Biochemistry

 Volume 13, Number 20 September 24, 1974

Triton X-100–4 M Urea as an Extraction Medium for Membrane Proteins. I. Purification of Chloroplast Cytochrome b_{559}†

Harinder S. Garewal‡ and Aaron R. Wasserman*

ABSTRACT: Extraction studies at pH 8 showed that 2% Triton X-100–4 M (or 2 M) urea, and only this medium, succeeded for the first time in quantitatively extracting all three chloroplast cytochromes (f, b_{559}, b_6) in stable form from ethanol-extracted chloroplast grana membranes. Assay of particulate cytochrome b_{559} was reliable only after dispersion by Triton–urea. Following a nonchromatographic binding of impurities to DEAE-cellulose in Triton–4 M urea containing dithiothreitol, cytochrome b_{559} was purified as a single electrophoretic species by preparative disc electrophoresis in 0.1% Triton X-100 (pH 8) in a highly selective (10%) polyacrylamide gel. Inclusion of 10% glycerol and 4 mM thioglycolate as a mobile sulfhydryl reagent were required to maintain the component as a single form during electrophoresis. When unprotected by thioglycolate, two to five additional (artifactual) b_{559} bands appeared in the gel possibly due to alteration by oxidation. Overall yield of this tightly bound membrane protein was about 30–50% of that originally present in ethanol-extracted particles. Prolonged stability of the cytochrome during storage requires dithiothreitol and 15% glycerol under a nitrogen atmosphere. The isolated cytochrome does not react with carbon monoxide and is completely reducible by ascorbate (pH 8) after autoxidation. Forms reducible by dithionite but not by ascorbate were not encountered. Cytochrome b_{559} is established as a b-type cytochrome in that its prosthetic group gives a reduced pyridine hemochrome identical with that of protoheme and the heme is quantitatively extractable by acid–acetone. The millimolar extinction coefficient for reduced b_{559} at its α peak ($A_{559} - A_{600}$) is 21 per mM heme per cm. The cytochrome is an intrinsically aggregatable molecule but is nondestructively dissociated by 0.1% Triton X-100 (pH 8) to a single form. The cytochrome is denatured but not disaggregated by 8 M urea or 6 M guanidine.

The extraction and purification of tightly bound membrane proteins with retention of their known properties has proved to be frustrating and challenging to many areas of biology. The chloroplast grana of higher plants contain three cytochromes—f (α absorption peak at 554 nm), b_{559} (α peak at 559 nm), and b_6 (α peak at 563 nm)—all tightly bound membrane proteins which are not released by extraction with salt solution or by prior delipidation of the membrane. Purification of these cytochromes was pursued in this laboratory with the primary objective of gradually developing general methods suitable for purification, proof of purity, and study of properties of all membrane proteins. Cytochromes offer many advantages in such studies, especially in ease of assay. In addition, loss of the characteristic α absorption peak of a cytochrome offers a convenient direct measure of cytochrome loss or modification in extraction media under investigation.

Purification of cytochrome f (Singh and Wasserman, 1971) and studies with pure membrane proteins (Singh and Wasserman, 1970) showed that disc electrophoresis with nonionic detergent could serve as a convenient test of aggregation for membrane proteins in clarified dispersions, as a final preparative step with the efficiency of a chromatographic method and as a much-needed method for analytical examination of purity and homogeneity of a membrane protein *without denaturation*. However, extraction of cytochrome f by aqueous 1-butanol–Triton X-100 (pH 8) resulted in irreversible loss of cytochromes b_{559} and b_6.

Lundegårdh (1962) observed an absorption peak in chloroplasts at 559 nm after reduction by dithionite or reduced pyridine nucleotide and designated it as cytochrome b_3, a component not exclusively confined to chloroplasts. Boardman and Anderson (1967) showed that the chloroplast component was

† 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.

ascorbate-reducible; they renamed it cytochrome b_{559} to avoid confusion with cytochrome b_3. Extraction of cytochrome b_{559} from particles has not been achieved.

Purification of cytochrome b_{559}, a relatively unstable protein, was pursued to develop a nondestructive general extraction medium for membrane proteins and to examine whether disc electrophoresis with nonionic detergent could serve in the purification and proof of purity and homogeneity of a relatively unstable protein. A preliminary communication from this laboratory (based on parts of the procedure developed here) reported a pure preparation of cytochrome b_{559} of lesser electrophoretic homogeneity (Garewal *et al.*, 1971).

Experimental Section

Materials

"Total Chloroplast Particle Fraction" (TCPF)[1] and washed P_1S (washed chloroplast grana membranes) were prepared from spinach leaves and stored frozen (−15°) in physiologically active form as previously described (Singh and Wasserman, 1971). DEAE-cellulose (Reeve Angel) was Whatman DE-11, medium fibrous powder.

Methods

Sonic Disruption. The instrument, manufactured by Blackstone Ultrasonics, Inc., consisted of probe Model BP2 with ⅝-in. probe tip and generator Model SS-2 capable of maximum power output of 200 W. It was set to position 40, out of a possible range of 100, and tuned to maximum pitch. Further details are given in Results.

Disc Gel Electrophoresis. The method of Davis (1964) was employed, but with the Triton X-100 modification and other conditions previously described (Singh and Wasserman, 1971), and the resolving gel buffer was 0.38 M Tris-HCl, pH 8 rather than 8.9 to increase cytochrome stability. Other conditions are noted, where appropriate, under Results. Location of protein bands in gels by staining and spectrophotometric location of cytochromes in gel slices were also described previously (Singh and Wasserman, 1971).

Spectrophotometric Assay of Cytochromes. Analyses by difference spectrophotometry of individual cytochromes in particles and during purification were performed essentially according to Hind and Nakatani (1970): for cytochrome b_{559}, ascorbate *vs.* hydroquinone; for cytochrome *f*, hydroquinone *vs.* ferricyanide; for cytochrome b_6, dithionite *vs.* ascorbate. (Ascorbate reduces cytochromes *f* and b_{559} but not cytochrome b_6; dithionite reduces all three cytochromes.) Absorption spectra during purification were recorded using a Phoenix–Chance scanning spectrophotometer whose response was linear down to its detection limit of about 0.0002 absorbance unit (1-cm cell). Accuracy of the absorbance values was checked by cross-calibration (using reduced cytochrome *c*) with a Cary Model 15 recording spectrophotometer. Absorbance spectra and absorbance data reported for pure cytochrome b_{559} were obtained with the Cary Model 15 spectrophotometer except for difference spectra at the temperature of liquid nitrogen (−196°) which were recorded on the Phoenix–Chance instrument equipped with low-temperature accessory (optical Dewar flask, 2-mm cells). One (absorbance) unit of cytochrome b_{559} is defined as that amount of pure reduced protein which when present in 1 ml gives an absorbance (absolute spectrum) of 1 (22°, 1 cm light path) at its α maximum at 559 nm relative to its absorbance at 600 nm. [One unit of the pure molecule assayed in a difference spectrum (reduced *vs.* oxidized) gives about 0.74 unit at 559 nm relative to the absorbance at 600 nm.]

Assays of Heme, Protein, and Chlorophyll. Heme was assayed as the reduced pyridine hemochrome (Appleby, 1969). Acid–acetone extraction of heme was performed by the method of Basford *et al.* (1957) but assayed as above. Protein determinations were made by the biuret method (Gornall *et al.*, 1949) with crystalline bovine serum albumin as standard; the biuret color of pure cytochrome b_{559} samples was corrected for heme contribution by an alkaline control containing cytochrome b_{559}. Chlorophyll content was determined by the method of Arnon (1949).

Preparation of "ethanol-extracted particles" at 0° from suspensions of TCPF (or P_1S) was performed as previously described (Garewal *et al.*, 1971).

Results

Experiments to Develop the Purification Procedure for Cytochrome b_{559}: Rationale. The experimental approach pursued three problems, of which the first two are classical requirements in protein purification: (1) *extraction* of the desired species into a clarified (*i.e.*, optically clear) aqueous dispersion, with quantitative retention of the desired protein(s) in the supernatant after centrifugation (centrifugation of the clarified suspensions at 30,000*g* for 30 min (followed, if desired, by 100,000*g* for 30 min) provided a convenient initial screening procedure to test the degree of extraction by various aqueous media); (2) *stability, i.e.*, retention of known molecular properties of the desired component(s) in the dispersion medium, at first for a brief time (1 hr) and then for a more extended time period (24 hr) which would certainly be a minimum time requirement for preparative purification steps employing such media; (3) *a molecular dispersion* of the desired component(s) (*i.e.*, as separate disaggregated molecules) either immediately susceptible to rational purification steps based on the separation of *molecules* or, failing that, susceptible to purification after an artfully improvised preliminary purification step. This third problem has been particularly difficult for membrane proteins in general in that purification of a clarified aqueous dispersion has often failed, possibly because the desired protein has been present in the form of polydispersed aggregates and/or in small membrane fragments. Accordingly, stable clarified dispersions of cytochrome b_{559} were examined by analytical disc electrophoresis in the nonionic detergent Triton X-100 (Singh and Wasserman, 1970, 1971) to determine whether cytochrome b_{559} behaved as a single electrophoretic species of small size demonstrably resolved from cytochromes *f* and b_6. In addition to their relevance to the purification of cytochrome b_{559}, assays of all three cytochromes were performed throughout these studies to indicate whether any extraction medium or purification step showed general promise for the methodology of membrane proteins.

Definitions. (1) Percentage survival ≡ percentage survival for time indicated in suspension of cytochrome b_{559} (or b_6 or *f*) = (total cytochrome b_{559} after addition of solubilizing agent/total cytochrome b_{559} before addition of solubilizing agent)100. (2) Percentage extraction = (total cytochrome b_{559} in supernatant after centrifugation/total cytochrome b_{559} in suspension before addition of solubilizing agent)100. (All values of percentage extraction for cytochromes b_{559}, *f*, and b_6 are relative to the amount of cytochrome found in the same aliquot of suspension using Triton–2 or 4 M urea medium, which was subsequently found to enable the most reliable assay, especially for cytochrome b_{559}.)

[1] Abbreviations used are: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TCPF, "total chloroplast particle fraction;" SDS, sodium dodecyl sulfate.

TABLE I: Effect of Supplementary Reagents on the Extraction of Cytochromes from "Ethanol-Extracted Particles" by 2% Triton X-100 [0.05 M Tris-HCl (pH 8) at 0°].

Reagent	% Survival[a] of Cytochromes in Suspension	%[a] Extraction of Cytochromes	Stability of Cytochromes *f* and b_{559} in Clarified Supernatants (0.002 M DTT; 24 hr)
Control: 2% Triton X-100 (0°)	100	30	100
Temperature:			
a. 22° (1 hr)	a. 100	a. 30–40	Not tested
b. 37° (1 hr)	b. 70–80%	b. 30–40	Not tested
0.01 M mercaptoethanol			
a. 0°	a. cyt. b_6 inactivated; cyt. *f* and b_{559} = 100%	a. 30	Not tested
b. 37°	b. cyt. b_6 inactivated; cyt. *f* and b_{559} = 70–80%	b. 30–40	Not tested
7 M urea	50–60 (1 hr)	Not tested	Not tested
5 M guanidine-HCl	40–50 (1 hr)	Not tested	Not tested
0.1% SDS			
a. 0°	*ca.* 50 (1 hr)	Not tested	Not tested
b. 37°	<20 (1 hr)	Not tested	Not tested
15% formamide	40 (2 hr)	Not tested	Not tested
Sodium thiocyanate			
a. 2 M	a. 50 (4 hours)	>80	a. 20
b. 1 M	b. 50–60 (4 hours)	>80	b. 20–30
c. 0.2 M	c. 60 (4 hours)	50	c. Not tested
0.001 M EDTA	100	30	Not tested
0.3 M KCl or NaCl (ionic strength)	100	20	Not tested
Guanidine-HCl			
a. 2 M	a. 100 (1 hr)	a. 100	a. 100
b. 1 M	b. 100 (1 hr)	b. 100	b. 100
Urea			
a. 4 M	a. 100 (2 hr)	a. 100	a. 100
b. 2 M	b. 100 (2hr)	b. 100	b. 100

[a] As defined in the text.

Extraction with Triton X-100; Difficulties in Reliable Assay. Washed chloroplast-grana (washed P_1S) or TCPF was extracted with ethanol so as to remove sufficient chlorophyll to permit optical assay of cytochromes b_{559}, *f*, and b_6. Storage of these ethanol-extracted particles as a divided frozen suspension at −10 to −20° in 0.05 M Tris-HCl (pH 8) required 15% Me_2SO as supplement, without which the content of cytochrome b_{559} was diminished. Sonication of ethanol-extracted particles for 2 min at 0° in a medium containing 2% Triton X-100 and 0.05 M Tris-HCl (pH 8) produced a clarified dispersion of the three chloroplast cytochromes. However, the percentage extraction of the cytochromes into a clear supernatant was usually only about 30% and was quite variable (a range of 20–80%). The range of Triton concentrations tested varied from 0.5 to 5%; a minimum of 1% Triton [a Triton to protein ratio of about 0.5 (g/g)] was required to give any extraction. Higher Triton concentrations did not significantly improve the extraction obtained with 1% Triton X-100. A 2% Triton concentration was arbitrarily chosen for further experimentation. Sonication times greater than 2 min (up to 10 min) did not improve the extraction; sonication by itself resulted in no loss of any of the cytochromes. Although the cytochromes were completely stable up to 4 hr in 2% Triton X-100, reliable assay of cytochrome b_{559} proved difficult in that its reduction by ascorbate often increased after a variable time lag of several minutes and the total b_{559} content was not satisfactorily reproducible with repeated clarified dispersions from a common source of ethanol-extracted particles. Since, in contrast, the total spectrophotometric content of all three cytochromes appeared to be relatively constant in different aliquots after reduction by dithionite, we presumed that some of the extracted cytochrome b_{599} might still exist in smaller particles incompletely permeable to ascorbate.

Extraction with Triton X-100 and Supplementary Reagents. Supplementation of 2% Triton X-100 (pH 8) with 1–2 M guanidine-HCl or 2–4 M urea were the only treatments producing both complete short term stability in the clarified suspensions and quantitative extraction of cytochromes b_{559}, *f*, and b_6 (Table I). Guanidine (1 or 2 M) or urea (2 or 4 M) without Triton X-100 produced poor extraction. Assay of cytochrome b_{559} (as well as *f* and b_6) proved completely reproducible in Triton-2 or 4 M urea or in Triton-1 or 2 M guanidine (either with clarified total suspensions or with supernatants) and *no lag was now observed in the reduction of* b_{559} *by ascorbate.* The content of cytochrome b_{559} in the extracts decreased upon storage at 0° beyond 1–2 hr and was reduced to 60–70% of original content by 24 hr. Cytochrome *f* was more slowly inactivated but the surviving *f* content was predominantly reducible only by dithionite and not by ascorbate. (Cytochrome b_6 content was stable even at 24 hr.) The addition of

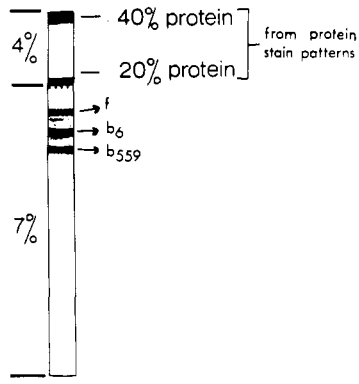

FIGURE 1: Disc gel electrophoretic pattern of initial clarified extract from ethanol-extracted particles. Sample was applied in the extraction medium of 2% Triton X-100–4 M (or 2 M) urea (pH 8) adjusted to either 10% sucrose or 10% glycerol before application. The electrode buffer and gels were supplemented with 1% Triton X-100 only. Electrophoretic time varied from 18 to 24 hr; shorter times produced even poorer resolution. The dotted line represents the border between the 4% stacking gel and 7% resolving gel. Non-cytochrome protein stain bands (at least three to four in number) occurring in the region of the cytochromes are omitted for reasons of clarity. Considerable cross-contamination occurred between the three cytochrome bands. Additional detail is furnished in the text.

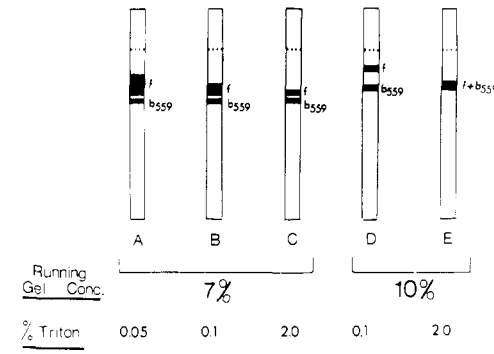

FIGURE 2: Disc gel electrophoretic separation of cytochrome b_{559} from cytochrome *f* as a function of resolving gel concentration and Triton X-100 concentration. The indicated concentrations of Triton X-100 were added as supplement to gels and electrode buffer. The electrode buffer and stacking gel (4% in all cases) were supplemented with 1 mM DTT; the resolving gels were each supplemented with 4 mM DTT. Duration of electrophoresis was about 18 to 20 hr. The dotted lines mark the border between stacking and resolving gels. In addition to the two cytochromes, two or three minor protein bands (not shown) were usually seen in the stain pattern outside the cytochrome region. Although several other combinations of resolving gel and Triton X-100 concentration were tested, only typical cases illustrating the basic trends observed are presented here. Additional information is furnished in the text.

2 mM DTT as reducing agent resulted in the complete survival (in the reduced form) of both cytochromes b_{559} and *f* in the extracts for at least 24 hr. Stability values (at 24 hr) of cytochrome b_{559} in the presence of DTT (shown in Table I) were approximate estimates corrected for the known small absorption of pure cytochrome *f* at 559 nm (Singh and Wasserman, 1971) and were made by comparison with fresh extracts supplemented with DTT in assay. (Cytochrome b_6 was inactivated by DTT or other thiols even in the absence of 2 M guanidine or 4 M urea but its loss did not increase the content of cytochromes *f* or b_{559}.)

Disc Electrophoresis of Initial Extract. Extracts of cytochromes b_{559}, *f*, and b_6 in Triton–1 or 2 M guanidine did not migrate satisfactorily in disc electrophoresis because of guanidine's high charge. Freshly prepared extracts of the three cytochromes in Triton–2 or 4 M urea were examined by disc electrophoresis with 1% Triton in the system. (No DTT was added to the extract so as to retain cytochrome b_6.) Figure 1 shows that all three cytochromes penetrated and migrated in the 7% resolving gel but a conclusion that the cytochromes had existed as separate molecular species in the dispersion was equivocal: electrophoretic stacking was slow (4–5 hr) and each of the resolved cytochromes showed cross-contamination from the other cytochromes. [Using Triton concentrations higher than 1% (up to 5%) or 1% Triton–2 or 4 M urea in the gel system did not improve these results.]

Preliminary Purification to Enable Subsequent Disc Electrophoresis. Preliminary purification of the 2% Triton–2 or 4 M urea extract (no DTT) *via* ammonium sulfate fractionation was ineffective in that over 90% of the total protein including 100% of all three cytochromes precipitated between 15 and 20% saturation of ammonium sulfate (0.05 M Tris-HCl (pH 8), 0°, experimental time of about 0.5–1 hr). Preliminary purification of cytochromes *f* and b_{559} was achieved by applying the original extract containing 2% Triton–2 or 4 M urea, 0.05 M Tris-HCl (pH 8) and 5 mM DTT to a column of DEAE-cellulose equilibrated with 2% Triton–2 M urea, 0.05 M Tris-HCl (pH 8), and 2 mM DTT and eluting with the column equilibration medium. Cytochrome b_6 was not present in the eluate and was likely bound and/or unstable to DTT. Cytochromes *f* and b_{559} did not bind to the column and emerged in a green eluate fraction. When the applied sample contained 4 M urea rather than 2 M, cytochromes *f* and b_{559} were recovered in smaller volume. Considerable amounts of red and brown material were visibly bound to DEAE-cellulose extending to ¾ the length of the column. Since cytochromes *f* and b_{559} were both in reduced form in the green eluate containing DTT, precise assay was not possible but the two cytochromes appeared to be quantitatively recovered after this binding step (relative to aliquots of applied extracts assayed in the presence or absence of DTT) and the eluted sample contained only about 3% of the applied total biuret protein. The eluted solution was concentrated with dry Sephadex G-25 and again examined by disc electrophoresis in 1% Triton X-100. In contrast to the original extract (*cf.* above) this sample stacked to a sharp red band within 1 hr and, in addition to one or two minor colorless protein bands, two visibly resolved major red bands were seen in the 7% gel; the faster moving band was cytochrome b_{559}, cytochrome *f* was a few millimeters behind it. It was empirically determined that *satisfactory electrophoretic stacking and resolution occurred (even with 100 to 200γ of protein) but only when binding of red and brown impurities to DEAE-cellulose was complete.* When these latter pigments extended beyond ¾ the length of the previous column (thus perhaps being incompletely removed), electrophoretic stacking and resolution was poor and was correlated with *small* amounts of brown pigments unable to penetrate the 4 and 7% gels. Failure of unequivocal molecular behavior in electrophoresis either by original extracts or the latter incompletely purified eluates thus suggested that the electrophoretic difficulty was attributable to gel blockage by a "trouble fraction" rather than to overloading of the applied sample (*cf.* Discussion).

Parameters in Disc Electrophoretic Separation. A 10% resolving gel containing 0.1% Triton X-100 achieved the best resolution of cytochromes *f* and b_{559} (Figure 2) and proved adequate in subsequent preparative electrophoretic experiments.

The stability of cytochrome b_{559} during storage and during electrophoresis was further examined using a preparation purified free of cytochrome *f* by small-scale preparative electro-

phoresis (10% gel, 0.1% Triton X-100). Cytochrome b_{559} solutions stored at 0° in 0.05 M Tris-HCl (pH 8) were completely stable for 48 hr when supplemented with 5 mM DTT and under N_2. After this time oxidation and subsequent inactivation was observed which could not be reversed with dithionite. Complete cytochrome stability up to 15 days was possible with further daily increments of 2 mM DTT. Neither ascorbate (1 mM, 5 mM) nor EDTA (1 mM) improved the stability obtained with DTT alone. Cytochrome b_{559} was completely stable for months when stored frozen (−20°) under nitrogen in 0.05 M Tris-HCl (pH 8)–5 mM DTT but only when either 15% Me_2SO or 15% glycerol was present. For routine storage (−20°) 15% glycerol was chosen. Inclusion of 10% glycerol as supplement in all gel media during preparative disc electrophoresis both sharpened the bands of cytochromes *f* and b_{559} and improved the recovery of cytochrome b_{559}.

Prevention by Thioglycolate of Artifactual Electrophoretic Forms. Cytochrome b_{559} purified free of cytochrome *f* by preparative electrophoresis (10% resolving gel containing 0.1% Triton X-100 and 10% glycerol as supplements) was examined by analytical disc gel electrophoresis (Figure 3, gel 1). Up to five bands were encountered in the stain pattern with a major b_{559} band (B) followed by three faster moving red bands and preceded by one very faint band (A). That the multiple bands might be artifacts produced by oxidation of a single form of b_{559} during electrophoresis (rather than "isozymes") was explored by introducing thioglycolate as a mobile (negatively charged) thiol stabilizing agent during electrophoresis. When a layer of 20 mM Tris-thioglycolate preceding the sample layer was run through the gels to reduce any oxidants in the gel, the pattern was improved (gel 2). The best pattern (gel 3) was achieved simply by supplementing the sample and the electrode buffer with 4 mM thioglycolate (no prelayer of thioglycolate was required). Only band A (less than 1% of the protein stain and barely detectable spectrophotometrically as b_{559}) and band B (over 99% of the protein stain and containing b_{559} spectrophotometrically) were present even after 20–24 hr of electrophoresis.

Purification Procedure. All operations were performed at 0° or in a 4° cold room. A typical preparation is evaluated in Table II. Additional procedural detail is given by Garewal *et al.* (1971) and Table II.

STEP 1: EXTRACTION OF CYTOCHROMES *f*, b_{559}, b_6. A suspension of ethanol-extracted particles was centrifuged (30,000*g*, 5 min); the pellet was then resuspended by hand-homogenization in 2% Triton X-100, 4 M (or 2 M) urea, and 0.05 M Tris-HCl (pH 8) and the particles were then sonicated (30–40-ml batches) for 2 min at 0°. (*Temperature* during sonication *should not* be allowed to *exceed 7–8°* even briefly. After sonication was completed, the dispersion *was immediately cooled in ice to 0–2°*.) After centrifugation (30,000*g*, 30 min), a second, optional centrifugation (90,000*g*, 30 min) allows the use of a smaller amount of DEAE-cellulose in the next step and the procedure is thus shortened.

STEP 2: BINDING OF IMPURITIES TO DEAE-CELLULOSE TO PERMIT SUBSEQUENT ELECTROPHORESIS. Spectrophotometric assays of eluted fractions were performed using the DTT-containing sample *vs.* ferricyanide-oxidized sample (both *f* and b_{559} are reduced by DTT and their absorption peaks overlap, but a semiquantitative assay of each cytochrome can be obtained). Whenever colored impurity bands (likely carotenoid-containing material) extended over the entire length of the column, *even slight traces* of the brown and orange-red impurities eluted with the cytochromes *resulted in failure of subsequent electrophoresis.* In such cases a second, smaller DEAE-cellulose column prior to electrophoresis was obligatory.

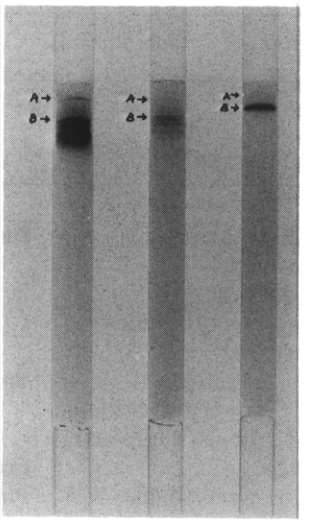

FIGURE 3: Effect of thioglycolate (as a mobile sulfhydryl stabilizing agent) on the disc electrophoretic homogeneity of pure cytochrome b_{559}. A 4% stacking gel (pH 6.7) and 10% resolving gel (pH 8) were both supplemented with 10% glycerol. The electrode buffer and stacking gel were supplemented with 1 mM DTT and 0.1% Triton X-100; the resolving gel contained 4 mM DTT and 0.1% Triton X-100. The sample was supplemented with 15% glycerol before application. (Since the stacking gels were very fragile, they had to be removed prior to photography but contained no protein bands.) Time of electrophoresis was 20–24 hr. Gel 1, the pattern obtained in the absence of thioglycolate. The positions of bands A and B (see text) are indicated. The faster moving bands seen in the figure are the bands C, D, and E referred to in the text. Gel 2, the pattern obtained when a layer of 0.1 ml of 20 mM Tris-thioglycolate was run through the gels to reduce any oxidants in the gels. The electrode buffer and sample were supplemented with 1 mM Tris-thioglycolate (in addition to 1 mM DTT). The positions of bands A and B are shown. Fewer faster moving bands and of lesser intensity were seen compared with gel 1. Gel 3, the pattern obtained when both the sample and electrode buffer were supplemented with 4 mM Tris-thioglycolate. Only band A (less than 1% of the total protein stain) and band B (over 99% of the protein stain) were seen in the gel.

STEP 3: PREPARATIVE DISC GEL ELECTROPHORESIS IN TRITON X-100. A 2-cm long 4% stacking gel and a 8-cm long 10% resolving gel were used. Both the stacking and resolving gel media contained 10% glycerol as supplement. Instead of the "normal" Davis (1964) pH value of pH 8.9 in the resolving gel, pH 8.0 was used since the cytochromes were found to be more stable at this pH. *The electrode buffer was supplemented with 4 mM Tris-thioglycolate and 1 mM DTT.* The sample was adjusted to 4 mM Tris-thioglycolate and applied beneath the electrode buffer using a 10-ml syringe fitted with long, thin rubber tubing. The electrode buffer with its thiol preservatives was changed every 16 hr during electrophoresis. Elution of b_{559} from its gel slice was performed by homogenizing the gel in 7 volumes of 0.05 M Tris-HCl, 5 mM DTT, and 15% glycerol (no detergent), stirring for 2 hr (under nitrogen), and centrifuging out the gel (30,000*g*, 1 hr). Foaming during the homogenization or later should be very carefully avoided as this inactivates the cytochrome. The pure cytochrome was concentrated using dry Sephadex G-25 and was best stored frozen (−20°) in 0.05 M Tris-HCl (pH 8), 5 mM DTT, and 15% glycerol under nitrogen.

The overall yield of pure b_{559} was high, about 30–50% of that present in ethanol-extracted particles. Either TCPF or washed P_1S (chloroplast grana) as the starting particle preparation gave the same pure b_{559} component.

TABLE II: Purification of Cytochrome b_{559}.

Fraction	Color	Volume (ml)	Total cyt. b_{559} (units)	Total Protein (mg)	Specific Content (units/mg protein)	Fold Purification
1. Particles						
a. Original chloroplast particles[a]	Dark green	274	16.9[b]	5754	0.0029	
b. Ethanol-extracted particles (in Triton–low urea)	Brownish	110	16.9	4180	0.0041	1.4
c. Supernatant after 30,000g centrifugation of Triton-4 M urea extract	Brownish green	95	16.9	1615	0.011	3.8
2. DEAE-cellulose eluate	Light green	215	c	53.6		
3. Preparative polyacrylamide electrophoresis, elution, and concentration by Sephadex G-25	Pink-orange	18.6	5.6[d] (7.6)	14.5	0.39 (0.52)[e]	134

[a] Chlorophyll concentration was 2.0 mg/ml. [b] This content could not be assayed but was an assumed value from the cytochrome b_{559} content in ethanol-extracted particles (1b). [c] This fraction could not be accurately assayed because of the presence of DTT (DTT reduces both cytochromes f and b_{559}). [d] Losses encountered after the previous analysis of step 1, as known from repeated analyses of the following individual preparative steps, were due to: (a) concentration by Sephadex G-25 of the eluate after DEAE-cellulose (70–80% recovery as assayed using quantifiable substances); (b) electrophoresis and elution (about 80% recovery); (c) concentration after elution (about 80% recovery); (d) the loss in step 2 (estimated indirectly as being no more than 12%). Thus none of the steps resulted in large losses. [e] Based on absolute spectra.

Evidence for Purity and Homogeneity. (1) (Strongest evidence) Analytical disc gel electrophoresis of b_{559} (in undenatured form) with 0.1% Triton X-100 in a 10% gel (Figure 3, gel 3) showed one b_{559} band accounting for over 99% of the protein stain. A slower moving component (band A), barely detectable spectrophotometrically as b_{559}, contributed less than 1% of the total protein stain. No cytochrome f or any other protein was detected in any section of the gel. Electrophoresis at pH values other than those employed here was precluded by the cytochrome's instability at pH 6 or below and at pH 8.9 or above. (2) Absence of cytochrome f was also indicated by the absorption spectra of pure cytochrome b_{559}: its difference spectrum at 22° is shown in Figure 4; its reduced absolute spectrum (22°) and difference spectrum at −196° were identical with those previously reported (Garewal *et al.*, 1971) for a preparation of equal purity but lesser electrophoretic homogeneity. (3) Only one sedimenting species was observed in analytical ultracentrifuge studies using the method of Yphantis (1964) and a medium containing 0.05 M Tris-HCl (pH 8), 5 mM DTT, 0.1% Triton X-100, and 15% glycerol. No additional component was observed at the bottom of the cell. The sample was quantitatively recovered after the experiments, thus indicating that the single species observed was not a denatured artifact. (4(Denaturation of the component in sodium dodecyl sulfate and mercaptoethanol followed by disc electrophoresis in SDS (Weber and Osborn, 1969) yielded only one sharp band (R_F 0.51) in a 20% gel.

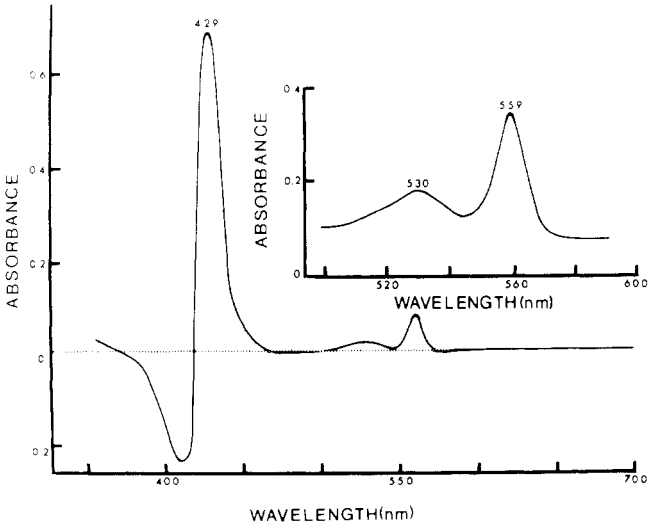

FIGURE 4: Difference spectrum (reduced *vs.* oxidized) of pure cytochrome b_{559} at 22°. The cytochrome was present in 0.05 M Tris-HCl (pH 8) containing 15% glycerol. The sample cuvet contained the cytochrome reduced in the presence of DTT and the reference cuvet contained an identical sample oxidized by a few colorless crystals of ammonium persulfate. The inset shows the α and β peaks in greater detail using a more concentrated sample.

Stability. Cytochrome b_{559} was perfectly stable for months when stored frozen under nitrogen at −20° in 0.05 M Tris-HCl (pH 8), 5 mM DTT, and 15% glycerol. Repeated freezing and thawing (where necessary) did not alter the cytochrome in any way. Liquid solutions of the cytochrome at 0° in the above medium (under N_2) were also stable for at least 2 weeks provided the DTT level was maintained. (Excessive bubbling during gassing with N_2 inactivated the cytochrome). Ascorbate (5 mM) as reducing agent could replace DTT but the time of stability at 0° was somewhat reduced. The cytochrome was stable at pH 7 (phosphate buffer) but was inactivated at pH 6 and pH 8.9. *The cytochrome was inactivated even at pH 8, 0° by commonly employed disaggregating agents such as 6 to 8 M urea, 4 M guanidine-HCl, and 0.1% SDS* even when supplemented with 5 mM DTT.

Absorption Spectra. The difference spectrum in the visible region (reduced *vs.* oxidized) at 22° is presented in Figure 4. Visible absorption spectra at 22° (either difference, absolute oxidized, or absolute reduced) were unaffected by the presence of Triton X-100. Absolute spectra at 22° and the difference

spectrum at −196° were identical with those previously reported for a preparation of equal purity but lesser homogeneity (Garewal *et al.*, 1971).

Ascorbate Reducibility. The pure component was fully reducible by sodium ascorbate (pH 8); sodium dithionite produced no further reduction. At no stage of the purification was any "dithionite-reducible" form of b_{559} observed (*i.e.*, a form reducible by dithionite but not by ascorbate) although some was observed when the pure sample became unstable occasionally during prolonged storage. The pure component showed no detectable reduction by hydroquinone *at pH 8.*

Nonreactivity with Carbon Monoxide. The reduced spectrum of cytochrome b_{559} (in the presence of DTT) remained unaltered after passing carbon monoxide through the sample (in the absence or presence of 0.1% Triton X-100).

Autoxidation. The pure cytochrome was autoxidizable (pH 8, 0°). Subsequent complete reduction by ascorbate (or alternatively by DTT or dithionite) was possible when performed within 1-2 hr, but longer storage in oxidized form led to irreversible inactivation.

Prosthetic Group and Millimolar Extinction Coefficient. The spectrum of the reduced pyridine hemochrome derived from cytochrome b_{559} was identical with that obtained from hemoglobin in the same set of analyses. The prosthetic group of b_{559} is thus a b-type heme (protoheme). All the b_{559} heme was extractable by acid-acetone at 0°, indicating that it is probably not covalently linked. These two properties, together with its native reduced α peak at 559 nm, thus firmly establish the pure component as a *b*-type cytochrome. The millimolar extinction coefficient for reduced b_{559} at its α peak (559 nm) (absolute spectrum) was calculated as 21 ($A_{559\,nm} - A_{600\,nm}$) per mmole of heme per liter per cm.

Reversible Reaggregation upon Removal of Triton X-100 (pH 8). To remove Triton X-100, 1 ml of pure b_{559} originally containing less than 0.1% Triton X-100 was dialyzed thoroughly against 1 l. of a dialysis buffer containing 0.05 M Tris-HCl, 2 mM DTT, and 15% glycerol (pH 8) under nitrogen. The dialysis was continued for 3 days at 4° with the dialysis fluid being changed every 24 hr. The cytochrome was completely recovered and *soluble* after dialysis. The sample was then examined by disc gel electrophoresis employing a 4% stacking gel and a 10% resolving gel under the conditions described in Figure 3, gel 3, except that Triton X-100 was omitted from the system (sample, gels, and electrode buffer). The cytochrome penetrated the stacking gel but stopped at the border between the stacking and resolving gels where it persisted as a dark red band despite 12 additional hr of electrophoresis. Subsequent analysis of the gel showed that all of the protein stain and all of the cytochrome content of the gel was confined to this band at the top of the resolving gel. The dialyzed, aggregated cytochrome could be subsequently quantitatively, nondestructively disaggregated by supplementation with 0.1% Triton X-100 in that electrophoresis in a system containing this detergent concentration resulted in the sample's penetration into the 10% resolving gel and migration in it as a single sharp red band.

Nondissociation by 8 M Urea or 6 M Guanidine-HCl (pH 8). Solutions of cytochrome b_{559} were dialyzed to remove Triton X-100 and shown to be quantitatively reaggregated upon examination in disc electrophoresis (*cf.* previous section). The samples were then incubated at 37° for 3 hr in a solution containing 0.05 M Tris-HCl (pH 8), 1% mercaptoethanol, and either 8 M urea or 6 M guanidine-HCl (under nitrogen). Both treatments resulted in the complete loss of b_{559} spectrum. Since the high charge of samples containing 6 M guanidine-HCl prevented satisfactory disc electrophoresis, samples incubated in guanidine were subsequently thoroughly dialyzed against 8 M urea-0.05 M Tris-HCl (pH 8) at 22°. Examination of b_{559} by disc electrophoresis containing 6 M urea throughout the system (but no Triton) revealed that neither prior treatment with urea nor with guanidine had dissociated b_{559}: in either case a single denatured protein "band" was seen at the border between a 4% stacking gel and a 10% resolving gel.

Discussion

Triton-4 M Urea (pH 8) as an Extracting Medium. The quantitative extraction of tightly bound membrane proteins into a *clarified* dispersion with retention of their known biological properties has heretofore been a difficult art not only for a variety of cytochromes and flavoproteins but for many other membrane proteins of biological interest. Our present studies have demonstrated that a combination of 2% Triton X-100 (a nonionic detergent) with low concentrations of urea (2-4 M) effectively extracted all three chloroplast cytochromes under nondenaturing conditions (pH 8, 0°). This extracting solvent had not previously been used successfully in the extraction and purification of membrane proteins with retention of their properties. All three cytochromes could be quantitatively maintained in stable form for at least 24 hr; cytochrome b_6 was stable without any stabilizing supplements but inactivated by thiols, whereas cytochromes *f* and b_{559} required a reducing environment (DTT) for complete stability. In addition to cytochrome b_{559} here, subsequent studies showed that cytochrome *f* (Garewal *et al.*, 1974) and cytochrome b_6 (Stuart and Wasserman, 1973) could each be purified to disc-electrophoretic homogeneity from an initial, clarified dispersion of ethanol-extracted spinach chloroplast grana in Triton-4 M urea. The present studies could not establish whether the three chloroplast cytochromes had been dispersed by Triton-4 M urea into a solution of separate molecules since direct disc electrophoresis of the original extract, although partially successful, failed to achieve an unequivocal resolution of the cytochromes. However, based on this work we postulated that Triton-4 M urea (pH 8) does produce a molecular dispersion of the cytochromes but that a high-molecular weight fraction or "trouble-fraction" in this dispersion somehow prevents satisfactory subsequent disc electrophoresis (perhaps by clogging the gel pores and preventing entry of even truly disaggregated molecules). Cytochrome b_6 and cytochrome *f* were subsequently shown to be present as separate molecules in the original extract in that each was satisfactorily chromatographed on a Bio-Gel A 1.5m molecular-sieving column in Triton-4 M urea with the "trouble fraction" excluded with the front (Stuart and Wasserman, 1973). Since cytochrome b_6 is not stable in the presence of thiols, the deletion of thiols from this column led to the inactivation of cytochrome b_{559}, which requires a reducing agent for stability. We have recently found that all three cytochromes can be chromatographed on Bio-Gel A 1.5m in Triton-4 M urea as separate single fractions when a nonthiol reducing agent is employed. Thus cytochrome b_{559} also existed as separate molecules in the original dispersion (A. L. Stuart and A. R. Wasserman, unpublished experiments). Subsequent studies have shown that dispersion with Triton-4 M urea (pH 8) followed by removal of trouble fraction permits disc electrophoretic separation without denaturation of most of the proteins (including several enzymes) from a variety of biological membranes (H. S. Garewal, A. L. Stuart, and A. R. Wasserman, manuscript in preparation). Consequently this medium consisting of a combination of the nonionic detergent Triton X-100 with low concentrations of urea (2-4 M) has potential as a general medium for the nondestructive solubilization of membrane proteins.

Binding of protein impurities on a column of DEAE-cellulose in Triton–2 M urea, although an effective purification step in the present procedure, is not a chromatographic procedure since cytochrome b_{559} (and cytochrome *f*) moved with the solvent front. Furthermore, we could not subsequently elute and resolve the bound impurities. Thus this step is not proposed as generally useful for purifying membrane proteins.

Preparative disc gel electrophoresis in nonionic detergent, previously devised for the complete purification of cytochrome *f* (Singh and Wasserman, 1971), has been shown suitable for the purification of cytochrome b_{559}, a relatively unstable protein. Inclusion of 10% glycerol and thioglycolate (an electrophoretically mobile sulfhydryl agent) as supplements during electrophoresis prevented losses and maintained the cytochrome as a single species. We have subsequently found that 10% glycerol is a generally beneficial stabilizing supplement during disc electrophoresis both for cytochrome b_6 (Stuart and Wasserman, 1973) and for mammalian membrane enzymes (H. S. Garewal, A. L. Stuart, and A. R. Wasserman, manuscript in preparation). Inclusion of thioglycolate prevented the formation (possibly *via* oxidation) of several artifactual bands of cytochrome b_{559} which might otherwise have been misinterpreted as "isozymes." The best electrophoretic purification of cytochrome b_{559} was accomplished using a 10% resolving gel and 0.1% Triton X-100; optimum values for each parameter required an empirical solution. Subsequent studies with cytochrome b_6 and other enzymes indicate that the optimum values for these parameters vary with each new protein to be purified. The required concentration of Triton X-100 during electrophoresis is possibly a net balance between the minimum amount needed for disaggregation of both the desired species and closely migrating contaminants and higher levels whose viscosity or density might impede resolution.

How Many Distinct Molecular Forms of Cytochrome b_{559} are There in Chloroplast Particles? Wada and Arnon (1971) have noted three forms of cytochrome b_{559} in spinach chloroplasts and inactivated chloroplasts. The forms have identical absorption spectra, but they differ from one another in their response to the added reductants dithionite, ascorbate, and hydroquinone. The low-potential form, L, is reducible by dithionite only; the middle-potential form, M, is reducible by ascorbate or dithionite but not by hydroquinone; the high-potential form, H, is reducible by hydroquinone or ascorbate or dithionite. In evaluating the complex literature on cytochrome b_{559} in particles we find it semantically desirable to adhere strictly to this nomenclature, as defined above. (1) L form—Wada and Arnon (1971) concluded that the L form arises from the M and H forms when chloroplasts lose their function as a result of drastic treatments; b_{559} was exclusively in the L form in acetone-extracted chloroplasts. Our own conclusion is that no reliable evidence supports an L form even in inactive particles, but that this form in particles is an artifact of assay due probably to the impermeability of ascorbate into inactive particles, especially particles extracted with organic solvents. As we have noted in Results, reliable assay of b_{559} in ethanol-extracted chloroplasts was achieved only in the presence of Triton–2 or 4 M urea (without any time lag), and the cytochrome is fully reducible by ascorbate (M form). Assay of these particles either directly or in Triton X-100 alone produced lower and quite variable quantities of the M form, and a lag in reduction of several minutes often occurred. (Triton alone produces a clarified dispersion probably consisting of smaller membrane fragments and not a dispersion of molecules.) Although we have not assayed b_{559} in heated or aged green chloroplasts, one could not preclude *a priori* an increased impermeability to ascorbate by such particles. Assay of the M form in such particles in Triton–2 or 4 M urea would be required to establish whether its content had been diminished. The absence of ascorbate-reducible b_{559} (M form) in acetone-extracted particles noted earlier by Boardman and Anderson (1967) and by our laboratory (Garewal *et al.,* 1971) was also an incorrect interpretation in that dispersion of such particles in Triton–2 or 4 M urea (pH 8) results in the complete immediate reduction of cytochrome b_{559} by added ascorbate. (2) H form—Wada and Arnon (1971) have concluded that this hydroquinone-reducible form predominates in freshly prepared green particles. Since functionally active chloroplasts can reduce cytochrome b_{559} in the dark, an untreated *vs.* ferricyanide control spectrum (absent in their paper) is necessary before one can conclude that the b_{559} spectrum was directly produced by hydroquinone. Cramer *et al.* (1971) have observed that the addition of hydroquinone to functionally active particles previously oxidized by ferricyanide produces the reduction of b_{559} (H form); the ratio of H to M form increased markedly as the pH dropped from 8.4 to 7.0. Although the existence of a hydroquinone-reducible form is tenable, an alternate argument might be entertained: excess oxidant inhibits or otherwise prevents b_{559} reduction in the dark. When hydroquinone reduces this added oxidant (and perhaps unknown physiological oxidants as well), physiological electron flow quickly is resumed and b_{559} (M form) is reduced. Thus there may be no H form; hydroquinone may act indirectly on the system rather than by a direct reduction of b_{559}. Wada and Arnon (1971) have observed that a number of treatments which lower the activity of chloroplasts (including the addition of Triton X-100) result in a conversion of H form to M form. Since all of these treatments reduce physiological electron flow, they might merely abolish the endogenous dark reduction of a single form of b_{559} (M form), rather than each treatment resulting in the conversion of H to M as the authors conclude. It would appear at the present time, despite the abundance of observations, that none of the available information compels acceptance of more than one form of b_{559} in chloroplast particles, likely the M form.

The purification procedure has isolated a cytochrome b_{559} preparation which does not combine with carbon monoxide; the cytochrome is also fully reducible by ascorbate either as dissociated molecules or when present as pure aggregates after depletion of Triton X-100. Each of the steps essentially achieved quantitative recovery; at the minimum no major form of the cytochrome was discarded or lost by accident. The extracting solvent of Triton–4 M urea (pH 8) has been used by us subsequently in extracting and purifying several delicate mammalian enzymes with retention of high enzymic activity (H. S. Garewal, A. L. Stuart, and A. R. Wasserman, manuscript in preparation). Cytochrome *f* thus far has been the only molecule whose properties were altered in Triton–4 M urea. Although cytochrome *f* was largely converted to a dithionite-reducible form in Triton–4 M urea (pH 8), it became fully ascorbate-reducible again after its removal from this medium (Garewal *et al.,* 1974). A possible objection to the final preparation which is potentially more serious is that the cytochrome might originally have been exclusively hydroquinone-reducible (H form) but was converted to the ascorbate-reducible form (M) in the primary step of preparing ethanol-extracted particles. (Ethanol extraction was performed merely to permit spectrophotometric assay.) However, this extraction and the overall procedure did not deplete the cytochrome of all lipid content (Garewal and Wasserman, 1974). In addition, subsequent more limited studies also indicate that most, perhaps all, of the cytochrome that can be purified from *undelipidated* chloro-

plast particles is in the M form (H. S. Garewal, A. L. Stuart, and A. R. Wasserman, unpublished experiments).

Cramer *et al.* (1971) have proposed that the M and H forms of cytochrome b_{559} in active particles are interconvertible, an attractive hypothesis whose validity might be tested *in vitro* using our pure preparation. Although we found no hydroquinone reduction at pH 8 (0.05 M Tris-HCl buffer and 10% glycerol), future investigations at lower pH or with other changes in solution might succeed in allowing reduction of the pure preparation by hydroquinone. It is clear from our studies of stability of the cytochrome that such future studies as well as any valid determinations of the redox potential of cytochrome b_{559} will require extraordinary care: the reducing agent must be removed quickly, at 0–5°, under nitrogen, and aliquots used for such experiments must not be permitted to remain oxidized for more than a few hours even at 0°.

Acknowledgment

We thank our former colleague Dr. Jasbir Singh for his advice and encouragement and Allan L. Stuart for permission to cite designated unpublished experiments.

References

Appleby, C. A. (1969), *Biochim. Biophys. Acta 172,* 88.
Arnon, D. I. (1949), *Plant Physiol. 24,* 1.
Basford, R. E., Tisdale, H. D., Glenn, J. L., and Green, D. E. (1957), *Biochim. Biophys. Acta 24,* 107.
Boardman, N. K., and Anderson, J. M. (1967), *Biochim. Biophys. Acta 143,* 187.
Cramer, W. A., Fan, H. N., and Böhme, H. (1971), *Bioenergetics 2,* 289.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121,* 404.
Garewal, H. S., Singh, J., and Wasserman, A. R. (1971), *Biochem. Biophys. Res. Commun. 44,* 1300.
Garewal, H. S., Stuart, A. L., and Wasserman, A. R. (1974), *Can. J. Biochem. 52,* 67.
Garewal, H. S., and Wasserman, A. R. (1974), *Biochemistry 13,* 4072.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177,* 751.
Hind, G., and Nakatani, H. Y. (1970), *Biochim. Biophys. Acta 216,* 223.
Lundegårdh, H. (1962), *Physiol. Plant. 15,* 390.
Singh, J., and Wasserman, A. R. (1970), *Biochim. Biophys. Acta 221,* 379.
Singh, J., and Wasserman, A. R. (1971), *J. Biol. Chem. 246,* 3532.
Stuart, A. L., and Wasserman, A. R. (1973), *Biochim. Biophys. Acta 314,* 284.
Wada, K., and Arnon, D. I. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 3064.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.