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The Chemical Composition of a Crystalline Bacteriochlorophyll–Protein Complex Isolated from the Green Bacterium, *Chloropseudomonas ethylicum**

J. P. Thornber and J. M. Olson

ABSTRACT: The amino acid composition and N-terminal residue of the bacteriochlorophyll–protein complex have been determined. The results reveal that the complex is very probably made up of four identical subunits, each of which contain 5 moles of bacteriochlorophyll. The molecular weight of such a subunit is 37,940; using this value and some previously published results the following physical properties of the complex have been calculated. The bacteriochlorophyll–protein concentration in the crystalline state is 0.43 g/cm^3; $\bar{V}$ = 0.764 ± 0.014 cm^3/g, molecular weight = (1.52 ± 0.04) × 10^5, molecular volume = (1.93 ± 0.08) × 10^5 Å^3; the specific volume of bacteriochlorophyll in the complex is about the same as that of crystalline chlorophyll a. The complex has been examined for the presence of components other than protein and chlorophyll; there was no other pigment or lipid but a trace of carbohydrate material was observed. Attempts to isolate a bacteriochlorophyll-peptide(s) by tryptic digestion of the denatured complex were not successful; the protein was removed from the pigment during proteolysis. The composition and properties of the complex show some striking similarities to those of a chlorophyll a containing protein of spinach beet.

The water-soluble bacteriochlorophyll–protein (Bchl-P)[1] complex of the green photosynthetic bacterium *Chloropseudomonas ethylicum* was isolated by Olson and Romano (1962). Olson (1966) reported that the complex had a molecular weight of (1.67 ± 0.17) × 10^5 g/mole and contained ~20 Bchl molecules. The biological function of the complex in the transfer of excitation energy has been demonstrated (Olson and Sybesma, 1963; Sybesma and Olson, 1963; Sybesma and Vredenberg, 1963) and there is some indication that *in vivo* the complex is associated with those molecules that take part in the primary photochemical reaction (Olson, 1964). An ellipsoidal model of the Bchl-P macromolecule *in vitro* is proposed (Olson *et al.*, 1968a) with a radial array of chromophore sites inside the ellipsoid (Olson *et al.*, 1968b). A detailed chemical analysis of the complex is reported in the present paper as a first step in elucidating the nature of chlorophyll–protein interactions and in understanding the nature of primary photosynthetic processes.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Bchl-P, bacteriochlorophyll–protein complex; Chl a, chlorophyll a.

Methods

Purification of the Bchl-P. The procedure as originally published (Olson *et al.*, 1963; Olson, 1966) has undergone slight modification. The protein is now prepared as follows. Step 1: *Cps. ethylicum* cells (480 g wet weight) are suspended in five volumes of water, solid Na_2CO_3 is added to give a 0.2 M solution (pH ~10), and the cells are broken by sonication. Step 2: A green fraction (Bchl plus chlorobium chlorophyll) is precipitated by the addition of 30 g of $(NH_4)_2SO_4$/100 ml of extract. Step 3 (Adapted from the method used by Chapman *et al.* (1968) for the purification of C-phycocyanin). The ammonium sulfate precipitate is mixed with 2.5 kg of diatomaceous earth (Celite 545) and the slurry is poured into a column (60 × 14 cm). A decreasing linear gradient of ammonium sulfate (35–0%) in 0.01 M sodium phosphate (pH 7.8) or Tris-HCl (pH 8.0) (15 l.) is passed through the column, and that portion of the eluted solution containing Bchl (6–8 l.) is collected and concentrated to 150 ml by ultrafiltration. Step 4: The solution is dialyzed against buffer to remove the remaining ammonium sulfate and then loaded onto a DEAE-cellulose column (60 × 14 cm), which is eluted with a gradient of NaCl (0–0.3 M) in 0.01 M buffer. Step 5: The eluted protein is concentrated by ultrafiltration and crystallized as described previously (Olson, 1966).

General Methods. The concentration of the complex in solution was determined from its absorbance at 371 nm (a = 9.0 (mg/ml)$^{-1}$ cm^{-1}). All spectrophotometric measurements were made with a Cary Model 14R spectrophotometer. Polyacrylamide gel electrophoresis was performed as described by Ridley *et al.* (1967). Nitrogen was determined by the micro-Kjeldahl method of Markham (1942).

Amino Acid Analysis. Constant-boiling HCl was added to protein samples (~3 mg) contained in thick-walled Pyrex tubes. The contents were cooled in a mixture of ethanol and solid CO_2 and the tubes were evacuated until the contents ceased to bubble. The tubes were then sealed under vacuum (<100 μ) and the complex hydrolyzed at 110° for 24–96 hr. An automatic amino acid analyzer (Beckman Spinco, Model 120) was used to determine the composition of the hydrolysates. Performic acid oxidized samples were prepared by the method of Moore (1963), and half-cystine and methionine estimated as cysteic acid and methionine sulfone, respectively; it was assumed that 94% of the half-cystine was recovered as cysteic acid (Moore, 1963).

Tryptophan Content. This was obtained from a spectrophotometric determination of the molar ratio of tyrosine:tryptophan in the protein. Samples of the protein crystals which had been used for the determination of the amino acid composition were dissolved in 10% urea solution and then dialyzed. Bchl was extracted from the solubilized protein with chloroform–methanol (1:1, v/v) and the precipitated protein was centrifuged and washed twice with the organic solvents to remove the last trace of pigment. The residue was dissolved in 0.1 N NaOH and the molar ratio of tyrosine:tryptophan was determined by the method of Beaven and Holiday (1952).

Amide Nitrogen. Crystals of the protein (~15 mg) were partially dissolved by dialysis against 0.01 M sodium phosphate (pH 7.8). HCl was added to the suspension to make the acid concentration 2 N. The suspension was heated at 100°, and aliquots were removed at periods between 0 and 4 hr. The ammonia released from the complex was estimated by the method of Marshall and Gottschalk (1966). Total nitrogen in the samples was also determined.

Percentage Nitrogen in Bchl-P. A solution of the protein was exhaustively dialyzed over a period of 4 days against 0.001 M acetic acid, and then lyophilized. The total nitrogen was determined in weighed samples of the protein which had been dried *in vacuo* at 100° for 3 days.

Concentration of Bchl-P in the Crystalline State. Two crystals (~0.7 × 0.2 × 0.2 mm) were cross-linked with 1% glutaraldehyde (Olson *et al.*, 1968a) to enable manipulation of the crystals for the determination of their linear dimensions by means of a calibrated reticle in an optical microscope. The crystals were then hydrolyzed for 24 hr with constant-boiling HCl, and the hydrolysate was analyzed as described under Amino Acid Analysis. The protein content of the crystals was calculated from the recovered micromoles of Asp, Thr, Ser, Glu, Ala, Val, Ile, Leu, and Phe, the composition of the 24-hr hydrolysate of noncross-linked crystals, and the complete amino acid analysis (Table I). The effect of glutaraldehyde on the amino acid analysis of the protein was checked by examination of the hydrolysate of a solution of Bchl-P in 1% glutaraldehyde; all the amino acids on which the protein content were based were obtained in approximately 100% recovery.

N-Terminal Amino Acid of the Protein. A solution of Bchl-P containing a known amount of protein (~10 mg) was adjusted to pH 8.5 with 5% sodium bicarbonate solution and fluorodinitrobenzene (50 μl) was added. The mixture was vigorously stirred for 2 hr at 37° in the dark. The solution was then made acidic and the precipitated DNP–protein complex centrifuged. The protein was washed three times with chloroform–methanol (1:1, v/v), and finally with ether. Different preparations of the yellow precipitate were hydrolyzed with constant-boiling HCl at 110° for 5, 16, or 48 hr, or with concentrated HCl at 110° for 5 hr. The DNP-amino acids were removed from the hydrolysates by extracting three times with ether; the ethereal extract was evaporated to dryness under a stream of nitrogen. The residual aqueous phase from the ether extraction was evaporated to dryness in a desiccator containing NaOH pellets. The DNP-amino acids in both phases were examined by thin-layer chromatography on Absorbosil 5 (Applied Science Laboratories, Inc., Pa.) using the solvent systems described by Brenner *et al.* (1961); acid-soluble DNP-amino acids were examined using a solvent, butanol–acetic acid–water (4:1:1 v/v). The quantity of any DNP-amino acid was determined from the absorbance at 360 nm of the eluted compound in 1% sodium bicarbonate solution (ϵ 15,500 M^{-1} cm^{-1}).

Lipid Content. Bchl-P (~50 mg) was precipitated

from solution with ammonium sulfate and centrifuged. The precipitate was extracted twice with each of the following solvents: chloroform–methanol (1:1, v/v) and ether and benzene. The combined extracts were concentrated by rotary evaporation under nitrogen and then chromatographed on thin-layer plates of Absorbosil 5 or cellulose which were developed with: (a) hexane–benzene (95:5, v/v), (b) chloroform–methanol–water (60:30:5, v/v), (c) ethyl acetate–carbon tetrachloride (60:40, v/v), (d) petroleum ether (bp 40–60°)–acetone–propanol (90:10:0.45, v/v), or (e) diisobutyl ketone–acetic acid–water (40:25:5, v/v). Lipids were detected on the thin-layer plates with chromic acid, rhodamine B, or phosphomolybdic acid solutions after exposure of the plate to iodine vapor. Carotenoids were isolated from solutions of the protein by the procedure of Davies (1965) and chromatographed with solvent c on Absorbosil 5.

Phosphorus Content. Preparations of Bchl-P were made from the bacterium using either sodium phosphate or Tris-HCl buffer; samples of both were examined for their bound phosphorus content. Solutions of known concentrations were exhaustively dialyzed against 0.002 M Tris-HCl (pH 7.8) before being precipitated either with ammonium sulfate or by boiling. The precipitated proteins (~10 mg) were centrifuged and their content of phosphorus was estimated by the method of Lowry and Lopez (1946).

Carbohydrate Content. Protein crystals were examined in two ways. (a) Approximately 10 mg of material was hydrolyzed with 2 N H_2SO_4 for 4 hr at 100° and the hydrolysate, which was neutralized with saturated barium hydroxide solution, was chromatographed on Whatman No. 1 paper with ethyl acetate–pyridine–water (8:2:1, v/v); monosaccharides were detected with alkaline silver nitrate solution (Trevelyan *et al.*, 1950); and (b) 2 mg of material was hydrolyzed with 0.2 N HCl and Dowex 50 resin in a sealed tube for 4 hr at 100°; one-tenth of the hydrolysate was analyzed by chromatography of the trimethylsilyl derivatives (Sweeley *et al.*, 1963) on a gas chromatograph (Barber-Coleman series 5000).

Digestion of Bchl-P with Trypsin. A solution of Bchl-P (~10 mg) was denatured by the addition of an equal volume of 50% acetic acid which had been adjusted to pH 3.2 with solid sodium acetate. The precipitated material was centrifuged and thoroughly washed with 0.2 M ammonium acetate (pH 8.5). Trypsin (Worthington Biochemical Corp.) was added to the precipitate suspended in 0.2 M ammonium acetate (pH 8.5) (trypsin–protein, 1:40, w/w), and the mixture was incubated at 37° for 4 hr. After this time the same quantity of trypsin was again added and the digestion was allowed to proceed for a further 15 hr. The course of the digestion was followed on an automatic titrator.

Results

Amino Acid Composition of the Complex. Table I shows the compositions of one of three series of hydrolysates which have been prepared from the crystalline protein; there were no significant differences between the series. The molar ratio of tyrosine:tryptophan was determined as 1.54 ± 0.06 on four different samples of the same batch of crystals. This value was used to calculate the tryptophan content given in Table I. As a check of the value obtained for the molar ratio, a mixture of tyrosine and tryptophan was made in the proportion in which they had been estimated to occur in the protein; the spectrum of this mixture in 0.1 N NaOH solution resembled very closely that of the protein in alkali.

The presence of Bchl during the hydrolysis of the protein made no difference to the amino acid composition; this was demonstrated by analysis of hydrolysates of the protein from which Bchl had been removed with chloroform–methanol (1:1, v/v). The amino acid composition of the material soluble in chloroform–methanol was also examined. The extract was evaporated and then hydrolyzed for 24 hr with constant-boiling HCl at 110°. The hydrolysate contained some of each amino acid found in Bchl-P but in a very different proportion; there were relatively larger quantities of Lys, Thr, Ser, Glu, Gly, and Ala, and smaller of His, Arg, and Ile than in the complex. If the extract was not hydrolyzed before examination on the amino acid analyzer, it was found that it still contained some amino acids and that other ninhydrin-positive substances were present which could not be free amino acids; these were presumed to be low molecular weight peptides. It was calculated that 0.4% of the protein moiety was soluble in chloroform–methanol; this value was derived from a knowledge of the weight of the complex before extraction and the amino acid analysis of the extract.

The minimum molecular weight of the protein was determined from the composition given in Table I. The approximate number of micromoles in the column of best values which is equivalent to one residue in the protein was determined by subtraction of the recovered micromoles of one amino acid from that of the next most concentrated. By using the frequently occurring lowest value obtained (~0.06 μmole/residue) the whole number of the least concentrated amino acid residues in the protein (half-cystine, methionine, histidine, and tyrosine) was predicted; thereby a more probable value of 0.0573 μmole/residue (2 SD = 0.0013) was obtained, and this ratio was applied to the best values to determine the number of residues of each amino acid in the minimum unit (Table I). The most likely molecular weight of such a unit is 33,430 (minimum, 33,320; maximum, 33,900).

The amide nitrogen content of the protein was 7.2 ± 0.2 μg per 114.1 ± 3.3 μg of total nitrogen. From the assumption that only Bchl, amide groups, and amino acids contribute to the total nitrogen of the complex, and from a knowledge of the Bchl:N ratio (Olson, 1966), it can be calculated that 7.2 ± 0.2 μg of amide nitrogen is derived from 101.7 ± 3.8 μg of protein nitrogen which is equivalent to 27.7 ± 1.9 moles of amide per protein subunit of mol wt 33,400. Such a unit would contain 5.06 ± 0.13 moles of Bchl. Thus the minimum unit of the complex has a most probable molecular weight of 37,940 (minimum, 37,830; maximum, 38,410) and contains 16.3% nitrogen. The nitro-

TABLE I: Amino Acid Composition of Bacteriochlorophyll–Protein Complex.

Amino Acid	Amt of Amino Acid Recov (μmoles)[a]						Best[c] Value	Residues/Subunit	
	Hydrolysis Time (hr)								
	24	48	72	96	Ox[b]	Ox[b]		Best Value/ 0.0573[d]	Nearest Integer
Lysine	0.910	0.906	0.905	0.911	0.896	0.893	0.908	15.9 ± 0.4	16
Histidine	0.394	0.395	0.392	0.403	0.387	0.380	0.396	6.9 ± 0.2	7
Arginine	1.017	1.016	1.020	1.041	1.007	1.005	1.024	17.9 ± 0.5	18
Aspartic acid	1.914	1.906	1.921	1.908	1.955	1.957	1.912	33.4 ± 0.9	33–34
Threonine	0.682	0.657	0.622	0.590	0.656	0.659	0.717	12.5 ± 0.3	13
Serine	1.191	1.096	0.945	0.843	1.072	1.046	1.319	23.0 ± 0.6	23
Glutamic acid	1.612	1.625	1.619	1.611	1.626	1.644	1.617	28.2 ± 0.8	28–29
Proline	0.864	0.859	0.857	0.861	0.848	0.836	0.860	15.0 ± 0.4	15
Glycine	1.975	1.984	1.981	1.951	1.981	1.989	1.973	34.4 ± 0.9	34–35
Alanine	1.047	1.056	1.048	1.060	1.050	1.055	1.053	18.4 ± 0.5	18–19
Valine	1.590	1.624	1.685	1.664	1.677	1.654	1.675	29.2 ± 0.8	29–30
Methionine	0.181	0.167	0.180	0.176	0.168	0.163	0.173	3.0 ± 0.1	3
Isoleucine	1.099	1.135	1.150	1.157	1.138	1.146	1.154	20.1 ± 0.5	20
Leucine	1.000	1.003	1.000	1.006	0.995	1.001	1.002	17.5 ± 0.5	17–18
Tyrosine	0.483	0.464	0.460	0.456			0.466	8.1 ± 0.2	8
Phenylalanine	0.863	0.860	0.853	0.849	0.864	0.855	0.856	14.9 ± 0.4	15
Cysteic acid					0.109	0.111	0.110	1.9 ± 0.1	2
Tryptophan							0.304	5.3	5
Amide[e]								27.7 ± 1.9	26–29

[a] In order to compare one analysis with another, each set of values was multiplied by a factor so that the sum of the recoveries of all amino acids except threonine, serine, valine, isoleucine, tyrosine, and cysteic acid were constant. [b] Ox: duplicate samples oxidized with performic acid and hydrolyzed for 48 hr. The values obtained were not used for the determination of the best values except those of cysteic acid and methionine, which was determined as methionine sulfone. [c] Averages of the values after 24-, 48-, 72-, and 96-hr hydrolysis were used for each amino acid except threonine, serine, valine and isoleucine. The values for threonine and serine were obtained by extrapolation of their recoveries to zero time, and those for valine and isoleucine were the mean of the 72- and 96-hr recoveries. [d] The number of micromoles equivalent to one residue of amino acid in a minimum molecular weight subunit were determined by dividing the sum of the best values for cysteic acid, methionine, histidine, and tyrosine by 20; a value of 0.0573 ± 0.0013 was obtained. [e] Amide: determined on separate samples (see text).

gen in a salt-free well-dried sample of the complex was determined as 15.6 ± 0.6%.

Concentration of Bchl-P in Crystals. Two cross-linked crystals with a combined volume of 0.0338 mm^3 (±3%) were found to contain between 14 and 15 μg of Bchl-P. This concentration of Bchl-P (0.43 g/cm^3) is equivalent to [Bchl] = 56 mM, which is in good agreement with the value of 60 mM found by Olson *et al.* (1968b).

N-Terminal Amino Acid of Bchl-P. Preparations of the DNP protein were made from several batches of Bchl-P solutions and were hydrolyzed under various conditions (see Methods). DNP-alanine was found to be the preponderant DNP-amino acid present in the hydrolysates. A quantity of 0.86 ± 0.04 mole per 37,940 g complex was determined for seven different preparations; traces of other DNP-amino acids were also present in the ether extracts, but no individual N-terminal amino acid occurred in any concentration greater than 3% of that of DNP-alanine. However one preparation from a 1-year-old batch of protein contained mostly DNP-glycine and in this case other DNP-amino acids totalled 25% of the amount of DNP-glycine. Trace DNP-amino acids were always identified as the DNP derivatives of leucine, valine, glycine, and serine; the full spectrum of DNP-amino acids was not present. Preparations made from protein immediately after its elution from the DEAE-cellulose column still contained a trace of DNP-glycine, but in a concentration less than 1% of that of DNP-alanine. Correction factors were applied to the recovered DNP-amino acids after their estimation to account for losses during hydrolysis; the factors were determined by taking known quantities of DNP-amino acids through the whole procedure: 61 ± 4% (four observations) for DNP-alanine (17-hr hydrolysis) and 43 ± 5% (four observations) for DNP-glycine (5-hr hydrolysis). Values for all DNP-amino acids estimated, except those of DNP-glycine, were corrected using the DNP-alanine recovery figure. Hydrolysis with concentrated ammonium hydroxide solution at 110° for 2 hr of the major DNP-amino acid isolated from DNP-Bchl-P and examination of the hydrolysate on an automatic amino acid analyzer con-

firmed the identity of the N-terminal amino acid as alanine.

Content of Lipid, Phosphorus, and Carbohydrate in Bchl-P. A. LIPID. There was no evidence of any carotenoid in the complex. Ether extracts of Bchl-P which had been saponified in 75% ethanol were examined in a spectrophotometer and no absorption in the region corresponding to that of carotenoids was observed; examinations of the extract on thin-layer chromatograms revealed the presence of phytol only.

No lipid material (polar or nonpolar) other than Bchl a, its oxidation or pheophytinized products, was observed in solvent extracts of Bchl-P when they were examined in any chromatographic system (see Methods). The investigations were carried out using such a concentration of material that 1 mole of lipid/mole of complex would have been detected. Attempts to confirm the absence of phospholipids were made by estimating the phosphorus bound to the protein. Preparations of Bchl-P made using Tris buffer were found to contain 1.3 ± 0.1 moles of phosphorus/mole of complex; however when phosphate buffer was used, it was estimated that 30–70 moles of phosphorus was bound to 1 mole of the complex. Thus, since the protein readily binds phosphorus, it seems more probable that the phosphorus found in the complex prepared using Tris buffer originated from the cell sap during breakage of the cells or from solutions used during isolation of Bchl-P rather than from a phosphorylated compound of the complex.

B. CARBOHYDRATE. No evidence was obtained for the presence of carbohydrates in hydrolysates of the complex by paper chromatography. Gas–liquid partition chromatography was also used to examine hydrolysates; no commonly occurring neutral monosaccharides were present, but a trace of unidentified material was eluted just before α-mannitol, a reference standard. This trace was estimated to represent 1 mole of mol wt 220/mole of complex.

Determination of Free SH Groups in the Complex. Reaction of the protein with *p*-mercuriobenzoate (PMB) was carried out as described by Isles and Jocelyn (1963). No PMB was bound to the protein on incubation together at room temperature. Confirmation that the two half-cystines in the hypothetical subunit (Table I) were linked by an S–S bridge was achieved by finding that the half-cystines were not carboxymethylated when the complex was treated with iodoacetic acid. The protein was dissolved in 2 M guanidine-HCl (recrystallized from methanol) and treated with a 100-fold excess of iodoacetic acid at pH 8.6 for 20 min; excess reagents were removed by dialysis and the protein was hydrolyzed with constant-boiling HCl at 110° for 24 hr. Examination of the hydrolysate on an automatic amino acid analyzer demonstrated that no carboxymethylcystine had been formed.

Tryptic Digestion of Bchl-P. The native complex was not digested by any proteolytic enzyme; however, the denatured Bchl-P was readily digested by trypsin, chymotrypsin, Nagarse, or Pronase. The method by which the complex was denatured had to be such that it would not alter the chlorophyll–protein interaction, *i.e.*, it would irreversibly denature the complex but would not pheophytinize the Bchl; pheophytinization may alter the position of binding of the pigment to the protein. Denaturation without loss of Mg^{2+} was achieved by the addition of a solution containing 50% acetic acid adjusted to pH 3.2 with solid sodium acetate. After tryptic digestion all the Bchl was still insoluble. The amino acid composition of the buffer-washed residue was very similar to that of Bchl-P (Table I); all the amino acids were present and in approximately the same molar ratio; however only 3% of the original protein material remained with the Bchl. Analysis of the DNP residue demonstrated that there were many DNP-amino acids, none of which was preponderant.

Discussion

The molecular weight of the Bchl-P complex was originally estimated to be 1.37×10^5 g/mole on the assumption of $\bar{V} = 0.74$ cm³/g (Olson *et al.*, 1963). A later estimate of $(1.67 \pm 0.17) \times 10^5$ g/mole was based on an experimentally determined $\bar{V}$ of 0.79 ± 0.02 cm³/g (Olson, 1966). The amino acid composition obtained at that time led to the postulation that the molecule consisted of seven subunits (mol wt 24,000) each of which contained 3 moles of Bchl. There has been no previous examination of the complex for the presence of components other than Bchl and protein, except for spectrophotometric studies which indicated that Bchl was the only pigment associated with the protein. Present studies have shown that there are only traces of other components in the complex and these represent much less than one-half of 1%; furthermore the determined percentage nitrogen value is close to that calculated for a complex consisting of Bchl and protein only; consequently we now believe that Bchl-P is composed essentially of bacteriochlorophyll a and protein.

The best interpretation of the data presented in this paper is that the complex is made up of four, and not seven, subunits. The minimum molecular weight which is a guide to the number of subunits in a macromolecule of known molecular weight was determined from the amino acid composition and the Bchl:nitrogen ratio of the complex; a value of 37,940 (minimum, 37,380; maximum, 38,410) was obtained. Four such subunits per complex is the only ratio which will coincide with the determined molecular weight of the complex. Confirmation of this number is provided by the analysis of the N-terminal amino acid of the protein; DNP-alanine occurred in the most likely proportion of 4 moles/mole of Bchl-P. Thus the theoretical molecular weight of the complex can be calculated assuming that the four subunits are identical (a reasonable assumption since only one species of N-terminal amino acid is present) and that each contains 5 moles of Bchl; a value of $(1.52 \pm 0.04) \times 10^5$ is obtained. From this value and the value for the effective molecular weight, $M_{\mathrm{eff},20,w} = (3.6 \pm 0.1) \times 10^4$ (Olson *et al.*, 1963), the molecular volume and the specific volume were calculated to be $(1.93 \pm 0.08) \times 10^5$ Å³ and 0.764 ± 0.014 cm³/g, respectively. The macromolecule now appears to be

TABLE II: Comparison of the Properties of the Bacteriochlorophyll–Protein Complex with those of a Chlorophyll a Containing Protein of Spinach Beet Chloroplasts (Thornber *et al.*, 1967b).

Properties	Bchl-P	Chl a–P	Difference (Chl *a*–P minus Bchl-P)
$s_{20,w}$	7.2	8–9[a]	
Minimum molecular weight of protein	33,400	33,300	
Chlorophyll (moles)/subunit	5 Bchl	4–4.5 Chl a[b]	
Carotenoid	None	1 mole/14 moles of Chl a[b]	
Lipid[c] content	None	None	
Carbohydrate content	Trace (unknown)	Trace (Glu,Gal,Man,Ara,Xyl)	
Electrophoretic mobility[a] (molecular size)	1.52×10^{-5}[d]	1.60×10^{-5}[d]	
Amino Acid Composition			
Residues/subunit	305	308	+3
Polar residues	*138*	*118*	*−20*
Acidic residues	*61*	*50*	*−11*
Asp	33	26	−7
Glu	28	24	−4
Basic residues	*41*	*35*	*−6*
Lys	16	9	−7
His	7	15	+8
Arg	18	11	−7
Hydroxyl residues	*36*	*33*	*−3*
Thr	13	16	+3
Ser	23	17	−6
Nonpolar residues	*167*	*190*	*+23*
Aliphatic residues	*139*	*158*	*+19*
Gly	34	34	0
Ala	18	30	+12
Val	29	19	−10
Ile	20	20	0
Leu	18	35	+17
Pro	15	15	0
Met	3	4	+1
Cys	2	1	−1
Aromatic residues	*28*	*32*	*+4*
Tyr	8	8	0
Pye	15	21	+6
Trp	5	3	−2

[a] Determined in the presence of detergent. [b] Removal of pigments from the complex may occur during isolation (see Thornber *et al.*, 1967a). [c] Other than pigments. [d] In cm^2/V per sec.

slightly smaller than previously indicated; the former values of molecular weight and volume were $(1.67 \pm 0.17) \times 10^5$ g/mole and $(2.19 \pm 0.33) \times 10^5$ Å^3 (Olson, 1966).

From the amino acid composition, the molecular volume and the specific volume of the protein alone were calculated to be $(1.63 \pm 0.04) \times 10^5$ Å^3 and 0.733 cm^3/g (Schachman, 1957). The specific volume of the Bchl in the complex was then computed to be 0.99 ± 0.13 cm^3/g. If, on the other hand, the specific volume of the Bchl in the complex is assumed to be the same as that for crystalline chlorophyll a (0.93 cm^3/g; Donnay, 1959), the molecular and specific volumes for the complex would be $(1.91 \pm 0.04) \times 10^5$ Å^3 and 0.757 cm^3/g, respectively. These calculations indicate that it is now reasonable to presume that the specific volume occupied by the chlorophyll in the complex is about the same as that occupied in chlorophyll crystals (*cf.* Olson, 1966).

The observation that solvent treatment of the com-

plex extracts some peptides and amino acids, and that traces of N-terminal amino acids other than alanine occur in the DNP protein preparations, is explained by postulating that during storage the Bchl-P is undergoing autodigestion possibly from the N-terminal end of the protein; this would give rise to amino acids and peptides and new N-terminal residues. The original N-terminal residue would be extracted as the DNP-amino acid or DNP peptide into the solvent washings during the preparation of the DNP protein; this loss is particularly noticeable in a 1-year-old batch of protein in which the sum of all N-terminal residues is still no greater than four residues per mole of complex although 75% of the N-terminal residues are glycine. The trace DNP-amino acids are not due to the presence of other proteins in the purified Bchl-P solutions since gel electrophoresis of the solution reveals only one protein zone.

A start has been made toward an elucidation of the nature of the binding of Bchl to protein by attempting to isolate chlorophyll-containing peptides from a tryptic digest of the denatured complex. The results were not encouraging since only a very small proportion of the protein remained with the Bchl molecules, and furthermore this residual material was a heterogeneous mixture of peptides and possibly undigested protein. It appears more probable that the peptides remaining with the residue are trapped within the precipitated chlorophyll rather than that they occur as Bchl peptides. The binding of chlorophyll to protein is thus a very weak linkage occurring more through folding of the peptide chain rather than by any strong electrostatic or hydrophobic bonding.

No other chlorophyll-containing proteins have been isolated whose chemical composition has been studied in such detail as that of Bchl-P. Thornber *et al.* (1967a,b) isolated two detergent-soluble chlorophyll–protein complexes from chloroplasts of higher plants and made preliminary studies of their chemical nature, and it is with the Chl a containing protein that the chemical composition of Bchl-P is compared in Table II. Of the two isolated complexes this one is chosen for comparison with Bchl-P since it appears likely that both are derived from photochemical systems carrying out the same function, *i.e.*, system I activity. There are some striking similarities between Bchl-P and the Ch a–protein in chlorophyll/protein ratio, mo'ecular weight of protein subunit, and the lack of any lipid other than pigments, but differences occur in the ratio of polar to nonpolar amino acids, carotenoid content, and the nature of the trace carbohydrate material; the difference in solubility of the two complexes is a consequence of these variations. Electrophoresis in polyacrylamide gels of a mixture of the two complexes in 1% sodium dodecyl sulfate solution gave only a very slight separation (Table II), which indicates that their molecular sizes are almost identical (Shapiro *et al.*, 1967). The Chl a–protein is slightly smaller, which may be the result of loss of pigments from the Chl a complex during its isolation (Thornber *et al.*, 1967b). The Bchl-P is not dissociated by the conditions used for the electrophoresis (J. P. Thornber, unpublished data). It is not unreasonable to assume that *in vivo* each subunit of the Chl a–protein may contain 5 moles of chlorophyll (Table II) which would be in exact agreement with Bchl-P. If this were the case, both complexes have the stoichiometric ratio Chl–Gly–Ile–Pro, 1:7:4:3, and this may give an indication of how the chlorophyll molecules are located in the complex; four isoleucine residues in close proximity along a peptide chain would form a suitable environment for the hydrophobic bonding of the phytyl chain of the chlorophyll molecule. Before it can be unequivocably stated that the two complexes are derived from an analogous class of proteins, *i.e.*, the differences in composition between the two can be explained by mutations which have occurred during evolution, much more detailed investigation is needed not on y of Bchl-P but also of chlorophyll-containing proteins of other photosynthetic organisms.

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A Cell-Free Amino Acid Incorporating System from an Extremely Halophilic Bacterium*

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ABSTRACT: A cell-free system has been developed from *Halobacterium cutirubrum* that will incorporate 10–100 $\mu\mu$moles/mg of ribosomes of each of 17 amino acids into hot trichloroacetic acid insoluble material. The system requires adenosine triphosphate, phosphoenolpyruvate, guanosine triphosphate, and ribosomes, is sensitive to pancreatic ribonuclease and puromycin, and will incorporate increased amounts of [^{14}C]phenylalanine in response to added polyuridylic acid. This evidence together with the nature of the labeled product suggest that incorporation of amino acids represents true polypeptide synthesis.

The optimum Mg^{2+} concentration for incorporation is 0.02–0.04 M, but the system is extremely halophilic in requiring nearly saturated salt and specifically 3.8 M KCl, 1 M NaCl, and 0.4 M NH_4Cl for maximum activity. The possible significance of this specificity is briefly discussed.

The synthesis of a protein depends on a series of molecular interactions which, by and large, are inadequately understood. One feature of these interactions that has been recognized for some time is the importance of cations. Binding of mRNA to ribosomes (Okamoto and Takanami, 1963a,b), the structural integrity of ribosomes (see, *e.g.*, Petermann, 1964; Gesteland, 1966) and of tRNA (Nishimura and Novelli, 1963; Lindahl *et al.*, 1966), and the activity of transfer enzymes (Gordon and Lipmann, 1967) all involve Mg^{2+} ions, which, in some instances, can be replaced by Ca^{2+} (Gordon and Lipmann, 1967). Binding of tRNA to ribosomes depends on NH_4^+ or K^+ (Spyrides, 1964).

Within extremely halophilic bacteria, the concentration of salt is very high (Christian and Waltho, 1962) so that ionic interactions between protein and nucleic acid components of the protein synthesizing system should be correspondingly reduced. Study of the mechanism of protein synthesis in these bacteria may therefore shed new light on the nature of the molecular interactions involved. Interesting observations have already been made on the ribosomes of one of these organisms, *viz.*, *Halobacterium cutirubrum* (Bayley and Kushner, 1964; Bayley, 1966a,b). To extend this work, a cell-free amino acid incorporating system from this bacterium was required. This paper describes such a system.

Materials and Methods

Preparation of Cell-free Extracts. Cells were grown at 37° under continuous aeration, with added oxygen, in a 150-l. stainless-steel fermentor in the medium described by Sehgal and Gibbons (1960), except that 10 ppm of Fe^{2+} (as $FeSO_4$) was added and the final pH was adjusted to 6.2. The concentrations of the principal inorganic salts in this medium were 4.3 M NaCl, 0.027 M KCl, and 0.08 M $MgSO_4$. Sterilized medium (100 l.), which had been centrifuged to remove the precipitate, was inoculated with a 5-l., 30-hr culture. After 19–20 hr the culture was cooled and the cells, in early log phase, were harvested in the cold. The cells were washed at 0° with centrifuging, once in a solution containing 4.3 M

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