Steiner, L. A., Okamura, M. Y., Lopes, A. D., Moskowitz, E., and Feher, G. (1974), *Biochemistry 13*, 1403.
Straley, S. C., Parson, W. W., Mauzerall, D. C., and Clayton, R. K. (1973), *Biochim. Biophys. Acta 305*, 597.
Takemoto, J., and Lascelles, J. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 799.
Thornber, J. P. (1970), *Biochemistry 9*, 2688.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Characterization of Reaction Centers from Photosynthetic Bacteria. II. Amino Acid Composition of the Reaction Center Protein and Its Subunits in *Rhodopseudomonas spheroides* R-26†

L. A. Steiner,* M. Y. Okamura,‡ A. D. Lopes, E. Moskowitz, and G. Feher

ABSTRACT: The amino acid composition of the reaction center protein from *Rhodopseudomonas spheroides* R-26, and of its three subunits, was determined. The protein, which is extracted from the bacterial membrane by detergent, contains a large proportion of apolar residues. The two smaller subunits are especially hydrophobic in composition and are probably associated with the lipid part of the membrane. The amino acid composition of reaction centers from *Rhodospirillum rubrum* G-9 was similar but not identical with that of reaction centers from *R. spheroides* R-26. Immunochemical tests showed that reaction centers from these two bacterial species are antigenically distinct.

The photochemically active reaction center, isolated from chromatophores of *Rhodopseudomonas spheroides* R-26, is composed of protein and a number of associated molecules including bacteriochlorophyll, ubiquinone, and iron. In the accompanying paper (Okamura *et al.*, 1974), we have presented evidence that the protein moiety consists of three subunits, present in equimolar amounts, with a composite molecular weight of about 70,000. Two of these subunits appeared to be essential for activity; removal of the third resulted in extensive inactivation at cryogenic temperatures but not at room temperature (Okamura *et al.*, 1973, 1974).

The present paper is concerned with a more detailed analysis of the reaction center protein and its subunits. Our principal objectives have been to determine whether this protein, derived from the bacterial membrane, has an unusual amino acid composition, whether the two "essential" subunits are independent, and whether they differ in overall composition from the third subunit. In addition, since iron has been postulated to be the primary electron acceptor (Feher, 1971; Leigh and Dutton, 1972), it was particularly important to establish whether the intact protein, and the individual subunits, contain residues of cysteine that might function as binding sites for iron. Finally, we hoped that these studies would provide an independent estimate of the minimum molecular weight and of the stoichiometry of the subunits.

The reaction center protein from the related photosynthetic bacterium, *Rhodospirillum rubrum* G-9, is also composed of three subunits (Noël *et al.*, 1972; Clayton and Haselkorn, 1972; Okamura *et al.*, 1974). In order to obtain additional information on the properties of the photochemically active proteins from the two species, we have compared their overall amino acid composition and their antigenic properties.

Materials and Methods

Preparation of Reaction Centers. The preparation of reaction centers from *R. spheroides* R-26 and *R. rubrum* G-9 is described in the accompanying paper (Okamura *et al.*, 1974). Reaction centers containing either radiolabeled half-cystine or tryptophan were obtained from bacteria grown in modified Hutner medium without yeast extract, peptone, or glutamate, but including either 100 μCi of L-[^{35}S]cystine/l. (Schwarz/Mann, sp act. 25 mCi/mmol) plus 200 mg of L-methionine/l. or 200 μCi of DL-[^{14}C]tryptophan/l. (International Chemical and Nuclear Corp., ring-2 labeled, sp act. 3 mCi/mmol). In each case, the other amino acids were included at a concentration of 10 mg/l.

Preparative Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. The procedure for preparative gel electrophoresis was essentially the same as that for analytical gel electrophoresis, discussed in the preceding paper, except that the load per gel was about 50 μg. After electrophoresis the gels were marked with a reference bristle and scanned at 280 nm. Sections corresponding to the protein bands were excised from 20 gels with a taut wire and template and macerated in a volume of buffer (50 mM Tris-Cl (pH 8.0)–0.1% sodium dodecyl sulfate) equal to 1.5 times the volume of the gel slices. The macerated gel sections were then placed into electrophoresis tubes (12 × 0.8 cm) sealed at the bottom with a 0.5 cm long plug of 10% polyacrylamide supported with a nylon net. The tubes were placed into concentric cylinders (2 × 2 cm) that were sealed at one end with a dialysis membrane and that contained the same buffer solution. The protein was eluted electrophoretically into these cylinders (15 mA/gel for 4 hr) and concentrated by vacuum dialysis in collodion bags (Schleicher and Schuell). The purity of the separated

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (L. A. S. and A. D. L.), and the Department of Physics, University of California, San Diego, at La Jolla, California 92037 (M. Y. O., E. M., and G. F.). *Received October 1, 1973.* Supported by National Science Foundation Grants GB-33402 and GB-33340X and National Institutes of Health Grant GM-13191.

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subunits was assayed by analytical sodium dodecyl sulfate gel electrophoresis. In addition, blank sections of gel were carried through the purification procedure in parallel to serve as background for amino acid analysis. Special care was taken to avoid contamination by handling the gels with gloves and forceps and by performing all operations under a protective plastic hood.

Amino Acid Analysis. Reaction centers (0.55 OD_{802} in 25 μl of 5 mM $NaHCO_3$ (pH 9.1)–0.1% LDAO[1]) were made 2.5 mM in phenol in 1.0 ml of 6 N HCl, and hydrolyzed under vacuum in a toluene bath at 110 ± 1° for 24, 48, and 72 hr. The samples were dried at 40° by rotary evaporation and taken up in 6.6 ml of 0.2 N sodium citrate buffer (pH 2.2). Analyses were carried out by an accelerated method (Spackman, 1967) in a Beckman amino acid analyzer (Model 120B) with a 3.6–4.6 mV range expander and 2.2-mm flow cells. For the determination of acidic and neutral residues, samples of 1.0 ml were applied to a 56-cm column of Beckman AA 15 resin and eluted at 53° with a buffer flow of 70 ml/hr using the standard 0.2 N sodium citrate buffers at pH 3.25 and 4.25. For the determination of basic residues, samples of 2.0 ml were applied to a 10-cm column of Beckman PA 35 resin and eluted at the same flow rate and temperature with 0.35 N sodium citrate buffer (pH 5.25). Standards (Beckman or Pierce) containing about 16 nmol of each residue were run alternately with experimental samples. For determination of tryptophan, cysteic acid, and ornithine, standards were prepared from the corresponding L-amino acids (Sigma).

The tryptophan content of reaction centers was determined after hydrolysis in thioglycolic acid (Matsubara and Sasaki, 1969). Since we found that recovery of tryptophan after hydrolysis of lysozyme was not quantitative, a radiolabeling method was used to measure the recovery. Reaction centers containing [^{14}C]tryptophan were prepared and were hydrolyzed in 6 N HCl made 5% (v/v) in thioglycolic acid. After hydrolysis, the sample was extracted with ether to remove thioglycolic acid, dried by rotary evaporation, taken up in the pH 2.2 buffer, and applied immediately to the 10-cm column. Another aliquot of the sample was applied to the same column and the effluent was collected in 1.0-min (about 1.1 ml) fractions directly from the column. One milliliter of each fraction was added to 10 ml of a mixture containing 1 part of BioSolv BBS3 (Beckman) and 5 parts of toluene scintillation solvent (8 g of butyl-PBD (Beckman) and 0.5 g of PBBO (Beckman)/l. of toluene). The radioactivity of the samples was measured in a liquid scintillation spectrometer (Beckman LS 250) and the fraction of the applied radioactivity eluting in the position of tryptophan was determined. A third aliquot of the sample was applied to the 56-cm column and the radioactivity of the eluted fractions was measured.

The half-cystine content of reaction centers was obtained after performic acid oxidation carried out according to Moore (1963). The recovery of cysteic acid after hydrolysis was determined using [^{35}S]cystine labeled reaction centers as described for tryptophan above. On the 56-cm AA 15 column, cysteic acid was not retarded. Since other ninhydrin positive or radioactive material might also elute at the column front, cysteic acid was also determined on a 0.9 × 37 column of the anion exchange resin DA-X4 (Durrum Chemical Corp., Palo Alto, Calif.). When the column was run at 53° using the 0.2 N sodium citrate buffer (pH 3.25), with a buffer flow of 70 ml/hr and ninhydrin flow of 35 ml/hr, cysteic acid emerged at 49 ± 1 min (after the other amino acids).

Preparations of reaction centers from *R. rubrum* G-9 were found to contain material that emerged with lysine in the usual analysis for basic residues. This material was resolved by running the 0.9 × 10 cm PA 35 column in 0.38 N sodium citrate (pH 4.80), at 25° with the usual flow rates. Under these conditions, the new peak emerged at 38 min, lysine at 45 min, NH_3 at 53 min, and histidine at 68 min. After histidine emerged, the temperature was raised to 53° and arginine was eluted about 45 min later. The elution position of the new peak was identical with that of L-ornithine.

The amino acid composition of the subunits was determined by similar procedures. The H subunit, obtained by sucrose gradient centrifugation, was dialyzed into 5 mM sodium phosphate (pH 8.0)–1% sodium dodecyl sulfate. Samples of 0.5 ml containing 0.13 OD_{280} were mixed with 25 μl of 0.1 M phenol and 0.53 ml of concentrated HCl and were hydrolyzed for 24 hr at 110°. The L and M subunits, obtained by polyacrylamide gel electrophoresis, were dialyzed into 5 mM sodium phosphate (pH 8.0)–0.1% sodium dodecyl sulfate, and samples of about 0.3 ml containing about 0.2 OD_{280} were hydrolyzed for 20 hr.

Immunological Analyses. Two rabbits were immunized with 3.7 OD_{802} of reaction centers of *R. spheroides* R-26 and were bled at 5 weeks. Two other rabbits were immunized with 1.4 OD_{802} of reaction centers of *R. rubrum* G-9, boosted 7 weeks later with 0.7 OD_{802} of the same antigen, and bled 9 days after the booster injection. All injections were with complete Freund's adjuvant (Difco) and were given into the footpads.

Double diffusion in two dimensions was carried out on microscope slides with apparatus supplied by Gelman. The medium was 1% Ionagar (Difco) in 0.1 M Tris-Cl (pH 8.9)–0.02% LDAO.

Miscellaneous. Sucrose gradient centrifugation, analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis, gel scanning, and determination of absorbancies were carried out as described in the accompanying paper (Okamura *et al.*, 1974).

Experimental Results

Amino Acid Composition of Intact Reaction Centers from R. spheroides R-26. Reaction centers were hydrolyzed for 24, 48, and 72 hr. Before hydrolysis, the absorbance of each sample at 802 nm was carefully measured, since the amino acid content was normalized to the optical absorbance. The average composition at each time point and "best values" are presented in Table I. In addition, the number of residues was calculated, assuming a mol wt of 73,000 for the reaction center protein (Okamura *et al.*, 1974).

A radiolabeling method was used to determine *tryptophan* since it is difficult to recover the residue quantitatively after acid hydrolysis. Reaction centers were obtained from bacteria grown in the presence of DL-[^{14}C]tryptophan. None of the radiolabel was found in bacteriochlorophyll. The protein was hydrolyzed in acid in the presence of thioglycolic acid, allowing a partial recovery of tryptophan. Of the total radioactivity found in the hydrolysate, 48% was eluted in the position of L-tryptophan on the 10-cm column; the amount of tryptophan determined by ninhydrin analysis was corrected accordingly, thus obtaining the value (4.32 mol %) shown in Table I. A small amount (6% of total) of radioactive material was eluted after tryptophan but did not correspond in position to any of

[1] Abbreviations used are: LDAO, *N*,*N*-dimethyllaurylamine oxide; $A_\lambda^{1\ cm}$, optical absorbance at wavelength λ in a 1-cm path length; OD_λ, the absorbance at wavelength λ (nanometers) in a 1-cm path length times the volume (milliliters) of the solution.

TABLE I: Amino Acid Composition of Reaction Centers from *Rhodopseudomonas spheroides* R-26.[a]

Amino Acid	nmol/ODV_{802}				mol %	No. of Residues for Mol Wt 73,000[c]
	24 hr[a]	48 hr[a]	72 hr[a]	Best Value[b]		
Lys	78	79	78 ± 1	78	2.69	17.9
His	69	69	68 ± 1	69	2.38	15.8
Arg	113	111	110 ± 2	111	3.83	25.4
Asp	201	200	198 ± 1	200	6.91	45.9
Thr	134	128	122 ± 1	140	4.83	32.1
Ser	131	121	108 ± 2	143	4.94	32.8
Glu	195	195	192 ± 1	194	6.70	44.5
Pro	179	172	175 ± 5	175	6.04	40.1
Gly	317	319	316 ± 2	317	10.95	72.6
Ala	297	303	305 ± 2	302	10.43	69.2
Val	178	183	188 ± 2	188	6.49	43.1
Met	74	75	78 ± 3	76	2.62	17.4
Ile	146	157	159 ± 2	159	5.49	36.4
Leu	309	315	318 ± 2	314	10.84	71.9
Tyr	87	90	92 ± 1	90	3.11	20.6
Phe	181	192	194 ± 2	194	6.70	44.5
$^1/_2$Cys[d]				21	0.71	4.7
Trp[e]				125	4.32	28.5
			Σ(nmol/ODV_{802}) = 2900		Σ residues = 663.4	

[a] For each time point, three samples were hydrolyzed and analyzed in duplicate. Thus, each value is the average of six determinations. The standard deviations for the 24- and 48-hr hydrolyses were similar to those shown for the 72-hr values. [b] "Best values" for threonine and serine were obtained by extrapolation to zero time of the 24-, 48-, and 72-hr hydrolyses. Best values for valine, isoleucine, and phenylalanine were taken as the values after 72 hr of hydrolysis. The other best values are the means of the 24-, 48-, and 72-hr hydrolyses. [c] Values are based on the calculated mean residue weight of 110 for the reaction center protein. [d] Average of eight determinations after performic acid oxidation corrected for loss of cysteic acid by radioactive method (see text). The standard deviation from the mean was 0.05 mol %. [e] Tryptophan was determined by analysis of reaction centers containing [^{14}C]tryptophan after hydrolysis in thioglycolic acid. Corrections for recovery were made as described in the text. The corrected values in two separate hydrolyses were 4.23 and 4.41 mol %.

the amino acids. Additional radioactive material (23%) was eluted before tryptophan, and 10% was removed by the NaOH wash. Accordingly, the sample was also applied to the 56-cm column. None of the eluted radioactivity corresponded in position to any of the standard amino acids. The additional radioactivity probably corresponds to degradation products of [^{14}C]tryptophan formed during hydrolysis.

The amount of *half-cystine* was determined from the cysteic acid content of reaction centers after performic acid oxidation. The "best value" shown in Table I (0.71 mol %) represents the mean of eight such determinations. Since the recovery of cysteic acid is not always quantitative (Moore, 1963), a radiolabeling method was again used to estimate the recovery. Three of the preparations of reaction centers were obtained from bacteria grown in the presence of L-[^{35}S]cystine and excess nonradioactive L-methionine. A single radiolabeled peak emerged in the position of cysteic acid; there was no radioactivity in the position of methionine sulfone. The recovery of the applied radioactivity in the position of cysteic acid was 95 ± 3%. The value for cysteic acid determined by ninhydrin analysis was corrected for the small loss. Since cysteic acid emerges in the void volume of the 56-cm column, it is possible that radiolabeled (or ninhydrin positive) impurities or degradation products also emerge in this position. To evaluate this possibility, an additional chromatographic method was developed, utilizing an anion exchange resin that retards cysteic acid. When the hydrolysate of performic acid oxidized reaction centers containing [^{35}S]half-cystine was applied to this column, a single radioactive peak emerged in the position of cysteic acid. Moreover, the value obtained by ninhydrin analysis agreed with that obtained on the 56-cm column under standard running conditions. Evidently, impurities or degradation products did not contribute to the cysteic acid values in the standard run.

From the total number of micromoles of amino acids per ODV_{802} (see Table I) and the average molecular weight per residue (110 for the reaction center protein), one can obtain the *extinction coefficient* for a solution containing 1 mg of protein/ml, *i.e.*, 1.0 ODV_{802} contains a total of 2.90 μmol or 0.319 mg of amino acid residues. Therefore, the absorbance $A_{802}^{1\,cm}$ of a solution of reaction centers (1 mg/ml) is 3.13.

Amino Acid Composition of Subunits of Reaction Centers. It was shown in the preceding paper that reaction centers can be dissociated into two units (LM and H) by centrifugation in a sucrose gradient, and into three subunits (L, M, and H) by sodium dodecyl sulfate–polyacrylamide electrophoresis (Okamura *et al.*, 1974). For the determination of the amino acid composition, the H subunit was prepared by centrifugation. The L and M subunits were prepared by gel electrophoresis. The appropriate bands were cut out after scanning the gels and the protein was eluted electrophoretically from the slices and hydrolyzed. The amino acid compositions were corrected for incomplete hydrolysis and partial destruction with correction factors derived from the 24-, 48-, and 72-hr analyses of intact reaction centers. In the case of the H subunit, a correction was made for a small amount of contamination (approxi-

TABLE II: Amino Acid Composition of Reaction Center Subunits from *R. spheroides* R-26.

Amino Acid	Reaction Center								Lysozyme	
	mol %			No. of Residues[c]			Sum of Residues in Subunits[d]	No. of Residues in Reaction Center[f]	No. of Residues	
	L[a]	M[a]	H[b]	L[a]	M[a]	H[b]			Found[a]	Expect[g]
Lys	2.4	0.9	5.1	4.5	1.9	13.3	19.7	17.9	5.9	6.0
His	2.3	2.1	2.3	4.4	4.5	6.0	14.9	15.8	1.0	1.0
Arg	3.3	4.0	4.4	6.2	8.5	11.5	26.2	25.4	10.8	11.0
Asp	6.3	6.4	8.2	11.9	13.6	21.5	47.0	45.9	21.8	21.0
Thr	5.2	4.4	4.7	9.8	9.3	12.3	31.4	32.1	6.9	7.0
Ser	4.4	5.6	5.0	8.3	11.9	13.1	33.3	32.8	9.9	10.0
Glu	4.8	6.5	8.2	9.1	13.8	21.5	44.4	44.5	5.3	5.0
Pro	5.9	4.7	7.8	11.2	10.0	20.4	41.6	40.1	2.0	2.0
Gly	12.0	11.7	10.3	22.7	24.8	27.0	74.5	72.6	12.2	12.0
Ala	9.7	9.9	10.5	18.4	21.0	27.5	66.9	69.2	12.1	12.0
Val	6.0	5.5	7.8	11.4	11.7	20.4	43.5	43.1	5.8	6.0
Met	1.6	3.1	2.0	3.0	6.6	5.2	14.8	17.4	2.0	2.0
Ile	6.2	5.1	5.4	11.7	10.8	14.1	36.6	36.4	5.8	6.0
Leu	11.2	11.9	9.9	21.2	25.3	25.9	72.4	71.9	7.7	8.0
Tyr	4.3	3.3	2.5	8.1	7.0	6.5	21.6	20.6	2.9	3.0
Phe	7.5	7.7	4.2	14.2	16.4	11.0	41.6	44.5	3.0	3.0
$^1/_2$Cys[e]	1.3	0.0	0.9	2.4	0.0	2.3	(4.7)	4.7		
Trp[e]	5.7	7.3	0.8	10.9	15.4	2.2	(28.5)	28.5		

[a] Eluted from sodium dodecyl sulfate–polyacrylamide gels and hydrolyzed in duplicate for 20 hr. Values were corrected for background from blank gels, and for partial destruction of serine and threonine, and for incomplete hydrolysis of valine, isoleucine, and phenylalanine as in Table I. The estimated average errors are ±10%. [b] Obtained by sucrose gradient centrifugation; hydrolyzed for 24 hr and corrected for partial destruction and incomplete hydrolysis as in footnote *a*. The estimated average error is ±5%. [c] Values based on the calculated mean residue weight (MRW) and the assumed molecular weight (MW) of each subunit: L, MRW = 111, MW = 21,000; M, MRW = 113, MW = 24,000; H, MRW = 107, MW = 28,000. [d] A 1:1:1 stoichiometry was assumed. [e] Obtained by radiolabeling method from reaction centers containing [^{35}S]cystine or [^{14}C]tryptophan (see text). [f] See Table I. [g] Composition of chicken egg-white lysozyme (Canfield, 1963).

mately 0.05 mol fraction of each subunit) by the L and M subunits. In the gel experiments, the amino acid content of equivalent lengths of blank gel was subtracted from the L and M subunits. For most residues the "blank" amino acid content was less than 0.1 nmol/cm of gel. For glutamic acid and alanine it was 0.3 nmol/cm, and for serine and glycine 0.5 nmol/cm. The correction amounted to 2–5% for most residues, except serine, where it ranged from 10 to 16%, and lysine, where it was 29% in the case of the M subunit, which is relatively deficient in lysine.

The amino acid compositions of the L, M, and H subunits are shown in Table II. From the amino acid content of the subunits and the intact reaction center, one can, in principle, determine the stoichiometry of the subunits. For the correct stoichiometry (specified by the indices *l*, *m*, and *h*) the average fractional difference, $\bar{\epsilon}_{lmh}$ (see Appendix, eq 4), between the amount of each residue in the intact reaction center and the sum of the amounts of that residue in the three subunits should be less than that for other stoichiometries. The procedure for calculating these differences and their errors is outlined in the Appendix; the results are summarized in Table III. It can be seen that the most probable stoichiometry (smallest value of $\bar{\epsilon}_{lmh}$) is 1:1:1. This is consistent with the results of the accompanying paper, although, in view of the relatively large errors, the confidence limit of the assignment in this work is considerably lower (see Appendix).

To assess the accuracy of the gel procedure, the amino acid content of a standard protein, lysozyme, was also determined after elution from polyacrylamide–sodium dodecyl sulfate gels. The values obtained are compared in Table II with the known composition of lysozyme and it can be seen that for most residues the agreement is very good. As an additional check on the procedure, the composition of the H subunit

TABLE III: Average Difference $\bar{\epsilon}_{lmh}$ between Residues in Reaction Center and Sum of Residues in Subunits for Different Stoichiometries of the Subunits.

Subunit Stoichiometry[a]			
l	*m*	*h*	$100\bar{\epsilon}_{lmh}$[b]
1	1	1	3.8 ± 1.1
1	2	1	4.3 ± 1.0
1	1	2	6.6 ± 1.5
2	1	1	4.5 ± 1.3
2	1	2	5.7 ± 1.5
2	2	1	4.8 ± 1.2
1	2	2	4.7 ± 1.1

[a] *l*, *m*, and *h* are the multiplicities of the subunits L, M, and H in the reaction center protein. [b] $\bar{\epsilon}_{lmh}$ is obtained from eq 4 in the Appendix. The standard deviations are the averages of the external and internal errors. (See eq 5 and 6 of the Appendix.)

was also determined after elution from polyacrylamide gels and was found to agree well with the analysis obtained after isolation of the subunit by sucrose density centrifugation.

The *relative* half-cystine and tryptophan contents of the L, M, and H subunits were obtained after electrophoretic separation of the radioactively labeled units by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Reaction centers labeled with either [^{35}S]cystine or [^{14}C]tryptophan were used for this purpose. The unstained gels were scanned at 280 nm, the bands corresponding to the three subunits were sliced out of the gels, and their radioactivity was determined. Results obtained with the ^{35}S-labeled reaction centers are shown in Figure 1. The absence of radioactivity in the M band indicates that this subunit is devoid of cystine. The recovery of the subunits from run to run was somewhat variable. Therefore, the specific absorbance of each component as determined in Table II of the preceding paper (Okamura *et al.*, 1974) was used, in conjunction with the measured integrated absorbance, to calculate the amount of each subunit in the gel. Thus, the relative amounts of half-cystine and tryptophan in the subunits could be obtained, as outlined in Table IV, despite the poor and variable recovery (~60% for [^{35}S]cystine and ~65% for [^{14}C]tryptophan). The absolute number of half-cystine and tryptophan residues in each subunit was calculated from their ratios in the subunits (Table IV) and the total amounts of half-cystine and tryptophan in the reaction centers (Table I).

Since the M subunit contained no half-cystine, the relative amounts of this residue in the L and H subunits could also be determined after separating the LM and H units by sucrose gradient centrifugation. The ratio obtained by this procedure was (Cys in H)/(Cys in L) = 0.95 ± 0.05, in good agreement with the ratio of 0.93 ± 0.12 determined by gel electrophoresis. The value obtained by the centrifugation method is more reliable, both because of its smaller statistical error and the greater recovery (98%) of the radioactivity.

Comparison of Reaction Centers from R. rubrum G-9 and R. spheroides R-26. The amino acid composition of reaction centers from the related bacterium *R. rubrum* G-9 was compared with that of reaction centers from *R. spheroides* R-26. As can be seen in Table V, the compositions were similar, but there were a number of significant differences, notably in the basic residues (lysine, histidine, arginine). In addition to the tabulated amino acids, the reaction center preparations from *R. rubrum* were found to contain variable amounts (0.5–1 mol %) of ornithine. This amino acid has been described in lipid constituents of a number of bacteria including *R. spheroides* (Gorchein, 1964); accordingly, the ornithine found in the reaction center preparation of *R. rubrum* is probably a contaminant.

A qualitative test of the structural relation of the reaction centers of the two bacteria was performed by immunological analysis. Antisera to both proteins were prepared in rabbits and allowed to react in immunodiffusion with the antigens. Each antiserum precipitated with the homologous antigen, but no cross-reaction could be detected (Figure 2).

Discussion

The reaction center protein, isolated from chromatophores of the photosynthetic bacterium, *R. spheroides* R-26, has been shown in the preceding paper to be composed of three subunits, present in equimolar proportion (Okamura *et al.*, 1974). The molecular weights of these subunits were estimated by polyacrylamide gel electrophoresis to be 21,000, 24,000, and

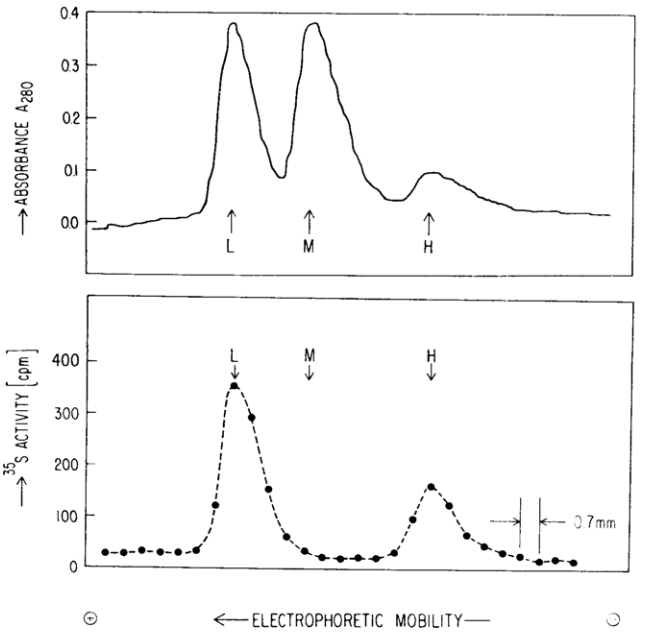

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoretograms of reaction centers containing [^{35}S]cystine. The top trace shows the absorbance at 280 nm and the bottom trace the ^{35}S radioactivity. The absence of activity in the M peak indicates that this subunit is devoid of half-cystine.

28,000. It was found that a complex consisting of the two smaller subunits was sufficient for activity at room temperature but that all three subunits were required for complete photochemical activity at cryogenic temperatures. In this paper, the amino acid compositions of the intact reaction center protein and of its three subunits have been determined. This work is an extension and refinement of the amino acid analyses presented in earlier reports (Feher, 1971; Feher *et al.*, 1971; Okamura *et al.*, 1973).

The most striking feature of the composition of the reaction center protein is its high content of apolar residues. This is consistent with its origin in the bacterial membrane and with its solubility properties. Indeed, the protein falls into the category of "integral membrane proteins" as defined by Singer and Nicolson (1972), *i.e.*, proteins that can be dissociated from the membrane only by detergents, organic solvents, or other "vigorous" reagents. Presumably, such proteins are embedded in the hydrophobic interior of the mem-

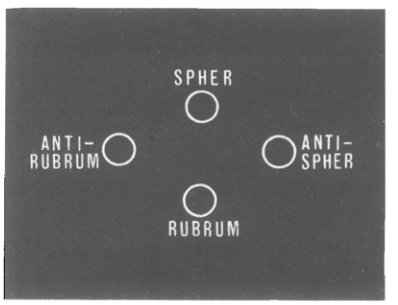

FIGURE 2: Immunodiffusion of reaction centers from *R. rubrum* G-9 and *R. spheroides* R-26 and their antisera. The antibodies in the antiserum to *R. rubrum* reaction centers were concentrated twofold by precipitating the immunoglobulins with ammonium sulfate and dissolving the precipitate in a volume of buffer corresponding to half the original serum volume. The antiserum to *R. spheroides* reaction centers was used without concentration. The absorbance at 802 nm of the *R. rubrum* reaction centers was 2.6 and that of the *R. spheroides* reaction centers was 2.0.

TABLE IV: Cystine and Tryptophan Content of Subunits of Reaction Centers from *R. spheroides* R-26.[a]

Subunit	Sp Absorb.[b]	[^{35}S]Cystine Gels					[^{14}C]Tryptophan Gels			
		Integ Absorb.	Integ Radioact	Recovery of Protein[c]	$^1/_2$Cys Content in Subunit[d]		Integ Absorb.	Integ Radioact	Recovery of Protein[c]	Trp Content in Subunit[d]
L	1.00	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00
M	1.08 ± 0.06	1.20 ± 0.10	0	0.97 ± 0.10	0		1.15 ± 0.10	1.32 ± 0.10	0.93 ± 0.10	1.42 ± 0.18
H	0.37 ± 0.03	0.29 ± 0.02	0.55 ± 0.02	0.59 ± 0.06	0.93 ± 0.10		0.27 ± 0.04	0.11 ± 0.02	0.55 ± 0.09	0.20 ± 0.05

[a] Values for the M and H subunits are expressed relative to those for the L subunit. [b] Integrated absorbance per gram of protein as determined in the preceding paper (Okamura *et al.*, 1974, Table II). [c] Obtained by dividing integrated absorbance by specific absorbance and multiplying by the ratio of the molecular weight of L to that of the subunit. [d] Obtained by dividing integrated radioactivity by recovery of protein.

TABLE V: Comparison of Amino Acid Composition of Reaction Centers from *R. rubrum* G-9 and *R. spheroides* R-26.[a]

Amino Acid	*R. rubrum* mol %	*R. spheroides* mol %	mol % *R. rubrum* / mol % *R. spheroides*
Lys	3.4	2.8	1.21
His	1.9	2.4	0.79
Arg	5.3	4.0	1.33
Asp	6.9	7.1	0.97
Thr	5.8	4.7	1.23
Ser	4.9	4.6	1.06
Glu	7.2	6.9	1.04
Pro	5.5	6.3	0.87
Gly	10.0	11.2	0.89
Ala	11.2	10.5	1.07
Val	6.4	6.3	1.02
Met	2.1	2.6	0.81
Ile	5.6	5.2	1.08
Leu	9.9	10.9	0.91
Tyr	3.1	3.1	1.00
Phe	5.8	6.4	0.91

[a] The values for *R. rubrum* are the averages of five 24-hr hydrolyses not corrected for partial destruction or incomplete hydrolysis of some residues. Tryptophan and half-cystine were not determined. The statistical errors are similar to those quoted in Table I. The values for *R. spheroides* are calculated directly from those in the first column of Table I. The values here are normalized to 95 mol %. (Based on results in Table I, it was assumed that half-cystine and tryptophan contribute 5 mol %.)

brane. The amino acid composition of a number of membrane and other proteins has been reviewed by Capaldi and Vanderkooi (1972). The "polarity" (defined as the fractional sum of Asp, $AspNH_2$, Glu, $GluNH_2$, Lys, His, Arg, Ser, and Thr) of most integral membrane proteins is between 29 and 40%, whereas most soluble proteins fall between 41 and 53%. On this scale, the polarity of the intact reaction center protein is 32%.

We have also determined the amino acid composition of the subunits of the reaction center, although with less precision than that of the intact protein. It is clear that the polar residues are not evenly distributed among the subunits, but occur preferentially in the largest, or H, unit. Thus, the sum of the polar amino acids is 38% in the H subunit, but only 29 and 30% in the L and M units; the difference is mainly the result of a decrease in the number of aromatic residues and an increase in the number of basic residues in the H unit. It is tempting to speculate that the greater polarity of the H unit, and its lesser importance for photochemical activity, may indicate that it is associated with the surface of the membrane, rather than with the lipid matrix.

A noteworthy feature of the amino acid composition is the distribution of lysine between the L and M subunits. The L, or smaller unit, has more than twice the amount of lysine found in the M subunit. Therefore, the smaller unit cannot be derived from the larger by simple proteolytic cleavage.

In the accompanying paper (Okamura *et al.*, 1974, Table II), it was found that the relative amount of Coomassie Blue bound to the three subunits (as determined by optical scanning) was not uniform. The differences in dye uptake can be correlated with the content of basic residues (Table II, this paper). Thus,

relatively more stain was bound by the H subunit than by the other two subunits.

The presence of half-cystine in the reaction centers is of interest because sulfhydryl groups may serve as binding sites for iron, a moiety that may play a role in the photochemical act as the primary electron acceptor. For example, chemical and X-ray crystallographic studies have shown that rubredoxin, the iron-binding protein of *Clostridium pasteurianum*, contains four residues of cysteine complexed with one atom of iron (Lovenberg and Williams, 1969; Watenpaugh *et al.*, 1971). In our earlier preliminary amino acid analysis we reported the presence of at most one residue of half-cystine (Feher, 1971), but this value was obtained without performic acid oxidation or radiolabeling and was too low. We now find 4.7 ± 0.3 residues of half-cystine per reaction center unit, based on a mol wt of 73,000. The half-cystine is evenly divided between the L and H subunits and is absent from the M subunit. Thus, the most likely value is two or three residues of half-cystine in each of the two subunits, or a total of four or six per reaction center particle. It is possible that the observed loss of iron from the reaction centers, when the H unit is dissociated (Okamura *et al.*, 1973), is due to the removal of binding sites provided by sulfhydryl groups of cystine residues. It must still be determined whether the half-cystine residues in the reaction centers are present in the reduced or in the oxidized form.

The determination of the amino acid composition of the reaction center protein and its subunits might allow an independent estimate of the molecular weights of these moieties and of the stoichiometry of the subunits. The composition of the intact reaction center protein has been obtained with a relatively high degree of precision but, with the exception of half-cystine, there are too many residues of each kind to permit estimation of minimum molecular weight. If the number of half-cystine residues is four per reaction center particle, the mol wt of the reaction center protein would be about 62,000 instead of 73,000; if the number of half-cystines is six per reaction center, the mol wt would be 93,000. (A value of five half-cystines is inconsistent with the finding that two of the subunits contain the same amount of this residue, whereas the third subunit is devoid of cystine.)

It is pertinent to compare these estimates of the molecular weight of the reaction center particle with those obtained from the molar extinction coefficient determined by independent methods. In recent reports, Reed and Peters (1972) and Straley *et al.* (1973) have found the extinction coefficient ϵ_{802}^{M} of the reaction centers to be 300 and 288 ± 14 mM^{-1} cm^{-1}, respectively. In the present work, we have found that 1.00 $OD_{V_{802}}$ of reaction centers contains 0.319 mg of amino acid residues. If ϵ_{802}^{M} is 2.88 × 10^5 M^{-1} cm^{-1}, then the mol wt of the reaction center protein would be about 90,000. This is greater than our estimate of 73,000 but corresponds approximately to the minimum value for the molecular weight if the total number of half-cystines in the reaction center is 6. The combined errors inherent in the determination of molecular weight (by gel electrophoresis), in the amino acid analysis, and in the optical absorbance could account for the observed discrepancy.

The subunits are of a more reasonable size for estimates of minimum molecular weight. However, it has been difficult to prepare pure L and M subunits in sufficiently large quantities. Consequently, the composition of the subunits has not been determined as precisely as that of the intact reaction centers; in particular, the infrequent residues on which minimum molecular weight calculations are based were the most uncertain. We are presently attempting to develop methods for obtaining the individual subunits on a larger scale.

In contrast to the calculations of the minimum molecular weights, the calculation of the stoichiometry from the composition of the subunits utilizes most of the residues. Accordingly, errors in individual residues make a relatively small contribution to the results. Thus, within the confidence limits discussed in the Appendix, the stoichiometry of 1 :1 :1 for the three subunits, which has been reported in the accompanying paper, also represents the best fit to the amino acid composition data.

The reaction centers of the photosynthetic bacterium, *R. rubrum* G-9, also consist of three subunits (Noël *et al.*, 1972; Clayton and Haselkorn, 1972; Okamura *et al.*, 1974). A comparison of these reaction centers with those from *R. spheroides* reveals a general resemblance in amino acid composition, although there are significant differences in a number of residues. In addition, the two proteins are antigenically distinct, since antisera to reaction centers from either organism did not cross-react with the other. The latter result is consistent with the finding that antisera to reaction centers from *R. spheroides* do not react with reaction centers from a variety of other photosynthetic bacteria (Clayton and Haselkorn, 1972). It will be of interest to determine whether these differences in amino acid composition and antigenicity are reflected in all three reaction center subunits, or whether they are confined to one or another component.

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Added in Proof

The nonphotosynthesizing mutant, *R. spheroides* 8-17, has been reported to contain a subunit with an electrophoretic mobility in polyacrylamide gels similar to that of the H subunit of *R. spheroides* R-26, but it does not contain moieties corresponding to the L and M subunits (Takemoto and Lascelles, 1973). We have confirmed these observations and have furthermore found that membrane fragments obtained from the 8-17 mutant precipitate with antisera to reaction centers of *R. spheroides* R-26. These findings are consistent with the hypothesis that at least a portion of the H subunit is exposed on the membrane surface. We are investigating the possibility of utilizing the 8-17 mutant to prepare specific antisera for the purpose of separating the LM and H subunits by immunochemical means. We thank J. Lascelles for supplying an inoculum of *R. spheroides* 8-17.

Appendix: Determination of Subunit Stoichiometry from the Amino Acid Composition

For each amino acid, the sum of the number of residues in the three subunits (L, M, and H) must equal the number of residues in the protein, *i.e.*

$$la_L{}^i + ma_M{}^i + ha_H{}^i = a_P{}^i \quad (1)$$

where, for the ith amino acid, $a_L{}^i$, $a_M{}^i$, $a_H{}^i$, and $a_P{}^i$ are the numbers of residues in the subunits L, M, H, and in the protein, respectively; l, m, and h are the multiplicities of the subunits in the protein, and it is these values that we wish to determine.

We define ϵ^i_{lmh}, the absolute fractional difference between the number of residues in the subunits and in the whole protein.

$$\epsilon^i_{lmh} = |la_L{}^i + ma_M{}^i + ha_H{}^i - a_P{}^i|/a_P{}^i \quad (2)$$

The standard error $\delta\epsilon^i_{lmh}$ in the determination of each ϵ^i_{lmh} can be obtained from the above expression by the usual procedure for combining uncorrelated errors (*e.g.*, Topping, 1962), *i.e.*

$$\delta\epsilon^i_{lmh} = \{l^2(\delta a_L{}^i/a_P{}^i)^2 + m^2(\delta a_M{}^i/a_P{}^i)^2 + h^2(\delta a_H{}^i/\delta a_P{}^i)^2 + [(la_L{}^i + ma_M{}^i + ha_H{}^i)/a_P{}^i]^2(\delta a_P{}^i/a_P{}^i)^2\}^{1/2} \quad (3)$$

where $\delta a_L{}^i$, $\delta a_M{}^i$, $\delta a_H{}^i$, and $\delta a_P{}^i$ are the average standard errors in the amino acid determinations. In this work these have been taken to be: $\delta a_L{}^i/a_L{}^i = \delta a_M{}^i/a_M{}^i = 0.1$, $\delta a_H{}^i/a_H{}^i = 0.05$, and $\delta a_P{}^i$ as given in Table I.

For each stoichiometry l, m, h we average the values of ϵ^i_{lmh} for all residues[2] and obtain the weighted mean $\bar{\epsilon}_{lmh}$ given by

$$\bar{\epsilon}_{lmh} = \sum_{i=1}^{n} \omega^i \epsilon^i_{lmh} \Big/ \sum_i^n \omega^i \quad (4)$$

where ω^i are weighting factors taken to be inversely proportional to the square of the standard errors [$\omega^i = (1/\delta\epsilon^i_{lmh})^2$]. The summation extends over the different amino acids; in the case of reaction centers, $n = 16$. We omit tryptophan and half-cystine from this calculation since the values for these residues in the subunits were based on the assumption of a 1:1:1 stoichiometry. For the correct stoichiometry, and in the absence of experimental errors, each ϵ^i_{lmh} and hence $\bar{\epsilon}_{lmh} = 0$. In the experimental situation, the most probable stoichiometry (*i.e.*, the most probable values of l, m, h) is the one that yields the smallest value of $\bar{\epsilon}_{lmh}$.

We now wish to determine the standard deviation of $\bar{\epsilon}_{lmh}$. There are two methods of computing this value. These lead to the external and internal errors and are given by Topping (1962)

$$\delta\epsilon^{ext}_{lmh} = \left[\frac{\sum_i^n \omega^i(\epsilon^i_{lmh} - \bar{\epsilon}_{lmh})^2}{(n-1)\sum_i^n \omega^i}\right]^{1/2} \quad (5)$$

and

$$\delta\epsilon^{int}_{lmh} = \frac{\left[\sum_i^n (\omega^i \delta\epsilon^i_{lmh})^2\right]^{1/2}}{\sum_i^n \omega^i} = \left[\frac{1}{\sum_i^n \omega^i}\right]^{1/2} \quad (6)$$

For normally distributed (random) errors $\delta\epsilon^{ext}_{lmh}/\delta\epsilon^{int}_{lmh} = 1.0$. We find an average ratio of 1.3 for the stoichiometries investigated, indicating that our estimates of errors have been fairly good.

From the amino acid composition data of Tables I and II, we have calculated the values of $\bar{\epsilon}_{lmh}$, $\delta\epsilon^{ext}_{lmh}$, and $\delta\epsilon^{int}_{lmh}$ for each of the seven stoichiometries. The results of these calculations are summarized in Table III. It can be seen that the most probable stoichiometry (smallest $\bar{\epsilon}_{lmh}$) is 1:1:1. In order to determine the confidence level of this assignment, we must calculate the probability that $\bar{\epsilon}_{lmh}$ of another stoichiometry is smaller than $\bar{\epsilon}_{111}$. This probability is approximately given by

$$P(\bar{\epsilon}_{lmh} < \bar{\epsilon}_{111}) = \frac{1}{\sqrt{2\pi}} \int_t^\infty e^{-\alpha^2/2} d\alpha \quad (7)$$

where

$$t = \frac{\bar{\epsilon}_{lmh} - \bar{\epsilon}_{111}}{[\delta\epsilon^2_{lmh} + \delta\epsilon^2_{111}]^{1/2}}$$

Using the above relation and the values of Table III, we determine that the probability that $\bar{\epsilon}_{121} < \bar{\epsilon}_{111}$ is 0.37, *i.e.*, the confidence level that 1:1:1 is the correct stoichiometry with respect to 1:2:1, is 63%. With respect to other stoichiometries the confidence level is higher.

[2] For a residue a^i that is present in equal mole fractions in the three subunits $\epsilon^i_{lmh} = 0$ for all stoichiometries; it should therefore not be used in evaluating $\bar{\epsilon}_{lmh}$. Consequently, a more sophisticated treatment should take this into account by weighting different residues according to their "information content." This was not done in this simple treatment.

References

Canfield, R. (1963), *J. Biol. Chem. 238*, 2691.

Capaldi, R. A., and Vanderkooi, G. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 930.

Clayton, R. K., and Haselkorn, R. (1972), *J. Mol. Biol. 68*, 97.

Feher, G. (1971), *Photochem. Photobiol. 14*, 373.

Feher, G., Okamura, M. Y., Raymond, J. A., and Steiner, L. A. (1971), *Biophys. Soc. Abstr. 11*, 38a.

Gorchein, A. (1964), *Biochim. Biophys. Acta 84*, 356.

Leigh, J. S., and Dutton, P. L. (1972), *Biochem. Biophys. Res. Commun. 46*, 414.

Lovenberg, W., and Williams, W. M. (1969), *Biochemistry 8*, 141.

Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 175.

Moore, S. (1963), *J. Biol. Chem. 238*, 235.

Noël, H., Van der Rest, M., and Gingras, G. (1972), *Biochim. Biophys. Acta 275*, 219.

Okamura, M. Y., Moskowitz, E., McElroy, J., Feher, G., and Steiner, L. (1973), *Biophys. Soc. Abstr. 13*, 270a.

Okamura, M. Y., Steiner, L. A., and Feher, G. (1974), *13*, 1394.

Reed, D. W., and Peters, G. A. (1972), *J. Biol. Chem. 247*, 7148.

Singer, S. J., and Nicolson, G. L. (1972), *Science 175*, 720.

Spackman, D. H. (1967), *Methods Enzymol. 11*, 3.

Straley, S. C., Parson, W. W., Mauzerall, D. C., and Clayton, R. K. (1973), *Biochim. Biophys. Acta 305*, 597.

Takemoto, J., and Lascelles, J. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 799.

Topping, J. (1962), Errors of Observation and Their Treatment, 3rd ed, London, Chapman and Hall.

Watenpaugh, K. D., Sieker, L. C., Herriott, J. R., and Jensen, L. H. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 359.