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Quantitative Dissolution of the Membrane and Preparation of Photoreceptor Subunits from *Rhodopseudomonas spheroides**

P. A. Loach, D. L. Sekura,† R. M. Hadsell,‡ and A. Stemer

ABSTRACT: An effective and optimal combination of three reagents commonly used for modifying the structure of proteins has been developed for causing quantitative dissolution of membrane structure. Alkaline solution, 1.5% Triton X-100, and 6 M urea at 0° for about 2 hr causes quantitative conversion of chromatophores from wild-type *Rhodopseudomonas spheroides* into Triton–protein complexes having particle weights of less than 200,000. No trap activity is lost as evidenced by light-induced absorbance and electron paramagnetic resonance changes. Good rates for photoxidation of exogenous cytochrome *c* also are demonstrated. Especially significant is the maintenance of a near-normal absorbance spectrum in both the bacteriochlorophyll and carotenoid regions in spite of the marked conversion into smaller units. Also, of possible importance and utility is the ability to cause reaggregation of the small units into chromatophore-like structures by the removal of excess detergent. Evidence is given for nearly total lipid displacement by the alkaline–urea–Triton treatment and subsequent sucrose density gradient centrifugation.

In the past 10 years much progress has been achieved in understanding the *in vivo* nature of the functioning light trap in bacterial photosynthesis. The following characteristic physical parameters can be associated with a functioning trap.[1] (1) Photoxidation of the primary electron donor molecule is attended by a loss in absorbance at wavelengths in the near-infrared region where bacteriochlorophyll has an intense band (Duysens, 1952; Duysens *et al.*, 1956; Clayton, 1962a,b, 1966), and by the formation of an unpaired electron (Androes *et al.*, 1962; Calvin and Androes, 1962) characteristic of a chlorophyll free radical (Mauzerall and Feher, 1964; Mauzerall, 1968; Felton and Linschitz, 1966; Fuhrhop and Mauzerall, 1968, 1969) which is easily measured by electron paramagnetic resonance. (2) Both measurable parameters referred to above can be easily reproduced in the dark by chemical oxidation (Goedheer, 1959a,b; Kuntz *et al.*, 1964; Loach *et al.*, 1963) with a midpoint potential of +0.44 V (Kuntz *et al.*, 1964; Loach *et al.*, 1963). (3) These normal light responses shown by all photosynthetic systems of bacterial origin, as well as "system I" of green plants and algae (Kok, 1956, 1961; Witt *et al.*, 1961; Beinert *et al.*, 1962; Beinert and Kok, 1964; Loach *et al.*, 1963), are also reversibly quenched at lower potentials (Kuntz *et al.*, 1964; Loach, 1966; Loach *et al.*, 1963, 1968). (4) The quantum yield for photoxidation of the primary electron donor has been accurately established by both measurable parameters and is the idealized value of 1.0 (Loach *et al.*, 1967, 1968; Parson, 1968; Beugeling, 1968; Bolton *et al.*, 1969); (5) The same light responses can be demonstrated to occur with good efficiency at temperatures so low that few molecular motions are possible (Arnold and Clayton, 1960; Androes *et al.*, 1962; Calvin and Androes, 1962), and the light-induced absorbance changes occur as rapidly as measuring devices can follow (Parson, 1968; Ke, 1969).

It now seems appropriate, therefore, to use these measurable parameters as an assay for a functioning light trap during simplification and purification procedures. In the past, less direct assays had to be used which admitted to the possibility of measuring nonbiological activities. The high concentrations of very reactive light absorbers such as carotenoids and chlorophylls can always complicate the measurement of physiologically authentic secondary reactions. Careful measurements of the quantum yields for such secondary reactions as pyridine nucleotide reduction are necessary before a normal biological role can be ascribed to them.

For the studies reported in this paper, we attempted to apply all of the following criteria as our assay for a functional

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[1] Abbreviations and definitions used are: AUT is used to denote those particles prepared using a combination of alkaline solution, high urea concentration, and the detergent Triton X-100. Trap is defined as that molecular site where absorbed light energy first causes the formation of oxidized and reduced molecular species. Photoreceptor subunit is defined as a protein–bacteriochlorophyll–trap (and possibly carotenoid) complex thought to exist as one of many different kinds of subunits in the membrane and isolable as a distinctive entity.

photoreceptor unit and trap activity. (1) The light-induced absorbance changes in the near-infrared region at 865 nm should be retained with high quantum yield. (2) The light-induced electron paramagnetic resonance signal should be retained with high quantum yield. (3) No significant change in overall absorbance by bulk bacteriochlorophyll should occur. (4) Coupling to a secondary reaction, the photoxidation of cytochrome *c*, should be demonstrable with high quantum yield. These criteria are at least as rigorous as those applied to most enzyme purification procedures since some involve following probes that are very sensitive to tertiary, and perhaps quaternary, structure as well as the central activity.

Some comment is appropriate regarding assay condition 3. From our initial observations (Loach *et al.*, 1968, 1969) with our membrane dissolution studies of chromatophores from both *Rhodospirillium rubrum* and *Rhodopseudomonas spheroides*, we became aware that it might be possible to prepare small protein-bacteriochlorophyll-trap complexes which are essentially unchanged relative to their original state in the membrane, except that they are considerably smaller. This early promise fit with our favored concept of membrane structure in which various kinds of lipoprotein subunits (Korn, 1966; Green *et al.*, 1967) interact with each other in a specific manner. The distinctive large shift in the bacteriochlorophyll bands to their long-wavelength positions characteristic of the *in vivo* state, together with their solubility in water and relative inertness in that *in vivo* state to organic solvents, strongly suggests to us that they are intimately and specifically associated with protein. Such specific association with protein would also allow more genetic control during membrane biosynthesis. The results reported herein, together with results from our studies of chromatophores from *R. rubrum* (Loach and Sakura, 1968; Loach and Walsh, 1969; P. A. Loach, R. M. Hadsell, D. L. Sekura, and A. Stemer, manuscript in preparation) validate adding a criterion as restrictive as condition 3.

Thus, membranes which have photosynthetic activity associated with them may be of a distinct advantage in studying structure and function since they not only contain well-defined activities at different levels of structure which can be readily measured (*e.g.*, trap photochanges and photophosphorylation) but these membranes also have built in probes for structural change, that is the bulk chlorophyll and carotenoid molecules. The major goal in the simplification procedures reported here has been to cause quantitative dissolution of the membrane into the smallest possible lipoprotein complexes, without seriously changing the bacteriochlorophyll environment and, of course, maintaining trap characteristics.

Successful fractionation and reconstitution (Keilin and King, 1960; Green *et al.*, 1961; Criddle *et al.*, 1962, 1966; Hatefi *et al.*, 1961, 1962; Kagawa and Racker, 1966; McConnell *et al.*, 1965; Tzagoloff *et al.*, 1967) of mitochondrial systems have stimulated many similar studies with photosynthetic materials (Komen, 1956; Bril, 1958, 1960; Clayton, 1962a,b; Boardman and Anderson, 1964; Anderson and Boardman, 1966; Kok, 1961; Garcia *et al.*, 1966a,b, 1968a,b; Cusanovich and Kamen, 1968; Reed and Clayton, 1968; Ke *et al.*, 1968; Thornber *et al.*, 1969), and have also given rise to new theories on the structure of membranes. To us the most attractive hypothesis is that supported by Green (Green and Goldberger, 1967) and by Korn (Korn, 1966) which suggests that the entire membrane is formed by association of relatively small protein–lipid subunits. Evidence for this consists largely in the observation of regular and repetitive small structures in electron micrographs of intact membranes (Blair *et al.*, 1963; Sjöstrand, 1963; Parsons, 1963; Park and Biggins, 1964; Holt and Marr, 1965) and in limited reconstitution experiments (Keilin and King, 1960; Kagawa and Racker, 1966; Hatefi *et al.*, 1962; McConnell *et al.*, 1965; Tzagoloff *et al.*, 1967). The results reported herein might be regarded as providing additional experimental support for this hypothesis. Three approaches which have been useful with mitochondrial membrane systems and protein subunit studies have been combined in the present study. One of these consists of incubation under alkaline conditions (Criddle *et al.*, 1962). Presumably the net negative charge on the protein constituents and other acid–base components will aid in disruption of the membrane. The second approach makes use of a detergent whose role may be to bond to the protein by hydrophobic interaction in place of protein–protein hydrophobic interaction (Green and Goldberger, 1967). The third utilizes high urea concentrations (6 M) to force rearrangements of protein tertiary structure, perhaps allowing the first two conditions to be more effective.[2]

Materials and Methods

The bacteria *R. spheroides* (No. 2.4.1.C, originally from R. Y. Stanier) were 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 (absorbance at 850 nm equals approximately 500). For use in some experiments the chromatophores were subjected to chromatography on Bio-Gel A-150m at pH 7 and the center two-thirds of the predominant chromatophore band was collected.

Triton X-100 is a product of Rohm and Haas and the radioactive sample used in these experiments was a gift.[3] In those experiments where radioactive ^{14}C bacteria were cultivated, *dl*-[3-^{14}C]malic acid was added at a final level of 10,000 cpm/ml to the standard growth media and the cells were harvested after 4-days growth. [^{33}P]P_i was purchased from New England Nuclear and used upon receipt. The Bio-Gel used in column chromatography was obtained from Bio-Rad Laboratories (Richmond, Calif.). Horse heart cytochrome *c* (type III), pepsin, trypsin (type I), bovine serum albumin, and coenzyme Q_6 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Indigotetrasulfonic acid was obtained from K & K Laboratories (Plainview, N. Y.).

The two samples of poly-γ-benzylglutamic acid used were purchased from Miles Laboratories (Elkhart, Ind.). The original samples had particle weights of 140,000 and 240,000 according to the supplier. For use in chromatography it was necessary to hydrolyze many of the γ-benzyl groups in order to ensure water solubility. This was accomplished by dissolving the sample in 1 N NaOH at room temperature and stirring for a 5–10-hr period. The per cent of γ-benzyl groups still attached to the polymer after the alkaline incubation was

[2] A preliminary report of the results contained herein was given at the 5th International Congress of Photobiology, Aug 26–30, 1968, at Dartmouth, N. H.

[3] We would like to express our appreciation to Dr. Sheldon N. Lewis for donating this material.

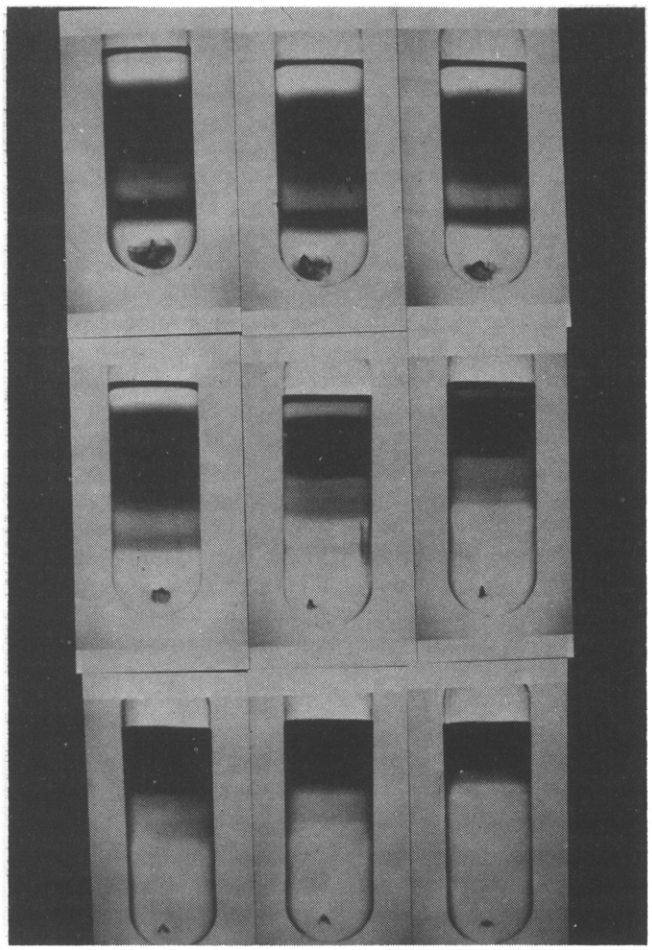


FIGURE 1: Behavior of chromatophores upon sucrose density gradient centrifugation under AUT conditions but with variable Triton concentration. From upper left to lower right, tubes have 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, and 3.0% Triton. All tubes contain 6 M urea and are at pH 11.5. Other details and description of the experiment are given in the text.

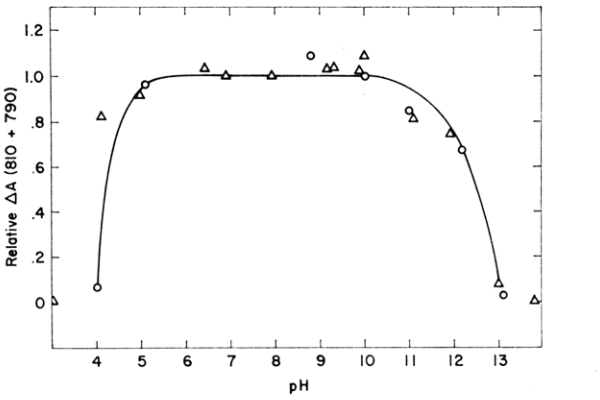


FIGURE 2: Variation of trap activity with pH. The activity measured was the photoproduced absorbance change at 790 and 810 nm. At each pH, an aliquot of a stock solution of chromatophores was added to a solution containing 1.5% Triton X-100, 7 M urea, and 2% sucrose. The final absorbance by the major near-infrared band was 50, the temperature for incubation was 25° and the total time for incubation was 15 min. A sample of this incubation mixture was then diluted 50-fold for measurement of the photochange. Buffers used for establishing the pH's indicated were 0.05 M potassium phosphate, 0.05 M potassium borate, and 0.05 M potassium acetate. The circles are the result of experiments with chromatophores from *R. rubrum*, and the triangles represent data obtained with chromatophores from *R. spheroides*.

determined by its ultraviolet spectrum after dialysis at pH 7. A corrected particle weight was then calculated and used for plotting the data of Figure 5.

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

A Tri-Carb liquid scintillation spectrometer (Series 314E, Packard Instruments Co., Downers Grove, Ill.) was used for measuring radioactivity in various cell fractions. The method of sample preparation for counting consisted of pipetting 0.10 ml of the material to be measured into 15 ml of 2:1 toluene–Triton containing 5 g/l. of Permablend II (98% 2,5-diphenyloxazole, 2% 1,4-bis[2-(5-phenyloxazolyl)]benzene) in a polyethylene bottle. This resulted in a clear solution which was then usually counted over a 10-min period of time.

Absorbance spectra and light-induced absorbance changes were measured with a Cary 14R recording spectrophotometer appropriately modified as described previously (Loach, 1966). More rapid kinetic parameters were measured using a kinetic spectrometer whose construction and operation have also been described (Loach and Loyd, 1966). The electron paramagnetic resonance signals were measured using a Varian E-3 spectrometer.

Results

Of the three parameters varied to determine the best reaction conditions, we initially tried to use just one at a time and tested the effect of the condition (*e.g.*, high pH) on chromatophore structure by subjecting the treated material to sucrose density gradient centrifugation. No single condition gave appreciable yields of smaller particles. Combinations of two conditions (*e.g.*, high pH + Triton X-100) did yield appreciable membrane dissolution. However, by introducing the third condition (*i.e.*, urea) a less extreme pH and lower concentrations of Triton could be used. Figure 1 shows the effect of varying Triton concentration from 0 to 3% at a constant pH of 11.5 and 6 M urea. The temperature was maintained at 0° for all operations, the tubes were spun at 110,000*g*, and the incubation time, including centrifugation, is normally 1.5 hr. The tubes all contained equal volumes (6 ml) of 2, 4, 6, and 16% sucrose which constituted the gradient. For Figure 1, 6 M urea and the appropriate Triton concentration were present throughout the *gradient*. Chromatophores (1.0 ml) suspended in water (optical density at 850 nm = 100) were placed on the top of the tube so that the reaction was initiated with centrifugation. The effectiveness of the Triton is clear from the progression observed.

A similar variation with pH was found. In these experiments, 6 M urea and 1.5% Triton were constant throughout the gradient. Higher pH clearly gave more complete conversion with the major changes in pattern occurring between pH 8 and 10 suggesting that the ε-amino groups of lysine residues

TABLE I: Activity and Yield of AUT Particles.

System Measured	% Recovd of Total Added
Bacteriochlorophyll	85 (850),[a] 105 (590)[a]
Bacteriocarotenoid	98 (520)
ΔA_{865}	95
ΔElectron paramagnetic resonance	92
Cytochrome c[b]	35

[a]Numbers in parentheses indicate wavelength for determination. While the bacteriochlorophyll bands in the near-infrared region are very sensitive to degradation or change in environment, the bands at 590 or 375 nm are much less so (compare with Figure 4). [b] This measurement was conducted with limiting light intensity so that the percentage activity is based on initial rates, and therefore reflects relative efficiency. This activity diminishes relatively rapidly on storage at 4°. The samples contained in a 1-cm, four-sided clear cuvet 0.15% Triton X-100, 1×10^{-4} M ferrocytochrome c, 1×10^{-4} M ubiquinone (Q_6), 0.05 M phosphate buffer at pH 7.5, and chromatophore or AUT particles for which the sum of the absorbances at 850 and 800 nm = +0.41.

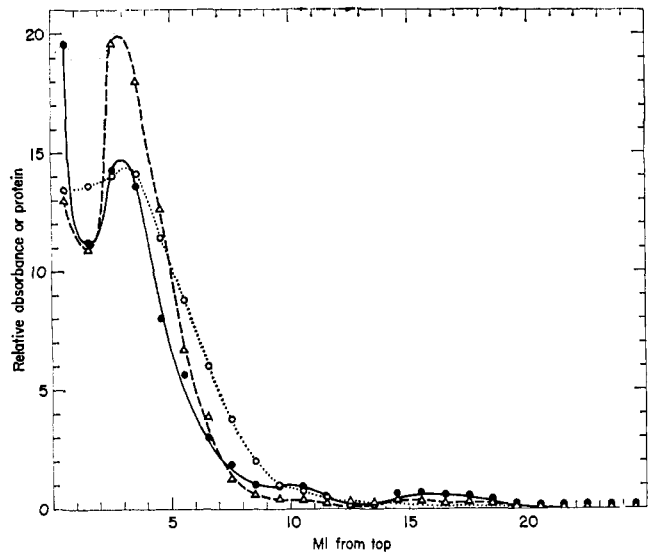


FIGURE 3: Protein and bacteriochlorophyll profile of a standard AUT sucrose density gradient tube. The solid line represents relative protein content and the dashed line the bacteriochlorophyll content after centrifuging the tube for 1 hr at 110,000g. The stippled line gives the protein content after centrifuging an identically prepared tube for 5 hr. The absorbance of the bacteriochlorophyll was measured at 850 nm while the color developed in the Lowry assay for protein was read at 500 nm.

in the protein are playing an important role. Maintenance of activity as a function of pH is shown in Figure 2.

The effect of varying the urea concentration was also systematically studied under conditions of constant 1.5% Triton and pH 11.5. The effect caused by urea on the sucrose density pattern developed was not as marked as the Triton affect shown in Figure 1 but it was still significant.

From these studies the optimal conditions for quantitative conversion of chromatophore material were near pH 11.0, 1.5% Triton X-100, and 6 M urea. A standard AUT preparation is therefore made using these conditions and the center 2–3 ml of the densely colored band is removed for immediate dialysis at pH 7 and 4°. This effectively removes urea and sucrose and changes the pH, but most of the Triton remains inside the dialysis sack. When AUT particles are referred to, this is the material just described as stored frozen or at 4°.

Protein and bacteriochlorophyll profiles for the sucrose density tubes of a standard AUT preparation are shown in Figure 3. As is also apparent in Figure 1, greater than 95% of the pigment is in the top third of the tube and peaks in the 2% sucrose fraction. From the protein analysis of this same AUT preparation nearly all of the protein is also in the top third of the tube. Longer centrifugation times (stippled line of Figure 3) give predominantly similar results except that significant degradation of bacteriochlorophyll begins to occur.

From a number of preparations over a 2-year period, typical results are that 95% of all protein and pigment of fresh chromatophores are accounted for in this AUT fraction. Use of aged chromatophores gives rise to a distinct pellet which grows larger with longer storage of the chromatophores. Some irreversible bacteriochlorophyll degradation occurs during the procedure. Table I summarizes the per cent recovery of pigment and activity under optimal conditions.

It is important to note that excellent trap activity is retained as measured by absorbance change at 791 or 865 nm, photoproduced electron paramagnetic resonance signal, or cytochrome c photoxidation. Quantum yields for ΔA and Δelectron paramagnetic resonance were measured by comparison with *R. rubrum* chromatophores used as an actinometer system (Loach and Walsh, 1969). The values were 0.3 for AUT and 0.6 for "detritonized" AUT (see below). The latter value compares very well with the value obtained for the original chromatophores (quantum yield = 0.7; Loach and Walsh, 1969). Cytochrome c photoxidation activity diminishes on storage at 4°. The photoproduced electron paramagnetic resonance signal and absorbance changes are relatively stable for several weeks if the sample is stored frozen at −20°.

Figure 4 shows the absorbance spectra of AUT particles and chromatophores. The fact that only a minor component of the long-wavelength bands of bacteriochlorohpyll is changed by the treatment, together with the minimal degradation which is also evident from the data of Table I, strongly argues that little change in the bulk pigment environment has occurred. The minor bacteriochlorophyll component whose spectrum is significantly affected is that whose peak in the chromatophores occurred at about 870 nm. This bacteriochlorophyll is presumably solubilized by the Triton (increase at 780 nm) and then partly degraded to colorless products in the presence of oxygen. Other experiments (P. A. Loach and R. L. Hall, manuscript in preparation) indicate that much of this minor bacteriochlorophyll component is loosely bound and not a necessary component of purified *R. spheroides* chromatophores. When Triton is removed (see below) from the AUT particles, about 50% of this shoulder is regained.

Three kinds of evidence have been obtained which bear on

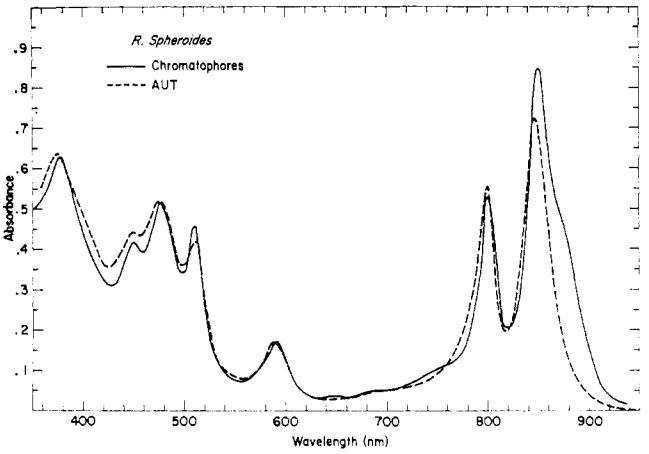


FIGURE 4: Absorbance spectra of chromatophores (solid line) and AUT particles (dashed line). The two spectra were normalized by assigning equal absorbancies at the 590-nm peak. In each case the curves plotted are the average of several typical preparations, 0.05 M phosphate buffer (pH 7.5), room temperature, 1-cm cuvets.

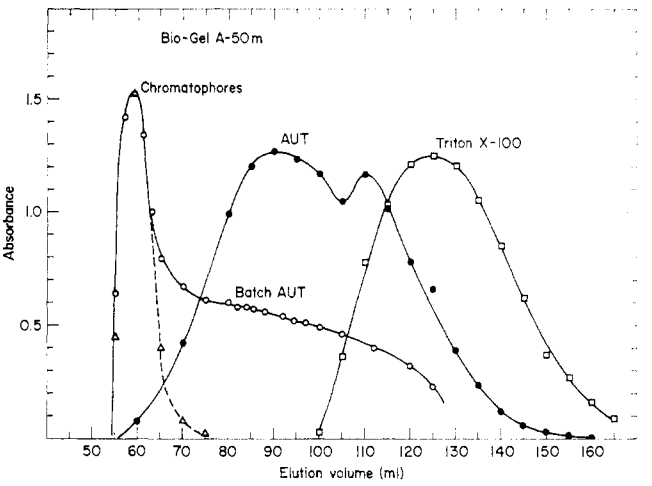


FIGURE 6: Gel filtration on Bio-Gel A-50m. The buffer used was 0.05 M phosphate at pH 7.5. The flow rate was maintained at 1.0 ml/min, the temperature was 2 ± 0.1° and the column size was 2.0 cm diameter × 40 cm long. Samples were applied in 1-ml volumes.

the particle size of the AUT preparation. The first is the centrifugation data. Additional evidence of this kind suggesting a small size was found when AUT particles were suspended in water (containing 0.1% Triton) and centrifuged overnight at 144,000*g*. Only a small portion was spun down.

A second kind of evidence has been obtained from gel filtration data. These results are shown in Figure 5. Note that all proteins which were run as standard markers were run in phosphate buffer containing 0.2% Triton X-100 at pH 7.5. Their effective radii are considerably larger in this nonionic detergent buffer than without it, but a nearly straight-line relationship is still obtained. From this comparison the AUT particles appear to be between 100,000 and 200,000 particle weight. No significant separation of protein and bacteriochlorophyll, or trap, occurs on this column.

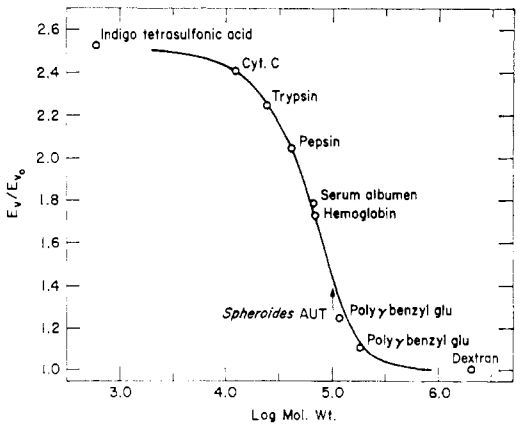


FIGURE 5: Gel filtration on Bio-Gel A-15m. The buffer used in all cases was 0.05 M phosphate (pH 7.5) containing 0.2% Triton X-100. Flow rates were maintained at 0.5 ml/min, the temperature was 1.5 ± 0.1° and the column size was 2.0 cm diameter × 40 cm long. All samples were applied in 1-ml volumes. Chromatophores were used as the void volume marker and were run immediately before and immediately after the protein or AUT sample.

The third kind of evidence consists of filtration data and is summarized in Table II. The behavior of the AUT particles with these Millipore filters is highly dependent on the concentration of the AUT particles. The preparation apparently aggregates significantly at high concentration (absorbancies above approximately 7.0 at 850 nm). Note that 75% of the normal AUT material passes through a 25-mμ filter; chromatophores will not pass through the 100-mμ filter and most are retained on 220-mμ filter. The AUT material passing through the 25-mμ filter has activity proportional to its protein and bacteriochlorophyll content. Included in the table are data for what we call a batch preparation wherein the AUT conditions are used but the centrifugation step is skipped. The protein and bacteriochlorophyll analysis of the sucrose gradient tubes (Figure 3) suggested that no real separation was occurring during that step. The results in Table II, however, show a significant difference in behavior for these two preparations. A marked difference in behavior is also apparent when these two preparations are subjected to chromatography on Bio-Gel A-50m (Figure 6). These last two kinds of

TABLE II: Millipore Filtration.

Pore Size (mμ)	AUT			Batch AUT		
	A_{on}	$A_{through}$	% Recov	A_{on}	$A_{through}$	% Recov
25	13.7	5.5	40			
	9.9	6.2	64			
	6.7	4.7	71			
	5.6	3.9	70	5.6	0.23	4.2
	2.0	1.4	70	2.0	0.110	5.5
	1.0	0.74	74	1.0	0.063	6.3
50	1.0	0.76	76	1.0	0.29	29
100	1.0	0.85	85	1.0	0.76	76

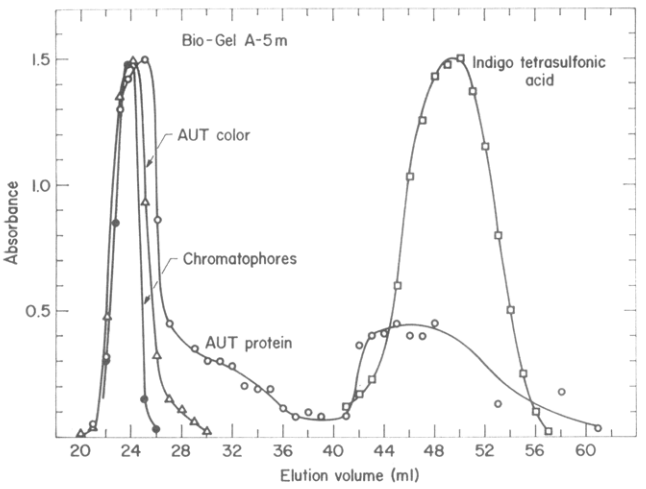


FIGURE 7: Gel filtration on Bio-Gel A-5m. The buffer used was 0.05 M phosphate at pH 7.5. The flow rate was 0.5 ml/min, the temperature was 2.5 ± 0.1° and the column size was 2 cm diameter × 40 cm long. Samples were applied in 1-ml volumes. Triton X-100 behaves similarly to the indigotetrasulfonic acid sample shown.

data suggest that batch AUT particles are larger than regular AUT particles, these latter of which have, as part of their preparatory procedure, been exposed to sucrose density gradient centrifugation. As will be seen below, the key factor seems to be lipid separation.

For all the detergent particle preparations we have studied, removal of Triton results in significant reaggregation of the AUT fraction. A column often used for detergent removal is Bio-Gel A-5m. Typical results with this column are shown in Figure 7. Although all of the pigment occurs in a band which is essentially excluded by the gel, only half of the protein stays with this band. All trap activity is also associated with the pigmented band. Note that the bulk of the Triton is well removed from the pigment band. This "detritonized" AUT preparation behaves like the original chromatophores when exposed to chromatography on Bio-Gel A-50m and will settle out of solution if they are left to stand for 3 or 4 days at 4°. Electron micrographs (Figures 8 and 9) routinely show these "detritonized" AUT particles to appear circular (or spherical) and quite chromatophore like in appearance. A typical time dependence for the reaggregation phenomena is illustrated in Figure 9. The "detritonized" AUT particles for Figure 9 (top) were fixed and stained within 1-hr elution from a Bio-Gel A-5m column. Those for Figure 9 (bottom) had stood at 4° for 2 days before fixing and staining. After 3–4 days, part of the samples often settled out of solution, and electron micrograph pictures showed only huge aggregates.

Some small amount of tightly bound Triton remains with the AUT particles after chromatography on Bio-Gel A-5m. Table III shows the results of several experiments in which ^{14}C-labeled Triton X-100 was used to prepare AUT. If the "detritonized" AUT of the first experiment listed was subjected to rechromatography on a fresh A-5m column, all the ^{14}C stayed with the pigment–protein fraction indicating tight binding. It is interesting to note that the batch preparation of AUT (bottom line) retains less Triton when exposed to this column. This is suggestive of a rebinding of natural

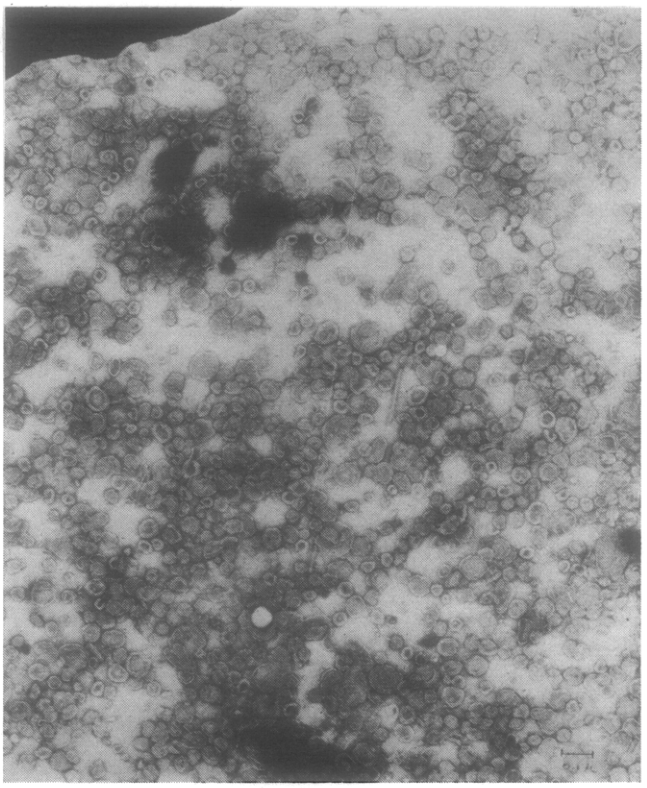


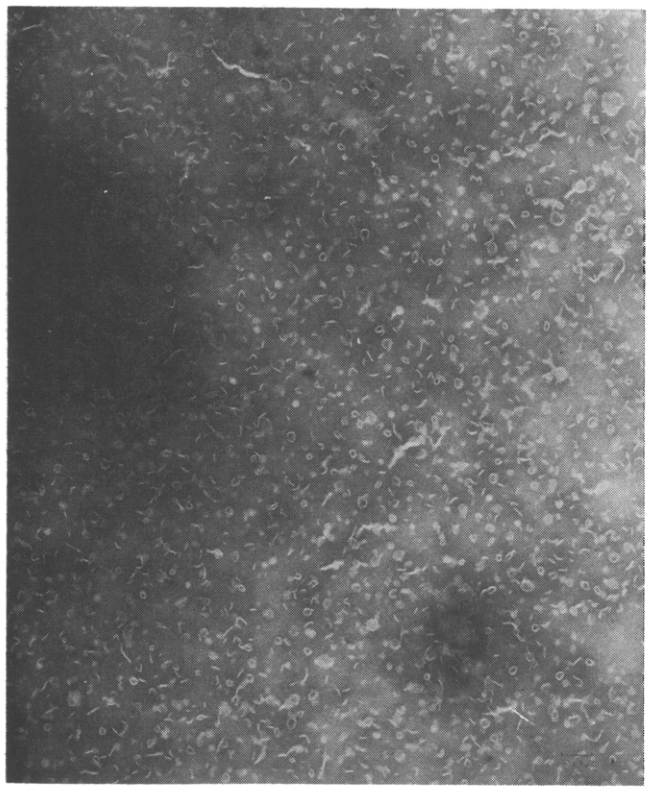
FIGURE 8: Electron micrograph of chromatophores prepared from *R. spheroides*. This chromatophore preparation was a standard preparation which was then further separated from other cellular material by gel filtration on Bio-Gel A-150m. Magnification, 90,000X. The average chromatophore size by our measurement is 800 Å in diameter. The sample was negatively stained with phosphotungstic acid.

lipids as the Triton concentration falls during chromatography. The middle line of Table III represents an experiment performed with Triton X-100 which was first itself subjected to chromatography on Bio-Gel A-5m. This experiment was conducted because a small quantity of fast-moving ^{14}C material existed in the Triton. This purified material gave results essentially identical with the unpurified Triton when it was used to make AUT. From Table III the weight ratio of Triton bound to total protein present approaches 1:2. This latter result suggested that the AUT incubation proce-

TABLE III: Tightly Bound Triton.[a]

	Triton X-100 (mg)	Protein[b] (mg)
AUT-5	0.0187	0.042
AUT-5[c]	0.0190	0.042
Batch AUT-5	0.0065	0.042

[a] Data for bacteriochlorophyll–protein complex after chromatography on Bio-Gel A-5m at pH 7. [b] The second and third samples listed were normalized to the same protein content as the first sample to aid comparison of the Triton content. [c] The Triton used for this experiment was first chromatographed on a Bio-Gel A-5m column.



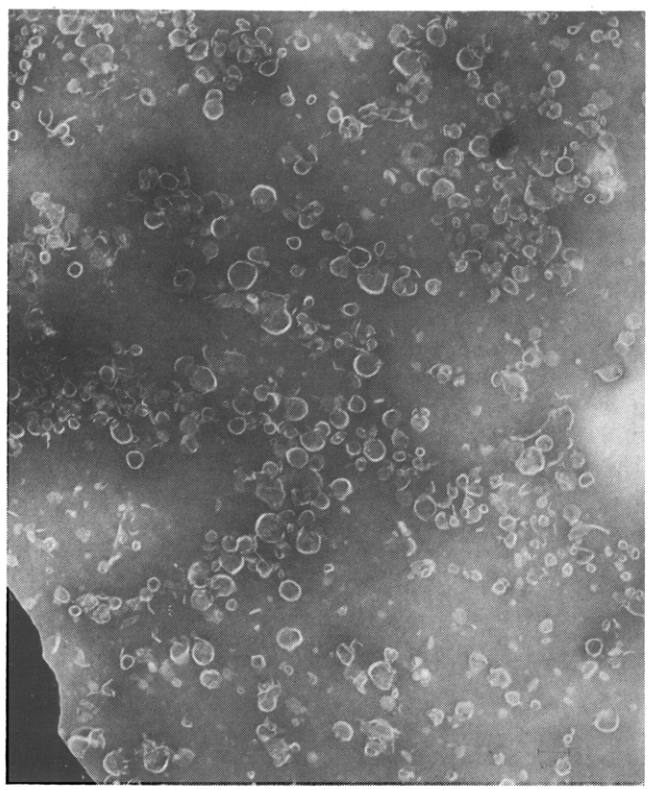


FIGURE 9: Electron micrograph of reaggregated AUT particles. Magnification and staining conditions are identical with those for the material of Figure 8. A standard AUT preparation was filtered through a 25 mμ Millipore filter and then "detritonized" on a Bio-Gel A-5m column. The resulting material was used within 1 hr for the top and after 2 days for the bottom picture.

dure had effected nearly complete displacement of the normal phospholipids of the membrane fragments (chromatophores) and the centrifugation step effectively separated this displaced lipid from the trap and bacteriochlorophyll fraction. Closer analysis of the sucrose density gradient tubes further confirms that such separation must be occurring.

Lipid analysis of the sucrose density tubes poses some serious problems because of the Triton present. To aid our initial analysis efforts we have grown our bacteria on [3-^{14}C]-malate and then prepared AUT with cold Triton. Figure 10 shows our results. The solid line of part A represents total activity and the dashed line is activity due to protein. Bacteriochlorophyll and carotenoid in the gradient are also shown. The gradient is somewhat lighter than those previously employed and consists of 8 ml of 1% sucrose, 8 ml of 2% sucrose, and 8 ml of 4% sucrose. The centrifugation time was 2 hr. By subtracting the sum of the counts per minute due to protein, bacteriochlorophyll, and carotenoid from the total counts per minute, good evidence is indeed obtained (part B) showing a high concentration of what must be lipid at the top of the tube. Preliminary experiments in which the original cells were grown on [^{33}P]phosphate further confirms this conclusion. It is expected that displaced lipids would tend to float. It should be noted that there is a large amount of colorless protein also at the top of the tube. Duplicate analysis agreed very well with these results.

Discussion

The AUT procedure, which was systematically arrived at for quantitatively causing dissolution of membrane material, appears to be extremely useful. The natural lipid material is apparently nearly completely displaced and a proportional amount of the nonionic detergent is tightly bound in its place (Table III). In addition, sufficient additional Triton (greater than 0.05%) must be present in the solvent to prevent significant reaggregation of the subunit material.

The best estimates of average particle weight (Figure 5) for the preparation are about 150,000 ± 50,000. Initial results using an analytical ultracentrifuge to measure $s_{20,w}$ and the diffusion coefficient for AUT in the presence of 0.05% Triton yield the value of 160,000 ± 20,000. Although no real effort was made in these experiments to separate that protein which does not have a function as part of the photoreceptor subunit, about half of the total protein was separated as very small (or low density) material by the sucrose density gradient (Figures 3 and 10), and about half of the remaining protein was separated on the "detritonizing" column (Figure 7). Consistent with this data are protein and bacteriochlorophyll analysis data from which the protein:bacteriochlorophyll weight ratio changes from 13:1 for chromatophores to 3.5:1 for "detritonized" AUT.

The quantitative conversion of all bacteriochlorophyll- (and carotenoid) containing material to a protein–trap–bacteriochlorophyll complex(es) whose size is only 150,000, without significantly affecting the spectrum of the major bacteriochlorophyll content or trap activities, provides the first really strong evidence that such a photoreceptor subunit exists in the *in vivo* membrane. The size of such a particle would be about right to fit with other types of subunits and give rise to the 50–80-Å substructure of chromatophores which can be seen by electron microscopy. Several possibilities exist for

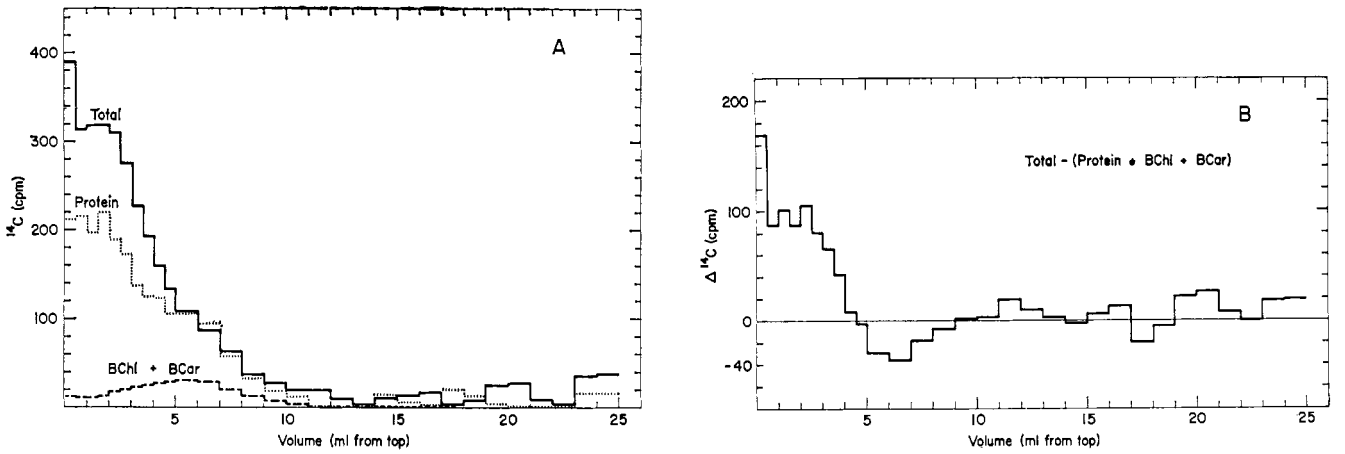


FIGURE 10: Sucrose density gradient pattern of ^{14}C-labeled chromatophores under AUT conditions. Only three sucrose densities were used as is described in the text. Urea (6 M) and Triton (1.5%) were present throughout and the pH was 11.0 buffered with 0.05 M potassium phosphate. The area under the protein curve was adjusted to 62% of the total area under the ^{14}C curve. This latter per cent was arrived at by consideration of the protein content of intact chromatophores (from analysis data), the fraction of carbon in protein versus the total carbon content, and assuming that the ^{14}C/carbon ratio is the same for all carbon-containing compounds. Similarly, the ^{14}C contribution due to bacteriochlorophyll and carotenoid was adjusted to 10% of the total ^{14}C. Protein analysis was performed using the Lowry assay and bacteriochlorophyll content was followed at 850 mμ. For scintillation counting 1 ml of each fraction was pipetted into the scintillation media. Controls gave an average background at 50 cpm which has been subtracted from the data shown in the figure.

assigning bacteriochlorophyll and carotenoid bands to the subunits present in the preparation. For example, each type of bacteriochlorophyll might exist in a different photoreceptor subunit. This would be compatible with earlier work where evidence has been given for separation of B870, on the one hand, and B850 and B800, on the other (Bril, 1958; Clayton, 1962a,b). However, as indicated in the Result section, B870 may well be a property of the aggregated (membranous) state only and not necessarily a separate complement of bacteriochlorophyll. B870 disappears (along with pertinent carotenoid changes) when Triton is added to chromatophores at pH 7, but upon removal of Triton the original spectrum is nearly restored (P. A. Loach and R. L. Hall, manuscript in preparation). If this bacteriochlorophyll component had merely been made soluble by the detergent one would have expected to have removed it during preparation, similar to phospholipid removal. Also, when fresh *R. spheroides* chromatrophores are exposed to size separation in a Bio-Gel A-150m column at pH 7 a larger amount of B870 is contained in the heavier fractions.

The existence of B850 and B800 on separate subunits seems unlikely at the present time for the following reason. The relative quantum yield for absorbance change of AUT *vs.* chromatophores is the same whether measured at 791 or 880 nm with the exciting light at 865 or 791 nm, respectively. The simplest explanation of this result is that a single unit contains B800, B850, and one trap. Physical data are consistent with the interpretation that the photoreceptor subunits are dispersed when at low concentration in 0.1% Triton solution so that they do not interact with each other. Thus the quantum yields mentioned should represent the average value for all traps.

If it is assumed that there are 50 bacteriochlorophyll molecules/photoreceptor subunit, then about one-third of the weight of this kind of AUT particle is accounted for. If the total compliment of carotenoid is also present in this same complex, then the sum of pigment weight would represent between one-third to one-half of the total. Thus, for a protein to bacteriochlorophyll ratio of 3.5 to 1 for the entire AUT fraction, perhaps about half of the AUT particles still present at this stage have no pigment associated with them and have a function which is not related to trapping light energy.

It may someday be possible to further dissociate the photoreceptor subunit complexes into single polypeptide–pigment complexes. These AUT particles are susceptible to succinylation, after which they are considerably slower to reaggregate when Triton is removed. Trap activity is highly unstable, however, after such treatment. The detailed studies of Olson (Olson and Romano, 1962; Olson *et al.*, 1969) suggest that a photoreceptor subunit may, in turn, consist of two to eight fundamental units.

A most interesting, and potentially exciting, result is the observation—both by gel filtration and electron microscopy—that reaggregation of AUT particles due to removal of excess Triton results in the quantitative formation of chromatophore-like structures (Figure 9, bottom). It is not surprising that membrane fractions will reaggregate when the solubilizing reagents are removed, but the tendency to form a distinctive structure carries with it the hope that it might be possible to reconstitute activities such as photophosphorylation, when all the appropriate coupling factors are present. None of the "detritonized" AUT preparations have so far been found to have such activity. This built-in tendency for the material to form a distinctive structure further suggests that the formation of chromatophores in the first place (that is, by sonically disrupting whole cells) may have been the result of a random reaggregation into the most stable structure of membrane subunits when the dislodging force (sonication) was stopped.

The results of the data reported in this paper may help in understanding some other detergent derived particles. It is easy to relate the present results to other detergent studies where activity was obtained in each of several heavy and light bands studied (Garcia *et al.*, 1966a,b, 1968a,b). These earlier preparations probably achieved only partial breakdown of

the membraneous structure since all fractions reported are considerably heavier than those presently documented. It is more difficult to relate the present results to other studies where inactive fractions are found (Garcia *et al.*, 1966a, 1968b; Reed and Clayton, 1968; Reed, 1969; Gingras *et al.*, 1968; Thornber *et al.*, 1969). Inactive fractions could arise for any of the following reasons: (a) loss of activity during preparation, (b) lack of redox control during activity assay, or (c) inactive photoreceptor subunits in the original membrane as a result of past mutations affecting the protein with which the trap and/or bulk pigments are associated. It is conceivable that each of the predominant bacteriochlorophyll peaks in a wide variety of bacteria are the result of a mutated gene yielding a variant polypeptide which binds or houses its complement of bacteriochlorophyll. In some bacteria (chromatium, for example) many of those variant photoreceptor subunits may exist together and still function. In the future it will be particularly interesting and informative to compare the photoreceptor subunits described here with reaction center preparations (Reed and Clayton, 1968; Reed, 1969; Thornber *et al.*, 1969; Gingras *et al.*, 1968). The latter preparations appear to be of a size similar to the AUT complexes when measured with detergent present (Thornber *et al.*, 1969). The reasons for the variable patterns in bacteriochlorophyll fractionation remains to be discovered.

The efficiency of light energy utilization for the AUT particles dispersed in a Triton-containing buffer is about one-third that of the original chromatophores and about one-half that of reaggregated AUT. Correspondingly the fluorescence caused by low-intensity exciting light with the Triton-dispersed units (51 relative units) is considerably greater than with either chromatophores (10 relative units) or "detritonized" AUT (14 relative units). These results suggest that the membrane condition, or reaggregated state, allows significant energy transfer from one photoreceptor subunit to another while the individual subunits dispersed in a Triton-containing buffer are essentially noninteracting.

It is clear that future work with the AUT preparation should include purification, that is, complete separation of other types of membrane subunits. Once this is achieved, analysis of the photoreceptor complex should limit considerably the possible molecules that may serve as primary electron acceptor in the trap site, and may result in other structural clarifications of this most important light-harvesting unit.

Acknowledgments

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Early Intermediates in the Degradation of α-Conidendrin by a *Pseudomonas multivorans**

Anne Toms and J. M. Wood

ABSTRACT: A *Pseudomonas* spp. tentatively identified as a *Pseudomonas multivorans* was isolated by elective culture with α-conidendrin as sole carbon and energy source. Two new organic compounds, 1,2,3-trihydro-6-hydroxy-7-methoxy-4-oxo-3-hydroxymethyl-2-naphthoic acid γ-lactone (I) and 4,6-dihydroxy-3-hydroxymethyl-7-methoxy-2-naphthoic acid γ-lactone (II), were isolated from culture filtrates and characterized by CH analysis, mass spectrometry, nuclear magnetic resonance, infrared spectrophotometry, and ultraviolet spectrophotometry. For compound I additional information was required in order to determine rigorously its structure owing to keto–enol tautomerization providing keto–enol mixtures. Consequently the *O*-acetyl- and 2,4-dinitrophenylhydrazone derivatives were prepared and the structure of each compound was determined by CHN analysis and mass spectrometry. Early intermediates in the oxidative degradation of α-conidendrin leading to a substituted 2-naphthoic acid γ-lactone for this *Pseudomonas* spp. are proposed.

Study of the microbial degradation of α-conidendrin can be related to the process of lignin decomposition in nature. α-Conidendrin is a lignin model compound containing two phenylpropane units and is believed to be synthesized by the condensation of dehydrogenated *trans*-ferulic acid with a quinone methide radical produced by dehydrogenation of a second molecule of coniferyl alcohol (Freudenberg and Geiger, 1963). It can also be regarded as a natural product since Erdtman (1944) succeeded in isolating it by direct extraction with acetone from spruce wood.

α-Conidendrin was first isolated by Lindsey and Tollens (1892) in the form of soft crystals in the oily residue extracted by ether from the spent liquor from a sulfite cook of spruce (*Picca abies*). Later, it was found to be readily isolated from the spent sulfite liquor of western hemlock (*Tsuga heterophylla*) (Pearl, 1945).

Several investigators (Konetzka *et al.*, 1952; Pratt *et al.*, 1953; Tabak *et al.*, 1959; Sundman, 1965) have isolated microorganisms of the genus *Pseudomonas*, *Achromobacter*, *Flavobacterium*, and *Agrobacterium* which will grow with α-conidendrin as sole source of carbon. Paper chromatog-

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