Quantitative Dissolution of the Membrane and Preparation of Photoreceptor Subunits from *Rhodospirillum rubrum**

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ABSTRACT: Application of the alkaline–urea–Triton membrane dissolution procedure to membrane particles from *Rhodospirillum rubrum* has given results completely compatible with those previously obtained for *Rhodopseudomonas spheroides*. Phospholipid material is nearly completely displaced by Triton X-100 in the preparation of these particles. The average particle weight of the derived material is about 100,000 in 0.2% Triton X-100 and the liberated photoreceptor subunits retain complete activity as measured by absorbance photochange, electron paramagnetic resonance photochange, and the quantum yield for these processes. It is demonstrated that these fully active bacteriochlorophyll–trap–protein complexes are obtained in quantitative yield from the membrane with no significant change in spectral properties. Thus, together with earlier results with the *R. spheroides* system, these data strongly support the concept that photoreceptor subunits exist in the membrane along with other kinds of lipoprotein subunits.

From preliminary analysis data it is shown that, of the transition metals present in the bacteria, only iron remains in these photoreceptor subunit preparations at a high enough concentration to play a role in the phototrap. The decrease in phototrap activity at lower potentials is measured and the results are shown to be consistent with an earlier suggestion which would assign this property to the primary electron acceptor molecule.

Now that several characteristic properties of a functioning bacterial reaction center, or phototrap, are known (Duysens, 1952; Duysens *et al.*, 1956; Vredenberg and Duysens, 1963; Arnold and Clayton, 1960; Clayton, 1962a,b, 1963; Androes *et al.*, 1962; Fuhrhop and Mauzerall, 1968, 1969; McElroy *et al.*, 1969; Goedheer, 1959; Kuntz *et al.*, 1964; Loach *et al.*, 1963; Loach, 1966; Loach and Sekura, 1968; Loach and Walsh, 1969; Parson, 1968; Cramer, 1969) and can be readily measured with suitable instrumentation (Duysens, 1952; Kok, 1956; Commoner *et al.*, 1956; Sogo *et al.*, 1957; Arnold and Clayton, 1960; Kuntz *et al.*, 1964) one can seriously pursue isolation and purification schemes.

Two different approaches toward such isolation have been undertaken in recent years. One of these, the earlier approach, made use of unmasking (Kok, 1961; Clayton, 1962a, 1963; Loach *et al.*, 1963) the phototrap at an early stage in membrane simplification. An extension of this effort focuses primarily on using selected detergents that work well with especially selected photosynthetic systems (Reed and Clayton, 1968; Reed, 1969; Thornber *et al.*, 1969; Gingras and Jolchine, 1969; Ogawa and Vernon, 1969; Vernon *et al.*, 1969). The ultimate goal for this approach seems to be to isolate a reaction center which is free of all components not essential to accomplishing the primary photochemistry. The initial thoughts (Clayton, 1962a,b; Reed and Clayton, 1968; Reed, 1969; Thornber *et al.*, 1969; Vernon *et al.*, 1969) were that the reaction center sought may necessarily have a required complement of electron carriers (cytochromes, quinones, etc.) associated with it in order for it to function.

A second, more recent, approach assumes that most of the bacteriochlorophyll in bacterial photosynthesis systems is deposited in very discrete and specific packets in the membrane and may be isolatable with distinct subunits of the membrane structure (Loach *et al.*, 1968–1970; Loach and Hall, 1970). In this case it is thought that nearly all bacteriochlorophyll molecules and, perhaps, the carotenoid molecules as well are an integral part of the unit and can be isolated with the phototrap as a relatively small (approximately 100,000 particle weight) bacteriochlorophyll–protein complex. This subunit complex in turn is viewed as consisting of four to eight smaller subunits, one of which has the phototrap pigments specifically attached to it. The two approaches may one day arrive at the same point if the phototrap–protein complex is obtained in pure form by each procedure.

In this paper we wish to report results obtained by applying the alkaline–urea–Triton procedure (Loach *et al.*, 1970) to chromatophores prepared from *Rhodospirillum rubrum*. The results are in excellent accord with those previously reported for chromatophores from *Rhodopseudomonas spheroides* and lend support to the existence of distinct entities which we have called photoreceptor subunits. Preliminary reports of this data have been given (Loach *et al.*, 1968, 1969; Loach and Hall, 1970).

Materials and Methods

The bacterium *R. rubrum* (No. 1.1.1) was propagated and chromatophores prepared from them as previously described (Loach *et al.*, 1963). The chromatophores were stored at 4° by resuspending in water at a high concentration

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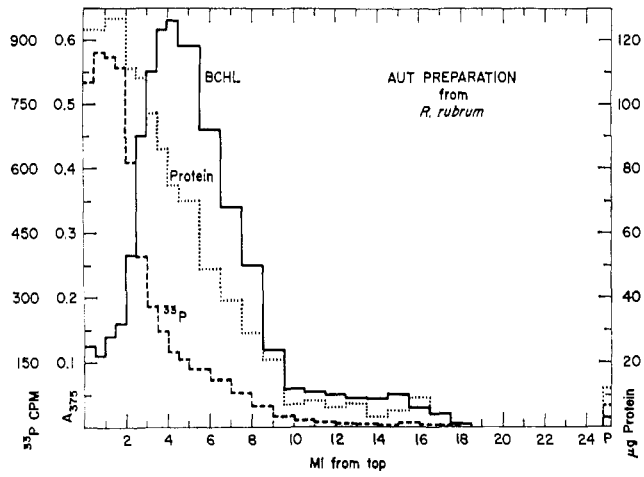

FIGURE 1: Analysis of centrifuge tube after centrifugation. Tube originally contained 8 ml of 4% sucrose, 8 ml of 2% sucrose, and 8 ml of 1% sucrose. All sucrose fractions contained 0.05 M phosphate buffer (pH 11.5), 3% Triton X-100, and 6 M urea. Chromatophores (1.0 ml; A_{880} = 100) in dilute glycylglycine buffer (pH 7.5) was initially layered on the top. Centrifugation time, including acceleration and deceleration, was 1 hr. Samples (0.060 ml) of each fraction were used for suspension in the scintillating media for determining ^{33}P content. Background counts have been subtracted from the plotted data. The bacteria used for chromatophore preparation in this experiment were grown on the usual media except [^{33}P]P_i was added to the phosphate buffer. The tube was drained from the bottom and 1- or 0.5-ml fractions collected as indicated. The content of the pellet is indicated by the letter P after the 25th ml. Several other experiments of this type gave nearly identical results.

(absorbance at 880 nm equals approximately 500). In some experiments the chromatophores were subjected to chromatography on Bio-Gel A-150 m at pH 7 and the center two-thirds of the predominant band collected for use.

Triton X-100 was obtained from Rohm and Haas (Philadelphia, Pa.). In those experiments where radioactive (^{14}C) bacteria were cultivated, *d,l*-malic acid-3-^{14}C (New England Nuclear) was added to the growth media at a final level of 10,000 cpm/ml. [^{33}P]P_i was also purchased from New England Nuclear and added to the growth media at a final level of 40,000 cpm/ml. We have found ^{33}P very useful in our studies because it has a softer β than ^{32}P and nearly twice the half-life. The Bio-Gel material used in column chromatography was obtained from Bio-Rad Laboratories (Richmond, Calif.).

Protein contents of samples were determined by the method of Lowry *et al.* (1951) and the nitrogen content of chromatophores was also determined by the microKjeldahl method (McKenzie and Wallace, 1964). Good agreement between results obtained by these two methods was found.

A Tri-Carb liquid scintillation spectrometer (Series 314 E, Packard Inst. Co., Downers Grove, Ill.) was used for measuring radioactivity in various cell fractions. The method of sample preparation was as previously reported (Loach *et al.*, 1970; Loach and Hall, 1970).

Absorbance spectra and light-induced absorbance changes were measured with a Cary 14R recording spectrophotometer appropriately modified as previously described (Loach, 1966). The redox titrations in the absence of air were conducted using techniques that have also been previously reported (Harbury, 1957; Loach and Calvin, 1962; Kuntz *et al.*, 1964; Loach, 1966). More rapid kinetic parameters were measured using a kinetic spectrometer whose construction and operation have been described (Loach and Loyd, 1966). The electron paramagnetic resonance signals were measured using a Varian E-3 spectrometer.

Disc gel electrophoresis was carried out using a Canalco unit (Model 66, Rockville, Md.). A variety of gels were prepared containing from 3.75 to 7.5% acrylamide. To develop color in the region of protein, the dye aniline blue black (Acid Black I, Matheson) or coomassie blue (Mann Labs) was used. An LKB electrofocusing apparatus (LKB Instruments, Inc., Rockville, Md.) was employed for determining the isoelectric point of various components and for electrophoresis experiments.

A Hitachi HuIIA electron microscope was employed for taking pictures of chromatophores, AUT particles, and reaggregated particles. The samples were negatively stained with 2% phosphotungstic acid at pH 6.0–7.5. Many pictures were taken over the entire grid and those included herein were selected as being representative.

Metal analyses were performed using a Varian-Techtron AA-5 atomic absorbance spectrometer (Varian Aerograph–Varian Techtron, Park Ridge, Ill.). Iron, copper, and manganese standards were run with each set of unknowns as well as controls to measure the effect of viscosity, Triton, buffers, lipids, proteins, and complex formation on the measurement. Excellent results were obtained with samples whose metal concentration was in the range of 1×10^{-6} to 8×10^{-6} M.

Results

The three parameters (pH, urea concentration, and Triton concentration) were varied systematically in the same manner as has been previously reported for *R. spheroides* (Loach *et al.*, 1970). For quantitative conversion of chromatophores from *R. rubrum* the best conditions were found to be pH 11.5, 3% Triton X-100, and 6 M urea at 0° for a period of 30–45 min, during which time the sample was being centrifuged through a discontinuous sucrose density gradient of 8 ml of 1% sucrose, 8 ml of 2% sucrose, and 8 ml of 4% sucrose. The Triton and urea are present throughout the gradient and the reaction is initiated by centrifugation. Use of freshly prepared chromatophores is required in order to obtain quantitative conversion. At the end of the centrifugation time the center (2–3 ml) of the densely colored band is carefully removed with a disposable pipet and sufficient solid KH_2PO_4 added to it to change the pH to approximately 7. Dialysis at 0° against 10^{-3} M phosphate buffer (pH 7.5) is then employed for 2–3 hr with four to six changes in the buffer against which the sample is dialyzed. This material is then kept frozen (−20°) and is referred to as the AUT preparation. The bacteriochlorophyll of *R. rubrum* AUT is considerably more susceptible to irreversible degradation than a similar preparation from *R. spheroides*. The spectrum of the stored AUT changes significantly over a period of a few days even when frozen. The *R. rubrum* chromatophores are also more difficult to break down than those from *R. spheroides* since a higher Triton concentration (and/or higher pH) is required to obtain a quantitative yield.

A typical profile of a sucrose density tube after centri-

fuging is shown in Figure 1. In this particular preparation the phospholipids were labeled with ^{33}P by growing the bacteria in a medium supplemented with [^{33}P]P_i. The results are in complete agreement with those obtained previously for *R. spheroides* (Loach *et al.*, 1970). That is, all of the protein, lipid, and color is in the top third of the tubes. Longer centrifugation time does not result in significantly further movement of the protein toward the bottom of the tube. From Figure 1 most of the phospholipid has been displaced from the membranous structure and is "floating" in the upper part of the tube. About one-half of the protein content is also in the upper few milliliters of the tube. No significant pellet is formed when really fresh chromatophores are used. The location of the colored band, the per cent conversion and the trap activity are very sensitive to the pH of the media. The pH stated is for phosphate buffer adjusted at room temperature and before Triton or urea was added. Dependence of the phototrap activity upon pH has been previously reported (Loach *et al.*, 1970). Good phototrap activity was retained between pH 5 and 11 at 0° for 1–2 hr.

The size of the material which is referred to as the AUT preparation was determined in the same way as was previously reported for the *R. spheroides* preparation (Loach *et al.*, 1970). When chromatographed on a calibrated Bio-Gel A-5m column with 0.2% Triton in the eluting buffer, it ran identically with the *R. spheroides* AUT preparation and was consistent with material having a particle weight near 100,000. Greater than 75% of the material passed through a 25 mμ Millipore filter. When the AUT preparation suspended in water was centrifuged at 110,000*g* for 18 hr (A_{880} = 2.0 with 0.2% Triton in the buffer) less than 10% spun down.

Attempts to take electron micrograph pictures when detergents are present have not given satisfactory results since the detergent coalesces on drying the sample and most of the biological material aggregates on the edges of detergent droplets. However, it is possible to get some idea of size from samples of AUT which are "deTritonized" by passage through a Bio-Gel A-5m column (Loach *et al.*, 1970). If a grid is prepared for electron microscopy immediately after the sample comes off such a column only small particles are in evidence as shown in Figure 2 (middle). If the "deTritonized" sample is left standing at 0° for a few hours, however, considerably larger particles are formed (Figure 2, bottom) and will settle out of solution after about 12 hr. These latter results are similar to those seen with "deTritonized" *R. spheroides* AUT but the reaggregation rate is about five times faster with *R. rubrum* material. This is perhaps consistent with the greater difficulty in breaking down the membranous particles of *R. rubrum*.

The absorbance spectrum of the AUT preparation is compared with that of chromatophores in Figure 3. The per cent recovery of the total originally present in chromatophores has routinely been between 70 and 85% when determined by following the band at 880 nm and 90–100% when calculating the yield according to the band at 590 nm. While the bacteriochlorophyll bands in the near infrared are very sensitive to degradation or change in environment, the band at 590 nm is much less so. The carotenoid region of the spectrum is also preserved with between 90 and 100% retention of this pigment absorbance as determined by following the band at 513 nm. We feel this is a most significant result

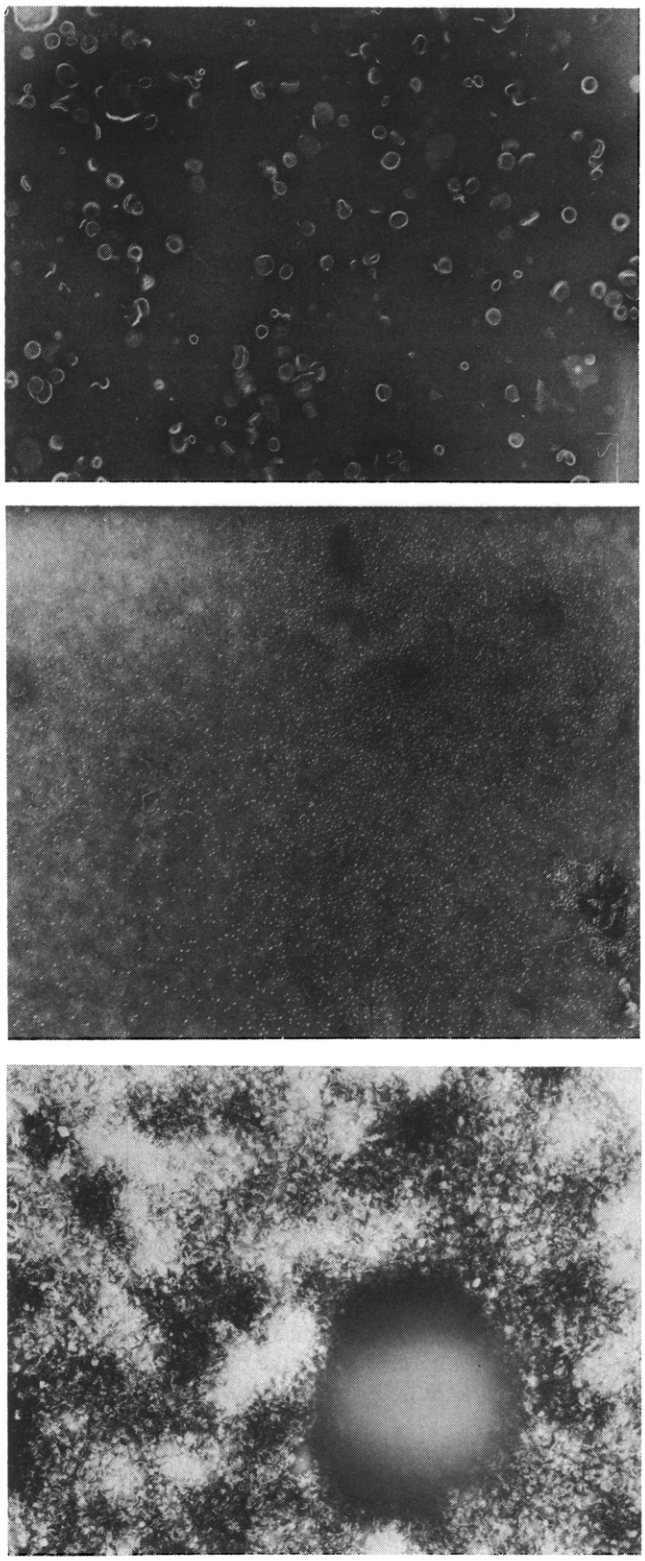

FIGURE 2: Electron micrograph of chromatophores (top), freshly deTritonized AUT (middle), and deTritonized AUT after standing for 1 day (bottom). The AUT preparation was filtered through a 25 mμ Millipore filter before deTritonizing on a Bio-Gel A-5m column (Loach *et al.*, 1970). The chromatophore preparation had been subjected to a Bio-Gel A-150m column and a sample corresponding to the maximum of the color peak was used for preparation of the grid. Magnification, 90,000×. The average chromatophore size is 800 Å in diameter by our measurement. The samples were negatively stained with phosphotungstic acid.

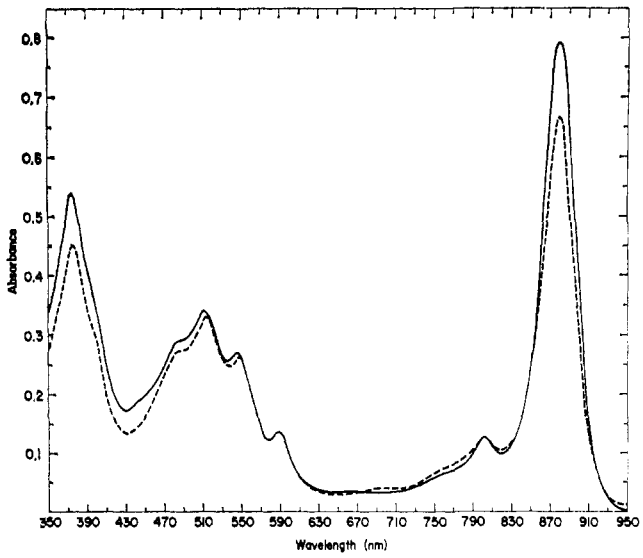

FIGURE 3: Absorbance spectra of chromatophores (solid line) and AUT (dashed line) prepared from *R. rubrum*. The AUT spectrum was normalized to the chromatophore spectrum by assigning an equal absorbancy at 590 nm. Both samples were in 0.05 M phosphate buffer (pH 7.5), 1-cm cuvets; room temperature.

TABLE I: Analytical Data.

	Protein: BChl[a] by Wt	Moles of Fe/Mole of $P_{0.44}$[b]	Moles of Cu/Mole of $P_{0.44}$[b]
Chromatophores	13.0	5.0	0.20[c]
		2.2[c]	
AUT	6.5	2.4	
deTritonized AUT	3.5		

[a] Protein was determined by the method of Lowry *et al.* (1951). The bacteriochlorophyll (BChl) content was estimated from the absorbance at 590 nm using a millimolar extinction of 20 cm^{-1}. [b] Iron and copper content were determined with the aid of an atomic absorbance spectrometer. Phototrap, or $P_{0.44}$ (Loach *et al.*, 1963), concentration was determined by measurement of the absorbance photochange at 865 nm of the various samples and using a millimolar extinction of 90 cm^{-1} (Loach and Sekura, 1968). [c] These samples of chromatophores were sonicated for 5 min in 0.001 M EDTA in 0.05 M phosphate buffer at pH 7.5. They were then dialyzed overnight against a large volume of the same buffer and then against several changes of demineralized water. This procedure did not affect trap activity and had only a limited effect on iron content, but greatly aided copper removal.

in the sense that there is little evidence for change in either the carotenoid or bacteriochlorophyll complement as a result of membrane dissolution. As mentioned previously, the spectrum will change rapidly (minutes) upon sitting at room temperature and slowly (days) at −20°.

The wavelength dependence of the photochanges in the near-infrared region of the spectrum is completely maintained in the AUT preparation as shown in Figure 4 (A). The per cent recovery of total activity varied between 50 and 100% as determined from absorbance photochange measurements at 865 nm. Also consistent with an active phototrap is the photoproduced electron paramagnetic resonance signal (see Figure 4B). Between 100 and 120% of the total activity originally present is accounted for by measurement of electron paramagnetic resonance photochanges. Thus, the phototrap is always found to be quantitatively accounted for if measured by electron paramagnetic resonance but the yield has varied from 50 to 100% by measurement of ΔA in the near infrared. Part of the reason for the sometimes lower activity as measured by absorbance photochange may be the result of our inability to saturate the photochanges with our present exciting light sources when the dark decay kinetics become very fast.

Some idea of the number of separable components present in the AUT preparation can be gained by observing the number of bands formed when it is subjected to disc gel electrophoresis. Figure 5 portrays a typical result of several disc gel runs. There were primarily two colored bands which separated and moved somewhat in the separating gel, some material that remained in the stacking gel and a very fast-moving minor component. The two colored bands in the separating gel contained most of the protein. The presence or absence of Triton in the buffer did not make a great deal of difference to the pattern developed.

Also shown in Figure 5 is a pattern obtained by column electrophoresis. Two colored bands are again evident in the developed part of the column plus some degraded pigments left at the origin. For purification and analysis, column electrophoresis has many obvious advantages over disc gel procedures. From other experiments in which the apparatus was used for electrofocusing, the sample was isoionic at a pH 4.3 and two slightly separated (but precipitated and inactive) colored bands could be discerned.

The electrophoresis result shown in Figure 5 is for a freshly prepared AUT sample. If the AUT material was stored frozen for several days, or aged in a solution at 4°, only one red-purple band occurs at a position which corresponds to the leading band shown in Figure 5. In this latter case a major protein band is found where the second (slower moving) red-purple band of Figure 5 is located. The reason for this variation on aging may be due to additional structure breakdown on freezing and thawing, or storage at 4°, with a high Triton concentration present (approximately 2% left after dialysis). Thus, in the freshly prepared and electrophoresed AUT material the photoreceptor subunit may be incompletely removed from other types of membrane subunits. The absorbance spectrum of either of the dense red-purple bands from freshly prepared and electrophoresed AUT material (Figure 5), or the single dense red-purple band from electrophoresis of a more aged preparation, is nearly identical with that of the original AUT sample. Some degraded bacteriochlorophyll is left at the origin. Excellent phototrap activity is retained in these dense red-purple bands (greater than 70% of the total activity added) whether measured by absorbance or electron paramagnetic resonance photochange. The wavelength dependence of the absorbance photochanges in the near infrared is also unchanged relative to that of the original chromatophores. Most importantly, the protein to bacterio-

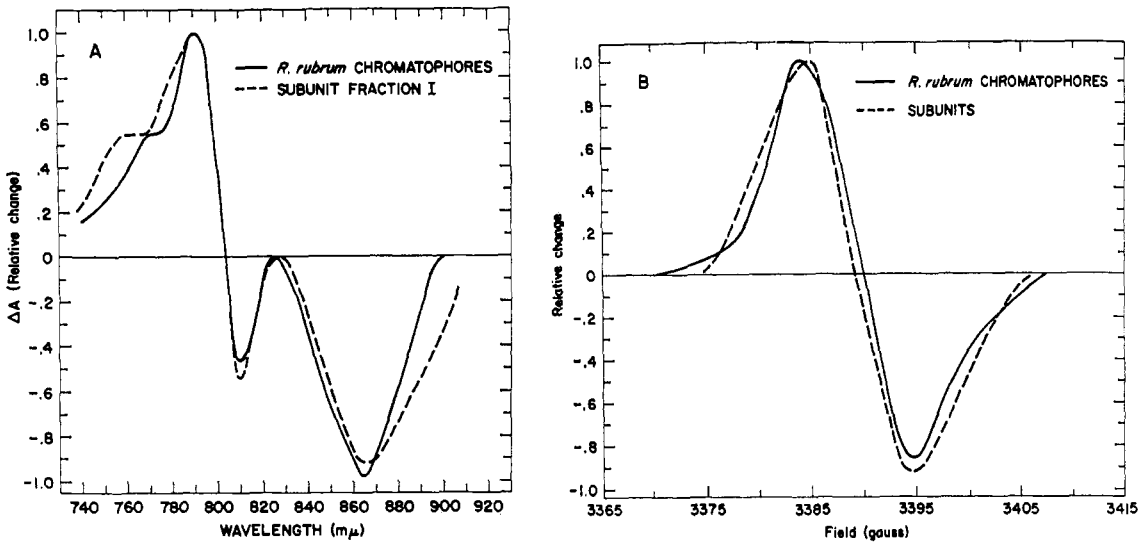

FIGURE 4: Photoproduced absorbance difference spectra (A) and electron paramagnetic resonance signal (B) of chromatophores (solid lines) and AUT (dashed lines) prepared from *R. rubrum*. Absorbance difference spectra were recorded using a Cary 14R recording spectrophotometer appropriately modified to admit a beam of exciting light perpendicular to the detecting beam (Kuntz *et al.*, 1964) with the 0–0.1 slide-wire. The electron paramagnetic resonance spectra were recorded with a Varian E-3 electron paramagnetic resonance spectrometer using 10-mW power and 5-gauss modulation amplitude. Absorbance at 880 nm was 0.5 for ΔA measurements and 5 for Δ electron paramagnetic resonance measurements. $K_4Fe(CN)_6$ was added to all samples to give a final concentration of 0.01 M before measuring activity. Saturating light intensities were used. Samples were suspended in phosphate buffer (pH 7.5) at room temperature.

chlorophyll weight ratio of the red-purple band is typically near 2:1 when more aged AUT preparations are used for electrophoresis.

A partial analysis of the AUT preparation is shown in Table I. "DeTritonized" AUT is considerably more stable toward degradative breakdown than the AUT and it can be stored without apparent change in absorbance or activity for many weeks. It will readily resuspend in 0.2% Triton and greater than 75% will then pass through a 25 mμ Millipore filter. This demonstrates a significant difference in behavior as compared with chromatophores: even though the "deTritonized" AUT appears as aggregated chromatophore-like particles, its redissolution into small complexes is readily achieved by merely adding detergent. As can be seen by the data of Table I about 75% of the protein present in the original chromatophores has been removed in deTritonized AUT.

No significant amount of copper is present in EDTA-washed chromatophores, which are still fully active, therefore these measurements were not extended to the AUT preparations. However, about 2 equiv of iron is still present in AUT particles even when prepared from EDTA-washed chromatophores. No significant manganese is present in the original chromatophores which is consistent with measurements by Kassner and Kamen (1968) on *R. rubrum* whole cells.

A comparison of the efficiency of utilization of absorbed light for causing phototrap changes is shown in Figure 6. Importantly, this preparation of AUT showed as high a quantum yield as chromatophores (Loach and Sekura, 1967, 1968). Results with reaggregated AUT are not shown but the efficiency of utilization of absorbed light is also the same as for chromatophores.

Examination of the decay kinetics (Figure 7) of the AUT preparation shows that although the half-time is considerably less for AUT than for chromatophores (0.13 sec *vs.* 6.0 sec), the process is still pseudo first order and is independent of temperature between 0 and 30°. This is consistent for phototraps which are thought to be well separated from each other and therefore noninteracting in this dilute solution.

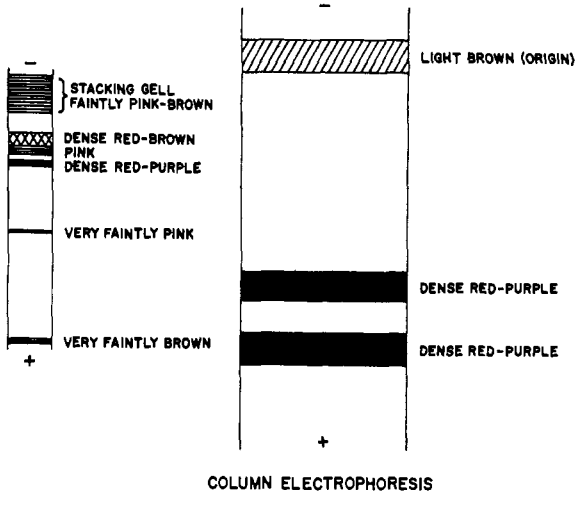

FIGURE 5: Behavior of AUT prepared from *R. rubrum* on disc gel electrophoresis (left) and column electrophoresis (right). For disc gel electrophoresis, gels with 5% acrylamide were used and the patterns developed at 1.5 mA/column for 2.5 hr at room temperature. The buffer used was Tris-glycine (pH 9.7). For column electrophoresis, the LKB 110-ml capacity electrofocusing column was used but with the pH buffered throughout at 8.2 with the pH 7–10 mixed ampholytes. A sucrose gradient was employed just as is usually employed in electrofocusing, and the AUT was applied near the top of the column. The temperature was 5° and the column was developed at 400 V for 11 hr.

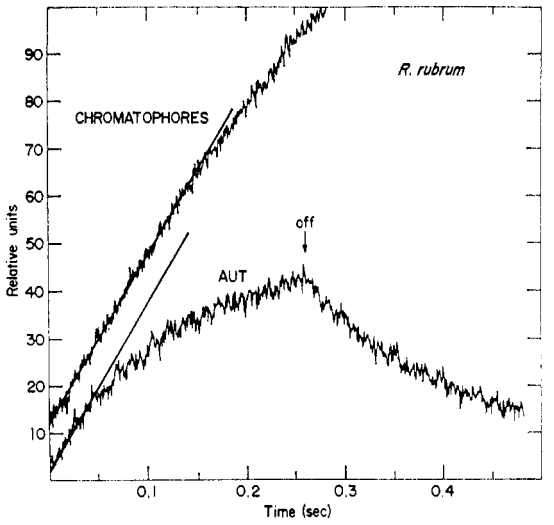

FIGURE 6: Comparison of the rate of absorbance photochange at 791 nm for chromatophores and AUT prepared from *R. rubrum*. Absorbance of both samples at 800 nm was 0.85 in a 1-cm cuvet. The exciting beam filters were a 7-69 Corning color filter, a 4-cm path water filter and a Baird Atomic B-9 narrow-band pass interference filter with maximal transmission at 881 nm. The light was allowed to fall on the sample at time zero. The detecting beam was at 791 nm and passed through a Baird Atomic B-9 narrow-band pass interference filter. All parameters were constant for both samples. The precision for conducting numerous experiments of this type or alternating the samples was within 5%. Similar results were obtained at 810 nm. The exciting beam intensity was adjusted so that the rate of onset of the absorbance change was about twice the rate of decay for the AUT sample. $K_4Fe(CN)_6$ was added to the chromatophore sample to give a final concentration of 0.01 M.

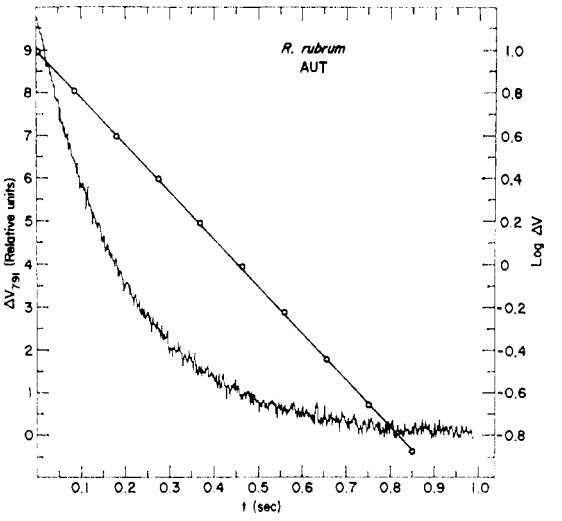

FIGURE 7: Decay kinetics of the light-induced absorbance change at 791 nm in AUT prepared from *R. rubrum*. The exciting beam was light from a tungsten lamp which had passed through a 4-cm path water filter and two 4-96 Corning color filters; its intensity was adjusted to just saturate the absorbance photochange. The left vertical axis is the measured change in voltage but is proportional to change in absorbance for these small changes. The appropriate vertical scale for the points defining the straight line is on the right side of the figure. Absorbance of the sample at 875 nm is 1.0 in a 1-cm cuvet; 0.05 M phosphate buffer (pH 7.5) room temperature.

Of considerable interest is the effect of environmental potential on the activity of the phototrap. For a membranous system as complex as chromatophores, certain reservations had to be made regarding the interpretation of the redox dependency at low potential. Because many known electron transport components are present in chromatophores, as well as excessive amounts of transition metals such as copper and iron, perhaps indicative of unknown electron transport components, a direct effect of a change in redox state of one of these components on the observable trap activity is a definite complication. While the AUT preparation is not sufficiently small to exclude binding of small molecules such as metals or quinones, it is certainly free from a direct effect of most of the components present in the chromatophore. Figure 8 shows that exactly the same reversible dependency on potential is obtained with dilute AUT in 0.2% Triton at pH 7.5 as with chromatophores and whole cells (Loach, 1966; Kuntz *et al.*, 1964; Loach *et al.*, 1963).

It is important to note that whereas the kinetics of $P_{0.44}^{+}$ restoration to its reduced form increase as air is removed and the potential is lowered with chromatophore systems (Loach *et al.*, 1963; Loach, 1966) no such change in kinetics is observed with the AUT preparations.

Discussion

Utilization of the AUT procedure for causing quantitative dissolution of *R. rubrum* membrane particles (chromatophores) has led to results completely compatible with those obtained with *R. spheroides* (Loach *et al.*, 1970). The phospholipid material is nearly completely displaced (Figure 1) and the AUT preparation remains dispersed only as long as the Triton concentration is higher than about 0.05%. The average particle weight is about 100,000–150,000 in 0.2% Triton and retains complete activity as measured by absorbance photochange, electron paramagnetic resonance photochange, and the quantum yield for $P_{0.44}$ oxidation.

The earlier systematic studies of Garcia *et al.* (1966) demonstrated limited effectiveness of Triton X-100 in causing breakdown of chromatophores from *R. rubrum*. In these studies a "light" fraction and a "heavy" fraction were obtained by reaction of chromatophores with Triton at pH 8.1, and then subjecting the material to sucrose gradient centrifugation. They found spectra and activity similar to that of the original chromatophores in both fractions. In comparison with the location of their "light" band, which came to rest in a 24% sucrose layer, the material reported in this communication does not sediment through a 2% sucrose layer at a corresponding *g* value. Electron micrographs of the light fraction of Garcia *et al.* show membrane fragments of considerable size, presumably even with detergent still present. We have duplicated the results of Garcia *et al.*, but as noted in the Results section, changing the pH from 8 to 11 and adding 6 M urea results in a quantitative yield of significantly smaller particles.

We consider the demonstration that fully active bacteriochlorophyll–protein complexes may be quantitatively prepared from membrane fractions with no significant change in spectral properties constitutes good evidence that photoreceptor subunits exist in the membrane along with other kinds of lipoprotein subunits. In this connection, the distinctive way in which reaggregation occurs when Triton is removed from the preparation (*cf.* Figure 2, bottom, and

Loach *et al.*, 1970) suggests that by a systematic approach, reaggregation may yield functional pieces of membrane with which the relationship between structure and function could be approached at a truly molecular level.

Unlike the AUT preparations of *R. spheroides* (Loach *et al.*, 1970) those with *R. rubrum* convert absorbed quanta into chemical potential as efficiently as the chromatophores from which they were prepared (Figure 6). This provides good evidence that the dispersed subunits all contain a complement of light-harvesting bacteriochlorophyll molecules and one phototrap. It would be most interesting to make similar measurements for the efficiency of utilization of light absorbed by the carotenoid molecules. We have not yet made this comparison quantitatively, but the relative efficiencies were similar, perhaps indicating that these molecules also exist in the same subunit as the bacteriochlorophyll and phototrap.

Although we feel it is premature to collect extensive analytical data on the composition of AUT preparations, some important conclusions may be drawn at this time. Of the three transition metals iron, copper, and manganese often present in the intact bacteria, manganese could previously be ruled out (Kassner and Kamen, 1968) as playing a vital role in the primary light-trapping act. The data we have obtained are consistent with that observation and, in addition, demonstrate that such a role for copper may be ruled out as well (see Table I). The advantages of choosing *R. rubrum* as the preferred photosynthetic bacteria to study, from the point of view of simplicity, is further underscored by the low metal content (but high trap activity) of the chromatophore preparation.

Of the 5 equiv of iron present per phototrap in the chromatophores (Table I), only a little over two are still present in the AUT preparation or EDTA washed chromatophores. The only data currently available in the literature on reaction center preparations for which a comparison might be made are those of Reed (1969) and Thornber *et al.* (1969). From the data of Reed, the amounts of iron, copper, and quinones were quite high relative to the phototrap concentration and are similar to the ratios originally present in the chromatophores. From the data of Thornber *et al.* the content of iron (in heme) was high (7:1) in that preparation; additional iron, copper, and quinone content were not given.

Much of the quinone material originally present in the *R. rubrum* chromatophore preparation has also been removed along with the phospholipids during the AUT preparation, but we have not yet performed sufficiently quantitative experiments to establish a valid minimal number.

Even though the AUT preparation is not regarded as a preparation of highly purified photoreceptor subunits these components are nevertheless fairly well separated from each other, and other kinds of membrane subunits, when dispersed as a dilute solution (A_{880} = <1.0) in Triton-containing buffer. Therefore, several physical measurements of phototrap properties compared with those measured in the chromatophore state are of interest. The kinetic profile and temperature independence for dark decay of the photochange at 791 nm (Figure 7) is consistent with an internal recombination of the primary oxidized and reduced species. The difference between these kinetics and those of chromatophores may have the following explanation. In membranous particles significant pools of redox components exist. The effect of the redox state of these pools on the decay rate of $P_{0.44}$ has been noted

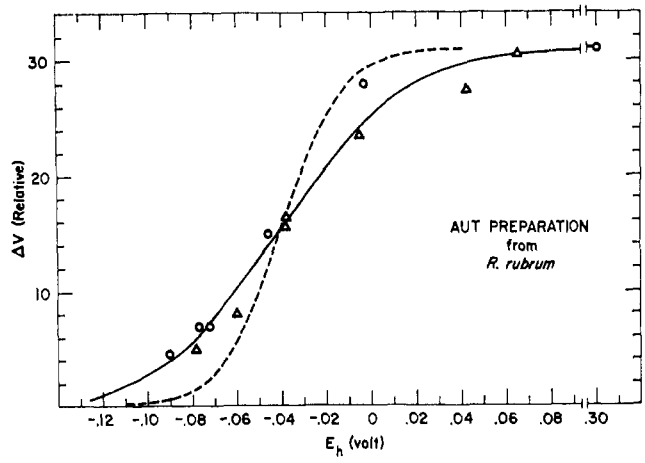

FIGURE 8: Low-potential redox dependence of absorbance photochanges for AUT prepared from *R. rubrum* as measured at 791 nm. Absorbance at 875 nm is 0.16. Phosphate buffer (0.05 M), pH 7.5; 0.2% Triton X-100; 5×10^{-5} M indigotetrasulfonic acid; 10^{-2} M $Na_2S_2O_4$ was used as reductant; air was used as oxidant. Temperature = 2°. The exciting light was that from a tungsten lamp filtered through two 4-96 Corning color filters and a 4-cm path water filter with an intensity sufficient to just saturate the photochanges. The curves drawn represent where data would have fallen for a simple one- (solid line) or two- (dashed line) electron process.

(Loach *et al.*, 1963; Loach, 1966). If the primary electron acceptor molecule normally feeds electrons into this pool at a very rapid rate, the light-oxidized $P_{0.44}$ would have to react with the pool in order to be restored to its reduced state. However, when the pool is either completely reduced, as has been observed during redox titrations (P. Loach, unpublished results), or if removed from the photoreceptor subunit during AUT preparation, the primary electron acceptor may directly back react with the primary electron donor. Consistent with this interpretation is the very close match between the decay kinetics for AUT, which are independent of potential, and those for chromatophores at or below 0 V (Loach, 1966).

The redox dependency of the absorbance photochanges (Figure 8) is found to be identical with those measured for chromatophores (Kuntz *et al.*, 1964; Loach, 1966). Since the number of possible interacting secondary components is considerably lowered in the case of the dispersed photoreceptor subunits, the likelihood of the redox dependency at low potential reflecting a chemical property of the primary electron acceptor molecule is considerably increased. It is worth noting that there seems to be a distinct trend relating the complexity of the titration curve (number of electrons transferred) and the simplicity of the material studied. Whole cells exhibit the same midpoint potential but approximate a four electron per redox unit process (Loach, 1966), chromatophores can display data most consistent with either a 2 or 1 electron per redox unit process (Loach, 1966) and the AUT preparations always approximate a one-electron per redox unit process (Figure 8). It might also be noted that whereas high ionic strength was required to ensure good reversibility for chromatophores, the AUT preparation shows excellent reactivity at low ionic strength. Subsequent to our earlier measurements at low potential (Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966) others have reported similar redox dependencies of absorbance

changes (Cusanovitch *et al.*, 1968; Nicolson and Clayton, 1969; Dutton, 1970) and fluorescence transients (Cramer and Butler, 1969; Cramer, 1969; Reed, 1969).

Whereas the data obtained by Nicolson and Clayton (1969) for the reaction center preparation derived from a carotenoidless *spheroides* mutant are in good agreement with our data on *R. rubrum* and *R. spheroides* systems (Loach, 1966) the fluorescence data obtained by Cramer (1969) is in good agreement for chromatophores of *R. spheroides* but the value reported for the low-potential titration in *R. rubrum* (E_{M7} = −0.15 V) was considerably lower. We have also titrated the variable portion of the fluorescence at low potential in the *R. rubrum* chromatophore system (unpublished data) and found good agreement with our titrations of absorbance photochange (Loach, 1966). Several possibly significant differences in conditions for our measurements and those used in other laboratories may exist. For example, we have found it necessary, when studying chromatophores, to conduct the experiment at high ionic strength in order to insure a reversible titration. In our hands, data from experiments at low ionic strength gave a significantly lower E_{M7} on reduction than on subsequent reoxidation (for example, see Figure 2 in Loach, 1966). The source and significance of the different values remain to be determined.

The absence of an electron paramagnetic resonance signal which can be readily assigned to the primary electron acceptor molecule (Loach *et al.*, 1963; Loach, 1966) (however, see McElroy *et al.*, 1970), together with the fact that the primary electron donor molecule is photooxidized by one electron in the primary act with a quantum yield near 1.0 (Loach and Sekura, 1967, 1968), leaves only limited possibilities as to the identity of the primary electron acceptor. The possibilities are even further limited if one assumes that the titration whose midpoint potential is −0.05 V does measure a property of this primary electron acceptor molecule. Because no absorbance change or electron paramagnetic resonance signal occurs in the dark (Loach *et al.*, 1963; Loach, 1966) which would suggest one-electron reduction of a bacteriochlorophyll molecule, nor would one expect to readily achieve such a reduction in the dark (Falk, 1964; Mauzerall and Feher, 1964, 1965), a transition metal such as iron (the only one present, see Table I) may play a crucial role. It is of interest that the absorbance changes centered at 763 nm noted some years ago (Loach, 1966) could belong to an iron–tetrahydroporphyrin complex, since the midpoint potential, molecular extinction, λ_{max} and the band shift upon change of oxidation state from Fe^{3+} to Fe^{2+} would be compatible with such compounds (P. Loach and F. J. Ryan, manuscript in preparation). The increase at 763 nm (usually a shoulder) is observed in all chromatophore, subunit, and reaction center preparations (Kuntz *et al.*, 1964; Reed, 1969; Thornber *et al.*, 1969, Sauer *et al.*, 1968; Loach *et al.*, 1970). It was previously suggested that the midpoint potential for this change in oxidation state was slightly low (−0.06 V compared with −0.02 V; Loach, 1966) to be ascribed to the hypothetical primary electron acceptor. Further experiments since that time, however, make it appear that the midpoint potential for the hypothetical primary electron acceptor molecule was somewhat too high. A slight correction downward is necessary because insufficient time at lower potential was allowed, in those experiments, for the primary electron acceptor molecule to become completely reoxidized in the dark before the next light period. We now feel the two midpoint potentials are within experimental error of having the same value.

Several organic molecules of biological importance have been suggested to play a role as the primary electron acceptor in bacterial photosynthesis. These include quinones (Clayton, 1962b) and pteridines (Fuller and Nugent, 1969). However the one electron reduction of either molecule would be expected to give rise to an organic radical which should be readily detectable by electron paramagnetic resonance. As mentioned above, no such radicals have been observed. All known redox equilibria involving the pteridine nucleus have considerably lower midpoint potentials (Clark, 1960) than those cited for shutting off the phototrap. Of course protein binding or metal interaction could affect these potentials significantly. The basis for assigning a primary role to quinones and pteridines has primarily been due to matching the absorbance photochanges in chromatophores, observed in the ultraviolet, with absorbance changes expected upon reduction of these molecules. Recent studies (P. Loach, R. A. Bambara, and F. J. Ryan, manuscript in preparation) indicate that the absorbance photochanges in the ultraviolet for *R. rubrum* and *R. spheroides* chromatophores cannot be assigned to these molecules.

From a comparison of the protein:bacteriochlorophyll weight ratio for the AUT preparation with that calculated for a hypothetical photoreceptor subunit containing 40 bacteriochlorophylls, 20 carotenoids, and a particle weight of 150,000 (Loach *et al.*, 1970) it would be expected that one-half to two-thirds of the protein present in deTritonized AUT is unrelated to the photoreceptor subunit. Initial results with column electrophoresis support this conclusion since several prominent and colorless protein bands were found and the photoreceptor subunit fraction had a protein bacteriochlorophyll weight ratio near 2:1. Thus, purification without loss of activity appears very promising and we find it preferable to await such purification before attempting extensive component analysis. Such analysis of purified material should eliminate much speculation about the nature of the primary electron acceptor molecule in bacterial photosynthesis.

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