassium acetate was also present and the chromatophore concentration was 0.22 mg bacteriochlorophyll/ml. 1 μg nigericin was either present (○———○) or absent (●———●). At the first (open) arrow, the light was switched on, and at the second arrow 1 μg valinomycin was added to the upper chamber.

absent not only in the usual Tris/acetate or Tris/phosphate reaction media but also when the chromatophores were suspended in similar concentrations of HEPES/KOH (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) or Tris/chloride. Our failure to observe a light-dependent ΔpH is not then a consequence of using an unusual suspending medium for the chromatophores.

Finally it is of interest that while K^+ and nigericin had no effect on the extent of SCN^- uptake, the combination nigericin, valinomycin and K^+ was effective in releasing all the SCN^- taken up in the light (Fig. 7). This result is consistent with previous work on the synergistic uncoupling effect of these two ionophores [18].

Determination of the phosphorylation potential (ΔG_p)

According to the chemiosmotic hypothesis of energy coupling [4], the sum of $\Delta\psi$ and ΔpH is termed the proton-motive force and can be equated with the free energy stored in ATP if it is assumed that the ATPase reaction is poised against the proton-motive force. Eqn. 3 expresses the relationship between the proton-motive force and the phosphorylation potential (ΔG_p):

$$\Delta\psi - 2.3\,RT\,\Delta pH = \frac{-\Delta G_p}{zF} \qquad (3)$$

where F is the Faraday constant and z the number of protons that are translocated across the chromatophore membrane for each ATP synthesised. z is postulated to have a value of 2 [4], although to our knowledge a direct mea-

TABLE II

THE PHOSPHORYLATION POTENTIAL IN *R. RUBRUM* CHROMATOPHORES

For determination of the phosphorylation potential, the reaction mixture contained in a final volume of 2 ml: 20 mM sucrose, 5 mM magnesium acetate, 1.7 or 2.5 mM phosphate/Tris, 0.2 mM sodium succinate and approx. 0.1 mg bacteriochlorophyll at pH 8.0, plus the other additions shown in the table. The samples were illuminated for 20 min with the same light intensity and at the same temperature (24°C) as used in the flow dialysis experiments. The rate of ATP synthesis catalysed by the chromatophores, approx. 1.5 μmol/min per mg bacteriochlorophyll, was such that a 20 min incubation was sufficient to allow a steady-state extent of phosphorylation to be reached. At the end of the incubation, 0.2 ml of 40% $HClO_4$ was added to the reaction mixture and, after standing this acid extract on ice for 10 min, the precipitated protein was removed by centrifugation. The supernatant was neutralised by addition of a predetermined amount of 0.25 M Tris in 10% KOH, and EDTA was also added to a final concentration of 2 mM. ATP in the neutralised extracts was determined with hexokinase and glucose-6-phosphate dehydrogenase, and ADP was assayed with pyruvate kinase and lactate dehydrogenase [37]. P_i was measured by the method of Hurst [38] in a Technicon Autoanalyser. In calculating ΔG_p a value for $\Delta G^{0'}$ of 7.8 kcal/mol was used [39].

Expt.	Other additions	Initial substrate concentrations (mM)			Final substrate concentrations (mM)			ΔG_p (kcal/mol)
		ATP	ADP	P_i	ATP	ADP	P_i	
1		1.7	0.25	1.7	1.95	0.044	1.5	14.1
2	10 mM KSCN	2.5	0.25	1.7	2.75	0.058	1.5	14.1
3		0	1.4	2.5	1.35	0.052	1.0	14.0
4	20 μM KSCN	1.3	0.25	2.0	1.5	0.070	1.8	13.5
5	12 mM KSCN + 20 μM methylamine hydrochloride	1.3	0.25	2.0	1.5	0.050	1.8	13.7

surement of the H^+/ATP ratio for chromatophores has not yet been reported. Data in Table II show that the chromatophores were capable of maintaining a phosphorylation potential of 14 kcal/mol under a variety of conditions.

The light-generated proton-motive force that was poised against the phosphorylation potential was found to be approx. 100 mV. This value was determined, in parallel to measurement of ΔG_p, in experiments where either 1.5 mM ATP or 1.5 mM ATP plus 0.25 mM ATP were added to the reaction mixture prior to illumination, provided that in the latter case the proton-motive force was measured after net ATP synthesis was complete. Thus 100 mV represents the magnitude of the proton-motive force when it is at "static head" (i.e. not driving any energy-linked reaction) so that the addition of concentrations of ATP and ADP at which no net ATP synthesis is occurring does not have any effect on the proton-motive force, which was unchanged when either ATP or ATP plus ADP were omitted from the reaction mixture. It is noteworthy that the reaction conditions for determination of the proton-motive force and the phosphorylation potential (Table II) were thus the same.

The proton-motive force was taken as comprising solely $\Delta\psi$, and thus was measured from the extent of SCN^- uptake, except where 10 or 12 mM KSCN was present in which case the proton-motive force was assumed to comprise ΔpH alone and so was measured from the extent of methylamine uptake. In each case the extent of uptake was estimated from the efflux of SCN^- or methylamine after turning off the light and adding 5 μM FCCP. The latter was added to prevent any ATP hydrolysis in the dark from generating a protonmotive force.

From Eqn. 3 it may be calculated, using the data given in Table II, and a value for the proton-motive force of 100 mV, that approximately five protons must be translocated for each ATP molecule synthesised if the ATPase is operating by a chemiosmotic mechanism.

Discussion

The proton-motive forces reported in the present paper are low compared with the phosphorylation potential that can be generated by the *R. rubrum* chromatophores. Determinations of both ΔpH and $\Delta\psi$ rely upon the estimate of the internal volume of the chromatophores (Eqns. 1 and 2), and therefore we must consider possible sources of error in our estimates of this volume. First we assume that all the internal volume is enclosed by coupled chromatophores that are capable of photophosphorylation. If the preparations of chromatophores contain substantial amounts of "uncoupled" chromatophores, or membrane preparations that are incapable of photophosphorylation because the orientation of the membrane is the same as in the whole cell (i.e. opposite polarity to the phosphorylating chromatophores), then the value taken for the internal volume in Eqns. 1 and 2 would need to be reduced. However, to produce a significant increase in $\Delta\psi$ or ΔpH, a large reduction in the internal volume is required, since a 2-fold decrease only raises $\Delta\psi$ or ΔpH by the equivalent of 18 mV. We have no direct evidence as to the extent of contamination of our chromatophores by non-phosphorylating membranes, but two lines of evidence suggest that our preparations are not unduly so contaminated. The

fact that the chromatophores maintained very high phosphorylation potentials (Table II) suggests that only a small proportion of uncoupled chromatophores are likely to be present. The rate of ATP hydrolysis catalysed by our preparations was typically 1.5 μmol ATP/mg bacteriochlorophyll per min (assayed in 50 mM Tris/chloride, pH 7.5, at 30°C with 5 mM $MgCl_2$ and 5 mM ATP), whereas the rate of ATP synthesis was between 1 and 2 μmol ATP/mg bacteriochlorophyll per min (assayed under the same conditions as employed for determination of the phosphorylation potential (Table II)). These values are similar to those reported by others (cf. ref. 27). If the ATP hydrolysis activity were mainly associated with the putative uncoupled chromatophores, then it is difficult to see how the high phosphorylation potentials of Table II could be attained. Presumably upon illumination the ATP hydrolysis activity of the chromatophores becomes ATP-synthesising activity. Thus either any "uncoupled" chromatophores lack ATPase activity or they are present in relatively small quantities. We have no evidence as to the possibility that we have a substantial fraction of non-phosphorylating membranes that are orientated with an opposite polarity to phosphorylating chromatophores. However, the similarity of the phosphorylation rate catalysed by our chromatophores and those of others (cf. ref. 27), at least suggests that our preparations are not grossly unusual in composition. We also note that there is immunological data and evidence from the electron microscope that chromatophore membranes are generally orientated in the sense that the ATPase is facing the suspending medium [5].

A second problem in estimating an internal volume could be that in chromatophores a significant fraction of the internal volume is occupied by the electrical double layer on the inner surface of the chromatophore membrane. However, this effect appears to be small, as, if we take a representative value of 0.8 nm for the thickness of this layer [28], then its volume is an insignificant fraction of the total internal volume of a chromatophore with a radius of 30 nm.

In contrast to the results in the present paper, there have been several previous reports of significant light-dependent pH gradients in chromatophores from *R. rubrum* [9,18,19]. Schuldiner et al. [9] found a light-dependent ΔpH of 1.8 units from the distribution of methylamine across the chromatophores membranes, which was measured using a precipitation method to separate the chromatophores from the suspending medium. We suspect that the precipitation step [9] may have resulted in an overestimation of the methylamine uptake, especially as a ΔpH of 1.1 units was measured even when the chromatophores were in the dark [9].

Jackson et al. [18] estimated a ΔpH of 1 unit from the difference between the concentrations of external KCl at which added nigericin caused no pH change in the medium when added to *R. rubrum* chromatophores in the dark and light. Comparison of their result with the present data is difficult as Jackson et al. [18] used rather different conditions of ionic strength and pH than were employed in our experiments.

Recently Leiser and Gromet-Elhanan [19] obtained evidence for a ΔpH of 2.6 units from the extent of quenching of 9-amino-acridine fluorescence using reaction conditions not very different from those used in the present work. Our

failure to observe a significant pH gradient may thus add weight to the previous criticisms that the fluorescence of 9-amino-acridine is not a reliable indicator of pH gradients [6,8] (but see refs. 29 and 30).

As previous work has suggested that a substantial ΔpH is formed when chromatophores are illuminated [1,9,18,19], we now consider some other possibilities as to why we fail to observe a ΔpH. The evidence for the permeability of the uncharged form of methylamine is that the chromatophores did accumulate a significant amount of methylamine when a permeant ion was present (Fig. 6). It is improbable therefore that methylamine was unable to equilibrate with any transmembrane pH gradient that was formed in the absence of a permeant ion. A lack of methylamine uptake in response to a pH gradient would be explicable if the chromatophore membrane is permeable to the charged form of methylamine. The charged form might, for instance, act as a K^+ analogue and thus be pumped out of the chromatophore lumen via a K^+ transport system. A consequence of such behaviour would be that methylamine, at low concentrations, should be an uncoupler which it is not known to be. Furthermore, as methylamine uptake can be observed in the presence of high concentrations of a permeant ion, a rapid transport of the charged form of methylamine from the chromatophore lumen seems unlikely.

Alternatively it may be that the chromatophores used in the present work have a higher internal buffering capacity than the chromatophores used by other workers; this would account in a simple way for our failure to observe a pH gradient. The number of protons that must be translocated to produce a membrane potential of 100 mV can be estimated at between 18 and 90 if a membrane capacitance of between 0.3 and 1.5 μfarad/cm^2 and a chromatophore radius of 30 nm are assumed [21]. It is plausible that this number of protons can be buffered within the lumen of the chromatophores thus giving no significant pH gradient.

Our estimate of the light-dependent $\Delta\psi$ in *R. rubrum* chromatophores is similar to the values obtained by (1) Schuldiner et al. [9] from the distribution of thiocyanate; (2) Pick and Avron [24] from the fluorescence of an oxacarbocyanine dye; (3) Leiser and Gromet-Elhanan [19] from the fluorescence of anilinonaphthalene sulphonate. The agreement of the data from thiocyanate distributions with methods (2) and (3) may, however, be fortuitous as reservations have been expressed about the calibration procedures for these dyes [24,31]. It is also of interest that membrane potentials in the range 100—120 mV have been reported for plasma membrane preparations from a number of bacteria [32].

Although the estimate of $\Delta\psi$ given in the present paper is similar to the value of $\Delta\psi$ determined in other systems, it is perhaps an unexpectedly low value especially as $\Delta\psi$ was found to be the sole component of the proton-motive force under most conditions. The question therefore arises as to whether the SCN^- distribution underestimates $\Delta\psi$, especially as in chromatophores from other photosynthetic bacteria such as *Rhodopseudomonas sphaeroides* or *Rhodopseudomonas capsulata* light-dependent membrane potentials of between 200 and 250 mV have been estimated in the steady state from the extent of the carotenoid band shift [1,21].

We consider that the data given in the present paper are consistent with the

requirement that SCN^- is a permeant ion (cf. refs. 24, 25 and 34) and does equilibrate across the membrane, and that the potential is not perturbed by the movement of SCN^-. With regard to the latter point it is recognised that the potential-driven uptake of the negatively charged SCN^- might tend to lower the potential. For an experiment (Fig. 2) in which 20 μM SCN^- is added to chromatophores, illumination results in uptake of approximately half the thiocyanate, and the concentration of SCN^- inside the chromatophores will be approx. 1 mM. If the radius of a chromatophore is 30 nm then 60 negative thiocyanate ions have been taken up by each chromatophore. As noted earlier a $\Delta\psi$ of 100 mV is established by net uptake of between 18 and 90 protons so that the uptake of 60 thiocyanate ions must be compensated by an extra and electrically balancing uptake of 60 protons if the $\Delta\psi$ is not to be reduced. Two lines of evidence argue that indeed the membrane potential is not reduced by SCN^- concentrations in the range 5—50 μM. First the same value for $\Delta\psi$ was obtained over this range although more SCN^- is taken up at higher concentrations. Second, the ΔG_p generated by the chromatophores was not reduced by the presence of SCN^- which also indicates that these concentrations of SCN^- were not reducing the membrane potential.

It is noteworthy that addition of antimycin to *R. rubrum* chromatophores abolished the light-dependent SCN^- uptake (Fig. 4). The carotenoid band shift, which is generally taken as an indicator of membrane potential, is only partially inhibited by antimycin [5]. Hence we are inclined to support the view of Baltscheffsky [5] that only the antimycin-sensitive component of the carotenoid shift should be used as an indicator of the steady-state membrane potential in the light, at least for *R. rubrum* chromatophores. The high values of the membrane potential obtained in chromatophores from other bacteria [1,21] were estimated from the extent of the carotenoid shift, but these values would be lowered if only the antimycin-sensitive component of the band shift were used.

From a total proton-motive force of 100 mV and a ΔG_p of approx. 14 kcal/mol it was calculated from Eqn. 3 that at least five protons must be translocated for each ATP molecule synthesised. The chemiosmotic hypothesis envisages that two protons are translocated for each ATP made [4], and some evidence has been obtained with chromatophores that agrees with this view [1,35]. Casadio et al. [1] reached this conclusion from a comparison of the proton-motive force with ΔG_p, but as discussed above they may have overestimated the proton-motive force by using the total extent of the carotenoid shift and relying on the quenching of 9-amino-acredine fluorescence to determine ΔpH.

Studies on the movement of protons during ATP synthesis following single flash excitation of chromatophores from *Rps. sphaeroides* have led Jackson et al. [35] to conclude that approximately two protons are translocated per ATP synthesised. The discrepancy between the kinetic work of Jackson et al. [35] and the present thermodynamic measurements cannot be explained at present, but it is noteworthy that Gräber and Witt [36] have found recently in chloroplasts that the H^+/ATP ratio may not be the same with single turnover flashes as during continuous illumination.

Note added in proof (Received February 6th, 1978)

A paper by Kakuno et al. [42] in which an estimate of 790 bacteriochlorophyll molecules per chromatophore of 60 nm diameter has belatedly come to our attention. Use of their data gives an internal volume of approx. 100 μl per mg of bacteriochlorophyll (cf. Table I).

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