Spectrophotometric Identification of the Pigment Associated with Light-driven Primary Sodium Translocation in \textit{Halobacterium halobium}*

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It has been recently proposed that in addition to bacteriorhodopsin, \textit{Halobacterium halobium} membranes contain a second light-reactive pigment, whose function is the translocation of sodium ions. The evidence is based on the presence of light-driven, uncoupler-insensitive, sodium ion extrusion and light-driven, uncoupler-enhanced, passive proton uptake in cell envelope vesicles prepared from \textit{H. halobium}. However, direct spectrophotometric demonstration of the existence of such a pigment had not been possible. \textit{H. halobium} strain ET-15 membranes lack spectroscopically detectable quantities of red carotenoids and bacteriorhodopsin. After such membranes are extensively illuminated (bleached) in the presence of hydroxylamine, addition of \textit{trans}-retinal causes the appearance of an absorption band at 588 nm, while the absorption band of free retinal decreases. The 588 nm band corresponds approximately to action spectra reported earlier for the light-driven sodium pump of \textit{H. halobium}, and its magnitude in partially bleached vesicle membranes is proportional to the extent of restoration of light-induced passive proton uptake after retinal addition. Hence, we have concluded that the 588 nm absorption band is associated with the sodium pump. From the amplitude of the absorption band and the estimated extinction coefficient (48,000 M$^{-1}$ cm$^{-1}$), the amount of the pigment in the ET-15 strain is calculated to be about 5% of the bacteriorhodopsin content of strain R-1. Although bleaching under the conditions employed, and reconstitution with retinal, are properties of bacteriorhodopsin, the 588 nm pigment is distinguished from the purple pigment by its red-shifted absorption band, by its lowered molar extinction coefficient, by its apparent lack of \textit{cis-trans} isomerization (dark adaptation), by a large pH-dependent blue shift in its absorption band (from 588 to 548 nm, with a pK of 9.6), and by its sensitivity to heating at 75°C for 5 min, for both transport function and retinal reconstitution.

The cytoplasmic membrane of \textit{Halobacterium halobium} contains at least two light-reactive pigments whose function is ion translocation. The better described of these is bacteriorhodopsin (for recent reviews see Refs. 1–9), which is a \textit{trans}-retinal-opsin complex with an absorption band at 588 nm. This protein is found in crystalline arrays ("purple membrane"), covering up to 50% of the cell surface, and its function has been identified as that of a light-energized pump for protons. Recently, Lindley and MacDonald (4) suggested that \textit{H. halobium} membranes contained a second, functionally distinct, pigment which acted as a light-driven pump for sodium ions. Action spectra obtained by Greene and Lanyi (5) and Weber and Probst* for this pigment indicated that it must absorb near 588 nm, and the data suggested that it was present in much smaller amounts than bacteriorhodopsin. The functional identification of the second pigment was aided considerably by the availability of bacteriorhodopsin-deficient mutant strains of \textit{H. halobium}. One of these was described earlier by Matsuno-Yagi and Mukohata (6), and contained red carotenoids but little or no bacteriorhodopsin, while another, isolated and characterized by Weber et al.* contained little or no interfering pigments which absorb above 500 nm.

When cell membrane vesicles prepared from \textit{H. halobium} are illuminated the proton movements observed reflect either the operation of the \textit{H}$^+$ pump (active proton extrusion) or the operation of the \textit{Na}$^+$ pump (passive proton uptake in response to the membrane potential created), or in some vesicle preparations, both. In rapidly sedimenting vesicles prepared from \textit{H. halobium} strain R-1, active proton extrusion predominates (7,9), while in the minor, slowly sedimenting vesicle fraction, both active and passive proton movements can be detected (5). In vesicles prepared from the bacteriorhodopsin-deficient \textit{H. halobium} strains only passive proton uptake can be observed (4,5). Proton uptake in these vesicles appears to be primarily through the \textit{F}$\sigma$ portion of the membrane ATPase, because it is inhibited by treatment with low concentrations of dicyclohexylcarbodiimide, but restored when an uncoupler is added (5).

The physiological role of the light-driven sodium pump is uncertain, since it does not appear to be the main \textit{Na}$^+$ extrusion mechanism in \textit{H. halobium} (5,9,10). On the other hand, Matsuno-Yagi and Mukohata (6) have described light-induced ATP synthesis in their bacteriorhodopsin-deficient strain, which they found to be accompanied by proton uptake. It

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R. E. MacDonald and E. V. Lindley, personal communication.
seems likely that such proton uptake in whole cells (5, 6) also seen transiently during the illumination of the bacteriorhodopsin-containing strain R-1, takes place in response to the increased membrane potential created by the extrusion of Na⁺, as in vesicles from bacteriorhodopsin devoid strains (4, 5). Since under these conditions the pH gradient across the cell membrane decreases, the increase in the electrochemical potential difference for H⁺ which activates ATP synthesis, should be the result of the operation of the sodium pump. Thus, it may be argued that the main function of the light-driven sodium pump in H. halobium is to energize the light-dependent synthesis of ATP.

This report describes the spectrophotometric identification of the pigment associated with the sodium pump in H. halobium strain ET-15, isolated by Weber et al. Vesicles prepared from this strain contain no more bacteriorhodopsin than 0.004 mmol/mg of protein (this report) and maybe much less, but possess about 0.13 mmol/mg of membrane protein of a retinal protein with an absorption band at 588 nm, which can be related to the operation of the sodium pump as assayed by following light-induced proton proton uptake. This pigment, like bacteriorhodopsin, is bleached when illuminated in the presence of hydroxylamine and can be reconstituted with added retinal, but various spectral properties, as well as its response altered pH and heat treatment clearly distinguish it from the other retinal-protein. Throughout this paper, we refer to the pigment as a light-driven sodium pump. This designation comes from the functional description of this membrane component, and should not be taken to imply a necessary structural analogy with the proton pump in the same organism, bacteriorhodopsin.

**MATERIALS AND METHODS**

Isolation and characterization of the "colorless" H. halobium strains ET-15 and W 5002-7 will be described elsewhere. These strains, as well as strain R-1, were grown in a peptone medium (11), and were used to prepare cell envelope vesicles by the sonication method (7). The vesicle membrane orientation was ascertained by assaying for NADH-monetidine oxidoreductase in the presence and absence of Triton X-100 (11). All preparations had 85 to 95% inside-out orientation. The vesicles were stored at 5 to 15 mg/ml of protein containing 0.2 M NaCl in the refrigerator, and retained transport activity for at least 3 to 4 weeks. Bacteriorhodopsin was prepared according to the method described by Oesterhelt and Stoeckenius (12). Unless otherwise indicated, all measurements were carried out in unbuffered 4 mM NaCl at pH 6.3 to 6.5. Protein was determined by the Lowry method (10).

The operation of the sodium pump was assayed by following light-induced proton uptake in the presence of the proton conductor, FCCP (4, 5). The pH change during the illumination of the stirred vesicle suspensions (3 ml, 1.35 mg/ml of protein in 4 mM NaCl) was measured at 30°C with a Beckman 2500B semimicro combination electrode, connected to a Corning model 12 pH meter. The signal was passed through a Tektronix 1A7A differential amplifier and recorded on a 10-inch strip chart recorder at 1/2 inch/min, set at a full scale deflection of either 0.25 or 0.50 pH unit. The electrode was wrapped with black tape to within 1/2 inch of the tip; its response to the illumination was insignificant, as shown by tests with the vesicles omitted or with vesicles from the light-unresponsive H. halobium strain W 5002-7. Illumination of the chamber was with a GE ELH 300 watt quartz-halogen lamp, with 7 cm of water, a Corning 3-69 cut-off filter, and an Optical Industries, Inc., heat-reflecting mirror interposed between light source and sample. Light intensities in the cuvette were about 2 x 10^6 erg-cm⁻²-s⁻¹, as measured with a Kettering model 68 radiant energy meter.

Bleaching of the pigments was by the method described by Oesterhelt et al. (14) for bacteriorhodopsin. The vesicles were suspended in 0.2 M hydroxylamine containing 2 mM retinal up to 20 to 20 ml of 0.2 M NH₂OH + 5 mM NaCl, adjusted to pH 7.0, and illuminated with two GE ELH lamps outfitted with Corning 3-69 cut-off filters, placed 10 cm from the source. After bleaching, the hydroxylamine was removed by sedimenting the vesicles at 35,000 g (1,000 rpm for 30 min in a Sorvall SS-34 rotor), and repeatedly washing with 4 mM NaCl, followed by suspension in 4 mM NaCl to a concentration of 4 mg/ml of protein. Extensive removal of the hydroxylamine was dependent on the bursting and rescaling of the vesicles during the transfer from 5 mM to 4 mM NaCl. Re-equilibration of the washed vesicles with 5 mM NaCl, followed by more washing with 4 mM NaCl better facilitated the complete removal of intravesicle hydroxylamine.

Difference spectra were determined in a Cary 14 spectrophotometer, equipped with a scattered transmission accessory, and connected to a Nicolet Corp. LAB 80 computer which allowed the digital recording, scaling, and subtraction of the traces. Slit width was less than 0.2 mm above 430 nm, and less than 0.5 nm throughout all scans. The scanning rate was 5 nm/s. The wavelength accuracy of the instrument was checked by determining spectra for purified light-adapted bacteriorhodopsin (580 nm) and reduced horse heart cytochrome c (550 nm). Base-lines, obtained with identical samples in the sample and reference cuvettes before additions, were always subtracted from the difference spectra. In order to show reproducibility, difference-base lines were obtained by subtracting one base-line from another, and are included in several of the reproductions of the spectra. In all cases the vesicle suspensions were contained in 3-ml capacity cuvettes (1 x 1 cm).

Absolute absorption spectra of the vesicles were determined in the Cary 14 spectrophotometer, but with the detection system replaced with an 8-inch diameter BaSO₄-coated, light-integrating sphere and an EM1958 QA photomultiplier. The signal was passed through a Hewlett Packard model 7561A logarithmic amplifier and collected in the Nicolet Corp. LAB 80 computer. In these experiments the spectrophotometer was used in the single beam mode, with the optical chopper disabled, and the slit set at 2 mm. The spectra were obtained by subtracting traces recorded with the sample out of the light beam from traces recorded with the sample placed in the beam. The vesicle suspensions were in 5-ml round cuvettes with 1-cm light path.

Hydroxylamine (HCl salt) and trans-retinal were purchased from Sigma Chemical Co. FCCP was from Pierce Chemicals.

**RESULTS**

Earlier reports (4, 5) indicated that the pigment associated with the primary light-driven Na⁺ transport system in H. halobium was present in quantities too small for direct spectrophotometric detection in turbid membrane suspensions. A method of detection of the pigment involved the use of difference spectroscopy occurred to us. Since the pigments appeared to be similar to bacteriorhodopsin, it seemed reasonable to expect that it could be bleached and reconstituted with retinal, using procedures developed for bacteriorhodopsin (14, 15). When retinal was added to bleached H. halobium ET-15 vesicles, the absorption spectrum of the sample changed; a new absorbance band appeared and the amplitude of the absorbance band of retinal became smaller. Fig. 1B contains difference spectra obtained between a bleached sample with added trans-retinal and another sample with the equivalent amount of solvent (methanol) added. The new absorption band, arising during the 24 min of incubation with retinal in Fig. 1B, is somewhat asymmetric and located at 588 ± 1.3 nm (nine determinations). The decrease of the retinal absorbance at 385 nm and the existence of an isosbestic point at 430 nm strongly suggest that the 588 nm pigment is reconstituted at the expense of retinal, i.e. that it is a retinal complex. In unbleached, but hydroxylamine-treated, vesicles the retinal absorbance did not change during the same time period (Fig. 1A). The magnitudes for the rise of the 588 nm absorption amplitude and the fall of the 385 nm retinal absorption are shown together in Fig. 2 for incubations with retinal up to 3 h. For the bleached sample the correspondence of the time course of the absorbance changes at the two wavelengths seem to be good. As Fig. 2 indicates, reconstitu-
Sodium Pump of Halobacterium halobium

Bleaching and retinal reconstitution of *H. halobium* R-1 vesicles was also performed (not shown). In these vesicles the predominant retinal-protein is bacteriorhodopsin. The time course of absorbance changes in the R-1 vesicles was similar to that in Fig. 1B, but the new absorption band which developed was at 562 nm. The blue shift from the expected 568 nm band for bacteriorhodopsin was due to dark adaptation of this pigment during reconstitution, which results in partial conversion to the 13-cis form of the retinal chromophore (summarized in Ref. 16), since light adaptation of the reconstituted membranes caused a red shift to 568 nm.

It was possible to calculate an extinction coefficient for the 588 nm pigment from the data in Fig. 2. The concentration of retinal in the reconstitution experiments was determined by obtaining absorption spectra for the retinal diluted into ethanol, and using the published extinction coefficient of 43,400 M⁻¹ cm⁻¹ in this solvent (17). The so calculated molarity of the retinal, its absorption in the presence of the membrane vesicles, and the ratio of the absorbance changes for the retinal pigment and the retinal were then used to calculate an approximate extinction coefficient. The calculated values depended somewhat on the length of incubation with retinal, since beyond the 1st h, the retinal absorbance fell less rapidly than the rise of the pigment absorbance (Fig. 2). This difficulty may be possibly caused by complexity in the reconstitution process. However, we feel that it is probably caused by an artifact, since after 1 h of incubation an absorbance shoulder at 370 nm of unknown origin appeared, which could have contributed to absorbance at 385 nm as well. In addition, the 588 nm pigment may have small but not negligible absorbance at 385 nm, as does bacteriorhodopsin (18). The extinction...
coefficients were therefore calculated from data for the 1st h of reconstitution. As a control, the molar extinction of bacteriorhodopsin was calculated from analogous data for R-1 vesicles. Correction was made for light-scattering, since the apparent extinction coefficient of purified bacteriorhodopsin added to a vesicle suspension was 11% greater than in 4 M NaCl by itself. The corrected extinction coefficient for bacteriorhodopsin was 61,500 ± 9,000 M⁻¹ cm⁻¹ (from four sets of determinations), as in Fig. 2, but with R-1 vesicles, which corresponds well with the generally accepted value of 63,000 M⁻¹ cm⁻¹ (19), but may be high because the position of the absorption band indicated that dark adaptation had taken place and the extinction coefficient of dark adapted bacteriorhodopsin is 12% lower (16). The estimated extinction coefficient of the 588 nm pigment was 48,000 ± 5,000 M⁻¹ cm⁻¹ (from three sets of determinations).

The amount of 588 nm pigment that can be reconstituted in extensively bleached ET-15 vesicles was calculated in retinal equivalents, using the above extinction coefficient. The amount was rather invariant: 0.075 ± 0.003 nmol/mg of protein in three different batches of vesicle preparations. The amount of bacteriorhodopsin reconstituted in an R-1 vesicle preparation was 2.2 nmol/mg of protein. Typical bacteriorhodopsin contents of such vesicles is 2.5 nmol/mg of protein (7), which agrees well with this result.

The functioning of the sodium pump in bleached and reconstituted ET-15 vesicles was followed by determining light-driven passive proton uptake in the presence of an uncoupler. Such H⁺ uptake had been shown earlier in these and other bacteriorhodopsin-deficient vesicles to take place in response to the electrical potential (inside negative) created during the electrogenic extrusion of sodium ions (4, 5). Vesicles bleached for different times were assayed for H⁺ uptake before and after addition of retinal. The rise in pH, measured during the illumination of the vesicle suspensions, reached steady state values within 3 min, and after the illumination was terminated the pH dropped to nearly its initial value within 4 to 5 min. This steady state pH change, together with the buffering capacity determined from HCl additions, was used to calculate the amount of protons displaced during illumination. It is evident from the results, shown in Table I, that increasing periods of bleaching progressively inactivated proton uptake activity, i.e. the functioning of the sodium pump. Addition of retinal to the bleached preparations caused a 20 to 25% recovery of the lost activity.

The appearance of the 588 nm absorption band after the addition of retinal was followed in the partially bleached preparations also. The time course for the rise of 588 nm absorption in these samples was similar to that shown in Fig. 2, but the amplitude of the absorbance change was dependent on the extent of bleaching. The initial rate of the absorbance change at 588 nm was approximately constant, so that the absorption amplitude reached its final value more slowly in samples bleached for longer time periods. The retinal-dependent increases in the light-induced proton movement (as in Table I) and the absorption increases at 588 nm (as in Figs. 1B and 2) in these samples were plotted against one another in Fig. 3. The linear relationship obtained between the reconstituted spectral and functional properties is consistent with the idea that the 588 nm absorption band belongs to a retinal-protein complex responsible for the electrogenic transport of Na⁺.

Bacteriorhodopsin undergoes slow (t½ = 20 min at room temperature) dark adaptation, reflecting the equilibration of cis-trans retinal isomers, which results in a blue shift of about 10 nm and an absorption amplitude decrease of 12%, reversed by illumination (16). The 588 nm pigment, reconstituted in ET-15 vesicles, showed no such dark adaptation.

The activity of the sodium pump in H. halobium vesicles was found earlier (5) to be sensitive to heating at 75°C for 5 min, while bacteriorhodopsin was not inactivated. The heat treatment did not alter the spectral properties of ET-15 vesicles.

**Table I**

**Reconstitution of light induced proton uptake in bleached H. halobium ET-15 envelope vesicles**

| Hours bleached | H⁺ uptake | Percent reconstitution
<table>
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<tr>
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<tr>
<td></td>
<td>Retinal (A) + Retinal (B)</td>
<td>B - A</td>
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<tr>
<td>0</td>
<td>15.6</td>
<td>15.6</td>
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<tr>
<td>1</td>
<td>13.7</td>
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<tr>
<td>2</td>
<td>9.0</td>
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<td>8</td>
<td>2.8</td>
<td>5.4</td>
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<td>15%</td>
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vesicles from the retinal reconstitution of the 588 nm band was depressed by at least 95%. In contrast, the retinal reconstitution of bleached ET-15 vesicles, placed an upper limit of 0.004 nmol/mg of protein on the sodium pump content of ET-15 vesicles. The magnitude of absorbance changes at other wavelengths in Fig. 5 cannot be identified at present. The shapes of the spectra, which change progressively with pH, suggest that the amplitude of the 600 nm difference band can be estimated in such experiments from the difference in absorption between 600 nm and 480 nm, rather than between 600 nm and the high wavelength end of the spectra, since the latter would not take into account nonspecific spectral changes probably due to changes in light-scattering. When the 600 minus 480 nm absorbance difference is plotted against the pH in the reference sample a titration curve with a midpoint at pH 9.6 is obtained (not shown). This pH presumably reflects a pK for the group(s) responsible for the alkalinization-dependent blue shift in the pigment absorption band.

![Graph showing relationship between reconstituted light-dependent passive proton uptake and absorbance increase in bleached Halobacterium halobium ET-15 vesicles.](image)

**Fig. 3.** Relationship between retinal-reconstituted light-dependent passive proton uptake and absorbance increase in bleached Halobacterium halobium ET-15 vesicles. The samples were partially bleached by illuminating for various lengths of time in the presence of hydroxyamine. Light-dependent proton uptake by the bleached vesicles, indicative of the activity of the sodium pump in the membranes, was determined before and 1 to 3 h after addition of trans-retinal, as in Table I. Changes in the amplitude of the 588 nm absorption band of the pigment after addition of the retinal were determined as in Figs. 1B and 2. Incubations with retinal were from 1 to 3 h, until the increment of the 688 nm band reached constant values.

Pigment differences. However, when bleached vesicles were first heated, the retinal reconstitution of the 588 nm band was depressed by at least 95%. In contrast, the retinal reconstitution of bleached vesicles from the bacteriorhodopsin-containing R-1 strain was virtually unaffected by prior heating. This difference in thermal sensitivity between the sodium pump pigment and bacteriorhodopsin allowed the estimation of bacteriorhodopsin content of ET-15 vesicles. The magnitude of absorbance increase at 562 nm during the reconstitution of the heated bleached vesicles placed an upper limit of 0.004 nmol/mg of protein on the purple pigment in ET-15 membranes.

When the pH of retinal-reconstituted bleached ET-15 vesicle suspensions was adjusted to values >9, the 588 nm absorption band shifted to 548 nm and diminished in amplitude (Fig. 4). The difference between the retinal-dependent spectra taken at pH 6.6 and 10.6 exhibits an absorption band at about 600 nm, as shown in Fig. 4. The ratio of the amplitudes of the original 588 nm band and the pH-dependent difference band at 600 nm is 1.5:1. When the pH of a bleached ET-15 vesicle suspension was adjusted to 10.4, addition of retinal caused the appearance of the 548 nm band directly. Neutralization of the vesicle suspensions with HCl completely reversed these spectral shifts. A pH-dependent shift in the absorption band of bacteriorhodopsin was not detectable when the pH of a suspension of purified purple membrane in 4 M NaCl was raised to about 11. Nor was such shift observed when bleached Halobacterium halobium ET-15 vesicles were reconstituted with retinal at neutral pH (yielding predominantly a dark-adapted bacteriorhodopsin spectrum), and the pH was then raised above 10, or when the reconstitution was carried out at pH 10.4.

The finding of a pH-dependent absorbance shift for the pigment associated with the sodium pump, which results in the appearance of a 600 nm absorption difference band, suggested that it should be possible to detect the pigment in unbleached membranes as well. The determination would be based on measuring the amplitude of the 600 nm band in difference spectra between samples adjusted to neutral and alkaline pH. Difference spectra were therefore obtained between two unbleached ET-15 vesicle suspensions, one kept at pH 6.65 and another whose pH was raised stepwise. As shown in Fig. 5, under these conditions an absorption band at 600 nm is indeed observed, whose amplitude increases with increasing pH in the reference sample. The pH-dependent absorbance changes at other wavelengths in Fig. 5 cannot be identified at present. The shapes of the spectra, which change progressively with pH, suggest that the amplitude of the 600 nm difference band can be estimated in such experiments from the difference in absorption between 600 nm and 480 nm, rather than between 600 nm and the high wavelength end of the spectra, since the latter would not take into account nonspecific spectral changes probably due to changes in light-scattering. When the 600 minus 480 nm absorbance difference is plotted against the pH in the reference sample a titration curve with a midpoint at pH 9.6 is obtained (not shown). This pH presumably reflects a pK for the group(s) responsible for the alkalinization-dependent blue shift in the pigment absorption band.

![Graph showing demonstration of pH-dependent blue shift in the retinal-induced difference spectrum of bleached Halobacterium halobium ET-15 vesicles.](image)

**Fig. 4.** Demonstration of a pH-dependent blue shift in the retinal-induced difference spectrum of bleached Halobacterium halobium ET-15 vesicles. Trace 1, difference spectrum between 24 h bleached vesicles, with and without added retinal, determined under the conditions described for Fig. 1B. The trace shows the 588 nm band, proposed to belong to the light-driven sodium pump in the membranes. Trace 2, after recording Trace 1 the pH of both sample and reference cuvette contents was raised from 6.6 to 10.6 by adding a few microdrops of 2 N NaOH, and another difference spectrum was recorded. The trace shows a blue shift in the retinal-dependent band (from 588 nm to 548 nm), and an amplitude decrease of about 45%. Subtracting Trace 2 from Trace 1 yields the third spectrum shown, which exhibits a distinct band at 600 nm.
The amount of 588 nm pigment in unbleached ET-15 vesicles were suspended to band in Fig. 5, and the ratio of 588 nm and 600 nm absorption, 4 and divided into sample and reference portions for difference spectroscopy. The pH of the reference cuvette contents was raised to the values indicated next to the traces by addition of microliter increments of 2 M NaOH. The sample cuvette received equal amounts of 4 M NaCl, and the difference spectra were recorded.

From the amplitude of the pH-dependent 600 nm difference band in Fig. 5, and the ratio of 588 nm and 600 nm absorption, the amount of 588 nm pigment in unbleached ET-15 membranes is estimated to be 0.137 nmol/mg of protein. When an extensively (24 h) bleached ET-15 vesicle preparation was tested for the pH-dependent absorbance changes described above, a small peak similar to that in Fig. 5 but located at 590 nm was observed. If the relationship between the absorbance difference at 600 and 490 nm and the amplitude of the 588 nm band due to the sodium pump still holds for such a preparation, about 0.064 nmol/mg of protein of the original 588 nm pigment can be calculated to remain after bleaching of the membranes. This quantity, plus the amount of pigment reconstituted with retinal, as determined by spectroscopic measurements, add up to 0.139 nmol/mg of protein, which is approximately the estimated original amount in unbleached membranes. Vesicles prepared from the light-unresponsive Halobacterium halobium strain W 5002-7 yielded virtually no difference of absorbance between 600 and 480 nm upon alkalization, but the absorption bands below 500 nm, found in the ET-15 membranes under these conditions (Fig. 5), were present. Thus, it appears that the 600 nm difference band, but not the other complex pH-dependent spectral changes, is associated with light-driven transport.

Another method for demonstrating the existence of the 588 nm absorption band in unbleached membranes proved to be difference spectroscopy using Halobacterium ET-15 vesicles, which contain the sodium pump, and W 5002-7 strain vesicles, which apparently lack it. The difference spectrum between such preparations (Fig. 6) indicates that the ET-15 membranes contain a number of absorbing species which are missing in W 5002-7 membranes. One of these absorbs near 590 nm, and should correspond to the pigment detected by retinal reconstitution, i.e. the sodium pump pigment. The other, larger absorption bands at lower wavelengths are presently unidentified, and they preclude the determination of a complete absorption spectrum for the pigment. It is significant to note, however, that the absorption band near 590 nm in Fig. 6 is evidence for the existence of the pigment in untreated ET-15 membranes. Using the extinction coefficient for this pigment, the difference spectrum in Fig. 6 yields 0.132 nmol/mg of protein, an amount which agrees with the estimation from the pH-dependent spectral changes (Fig. 5).

All of the spectra discussed so far were obtained by difference spectroscopy. It was desirable to determine also the absolute absorption spectra of the vesicle preparations near 590 nm, without including any contribution from light-scattering. The use of the light-integrating sphere, described under "Materials and Methods," made it possible to measure such absolute spectra, and these are shown in Fig. 7 for ET-15 vesicles at pH 6.5 and 10.6. At the lower pH no distinct band at 588 nm is seen, but rather a broad shoulder to a large absorption band near 423 nm. After raising the pH, however, a substantial decrease in absorbance near 590 nm occurs, yielding a difference spectrum with a band near 600 nm, similar to those detected under similar conditions by difference spectroscopy (Figs. 4 and 5). Using the relationship between the amplitudes of the 600 and 588 nm bands (from
The absorption spectrum of bacteriorhodopsin in the visible region is largely unchanged between pH 4 and 11, but the spectrum of the sodium pump pigment exhibits a large, reversible blue shift (from 588 to 548 nm) and amplitude decrease (about 45%) at a midpoint pH of 9.6 (Figs. 4 and 5). Therefore, it appears that the absorption band of the chromophore of the sodium pump, but not of bacteriorhodopsin, is dependent on relatively easily dissociated proton(s).

The absorption spectrum of bacteriorhodopsin, as well as its ability to reconstitute with retinal after bleaching and to translocate protons, is remarkably insensitive to heating. This thermal stability is undoubtedly a consequence of the rigid, highly helical structure of the apoprotein (20-22). In contrast, the light-driven sodium pump in vesicle preparations is inactivated by heating at 75°C for 5 min (5). This observation is consistent with the heat sensitivity of light-driven proton uptake in intact bacteriorhodopsin-deficient cells of *H. halobium* (6), a process which probably also reflects the activity of the sodium pump. We now find that the retinal reconstitution of the bleached apoprotein of the sodium pump does not occur in heat-treated membranes. It appears from these results that the structure of the sodium pump allows more thermal disruption than that of bacteriorhodopsin.

We feel, however, that in spite of the obvious differences between the two pigments of *H. halobium*, discussed above, the sodium pump belongs in the same general category of retinal proteins as does bacteriorhodopsin. The finding that the sodium pump pigment can be bleached with hydroxyamine during illumination tends to argue that the reaction of hydroxyamine with a deprotonated Schiff-base during the bleaching of bacteriorhodopsin (14) may occur also in the sodium pump pigment. The finding that the pigment is reconstituted with retinal with respect to activity and spectral properties suggests that the sodium pump pigment is also a retinal-protein complex. We do not rule out the possibility, however, that the original chromophore in this pigment is a retinal analogue, rather than retinal, or that reconstitution includes some chemical modification of the added retinal by membrane-bound enzymes.

The number of protons displaced per molecule of retinal chromophore in illuminated *H. halobium* ET-15 vesicles is calculated to about 120. Thus, assuming one charge per Na+ transported, we conclude that steady state in these vesicles is reached after more than 100 transport cycles for each sodium pump molecule. The corresponding number for bacteriorhodopsin in *H. halobium* R-1 vesicles at the same light intensity is about 20 (9).

The results reported here raise a fundamental question about the nature of the sodium pump of *H. halobium*, to be answered once it is available in purified form. One alternative is that the sodium pump may be structurally analogous to bacteriorhodopsin, but translocates Na+ instead of H+. On the other hand, the sodium pump may be a complex between a proton-translocating, bacteriorhodopsin-like pigment and a sodium/proton antiporter, with tight coupling between these two components. In either case, the structure of the opsin component of the sodium pump is likely to be different from that of bacteriorhodopsin.

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