Triton X-100–4 M Urea as an Extraction Medium for Membrane Proteins. II. Molecular Properties of Pure Cytochrome b_{559}: A Lipoprotein Containing Small Polypeptide Chains and a Limited Lipid Composition†

Harinder S. Garewal‡ and Aaron R. Wasserman*

ABSTRACT: The molecular composition of electrophoretically homogeneous preparations of chloroplast cytochrome b_{559} was examined. The partial specific volume was 0.91; the hydrodynamic molecular weight, likely as a complex with Triton X-100, was 117,000. The equivalent dry weight per mole of heme, using Triton-depleted samples, was 111,000. The protein content (41%) was equivalent to 45,900 g/mol of heme. Since only one size of polypeptide chain was found [5600(±1000)] about eight small chains were calculated to be present per one heme and thus per molecule. N-terminal analysis revealed at least three kinds of polypeptide chains (Glu, Asp, and Thr as N-terminals). The content of noncovalently linked lipids 56(±6)%, consisted of only four lipid components, thus representing a simple lipid composition relative to that of the original membranes. Only two nonpolar lipids were found: chlorophyll *a* (3%, 4 mol/heme) and β-carotene (1.6%, 3 mol/heme); tocopherols, plastoquinones, and chlorophyll *b* being absent. Two unknown polar lipids, one major (about 40%) and one minor (about 5–8%), comprised most of the lipid found. The Triton X-100 content was less than 5–6% and migrated as a single spot different from those of the two unknown polar lipids. Neither polar lipid was a glycolipid. The major polar lipid was not a phospholipid, but the minor component might have been a phospholipid. Each of the unknowns migrated chromatographically like unknown polar lipids of chloroplast-grana membranes. Cytochrome b_{559} contained no non-heme iron and no detectable hexose. Triton X-100–4 M urea (pH 8), the extracting solvent used to isolate cytochrome b_{559}, is suggested as potentially useful for isolating membrane lipoproteins in undenatured form.

In a companion publication we reported the isolation of an electrophoretically homogeneous preparation of a relatively unstable chloroplast membrane protein, cytochrome b_{559} (Garewal and Wasserman, 1974). Since little is known about the molecular composition of individual membrane proteins of proven purity and of recognizable biological properties, studies of molecular properties of cytochrome b_{559} were undertaken. Additional objectives were the possible relevance of the results to photosynthesis and to determine whether reliable studies of molecular properties were feasible with preparations obtained using the nonionic detergent Triton X-100. The characteristic absorption spectrum of a cytochrome offers many advantages in studies of molecular composition which are not afforded by colorless proteins or enzymes.

Methods

Purification of cytochrome b_{559} and definition of 1 (absorbance) unit have been described (Garewal and Wasserman, 1974). All analyses were performed on pure monodisperse preparations of cytochrome b_{559}. Chloroplast grana (used in studies of lipids) were prepared and stored as described by Wasserman and Fleischer (1968).

Content of Heme and Total Iron. Heme was determined as the reduced pyridine hemochrome (Appleby, 1969). Total iron was assayed by the method of Doeg and Ziegler (1962) using pure iron wire as standard.

Determination of Partial Specific Volume and Molecular Weight. The deuterium oxide (D_2O) method of Edelstein and Schachman (1967) was employed. Samples of b_{559} (0.2 mg of biuret protein/ml) were in a medium containing 0.05 M Tris-HCl, 2 mM DTT,[1] 15% glycerol, and 0.1% Triton X-100 (pH 8) which had been made to 50% D_2O (or no D_2O) by dialysis under nitrogen for at least 24 hr against large volumes of an identical medium containing either 50% D_2O or no D_2O. Corresponding dialysis fluids were used in the reference cells. Analytical ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge at 45,000 rpm and at a temperature of 3°. Sedimentation equilibrium was reached in 48–50 hr. Densities were determined by weighing, using a 10-ml pycnometer.

Determination of Protein, of Total Dry Weight, and Dry Weight of Total Lipid Using Samples Depleted of Triton X-100. Solutions of cytochrome b_{559} initially containing about 0.1% Triton X-100 were exhaustively dialyzed against about 1000 volumes of 0.05 M Tris-HCl, 15% glycerol, and 2 mM DTT (pH 8) under N_2 at 4° for a total of 72 hr with the dialysis buffer changed every 24 hr. Cytochrome b_{559} was stable during the dialysis period in that it was quantitatively recovered afterwards. Aliquots of the dialyzed samples were then dried for several days to constant weight in a vacuum oven at 70°; weighings were performed on a Cahn electrobalance (Fisher Scientific Co.) sensitive to 2 μg. Aliquots of the final dialysis buffer were similarly dried to constant weight and their weight was subtracted from the sample weight; 1-ml samples containing 0.35 unit of b_{559} gave 1.88 mg of net dry weight of b_{559}/ml. The accuracy of the overall procedure for dry weight

† From the Department of Biochemistry, McGill University, Montreal, Quebec, Canada. *Received February 1, 1974.* Supported by Grant MA-4385 (To A. R. W.) from the Medical Research Council of Canada.

‡ Supported by a Studentship from the Medical Research Council of Canada. This work constituted part of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree at McGill University. Present address: Harvard Medical School, Boston, Mass. 02115.

[1] Abbreviations used are: DTT, dithiothreitol; MGD, monogalactosyl diglyceride; DGD, digalactosyl diglyceride; SDS, sodium dodecyl sulfate.

determination was examined by drying several aliquots of a solution of crystalline bovine serum albumin (1 mg/ml) and found to be approximately 4%.

Protein determinations on samples dialyzed as above (or undialyzed) were performed by the standard biuret procedure of Gornall *et al.* (1949) which was adapted to the micro range (50 μg to 1 mg) by recording the absorption spectrum of the biuret color on a Phoenix-Chance spectrophotometer (J. Singh and A. R. Wasserman, unpublished procedure). The color given by an alkaline control of b_{559} was subtracted from the biuret color values. A standard curve in this microgram range was obtained using crystalline bovine serum albumin as standard.

Content of Triton X-100 in dialyzed samples was determined by the microcolorimetric method of Garewal (1973). Although a standard curve with Triton X-100 followed Beer's law only down to 40 μg, 20γ of Triton X-100 gave a significant color yield. In control determinations, additions of 40 μg of Triton X-100 to samples containing about 264 μg net dry weight of b_{559} which had previously given no detectable color due to Triton gave the expected Triton color yield thus indicating that the cytochrome does not interfere with colorimetric estimation of Triton X-100.

Total lipid of dialyzed cytochrome b_{559} samples was extracted by the method of Bligh and Dyer (1959). Care was taken to protect the lipids from light and the overall procedure was performed without undue delay. The final lipid extract, in chloroform, was dried to 0.1 ml under a stream of N_2 and transferred to a preweighed aluminum weighing container. To ensure quantitative transfer of the sample, the original container was rinsed successively with 5 aliquots of chloroform (5 ml, 5 ml, 3 ml, 1 ml, 1 ml) each of which was similarly dried to 0.1 ml and transferred to the aluminum weighing container. The combined solutions (about 0.6 ml) were dried at 37° under vacuum for 1–12 hr to constant weight on a Cahn electrobalance. A dialysis blank of the same volume as the sample and similarly transferred was dried to constant weight, and its weight was subtracted from that of the sample. The accuracy of the entire procedure (including that of quantitative transfer and drying) was examined using standard solutions of lecithin in chloroform and was found to be reliable to within ±5%.

Size of polypeptide chains of cytochrome b_{559} was determined by dissociation in sodium dodecyl sulfate and mercaptoethanol followed by disc electrophoresis in a 10% gel (Weber and Osborn, 1969). Modifications of acrylamide and cross-linker (methylenebisacrylamide) concentrations required in disc electrophoresis are indicated in the appropriate section of Results. The term "normal amount of cross-linker" refers to the ratio of methylenebisacrylamide to acrylamide used by Weber and Osborn (1969).

Amino Acid Analysis. Glycerol in the samples was found to interfere in these analyses and was removed from the cytochrome b_{559} samples by dialysis at 5° against 5 mM DTT–0.05 M Tris-HCl (pH 8) under N_2. Samples were hydrolyzed with 6 N HCl at 110° for 24 hr in sealed evacuated tubes, adjusted to pH 2 on a pH meter, and analyzed on a Beckman 120C amino acid analyzer. Separate analyses for cysteine and methionine (measured as cysteic acid and methionine sulfone, respectively) were performed on a 48-hr hydrolysate of the performic acid-oxidized protein (Hirs, 1967).

N-Terminal Analysis. The procedure, suggested to us by Dr. Edward Meighen, was essentially that of Gray (1972) which is based on thin-layer chromatography of dansylated amino acids on polyamide sheets (Woods and Wang, 1967). Differences in procedure from that of Gray were as follows. *Dansylation of protein:* (1) the sample was used without prior oxidation by performic acid, (2) addition of *N*-ethylmorpholine was omitted since the sample was not insoluble at this point, (3) the dansyl chloride solution contained 5 mg of the reagent/ml of acetone rather than 25 mg/ml of dimethylformamide. *Hydrolysis:* (1) the dansylated protein was precipitated with 10% trichloroacetic acid (rather than acetone) and the precipitate washed with 1 ml of 1 N HCl (rather than acetone). The washed precipitate was dried *in vacuo* at 50–60° prior to proceeding with hydrolysis in 6 N HCl as described by Gray. *Chromatography:* 5 cm × 5 cm polyamide cards were sufficient for rapid, efficient resolution of standard dansylated amino acid mixtures which were cochromatographed on the reverse side of the cards from that of the sample. Solvent systems I, II, and III were those used by Gray. An additional system (IV), pyridine–glacial acetic acid (pH 4.58 buffer), was employed to resolve Dns-Arg from Dns-ε-Lys. Chromatography in system I was followed by sequential chromatographic development in a second direction using systems II, III, and IV.

General Procedure for Thin-Layer Chromatography of Lipids on Silica Gel. Glass plates were prewashed in acetone and dried in air. Silica gel H (plain, no binder, Brinkmann Instruments Inc.) was spread as a slurry of 50 g of silica gel in 115 ml of 0.01% sodium carbonate to give a thickness of 0.25–0.50 mm for analytical plates (20 cm long) and 0.75–1.00 mm for preparative plates (20 × 20 cm). The plates were allowed to dry at room temperature for 1 hr and then dried at 120° for 2 hr. Preparative plates were prewashed to remove any traces of lipid by running them in chloroform–methanol (1:1) which was followed by drying as above. Lipid samples were spotted on silica gel plates in a nitrogen box. Lipid chromatography was performed in all-glass sealed tanks whose air atmosphere had been preequilibrated with the appropriate solvent system for 1–2 hr. Ascent of the solvent system in chromatography to within 1–2 cm of the top of the 20 cm plates generally was complete within 40–50 min. The developed plates were then allowed to dry in air for about 5 min; staining with specific reagents (where designated) was always performed immediately after drying.

General Detection of Lipids on Silica Gel Plates. Charring with sulfuric acid was performed by spraying the plates lightly with aqueous 30–40% (v/v) sulfuric acid and heating the plates on a hot plate at about 200° for 5–10 min. All the lipids were detected as charred black spots after this procedure. (Charring was also performed as a sensitive check on plates where prior specific staining had shown no positive unknown spots so as to ensure that unknowns were not underloaded.)

Preparation of Total Lipid Extract. Solutions of cytochrome b_{559} were thoroughly dialyzed to remove Triton X-100 by the procedure described earlier in this section. (The presence of large amounts of Triton X-100 in undialyzed samples interfered with lipid estimations, especially detection of polar lipids on thin-layer plates.) Quantitative extraction of total lipids was performed on dialyzed samples by the method of Bligh and Dyer (1959); care was taken to exclude as much light as possible and to work quickly so as to protect any labile lipids. The dry samples of total lipid were dissolved in absolute ethanol and stored at −15° under nitrogen in the dark (the ground-glass stoppered tubes were covered with aluminum foil).

Analyses of Neutral Lipids. The spectrum of the yellowish total lipid extract in ethanol was recorded from 250 to 700 nm on a Cary Model 15 recording spectrophotometer to give a qualitative indication of the possible neutral lipids present. Thin-layer chromatography of extracts of total lipid was performed in the dark on silica gel plates with the indicated solvent systems of Kushwaha *et al.* (1972): *Quinone-vitamin A*

system (0.1% diethyl ether in chloroform)—retinal, retinol, coenzyme Q_6 (ubiquinone), α-tocopherol, vitamin K_1, and Triton X-100 (colorless) were spotted simultaneously as standards and were positively detected after chromatography both by visible inspection and by charring. *Carotenoid system* (0.5% ethyl ether either in hexane or in petroleum ether)—standards included β-carotene, an extract from red tomatoes containing β-carotene, and Triton X-100.

Quantitation of Neutral Lipids. The entire total lipid extract derived from 5 ml of dialyzed cytochrome b_{559} (0.75 total unit) was applied as a streak along the origin of a preparative thin-layer plate. Chromatography (in the dark) was performed by the carotenoid solvent system described above. After drying the plate in air, the resolved yellow region of the plate (β-carotene) was scraped off and eluted with acetone; silica gel was removed by sedimentation in a clinical centrifuge. The section from the origin up to (but not including) the yellow region of the plate was also scraped off as a single fraction and eluted in acetone. (Chlorophyll remains at the origin in the chromatographic solvent system.) The β-carotene fraction was dried under a stream of nitrogen, dissolved in 1 ml of light petroleum, and its visible absorption spectrum was recorded on a Phoenix–Chance spectrophotometer. Content of β-carotene was calculated using the extinction coefficient $E_{1\text{ cm}}(1\%)$ at 451 nm = 2500 (Goodwin, 1965). The chlorophyll-containing fraction was similarly eluted in acetone, concentrated to 1 ml, and its visible absorption spectrum recorded. Content of chlorophyll *a* was calculated using the equation, chlorophyll *a* (mg/l.) = $12.7A_{663} - 2.69A_{645}$ (Arnon, 1949).

Analyses for Polar Lipids. Thin-layer chromatography on silica gel plates employed the solvent system chloroform–methanol–water (65:25:4, v/v) (Lepage, 1964) which was found to give the best resolution for the polar lipids we encountered. In this system neutral lipids move with the front. Control experiments using a total lipid extract of P_1S (spinach chloroplast-grana membranes) gave good resolution of the major known polar lipids. The relative quantities of individual colorless, polar lipid spots derived from cytochrome b_{559} were estimated by inspection after charring with sulfuric acid; these approximate estimates were relative to charring density given by known amounts of applied standards. Staining on silica gel plates for specific constituents of polar lipids was performed as follows: periodate–Schiff stain for glycol groups (Shaw, 1968) with MGD as standard; phosphate stain (Vaskovsky and Kostetsky, 1968) with lecithin, cardiolipin, and phosphatidic acid as standards; α-Naphthol stain for sugars (Siakotos and Rouser, 1965) with MGD and a total lipid extract of spinach leaves as standards. This extract contained, in addition to other lipids, MGD, DGD, and sulfoquinovosyl diglyceride which are known constituents of higher plant chloroplasts (Sastry and Kates, 1964). The steroid stain was that of Lowry (1968) with cholesterol as standard; the characteristic charring behavior of cholesterol and cholesterol esters after spraying with sulfuric acid was also examined (Shipski and Barclay, 1969). The ninhydrin stain for free amino groups of phospholipids (Shipski and Barclay, 1969) gave positive results when previously checked using phosphatidylserine and phosphatidylethanolamine as standards. Except for the ninhydrin staining, each of the previous standard compounds was chromatographed on the same plate as the unknown and gave a positive reaction with the appropriate specific stain.

Preparation of "Control Extract" from Preparative Electrophoretic Gel for Use in Polar Lipid Analysis. The preparative disc electrophoretic gel on which cytochrome b_{559} had been purified was cut to give a control slice identical in size with the b_{559} band but containing no protein. Using the procedures previously employed for cytochrome b_{559}, this "gel control" was eluted, concentrated (but not dialyzed), extracted to produce a total lipid extract, and then examined by thin-layer chromatography for polar lipid content.

Results

Content of Heme and Total Iron. Heme content (as the reduced pyridine hemochromogen) was 48.5 mμmol of heme per absorbance unit of cytochrome b_{559}. Total iron content was 47(±3%) mμmol/absorbance unit of b_{559}. The equivalence of heme and total iron content indicates that b_{559} contains no non-heme-iron.

Determination of Partial Specific Volume ($\bar{v}$) and Molecular Weight by Analytical Ultracentrifugation. Cytochrome b_{559} in 70% D_2O did not reach sedimentation equilibrium even after 3 days of centrifugation and was presumably floating in this medium. Experiments using 50% D_2O or no D_2O gave satisfactory results in that plots of log J *vs.* r^2 were completely linear. Fringes on the photographic plates could be read to the bottom of the cell and indicated that no heavier species had accumulated there. The cytochrome samples were quantitatively recovered after the determinations. Thus the cytochrome in 0.1% Triton X-100 was both monodisperse and stable during the determinations. Calculations using the slopes of the experimental lines gave a value of 0.91 ml/g for $\bar{v}$ and a molecular weight of 117,000. (The arbitrary assumption of a $\bar{v}$ of 0.73, which is often made for a protein sample, gives a calculated molecular weight of only about 40,000. However, it is noteworthy that when pure b_{559} was run on a Sephadex G-100 column in the same aqueous 0.1% Triton medium used in ultracentrifugation, its migration indicated a molecular weight greater than that for bovine serum albumin, *i.e.*, over 70,000.)

Analyses for Protein and Dry Weight and Corresponding Equivalent Weights per Mole of Heme; Content of Total Lipid. Analyses were performed on aliquots of solutions depleted of Triton X-100 by dialysis and showed that 1 unit of b_{559} ($\Delta A_{559-600} = 1$) corresponded either to 2.2 mg of biuret protein or to 5.4 mg of dry weight. The corresponding equivalent weights were 45,900 g of protein/mol of heme and 111,000 g of dry weight/mol of heme. Cytochrome b_{559} thus contains 41% protein and, by difference, 59% of unknown non-protein substance(s). Microcolorimetric analyses of aliquots of solutions used for determinations of dry weight showed no detectable amounts of nondialyzable Triton X-100 remaining. Since the method can detect as little as 20 μg of Triton X-100, it was calculated conservatively that Triton X-100 contributed a *maximum* of 7% to the total dry weight of b_{559}. Extraction of total lipid from aliquots of dialyzed samples and subsequent direct determination of dry weights of total lipid showed that b_{559} contains 56(±6)% lipid. Within experimental limits this lipid content matches that of the unknown non-protein substance(s) (59%) found by difference.

Calculation of Size of Native Monomer and Number of Hemes per Molecule. The close correspondence of the equivalent weight of 111,000 g of dry weight/mol of heme (found with samples depleted of Triton X-100) with the hydrodynamic molecular weight of 117,000 (found in the presence of 0.1% Triton X-100) indicates that b_{559} was dissociated by 0.1% Triton X-100 to a stable monomer of about 111,000–117,000 and that there is only one heme per molecule.

Carbohydrate Content. Analyses by the cysteine–sulfuric acid method of Ashwell (1957) showed no hexose was present. Since the method quantitatively estimates as little as 25 μg of hexose/ml, the b_{559} samples contained less than 1.5% hexose.

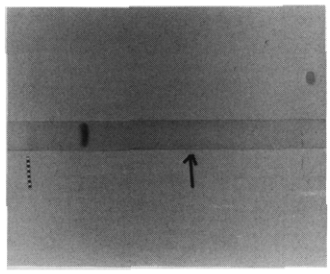

FIGURE 1: Disc gel electrophoresis in sodium dodecyl sulfate of the polypeptide subunits of cytochrome b_{559}. A 20% polyacrylamide gel of double the normal amount of cross-linker was employed. The applied cytochrome sample had been denatured by incubation in sodium dodecyl sulfate and mercaptoethanol as described in the text. The arrow marks the position where the dye-front occurred before staining. The dotted line indicates the top of the gel.

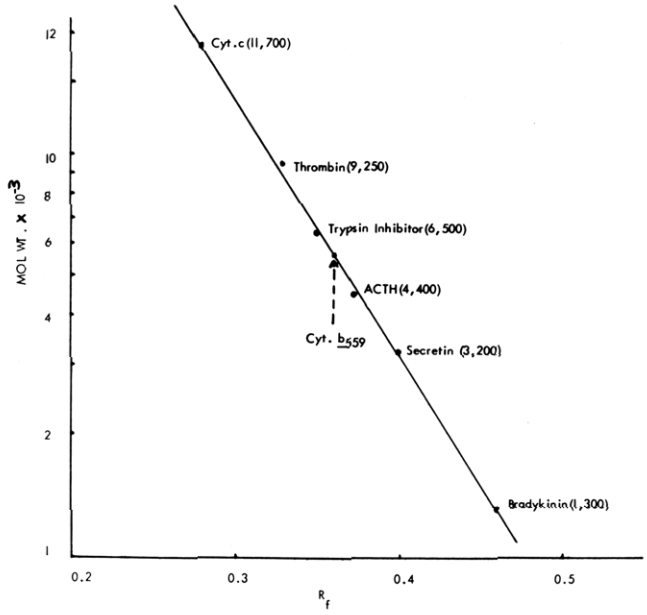

FIGURE 2: Determination of the molecular weight of the polypeptide chains of cytochrome b_{559} using a 20% gel with double the normal amount of cross-linker. Treatment and electrophoresis of the sample and pure calibrating proteins was that used in Figure 1.

(The anthrone method for carbohydrates (Strauss *et al.*, 1971) could not be reliably used due to interference by tryptophan in the protein.)

Size of Polypeptide Chains. After denaturation and dissociation in sodium dodecyl sulfate and mercaptoethanol the sample migrated in disc electrophoresis (10% resolving gel) as a single band with a mobility of 0.90 (0.85–0.95 in repeated experiments). This gave a preliminary indication that the molecular weight of individual chains was likely less than 10,000. (To eliminate the possibility that the small subunits were produced by proteolysis during incubation with SDS as recently reported for hexokinase (Pringle, 1970), the sample was incubated in a control experiment for 3 min at 100° immediately after the addition of SDS and mercaptoethanol. This treatment destroys any proteolytic activity (Pringle, 1970). However, as before, only a single band with an R_F of about 0.90 was observed in a 10% gel.) Prior extraction of lipid from b_{559} by the method of Bligh and Dyer (1959) followed by incubation in SDS and mercaptoethanol also gave the same fast moving band (R_F 0.90) in a 10% gel. Thus the presence of lipid in the original sample had not in some way caused hypothetically large polypeptides to move more rapidly. When mercaptoethanol was omitted from the incubation medium, the mobility of the sample was unchanged, indicating that disulfide bridges between chains in the molecule were probably not originally present. The use of a 14% gel after incubation of the sample in SDS–mercaptoethanol produced a single band whose mobility (ranging from 0.85 to 0.90) was still too high for satisfactory estimation of chain size. The use of a 20% gel gave a single band whose mobility (about 0.51) was sufficiently retarded but the calibration graph of known proteins was not linear. A 20% gel with twice the normal amount of cross-linker produced a single band (Figure 1) with an R_F of 0.36 and a linear calibration graph with standard proteins (Figure 2). The size of polypeptide chains in b_{559} was 5600 (±1000). (The experimental uncertainty of ±1000 (or ±18%) that was encountered is entirely due to the sharp slope of the calibration line and the intrinsic experimental errors in measuring the R_F values.) Supporting evidence that the size of polypeptide chain was indeed small was obtained by chromatographing samples incubated at 37° in 1% SDS and 1% mercaptoethanol on a column of Sephadex G-100 in 0.05 M Tris-HCl, 0.1% SDS, and 0.1% mercaptoethanol (pH 8) at 4°. Only a single protein peak was eluted whose K_{av} value (0.64) indicated a chain size considerably smaller than that for cytochrome *c* (12,000, K_{av} = 0.52).

Calculation of the Number of Polypeptide Chains. Division of the protein equivalent weight, 45,900 g of protein/mol of heme, by the size of polypeptide chain, 5600 (±1000), indicates that about 8 chains of identical size (7 to 10) exist per mole of heme and thus per molecule (monomer) of b_{559}.

Amino Acid Composition. A preliminary analysis is presented in Table I. An interesting feature is the relatively high content of the hydroxylated amino acids Ser and Thr (25 residues per total of 119 residues). If Asp, Asn, Glu, Gln, Lys, Ser, Arg, Thr, and His are classified as polar residues and the remaining amino acids as nonpolar [a classification similar to that of Hatch and Bruce (1968)], the percentage of nonpolar residues is calculated as at least 60% (Trp being unknown). The number of residues of each of the amino acids per 1 heme (and thus per molecule) was not determined, but may be about four times the values shown in Table I since the biuret protein weight per 1 heme (45,900 g) is about 3.7 times the total residue weights per 1 Cys (12,450 g).

N-Terminal Analysis. In addition to spots for dansyl hydroxide, dansyl-*O*-Tyr (internal Tyr) and dansyl-ε-Lys (internal Lys), three different N-terminal residues, Glu, Asp, and Thr, were found in cytochrome b_{559} suggesting that *at least three* different polypeptide chains are present in the molecule.

TABLE I: Amino Acid Composition of Cytochrome b_{559}.[a]

Amino Acid	Residues per 1 Cys.	Amino Acid	Residues per 1 Cys.
Lys	2	Ala	9
His	0	Val	7
Arg	4	Ilu	9
Asp (Asn)	9	Leu	11
Thr	10	Tyr	2
Ser	15	Phe	10
Glu (Gln)	7	Cys	1
Pro	9	Met	2
Gly	12	Trp	Not analyzed

[a] Based on a 24-hr acid hydrolysis and the performic acid oxidation method for Cys and Met.

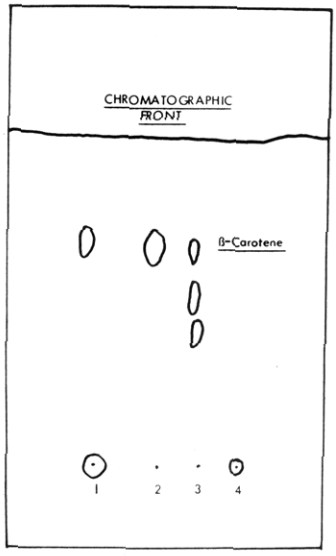

FIGURE 3: Thin-layer chromatography on silica gel of the total lipid extract of cytochrome b_{559} using the carotenoid solvent system [0.5% ethyl ether in either hexane or petroleum ether (v/v)]. Room lighting was switched off during the course of this experiment. Spot 1 refers to cytochrome b_{559}, spot 2 was pure β-carotene, spot 3 was an extract from red tomatoes containing β-carotene, and spot 4 was Triton X-100.

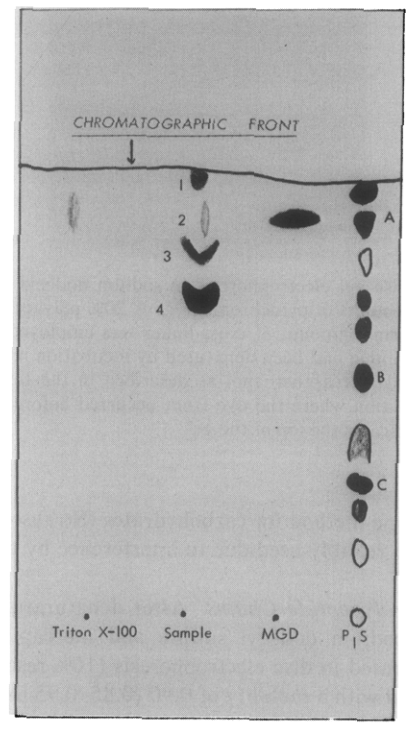

FIGURE 4: Polar lipid analysis by thin-layer chromatography on silica gel of the total lipid extract of cytochrome b_{559}. The solvent was chloroform–methanol–water (65:25:4, v/v). MGD was pure monogalactosyl diglyceride; sample signifies the total lipid extract of cytochrome b_{559}. Neutral lipids (spot 1) move with the front in this solvent system. P_1S represents a total lipid extract of photosynthetically active washed chloroplast grana which contained monogalactosyl diglyceride (spot A), digalactosyl diglyceride (spot B), and glycosulfolipid (spot C).

From the previous data obtained from SDS–polyacrylamide gels, these chains are all of the same small size ([5600 (±1000)]).

Analyses for Neutral Lipids. Preliminary spectrophotometric examination of the yellow total lipid extract indicated no quinones in the ultraviolet region nor any other uv-absorbing lipid. Absorption by residual Triton X-100 at 278 and 284 nm indicated that Triton could account for less than 10% of the mass of the cytochrome b_{559} preparation. The visible spectrum (not shown) indicated the likely presence of β-carotene (at about 450, 420 nm) and chlorophyll (about 660 nm). (Whether both chlorophyll *a* (665 nm) and chlorophyll *b* (650 nm) were present was not certain.) Examination of the total lipid extract by thin-layer chromatography using the "carotenoid system" showed only one spot, either by color (yellow) or after charring. The spot migrated identically with that of standard β-carotene (Figure 3). The origin was greenish before charring suggesting the possible presence of chlorophyll which, like Triton X-100 (colorless) and polar lipids (colorless), does not move in this system. Chromatography of total lipid extract in the "quinone–vitamin A system" showed no detectable quinones. Only one spot was observed both directly (pale yellow) and on charring with sulfuric acid. This spot moved with the front, as does β-carotene in this solvent system. Again, the origin had a greenish spot, possibly chlorophyll, which blackened on charring. (Triton X-100 and polar lipids also do not move in this system.)

Preparative thin-layer chromatography of total-lipid-extract in the "carotenoid system" was used to prepare a pure "β-carotene" fraction and a second fraction (*cf.* Methods) presumably containing chlorophyll as well as colorless substances at the origin. The visible absorption spectrum of the "β-carotene" fraction was identical with that of known β-carotene (Goodwin, 1955). From spectrophotometric assay, one molecule of cytochrome b_{559} contained 3 (3.2) molecules of β-carotene. On a weight basis (mol wt of β-carotene 536) β-carotene comprised only 1.6% (1.56%) of the dry weight of cytochrome b_{559}. The absorption spectrum of the second fraction (green) showed one symmetrical peak at 663 nm identical with that of chlorophyll *a*. The absence of chlorophyll *b* was concluded from the lack of any absorption peak or shoulder near 650 nm. From spectrophotometric assay, the content of chlorophyll *a* was found to be 4 molecules per molecule of cytochrome b_{559}. On a weight basis (mol wt chlorophyll *a* 892), chlorophyll *a* comprised only about 3% (3.24%) of the dry weight of cytochrome b_{559}. Thus β-carotene and chlorophyll *a* together comprised only about 5% of the mass of the cytochrome b_{559} preparation.

Analyses for Polar Lipids. Thin-layer chromatography of the total lipid extract derived from b_{559} showed one major polar lipid (spot 4) and one minor polar lipid (spot 3) (Figure 4). Based on intensity after charring with sulfuric acid, spot 4 constituted about 70% of the total mass of lipid spots and hence about 40% of the dry weight of cytochrome b_{559}; spot 3 constituted about 10–15% of the total lipid and about 5–8% of the weight of cytochrome b_{559}. Since neutral lipids move with the front in the solvent system employed [chloroform–methanol–water, 65:25:4 (v/v)], spot 1 (visibly yellow-green) likely contained the β-carotene and chlorophyll *a* now known to be present in the extract. (If the extract contained any free fatty acids resulting from any hydrolysis of the lipids of cytochrome b_{559}, spot 1 would also contain any such free fatty acids.) Spot 2, from its R_F and its shape, was identical with that of Triton X-100. The mass of spot 2 indicated that Triton X-100 represented less than 10% of the total lipid content and less than 5–6% of the total weight of cytochrome b_{559}. Lengthening the time of chromatography improved the resolution of spot 3 (minor polar lipid) thus confirming its existence as a separate spot and its approximate amount. Relative to the migration of the three characterized chloroplast polar glycolipids (MGD, DGD, and sulfoquinovosyl diglyceride) in this solvent system, the migration of the unknown polar lipids (spots 4 and 3) was distinctly slower than that of standard MGD but faster than that of

TABLE II: Lipid Analysis of Cytochrome b_{559}.

Lipid	Methods of Detection and Identification
A. Neutral Lipids	i. Spectrophotometric analysis (qualitative and quantitative)
1. β-carotene (3 moles/mole cyt. b_{559})	ii. Thin-layer chromatography in 0.5% ethyl ether in hexane
2. Chlorophyll *a* (4 moles/mole cyt. b_{559})	i. Spectrophotometric analysis (qualitative and quantitative) ii. Thin-layer chromatography in 0.5% ethyl ether in hexane (stayed at origin as a green spot)
3. Absence of quinones, vit. K, etc.	i. Spectrophotometric analysis ii. Thin-layer chromatography in 0.1% ethyl ether in chloroform

	Sulfuric Acid Charring	Phosphorus	Periodate–Schiff	Ninhydrin	Sugar (α-Naphthol)	Steroid
B. Polar Lipids[a]						
1. Spot 3 (see Figure 4)	+	+(?)	−	−	−	−
2. Spot 4 (see Figure 4)	+	−	−	−	−	−

[a] All tests performed on thin layer chromatograms developed in chloroform–methanol–water (65:25:4, v/v).

DGD and sulfoquinovosyl diglyceride. Efforts to characterize the unknowns by specific stain tests on thin layer plates are summarized in Table II together with a summary of the neutral lipid analyses. None of these tests gave any positive indication of the nature of the unknown polar lipids. Although the minor polar lipid (spot 3) moved with an R_F similar to that of phosphatidic acid or of cardiolipin, its identity as a phospholipid was equivocal since it gave only a faint blue color after staining for phosphate. Especially surprising was that, in contrast to the three known chloroplast glycolipids mentioned, neither of the two polar lipids in cytochrome b_{559} contained a sugar moiety. The possibility that the two unknowns were artifacts, *e.g.*, derivatives of Triton X-100 or of components in polyacrylamide gels was explored by examining the polar lipid content of a control extract eluted from a gel slice following preparative disc electrophoresis. Thin-layer chromatography, however, revealed only one spot, that corresponding to Triton X-100. Analysis of the polar lipid components of photosynthetically active, washed chloroplast grana (washed P_1S), which had not been exposed to Triton X-100 or urea, is shown in the charred thin-layer plate of Figure 4. In addition to three spots whose migration corresponds to those of the characterized polar lipids sulfoquinovosyl diglyceride, DGD, and MGD, several partly resolved spots of uncharacterized native polar lipids are seen whose mobility corresponds to the two polar lipids found in cytochrome b_{559}.

Discussion

Molecular Weight and $\bar{\nu}$. The hydrodynamic molecular weight, 117,000, was determined in the presence of Triton X-100 and is very likely that of a cytochrome b_{559}–Triton X-100 complex. The equivalent dry weight per mole of heme, 111,000, was determined on samples depleted of Triton X-100 [and hence pure aggregates (Garewal and Wasserman, 1974)]; this figure is thus likely a more reliable figure for molecular weight. Although direct spectrophotometric analysis for Triton X-100 detected none present, experimental sensitivity limits could not exclude a Triton X-100 content of 7% or less and the equivalent weight (and hence the molecular weight) might be as low as 103,000. (Given the latter figure, the percentage of protein would rise only to 47% from the observed value of 41%.) Of importance here is that the amount of bound Triton introduces an error in the hydrodynamic molecular weight which is either negligible within experimental error, or 6% (117,000 *vs.* 111,000) or 14% (117,000 *vs.* 103,000) but not large enough to preclude acceptance of 111,000 as an approximate size for the monomer of cytochrome b_{559}. The term monomer as we use it designates the smallest size retaining all known biochemical properties; in this instance cytochrome b_{559} in Triton solution retains its spectrum and can be reduced by ascorbate.

The experimental value for $\bar{\nu}$, partial specific volume, of 0.91 is outside the range (0.72–0.76) expected for a molecule composed entirely of protein. Given the experimental protein composition of 41% and an assumed $\bar{\nu}$ for protein of 0.73 and a lipid composition of 59% and a partial specific volume of at least 1.0 for lipid, a $\bar{\nu}$ for cytochrome b_{559} can be calculated as at least 0.89. Hence, massive amounts of Triton X-100 need not be invoked to explain the experimental $\bar{\nu}$ of 0.91 for a cytochrome b_{559}–Triton X-100 complex.

Polypeptide Chains. Determination of chain size by disc electrophoresis in sodium dodecyl sulfate was introduced by Shapiro *et al.* (1967) and modified by Weber and Osborn (1969). As used here the method is extended below 10,000 mol wt to a small chain size, 5600 (±1000), and appears satisfactory in that a calibration line was obtained when a 20% gel with double the normal amount of cross-linker were employed. Migration of the subunit as a small polypeptide occurred even after extraction of lipid from the samples and also when digestion was performed at 100° to prevent any hypothetical proteolytic digestion. Additionally, had proteolysis of hypothetically large units occurred, one would expect a heterogeneous, diffuse set of polypeptide bands. However, like any previous polypeptide determination by this method, one cannot yet exclude the possibility of small amounts of covalently linked lipid or other non-protein substances present on the polypeptides which might conceivably make the chains more compact and thus lead to higher mobility and a spurious estimation of chain size. Based on present data of chain size and total protein content in cytochrome b_{559}, there are about eight (7 to 10) protein chains of uniform *size* per mole of heme, with N-terminal analysis supporting at least three chemically different chains. Since the chains are of uniform size, a separation based on charge would be required. Hence, a solubilization without SDS would be a

precondition to a direct demonstration, perhaps *via* disc electrophoretic separation, of the eight chains calculated to be present. Preliminary efforts using 10 M urea containing 1% mercaptoethanol (22, 37, and 50° for 24 hr) have failed to solubilize the polypeptide chains of cytochrome b_{559} after extraction of lipid. (The original, nondelipidated preparation of b_{559} was not dissociated by high urea (Garewal and Wasserman, 1974).)

Lipid Composition. Boardman (1968) lists 20 known lipids (and additional unknowns) as constituents of spinach chloroplast lamellae. Of this large number only four lipids—chlorophyll *a*, β-carotene, and two unknown non-glycolipids—were found in the homogeneous cytochrome b_{559} preparations. The three known polar glycolipids (MGD, DGD, and sulfoquinovosyl diglyceride) present in major amounts in chloroplasts were absent as were plastoquinones, tocopherol quinones, the carotenoids lutein, violaxanthin and neoxanthin, and chlorophyll *b*. Thus, from its simple lipid composition (as well as from its protein composition) the preparation does not appear to be a nondescript piece of the original membrane. In addition to chlorophyll *a* (3% of the molecule) and β-carotene (1.6%) a major (about 40%) and a minor (about 5–8%) unknown polar lipid species are present plus less than 5–6% of Triton X-100. Within experimental error the combined weights of these species match the direct lipid dry weight of 55(±6)%. The two unknown polar lipid spots appear to migrate in chromatographic regions of unknown lipids extracted from chloroplast grana which had not been exposed to Triton X-100. Additionally, a control exposing Triton X-100 to the chemistry of polyacrylamide gel formation and subsequent electrophoresis yielded no thin-layer chromatographic spots other than that for Triton X-100 itself thus further suggesting that the two unknown polar lipids may be native components rather than Triton derivatives artifactually produced by gel electrophoretic procedures.

Since cytochrome b_{559} was prepared from ethanol-extracted particles, could its simple lipid composition simply be ascribed to selective prior removal (based on solubility) of all other chloroplast-lipid species? This would predict a lipid complement of cytochrome b_{559} with a *narrow* range of solubilities in organic solvents. On the contrary, the lipids found in the preparation represent as wide a range of lipid types (from the neutral β-carotene through chlorophyll *a* to the unknown polar lipids) as is found in the remainder of the chloroplast lipids.

Preliminary efforts in solvent extraction of the particles by 90 and 100% ethanol, more exhaustive than that reported here, or ethanol extraction followed by hexane extraction have resulted in virtually complete loss of the cytochrome even when performed at −15°. This would suggest that the lipid composition described is the minimum needed for retention of the cytochrome's spectrophotometric properties. The native lipid composition could conceivably be greater both in the amounts of those lipids reported as well as in additional lipid species not essential to the spectrum of cytochrome b_{559} or its reducibility by ascorbate. Studies on electrophoretically homogeneous preparations of chloroplast cytochrome b_6 (Stuart and Wasserman, 1973) show that this cytochrome, also extracted initially with Triton–4 M urea (pH 8) from ethanol-extracted particles, also contains a very limited lipid composition which differs from that found in cytochrome b_{559} (A. L. Stuart and A. R. Wasserman, manuscript in preparation). Direct proof of the requirement for lipid in the molecular structure or function of cytochrome b_{559} would likely require synthesis since our present information is circumstantial. The possibility of Triton–4 M urea (pH 8) as a general medium for extracting membrane lipoproteins is nevertheless at present an attractive one.

Lack of Chlorophyll a and β-Carotene Absorption Regions in the Cytochrome b_{559} Spectra. Calculations based on the molecular composition data of cytochrome b_{559} indicate that for each absorbance unit at 559 nm contributed by the heme (in aqueous solution), about 13 absorbance units of chlorophyll *a* at 663 nm (in 80% acetone) and 21 absorbance units of β-carotene at 451 nm (in light petroleum) should occur. However, the absolute absorption spectra of b_{559} in aqueous solution (reduced or oxidized, *cf.* Garewal *et al.*, 1971) do not show significant absorption peaks at 663 or 451 nm. Although the four molecules of chlorophyll *a* and three molecules of β-carotene are likely in bound form within a single b_{559} molecule in aqueous solution, the lack of these chromophoric entities in the spectrum of intact b_{559} appears nevertheless enigmatic.

Lipid and Photosynthetic Role of Cytochrome b_{559}. Evidence from chloroplast studies in several laboratories [Knaff and Arnon, 1969; Erixon and Butler, 1971; Floyd *et al.*, 1971] has shown that the oxidation of cytochrome b_{559} occurs even at the temperature of liquid nitrogen and is mediated by light absorbed by chlorophyll. If this is a primary photochemical event, *i.e.*, independent of temperature, it would require structural continuity between the light-absorbing lipid(s), cytochrome b_{559}, and the electron acceptor. If the chlorophyll *a* and β-carotene found in the cytochrome b_{559} preparation are an integral part of the cytochrome molecule, possibly present with a single heme at the "hydrophobic" core, then the light absorption and photooxidation which ejects an electron from cytochrome b_{559} might occur entirely within the cytochrome b_{559} molecule.

Our studies suggest that homogeneous cytochrome b_{559} is a lipoprotein molecule of 110,000 mol wt containing one heme (noncovalently linked), about eight small polypeptide chains (each of 5600), 4 mol of chlorophyll *a*, 3 mol of β-carotene, about 40% by weight of a major, unknown polar lipid, and about 5–8% of a minor, unknown polar lipid. If correct, the biochemical composition of cytochrome b_{559} appears to be almost as complex and interesting as the unusual role of this molecule in the more complex process of photosynthesis.

Acknowledgments

We thank Mr. Leonard Bazar for assistance in operating the analytical ultracentrifuge and Dr. Edward Meighen for guidance in N-terminal analysis and determination of molecular weight. The SDS–polyacrylamide gel procedure for the estimation of molecular weight of small polypeptides (Figure 2) was developed in collaboration with Allan L. Stuart of our laboratory. The lipid chromatographic analyses were performed in the laboratory of Dr. Morris Kates (University of Ottawa); his advice and the patient help of his colleague, Dr. S. Kushwaha, are gratefully acknowledged, although the conclusions drawn are our own responsibility.

References

Appleby, C. A. (1969), *Biochim. Biophys. Acta 172*, 88.
Arnon, D. I. (1949), *Plant Physiol. 24*, 1.
Ashwell, G. (1957), *Methods Enzymol. 3*, 81.
Bligh, E. G., and Dyer, W. L. (1959), *Can. J. Biochem. 37*, 911.
Boardman, N. K. (1968), *Advan. Enzymol. 30*, 1.
Doeg, K. A., and Ziegler, D. M. (1962), *Arch. Biochem. Biophys. 97*, 360.
Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.

Erixon, K., and Butler, W. L. (1971), *Photochem. Photobiol. 14,* 427.
Floyd, R. A., Chance, B., and Devault, D. (1971), *Biochim. Biophys. Acta 226,* 103.
Garewal, H. S. (1973), *Anal. Biochem. 54,* 319.
Garewal, H. S., Singh, J., and Wasserman, A. R. (1971), *Biochem. Biophys. Res. Commun. 44,* 1300.
Garewal, H. S., and Wasserman, A. R. (1974), *Biochemistry 13,* 4063.
Goodwin, T. W. (1955), *in* Modern Methods of Plant Analysis, Vol. III, Paech, K., and Tracey, M. V., Ed., Heidelberg, Springer-Verlag, p 272.
Goodwin, T. W. (1965), Chemistry and Biochemistry of Plant Pigments, London, Academic Press, p 489.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177,* 751.
Gray, W. R. (1972), *Methods Enzymol. 25B,* 121.
Hatch, F. T., and Bruce, A. L. (1968), *Nature (London) 218,* 1166.
Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 59.
Knaff, D. B., and Arnon, D. I. (1969), *Proc. Nat. Acad. Sci. U. S. 63,* 956.
Kushwaha, S. C., Pugh, E. L., Kramer, J. K. G., and Kates, M. (1972), *Biochim. Biophys. Acta 260,* 492.
Lepage, M. (1964), *J. Chromatogr. 13,* 99.
Lowry, T. W. (1968), *J. Lipid Res. 9,* 397.
Pringle, J. R. (1970), *Biochem. Biophys. Res. Commun. 39,* 46.
Sastry, P. S., and Kates, M. (1964), *Biochemistry 3,* 1271.
Shapiro, A., Viñuela, E., and Maizel, J. (1967), *Biochem. Biophys. Res. Commun. 28,* 815.
Shaw, N. (1968), *Biochim. Biophys. Acta 164,* 435.
Shipski, V. P., and Barclay, M. (1969), *Methods Enzymol. 14,* 544.
Siakotos, A. N., and Rouser, G. (1965), *J. Amer. Oil Chem. Soc. 42,* 913.
Strauss, J. H., Burge, B. W., and Darnell, J. (1971), *J. Mol. Biol. 47,* 437.
Stuart, A. L., and Wasserman, A. R. (1973), *Biochim. Biophys. Acta 314,* 284.
Vaskovsky, V. E., and Kostetsky, E. Y. (1968), *J. Lipid res. 9,* 396.
Wasserman, A. R., and Fleischer, S. (1968), *Biochim. Biophys. Acta 153,* 154.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133,* 369.

Oxygen-Linked Association–Dissociation of *Helix pomatia* Hemocyanin†

Roel van Driel* and Ernst F. J. van Bruggen

ABSTRACT: Hemocyanin of *Helix pomatia* (molecular weight 9×10^6) contains approximately 180 oxygen-binding sites. Oxygen-binding and oxygen-linked association-dissociation phenomena of *Helix pomatia* α-hemocyanin at high ionic strength (I = 1.1) (pH 8.2) have been investigated. Oxygen binding is cooperative. Under oxygen-free conditions 65% of the protein is present as half molecules (molecular weight 4.5×10^6) and 35% as one-tenth molecules (molecular weight 0.9×10^6). Upon oxygen binding an abrupt, complete dissociation of the half molecules into one-tenth molecules is observed. Evidence is presented that both the associated and the dissociated states bind oxygen noncooperatively, the observed cooperativity being almost completely due to the ligand-linked dissociation. The oxygen pressures at half-saturation of the dissociated and associated proteins are approximately 4 and 40 mm, respectively. These oxygen-binding properties are compared to those under more physiological conditions. It is suggested that formation of a closed ring of five one-tenth subunits (*i.e.* association of subunits to a half molecule) is a necessary condition to constrain the protein to a state with low oxygen affinity.

The structure and oxygen-binding properties of α-hemocyanin of the Roman snail, *Helix pomatia,* and other molluscs have been studied extensively in the past few years (Van Holde and Van Bruggen, 1971; Er-el *et al.,* 1972; Mellema and Klug, 1972; Brouwer and Kuiper, 1973; Van Driel, 1973; Siezen, 1973). *Helix pomatia* hemocyanin has a molecular weight of 9×10^6, and contains about 180 oxygen binding sites. Under proper conditions of pH, ionic strength, and divalent cation concentration, half, one-tenth, and one-twentieth molecules can be observed (Siezen, 1973).

Siezen (1973) presented evidence that one-tenth subunits, under liganded conditions, can exist in two grossly different conformations. At low ionic strength (I = 0.1) the structure is rather loose, whereas at high salt concentrations (I = 1) the subunit is more compact. At low ionic strength, pH 8.2, one-tenth subunits bind oxygen noncooperatively. This paper reports the oxygen-binding properties of hemocyanin at high ionic strength, pH 8.2. Under these conditions oxygen binding is cooperative and is linked to a monomer-pentamer equilibrium between one-tenth and half molecules. The oxygen affinity of the pentamer is about tenfold lower than that of the monomer, and is similar to the affini-

† From the Biochemisch Laboratorium, Rijksuniversiteit, Groningen, The Netherlands. *Received March 22, 1974.* This work was supported by the Netherlands Foundation for Chemical Research (S.O.N.), with financial aid from the Netherlands Organization for Advancement of Pure Research (Z.W.O.). This is paper XV in a series entitled Structure and Properties of Hemocyanins.