STUDIES ON FRACTION I PROTEIN

ERRATUM

Read "Triticale" for "triticales".

STUDIES ON FRACTION I PROTEIN

By

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SCOPE AND CONTENTS: Studies on the isolation and purification of Fraction I protein from various plants are described. Clear differences in the electrophoretic mobilities of Fr I from various species were observed. The genetic implication of observations on the electrophoretic mobilities of Fr I from wheat, rye and triticales are discussed. It is suggested that a non-chromosomal gene codes for Fr I. Conclusions are drawn from the fingerprint, N-terminal amino acid, and amino acid analysis studies. The presence of ribulose diphosphate carboxylase activity in Fr I protein is also investigated.

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HISTORICAL

In 1947, Wildman and Bonner found that 75% of the soluble protein of spinach leaves migrated as a single component in free boundary electrophoresis (1). This conspicuous protein component, designated Fraction I (Fr I), was later found to have a sedimentation constant of 18 S (2) and to possess ribulose diphosphate carboxylase (carboxydismutase) activity (see section 3). Evidence which indicates that Fr I protein is ribulose disphosphate carboxylase is reviewed in section 3 (p. 22). A second, smaller heterogeneous protein fraction (Fraction II) had a sedimentation constant of 4 S.

Fr I protein has since been found to occur in a wide variety of green plants in amounts varying from 23-50% of the total protein in whole cytoplasmic extracts. The most comprehensive survey for the presence of Fr I is that carried out by Dorner et al. using analytical ultracentrifugation and immunology (3).

Properties of Fraction I Protein

1. Molecular Weight:

The values which have been suggested for the molecular weight of Fr I protein and for carboxydismutase are summarized in Table I. The molecular weight suggested by Wildman (4) is at best an estimate as it is based on osmometry, a method which is subject to large errors. The value obtained by Eggman et al. (5) is subject to question on two

Table I

A Summary of Molecular Weight Values for Fraction I Protein and Carboxydismutase

Sample	Source	Author	Sedimentation Coefficient (S)	Diffusion Coefficient ($cm^2/sec \times 10^{-7}$)	Partial Specific Volume (ml/g)	Molecular Weight	Reference
Fraction I Protein	tobacco	Wildman et al.				>200,000[a]	4
	spinach, tobacco	Eggman et al.	$S_{w,20}$ = 17.9-19.5	-	0.69	375,000[b]	5
	tomato, glutinosa, tobacco, gherkin, cocklebur, spinach, pea	Singer et al.	S = ~18	-	-	600,000	7
	clover	Lyttleton	S_{20} = 16.2-16.6	$D^o_{w,20}$ = 2.60-2.75	-	590,000-620,000[b]	6
	bush bean	Kupke	S^o_{20} = 18.5				8
	spinach-beet	Thornber et al.				583,000[e] 547,000[e]	9
			$S^o_{w,20}$ = 18.29		0.737		10
	spinach	Pon	$S^o_{w,20}$ = 18.71	$D_{w,20}$ = 3.01		559,000[b] 475,000[c]	11
	spinach-beet	Ridley et al.	$S^o_{w,20}$ = 18.30		0.744	585,000[e] 561,000[e]	12
Carboxy-dismutase	spinach	Weissbach et al.	S_{obs} = 17	D_{obs} = 5.5		300,000(est)	13
	New Zealand spinach	Pon	$S_{w,20}$ = 18	D_{obs} = 1.6		300,000	14
	spinach	Trown	$S^o_{w,20}$ = 18.57	$D^o_{w,20}$ = 2.93	0.73	550,000[b] 560,000[d] 515,000[c]	15
	spinach	Paulsen and Lane	$S^o_{w,20}$ = 21.0			557,000[c]	16

a = osmotic measurement, b = sedimentation velocity, c = sedimentation equilibrium, d = Archibald technique (17), e = approach to equilibrium (Ehrenberg) (18).

counts. First, the material contained 11.2% nucleic acid, and second, its diffusion coefficient was estimated because the protein formed aggregates in solution making it impossible to measure the coefficient directly. In the material used in Lyttleton's determinations (6), RNA was absent and ultracentrifugal analyses made before and after the diffusion measurements showed that neither aggregation nor breakdown into smaller units had occurred. Previous attempts to determine diffusion constants had been impeded by dimer formation. The values reported for the diffusion coefficients by Weissbach et al. (13) and Pon (14) and for the sedimentation coefficient by Weissbach et al. (13) were not corrected to standard conditions for temperature (20°C) and solvent (water).

In 1965, Trown used three different methods to determine the molecular weight of carboxydismutase from spinach leaves (15) (see Table I). The diffusion coefficients were determined using the Rayleigh interference optical system and the partial specific volume was calculated from the densities of solutions of the protein. Trown states that the most accurate molecular weight determination was 515,000 ± 10,000. It was obtained from a sedimentation equilibrium experiment and is probably high as he allowed insufficient time to reach equilibrium (11). If the molecular weight calculated by Lyttleton (6) using an assumed partial specific volume is recalculated using the value determined by Trown (15) for the partial specific volume of carboxydismutase, the molecular weight obtained (550,000-574,000) is in reasonable agreement with those obtained by Trown (15), Thornber et al. (9,10), Paulsen and Lane (16), Pon (11) and Ridley et al.

(12) (see Table I).

Pon investigated the molecular weight of spinach Fr I by means of sedimentation equilibrium experiments (11) (see Table I). A limiting molecular weight at the meniscus of 475,000 ± 11,000 was indicated and limited evidence suggested that a small amount of a second component with a molecular weight of 545,000 might be present in some preparations. Pon (11) stated that the first component might be responsible for the $S^{o}_{w,20}$ (extrapolated) of 18.7 S and that the second component might have an $S^{o}_{w,20}$ of greater than 20 S and might explain the unusually high $S^{o}_{w,20}$ found by Paulsen and Lane (16). Pon (11) was unable to conclude whether one or both components contained the carboxydismutase activity (11). He suggests three factors which may account for the discrepancies in the molecular weights reported: (1) the $S_{w,20}$ decreases with increasing protein concentration, (2) the sedimentation coefficient is not a constant but rather a function of the time of sedimentation and (3) during the run, the protein concentration decreases with time thus causing the $S_{w,20}$ to appear higher than it is. Also, sedimentation velocity experiments require the determination of the diffusion coefficient and correction of this coefficient to the standard or reference state holds only if the temperature differential is a few degrees. Thus, the $D_{w,20}$ calculations of Pon (11) and Trown (15) would not be very accurate. Finally, a small error could occur in the determination of the initial concentration of protein (c_o) in sedimentation equilibrium runs as c_o is determined from a separate run using a capillary-type double sector cell. The c_o in the actual equilibrium experiment

might be less than in the synthetic boundary run resulting in a calculated molecular weight that is less than the true value. Pon concludes that the most probable molecular weight for Fraction I is 475,000 (11).

Some of the factors resulting in discrepancies have already been stated. It is possible that there are errors inherent in the correction of the sedimentation coefficients for the viscosities of the media used as the media have been many and varied. Small but different amounts of impurities in the preparations of the various authors may have resulted in different values for the concentration of the Fr I protein and this may also be a contributing factor to the discrepancies seen in the molecular weight values.

It can be seen that recent determinations of the molecular weight of Fraction I or RuDP carboxylase from spinach (11,13,15,16), New Zealand spinach (Tetragonia expansa) (14), spinach-beet (9,10,12) and clover (6), while not identical, are in reasonable agreement. While the molecular weights of Fraction I from various species may not be identical, no firm conclusion on this point can be drawn from published data.

2. Subunits:

Many proteins with high molecular weights are composed of subunits (19). Several proteins exhibit two or more different N- or C-terminal amino acids per mole of protein indicating that the protein is composed of two or more peptide chains (subunits) (21-23). For example, comparison of the number of ninhydrin positive spots on

fingerprint patterns of a tryptic digest of muscle α-glycerophosphate dehydrogenase with the number of basic amino acids per molecule of protein suggests that the protein is a dimer (20). Enzymes have been dissociated into subunits by treatment with agents such as urea, guanidine hydrochloride, detergents, alkali or acids (24-26). The subunits may be identical or nonidentical. Thus, dissociation of beef glutamic dehydrogenase leads to identical subunits (26) while the five isozymes of the lactic dehydrogenases are formed by various combinations of two nonidentical subunits (27-29).

The high molecular weight of Fr I suggests that it too may be made up from subunits. Published information on the possible subunit structure of Fr I protein is summarized in Table II. Haselkorn et al. (30) felt that the simple general considerations that led Watson and Crick (31) to their subunit model for the construction of protein shells of viruses also suggested that Fr I must be made up of subunits. The unpublished data of Rees (cited by Haselkorn et al. (30)) based on the amino acid composition of Fr I and the number of tryptic peptides obtained from Fr I have been taken to indicate that the protein consists of 24 identical subunits. Haselkorn et al. suggest, on the basis of electron micrographs, that Fr I from Chinese cabbage consists of a cube 120 Å along each edge containing 24 subunits, three along each edge of the cube and one in the centre of each of four faces (30). Mathematical calculations corroborate the data. The subunit model for the construction of protein shells of viruses does not hold for Fr I. To account for the distortion required to place a subunit on a vertex, it is proposed that subunits are either capable

Table II

Possible Subunit Structure of Fraction I Protein

Author	Method of Dissociation into Subunits	Sedimentation Coefficient of Subunits	Molecular Weight of Subunits	Number of Subunits Per Protein	Number of Subunit Types	Reference
Haselkorn et al.	-	-	-	24	1	30
Trown	0.001 M sodium dodecyl sulfate (SDS)	$\sim$ 2.5	-	-	-	15
Pon	pH 6.31 or low protein concentration	-	-	-	4?	11
Ridley et al.	pH 11.0	3.4 S and 12 S	24,427*	24*	2	12
	pH 11.5	S = 4 S			1	
	8 M urea	$S_{w,20}$ = 4.2 S			1	
	70% acetic acid	$S_{w,20}$ = 2.6-2.8 S			1	
	33% aqueous sodium dodecyl benzene sulfate	S_{20} = 4.8 S			1?	
Rutner and Lane	1 mM SDS	S_{20} = 3.0 S	heterogeneous		2	
	1 mM SDS, aminoethylation, chromatography on Sephadex G-100 with elution by 0.5% SDS	S_{20} = 3.0 S			2	32
		S_{20} = 1.8 S				

* Based on the amino acid composition of Fr I.

of making several kinds of "bonds", or that the subunits undergo considerable confirmational changes as a result of location in the completed particle.

Trown (15) and Pon (11) observed the dissociation of spinach Fr I into subunits (see Table II). Pon stated that at pH 6.31, Fr I separated into four distinct components on moving boundary electrophoresis (11). This was the first indication that the subunits might not be identical.

Ridley et al. determined the amino acid composition of spinach-beet Fr I protein and obtained a minimum molecular weight of 24,427 (12). This observation is consistent with the hypothesis that Fr I consists of 24 subunits. Ridley et al. used various agents to dissociate Fr I protein (see Table II) and stated that initial studies by gel electrophoresis in the presence of such agents indicated that the subunits had identical mobilities (12).

Rutner and Lane observed that electrophoresis of spinach Fr I protein in the presence of SDS indicated the existance of two distinct components (32). This is in direct contrast to the observations of Ridley et al. (12) but the latter authors did not describe the type of electrophoretic support or buffers used.

Rutner and Lane also dissociated Fr I in SDS, blocked the sulfhydryl groups by aminoethylation with ethylenimine and subjected the protein to chromatography on a Sephadex G-100 column (32). Elution with buffer containing 0.5% SDS gave two well-resolved peaks. The ratio of the total absorbancies at 280 mμ of the faster-moving peak to the slower-moving peak was 1.9. Amino acid analysis indicated

that the two peaks constituted different polypeptides and that large compositional differences existed between the two subunits. The S_{20} values of the subunits are indicated in Table II.

A variety of agents have been used to give subunits with differing sedimentation coefficients. These coefficients have not been corrected to the standard state (zero concentration, 20°C, solvent-water) and thus are hard to compare. From the work of Rutner and Lane (32), it appears that Fr I consists of at least two nonidentical types of subunits but, at this time, there is no proof that Fr I contains only 24 subunits and only two types of subunit.

3. Dimer Formation:

With the exception of the Fr I preparations described by Lyttleton (6), Paulsen and Lane (16) and Rutner and Lane (32), a second, faster-moving component with an $S_{w,20}$ of 24 is seen upon ultracentrifugation. The 24 S impurity appears to be an aggregate of the 18 S material as its concentration relative to that of the 18 S component increases with repeated high speed centrifugation in which the Fr I is "pelletted" or after freezing and thawing (2,5,33). The sedimentation constant of the Fr I and the 24 S component are in the ratio of $1/\sqrt{2}$ as might be expected for the sedimentation constants of monomer and dimer (2). Components having the sedimentation constants expected for the trimer and tetramer have also been observed (2). Mendiola and Akazawa suggested that the slower-moving band observed on starch gel electrophoresis represented an aggregate since it increased in intensity and width during purification and upon

concentration and freezing of the protein solution (34). Trown stated that electron micrographs (unpublished) of spinach Fr I showed the presence of monomers, dimers and trimers in approximately the same ratio as is found for the 18, 26 and 32 S components (15). The trimers appeared to be linear aggregates. Haselkorn et al. also have mentioned the occasional occurrence of linear aggregates in electron micrographs of Fr I from Chinese cabbage leaves (30).

Thornber et al. obtained a single band on polyacrylamide gel electrophoresis of purified Fr I protein in the presence of cysteine (9,10). In the absence of cysteine, additional components thought to be aggregates were observed. The aggregates did not revert to monomers upon treatment with cysteine.

Pon observed the appearance of a 26 S component after storage of Fr I protein for a long period or in the absence of cysteine or glutathione (11). The aggregated forms of Fr I were found to migrate on moving boundary electrophoresis with the same mobility as the monomer. Chromatography of the protein on Sephadex G-200 resulted in the elution of Fr I dimer from the Sephadex just before the monomer.

4. Non-Protein Groups Associated with Fr I:

(a) Auxin Binding and Phosphatase Activity:

Wildman and Bonner reported that all the auxin* of spinach leaves was "bound" to Fr I and that Fr I preparations also possessed phosphatase activity (1). Neither bound auxin nor phosphatase activity

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* Defined by the authors (1) as those chemical compounds which produce curvature in the Avena coleoptile assay.

could be separated from Fr I preparations by repeated ammonium sulfate precipitation. The phosphatase activity was lost upon denaturation of the protein. Since the phosphatase attacked a variety of phosphorylated substrates and it was not possible to show a stoichiometric ratio of auxin to Fr I, the authors suggested that the preparation might not be homogeneous. It is possible that contaminants were responsible for the apparent auxin binding and phosphatase activity as the method of preparation consisted simply of blending the leaves in water and forcing them through a colloid mill followed by a combination of low-speed centrifugation and ammonium sulfate precipitation. Auxin and phosphatase activity were not present in the preparations of Weissbach et al. (13) and Mayaudon (35) respectively, nor have bound auxin or phosphatase activity in Fr I preparations been mentioned in later publications.

(b) Nucleic Acids:

In 1949, Wildman et al. found Fr I preparations to contain 0.3-0.5% of the total phosphorus of the spinach leaf and to contain or be associated with substantial amounts of purine and pentose (36). Sixteen percent of the weight of an acid extract of tobacco leaves was adenine, pentose and phosphorus in the molar ratio of 1:1:2 (37). This is the molar ratio for ADP and not that expected for a nucleoprotein. The work with tobacco was done with whole leaves (37) while that with spinach was done with whole leaves or with Fr I preparations partially purified by relatively crude methods (36). In somewhat purer Fr I preparations from spinach and tobacco leaves, Eggman et al. demonstrated the presence of ribonucleic acid but the amount of

nucleic acid per molecule of 19 S component was variable (5). Mayaudon obtained similar results with Fr I preparations from New Zealand spinach (35).

Improved methods of purification have shown that Fr I is not a nucleoprotein. In 1956, Lyttleton showed that Fr I from clover was not associated with significant amounts of RNA (6). Also, there was no correspondence between the RNA concentration and the $S_{w,20}$ of Fr I. Weissbach et al. also obtained spinach Fr I preparations lacking nucleic acids (13). In 1958, Lyttleton and Ts'o obtained a 95% pure Fr I pellet after ultracentrifugation. No RNA could be detected in this spinach leaf preparation (38). Mendiola and Akazawa demonstrated that in a relatively pure preparation of rice Fr I the nucleic acids moved through a Sephadex G-200 column just ahead of the Fr I peak (34). Thus, Fr I from rice leaf is not a nucleoprotein but the nucleic acids are present in crude preparations of the protein. Racusen et al. observed that Fr I purified from bean, pea or oats by DEAE-cellulose chromatography was free of RNA (39). Thornber et al. (9,10) and Paulsen and Lane (16) found that pure preparations of Fr I protein from spinach-beet or spinach did not contain nucleic acids. Likewise, the spinach Fr I preparation of Pon was stated to be free of nucleic acids on the basis of an absorbance ratio $OD_{280:260}$ of 1.80 (11).

(c) Pyridine Nucleotide:

Haselkorn et al. noted that Fr I protein isolated from some preparations of Chinese cabbage leaves was associated with material having an absorption spectrum characteristic of reduced pyridine

nucleotide (30). No triosephosphate dehydrogenase activity was present. The significance of the bound pyridine nucleotide remains unknown.

(d) Protochlorophyll and Chlorophyll:

When certain so-called "active" protochlorophyll-protein complexes are irradiated with visible light, the protochlorophyll is converted to chlorophyll (33). Krasnovsky and Kosobuskaya (40) and Boardman (33) extracted such a complex from dark grown bean leaves. Several authors have noted the resemblance of the protein of the active protochlorophyll-protein complex and Fr I (8,15,33,41).

The sedimentation constants, diffusion coefficients, partial specific volumes and molecular weights of the protein from the complex and of Fr I are virtually identical (Table III). Both Fr I and protochlorophyll holochrome are insoluble below pH 6.0. Electron micrographs of Fr I and the holochrome appear identical. Freezing and thawing of solutions of Fr I or protochlorophyll holochrome results in a change from a single 18 S schlieren peak to three peaks corresponding to 18, 26 and 32 S. Electron micrographs indicated that the new schlieren peaks represented dimers and trimers of the protein (15,33).

Dissimilarities between Fr I and protochlorophyll holochrome have also been observed. Fr I protein had a higher mobility in glycine buffer than did either of the components observed on moving boundary electrophoresis of the protochlorophyll-protein complex (33). The addition or removal of a few molecules of an enzyme of 20,000 to 50,000 molecular weight to a large protein like Fr I during isolation

Table III

Physical Properties of Carboxydismutase, Fraction I Protein, and Protochlorophyll Holochrome (from Trown (15))

	Sedimentation Coefficient (S)	Diffusion Coefficient ($cm^2/sec \times 10^{-7}$)	Partial Specific Volume (ml/g)	Molecular Weight	pH at Which Electrophoretic Mobility toward Anode Was Observed	Particle Dimensions (from electron micrographs) (A)
Fraction I protein	$S_{w,20} = 17.9\text{-}19.5$[a]	$D^{\circ}_{w,20} = 2.60\text{-}2.75$[b]	0.69[c]	600,000 (est)[d,e]	pH 7.2[a]	100 x 200[f]
	$S_{20} = 16.2\text{-}16.6$[b]			375,000[a]		120 x 120[n]
	$S^{\circ}_{20} = 18.5$[g]			595,000-620,000[b,d]		
Carboxydismutase	$S_{obs} = 17$[h]	$D_{obs} = 5.5$[h]	0.73[i]	300,000 (est)[h]	pH 7.7[h]	100 x 200[f]
	$S_{w,20} = 18$[j]	$D_{obs} = 1.6$[j]		515,000 ± 10,000[i]	pH 6.9[j]	~100[i]
	$S^{\circ}_{w,20} = 18.57$[i]	$D^{\circ}_{w,20} = 2.93$[i]			pH 7.9[i]	
Protochlorophyll	$S_{20} = 15.3\text{-}16.2$[k]	$D^{\circ}_{w,20} = 2.7$[l]	0.73[l]	400,000-700,000[m]	pH 9.6[l]	100-110[l]
holochrome	$S^{\circ}_{w,20} = 18.0$[l]			600,000 ± 50,000[l]		

[a] Eggman et al. (5). [b] Lyttleton (6). [c] Sample contained 11.2% nucleic acid. [d] Molecular weights calculated assuming $\bar{v} = 0.75$ ml/g. [e] Singer et al. (7). [f] Park and Pon (42). [g] Kupke (8). [h] Weissbach et al. (13). [i] Trown (15). [j] Pon (14). [k] Smith and Kupke (41). [l] Boardman (33). [m] Smith (43). [n] Haselkorn et al. (30).

would have little effect upon the sedimentation coefficient and molecular weight of Fr I but might affect its electrophoretic mobility. It is possible that Fr I plus a protochlorophyll-protein complex form the protochlorophyll holochrome isolated. This might explain the observation that the mobilities of Fr I and the complex differ on moving boundary electrophoresis (33).

Thornber et al. compared the amino acid analyses of an 18 S holochrome from bean tissue and Fr I protein (10). There is a reasonable similarity between the amino acid compositions of the holochrome and Fr I but the compositions do differ in certain respects. Ridley et al. suggest that the dissociation of Fr I and the complex is similar (12). Fr I dissociates upon treatment with alkali, urea, acetic acid or detergent to give a rather heterogeneous mixture of subunits (2.6-4.8 S) while the protochlorophyll-protein complex dissociates at approximately pH 9.5 or with urea to give subunits of 2-5 S (54). The dissociated protein holochrome was still active (8). It should be noted that in contrast to the results found with the protochlorophyll-protein complex Fr I protein does not dissociate at pH 9.0 or 10.0 (12).

Fraction I also forms a complex with chlorophyll (8). As different chlorophyll-protein complexes can be prepared (47,55,56) (Table IV), the value of the observation that Fr I forms a complex with chlorophyll is questionable. No reproducibly obtainable and generally agreed upon chlorophyll-protein fragment has resulted from attempts to solubilize the chloroplast membranes with surface-active agents (Table IV).

Table IV

Properties of Chlorophyll-Protein Complexes Isolated from Chloroplasts (from R. S. Criddle (47))

Source	Solubilizing agent	Conc. of added sol. agent	Chlorophyll to protein ratio w/w	S	Mol. wt.	Ref.
Spinach	Digitonin	2.5%	1/6.1	13.5	265,000	44-6
Spinach	Bile Salts	3.0	1/6.1	13.5	265,000	44-6
Spinach	Deoxycholate	0.25-0.5	1/6.1	13.8	265,000	44-6
Spinach	SDS	0.25-2.5	1/6.1	2.6-1.7	-	44-6
Euglena	Digitonin-Naccinol	1-2	1/40	2.7	57-65,000	48,53
Poterichromonas	Digitonin-Naccinol	1-2	1/40		27-50,000	48
Spinach	Dupanol-Span-80	1		5.5	1.3×10^6	49
Spinach	Dodecylbenzene sulfonate	1/2	whole chloroplast	2.9-3.5		50
Spinach	Triton X-100	1	1/0.9-1.1			51
Trifolium repens	α-Picoline	50	1/9.6		19,200	52

Criddle has prepared a chlorophyll-structural protein complex *in vitro*, thus demonstrating the ease of formation of complexes during isolation (47). Wolken (53) has formed chlorophyll-protein complexes *in vitro* with such proteins as gelatin, bovine serum albumin and γ-globulin and has found the spectral properties of the complexes formed *in vitro* similar to the properties of the chloroplast complex.

Boardman calculated that there was one molecule of protochlorophyll per Fr I protein molecule (33) while Kupke's results (corrected to a molecular weight of 600,000 for Fr I) indicate that there are four protochlorophyll molecules for every Fr I molecule in the complex (8). Also, in a complex similarly prepared from green leaves from bush bean, there appear to be 72-81 molecules of chlorophyll per mole of Fr I protein (8). Thus, the number of protochlorophyll or chlorophyll molecules per mole of Fr I is variable.

Bailey *et al.* have reviewed work demonstrating that the bulk of the chlorophyll was present in the free state (56). Furthermore, it has been shown that probably not all of the active protochlorophyll in the holochrome preparation is bound to the protein in the same way (57,58) and that ultraviolet light converts only 30% of the transformable protochlorophyll to chlorophyll *a* (59).

Ohad *et al.* have described a mutant of *Chlamydomonas reinhardi* (y-1) which is unable to synthesize chlorophyll in the dark (60). When the cells are grown in the dark, chlorophyll is diluted rapidly through cell division whereas RuDP carboxylase continues to be present in relatively high concentrations. When dark-grown cells are exposed to continuous illumination, chlorophyll

is synthesized rapidly whereas only relatively small changes occur in the already present RuDP carboxylase (61). Thus, the formation of chlorophyll and RuDP carboxylase are independent and the lack of chlorophyll for complex formation does not appear to affect the production of enzyme.

Until the enzyme activity found for Fr I protein can be associated with the complex and until dissimilarities between Fr I and the holochrome can be explained, it cannot be claimed unequivocally that the two proteins are identical.

(e) Carbohydrates and Lipids:

Recently, Akazawa et al. claimed that Fr I protein isolated from rice leaves is a glycoprotein (62). Several samples of purified Fr I were subjected to electrophoresis on a block of polyacrylamide gel. The block was then halved and one half was stained for protein with Amido Black while the other half was stained for carbohydrate with fuschin-sulfite. The Fr I component was found to contain carbohydrate. Upon hydrolysis of the purified Fr I by "Pronase", a non-specific protease, galactose, arabinose and some glucose, rhamnose and ribose (or xylose) were identified by paper chromatography. Thornber et al. reported the presence of lipid and carbohydrate in their preparations of Fr I from spinach-beet but suggest that, particularly when whole leaf extracts are used as the starting material rather than aqueous chloroplast washes, their presence may have arisen during the isolation procedure (9,10). Thornber et al. (10) also reported that following polyacrylamide gel electrophoresis, the Fr I and dimer (26 S) bands could be stained with Lipid Crimson and

gave a positive reaction for ketose in the cysteine-carbazole reaction (63). Unlike Akazawa et al. (62) who reported that galactose and arabinose were associated with Fr I protein, Thornber et al. (10) have shown the presence of glucose, fructose, glucosamine and a trace of galactosamine.

Only traces of carbohydrates were found in the spinach Fr I protein preparations of Paulsen and Lane and these traces may have been due to carbohydrate carry-over from the steps involving Sephadex G-25 and DEAE-cellulose (16). Dialyzed samples of Fr I were analyzed by the quantitative phenol-sulfuric test (64). As no more than the equivalent of 2 moles of monosaccharide were present per mole of Fr I, they conclude that Fr I does not contain an oligosaccharide component (16). Pon also found that a carbohydrate test on Fr I protein was negative (11). Furthermore, Pon observed that the partial specific volume of Fr I differs from that of lipoproteins suggesting that Fr I is not a lipoprotein.

Ridley et al. found that lipid was present infrequently and felt that it became bound to the protein during isolation (12). Carbohydrate and material giving rise to ash (4%) were always present and probably accounted for the 16% non-nitrogenous material associated with the preparations. Glucose and xylose, small amounts of galactosamine, and trace amounts of fructose (or arabinose) and galactose were observed after hydrolysis of the protein. It is suggested that this non-protein material, like the lipid, might have become adsorbed on the protein molecule during the isolation procedures (12).

The Subcellular Location of Fraction I Protein

The early workers believed that Fr I was located in the leaf cytoplasm (1). However, Lyttleton and Ts'o showed that Fr I could be obtained from chloroplasts (38). Further evidence has been provided by Heber *et al.* that in tobacco, *Tetragonia* and spinach leaves, Fr I is associated entirely with the chloroplasts (65). Furthermore, Lyttleton found that Fr I was present only in photosynthetic tissues or in tissue, such as etiolated leaves, capable of photosynthesis after exposure to light (66). Fr I was present in small amounts in etiolated leaves and increased in amount when the dark-grown plant was exposed to light. Fr I is not produced in the roots of wheat or ryegrass or in the leaves of albino ryegrass (66).

Akazawa *et al.* mentioned that both Fr I and the lamellar structural protein of the grana are glycoproteins but offered no evidence that the two proteins were identical (62). Thornber *et al.* published an abstract claiming that some Fr I protein in the organelle is firmly bonded to the water-insoluble material (67). This claim was based on amino acid analyses and peptide maps of Fr I and a protein fraction from the lamellae of chloroplast grana of spinach-beet (*Beta vulgaris*) leaf homogenate. As the lamellar protein fraction contained two major components (6 S and 18-19 S) and at least four minor components, it is difficult to see how meaningful amino acid analyses and fingerprints could be obtained.

In 1966, the same group published the amino acid compositions of lamellae, lamellae fractions obtained by phenol and acetic acid treatment, and Fr I from spinach-beet leaves (56). There were

significant differences in the molar ratios of some of the amino acids present in the lamellar and Fr I proteins. Bailey et al. (56) have subjected the sodium dodecylbenzenesulphonate extracts of lamellae fractions to polyacrylamide gel electrophoresis. Ten bands are distinguishable after staining with Amido black. Thus, the lamellae fractions are not homogeneous and it is hard to see how valid comparisons can be made with Fr I protein.

Further attempts to gain some insight into the location of soluble constituents by comparing the components in solution after various degrees of lamellar disruption were made by the same group (12). Fraction I was released very readily from the osmotically broken chloroplasts indicating that it was not associated with the lamellar structure. These results are in direct contrast to the group's earlier data.

While many workers have isolated a lamellar fraction which contained many proteins, Criddle has isolated a specific lamellar protein fraction from spinach stated to function only in a structural role (47). This protein which was prepared in 0.5 M urea and 0.1% SDS, has an $S_{w,20}$ of 2.3 and a molecular weight of 25,000. The amino acid analysis of the structural protein is generally similar to analyses reported for lamellar proteins by other workers (47). Differences are seen in the mole percentages for aspartic acid, glutamic acid, methionine, tyrosine, phenylalanine, lysine and arginine. Thus, the purified structural protein differs slightly from Fr I in amino acid composition. The structural protein has a single N-terminal and C-terminal amino acid (47) but is stated to

have at least two nonidentical subunits (32). Since chloroplast structural protein *in vitro* has been shown to bind chlorophyll, horse heart myoglobin, lipid and ATP (47), it is quite possible that it may also bind Fr I during the isolation procedure.

The Catalytic Properties of Fr I Protein

In 1952, Calvin and Massini postulated that in photosynthesis, CO_2 and ribulose diphosphate react to give phosphoglyceric acid (68). Two years later, Quayle *et al.* obtained a cell-free extract from *Chlorella* which catalyzed the carboxylation of ribulose-1,5-diphosphate (RuDP) to give 3-phosphoglyceric acid (PGA) (69). Weissbach *et al.* observed a similar reaction with spinach leaf extracts (70).

In 1956, Weissbach *et al.* used ammonium sulfate precipitation and treatment with aluminum hydroxide to obtain a very pure preparation of the enzyme responsible for the carboxylation of RuDP, i.e., ribulose-1,5-diphosphate carboxylase (EC4.1.1.39, also known as carboxydismutase) (13). Their preparation was devoid of phosphoribulokinase, transketolase and transaldolase activity. In the presence of RuDP and ribulose diphosphate (RuDP) carboxylase, carbon dioxide was incorporated into the carboxyl group of the phosphoglyceric acid. With RuDP-1-^{14}C, the isotope appeared in the β-carbon atom of PGA. Ribulose-1,5-diphosphate carboxylase activity has since been reported in a wide variety of plants (14).

Jakoby *et al.* independently isolated RuDP carboxylase from spinach in the same year (71) while Mayaudon *et al.* isolated the carboxylase from *Tetragonia expansa* about the same time (72). The following year, Dorner *et al.* pointed out that the sedimentation

constant, sensitivity to acidic conditions (below pH 6.0) and molecular weight of tobacco RuDP carboxylase closely resemble the corresponding properties of Fr I (73). Mayaudon showed by using a separatory cell in an analytical ultracentrifuge that RuDP carboxylase activity was associated with Fr I (35). On the basis of physical and enzymic properties, RuDP carboxylase activity has been found to be associated with Fr I protein by various workers (see Table V).

In 1961, Van Noort et al. purified spinach and tobacco Fr I and found that their preparation contained RuDP carboxylase and two other enzyme activities, namely, phosphoriboisomerase and phosphoribulokinase activity (75). Phosphoriboisomerase is responsible for the isomerization of ribose-5-phosphate to ribulose-5-phosphate. Phosphoribulokinase catalyzes the phosphorylation of ribulose-5-phosphate to ribulose-1,5-diphosphate which is the substrate for RuDP carboxylase. Mendiola and Akazawa obtained similar results with a Fr I preparation from rice leaves (34).

Until 1964, it was believed that Fr I exhibited three enzyme activities. Van Noort and Wildman (74), demonstrated that the isomerase and kinase could be separated from the carboxylase (Fr I). They purified Fr I from tobacco leaves by differential ultracentrifugation and found all three enzymic activities to be present (74). After further purification with Sephadex G-75 and DEAE-cellulose, the Fr I protein still contained all three enzyme activities. Tobacco Fr I antiserum was added to the Fr I preparation and, in the antibody-excess zone of the quantitative precipitin curve, all the Fr I protein in solution formed an antigen-antibody complex

Table V

Plants in Which RuDP Carboxylase Activity
is Known to be Associated with Fr I Protein

Plant	Author	Reference
1. Spinach (*Spinacia oleracea*)	Lyttleton and Ts'o	38
	Park and Pon	42
	Pon	11
	Van Noort and Wildman	74
	Trown	15,Table III
	Paulsen and Lane	16
2. New Zealand Spinach	Mayaudon	35
(*Tetragonia expansa*)	Pon	14
3. Spinach-beet	Thornber *et al.*	9,10
(*Beta vulgaris*)		
4. Chinese cabbage	Haselkorn *et al.*	30
(*Brassica pekinensis*)	Ridley *et al.*	12
5. Pea (*Pisum sativum*)	Racusen *et al.*	39
6. Oat (*Avena sativa*)	Racusen *et al.*	39
7. Bean (*Phaseolus vulgaris*)	Racusen *et al.*	39
8. Rice (*Oryza sativa*)	Mendiola and Akazawa	34

which was removed as a precipitate by low speed centrifugation. The Fr I antiserum was shown to have precipitated only the RuDP carboxylase and to have effected a separation of this enzyme from phosphoriboisomerase and phosphoribulokinase. The isomerase and kinase remained in the supernatant. The recovery of the carboxylase activity was poor and a stimulation in the RuDP carboxylase activity (soluble) was observed upon centrifugation of the control tube containing normal serum.

Trown made a nearly homogeneous preparation of RuDP carboxylase from spinach leaves by ammonium sulfate fractionation and repeated gel filtration on Sephadex G-200 (15). The purified enzyme showed no significant isomerase or kinase activity. Thus, it appears that in at least spinach and tobacco Fr I protein contains solely RuDP carboxylase and does not contain phosphoriboisomerase or phosphoribulokinase activity.

Steer et al. obtained Fr I protein from oat leaves and showed that it was unable to incorporate $^{14}CO_2$ into PGA using R-5-P as the substrate (76). They suggest that the protein (Fr I) is carboxydismutase, free of the kinase-isomerase enzymes, but since they did not assay specifically for RuDP carboxylase, they cannot be absolutely sure that it was present in their final preparation.

Criddle (47) investigated the formation of molecular complexes among carbon cycle enzymes paying particular attention to the enzyme activities which might be associated with Fr I protein. Most procedures used for the preparation of Fr I favour dissociation of proteins into subunits. In an attempt to overcome this problem,

Criddle (47) used the method of Cohen (77) which allows the sedimentation of a "working enzyme" to be followed in the analytical ultracentrifuge using directly the crude extract of broken chloroplasts. An enzyme solution is layered over a substrate solution in a synthetic boundary cell and the reaction catalysed by the enzyme as it migrates to the bottom of the cell during centrifugation (or a coupled reaction) is followed as an optical density change. Table VI summarizes the sedimentation coefficient determinations for some chloroplast carbon cycle enzymes and it is seen that all of the enzymes tested move with sedimentation coefficients much slower than that of Fr I protein. These enzymes are probably not associated with RuDP carboxylase in an enzyme complex.

Ribulose diphosphate carboxylase was eluted from Sephadex G-200 (9,10) or DEAE-cellulose (16) just before the isomerase and kinase but there was some overlap of activity. The isomerase and kinase were completely removed from the carboxylase by chromatography on Hypatite C (16).

Fr I protein prepared by Pon possessed RuDP carboxylase activity but negligible isomerase activity (11). Thornber et al. showed that Fr I obtained from isolated chloroplasts contained RuDP carboxylase activity but no isomerase or kinase activity whereas when whole leaf extracts were used as starting material some isomerase and kinase activity was present in apparently homogeneous 18 S component preparations in addition to the carboxylase activity (10). They suggest therefore that while Fr I can be obtained rapidly and in large quantity from leaf cytoplasm, a product characteristic

Table VI

Summary of Sedimentation Coefficient Determinations for Chloroplast Carbon Cycle Enzymes (from Criddle (47))

Enzyme	Method used to follow activity	$S_{w,20}$
Fructose-1,6-diphosphate aldolase	Coupled with TPD, NADH formation	6.5
Glyceraldehyde-3-phosphate dehydrogenase (TPD)	NADPH formation	6.9
3-Phosphoglycerate kinase	Phenol red indicator change	3.5
Ribosephosphate isomerase [1]	Phenol red indicator change	7.1
Phosphoribulokinase [1]		

[1] Determined simultaneously.

of the species as it exists in the leaf is more likely to be obtained by purification of the protein from isolated chloroplasts.

It is almost certain that Fr I is RuDP carboxylase. However, carboxylase activity has been demonstrated in proteins other than Fr I. Anderson et al. isolated RuDP carboxylase from Rhodospirillum rubrum, a photosynthetic organism which, like all such organisms, lacks true chloroplasts (78). The $S_{w,20}$ value, estimated by sucrose gradient analysis, was 5.6 S while the molecular weight estimated by chromatography on Sephadex G-200 was 120,000. Neither isomerase nor kinase activity was measured. It is stated that bacterial RuDP carboxylase does not have the apparent structural complexity of the chloroplast enzyme (Fr I).

Santer and Vishniac observed that crude extracts of Thiobacillus thioparus, an autotrophic, non-photosynthetic sulfur bacterium, can convert RuDP and CO_2 to PGA (79). Identical results were obtained by Trudinger with crude extracts of Thiobacillus denitrificans (80). The enzyme responsible was not isolated.

Fuller and Gibbs studied the intracellular and phylogenetic distribution of RuDP carboxylase (81). It was noted that the carboxylase was present in all the photo-autotrophs tested, in Astasia, a naturally colourless species which is otherwise similar to Euglena, and in Escherichia coli grown on D-xylose and carbon dioxide. The carboxylase was also present in Chromatium, strain D, when the organism was grown either autotrophically or heterotrophically.

METHODS

Plant Material:

Spinach was obtained at a local supermarket. Peas (*Pisum sativum* var. Little Marvel) and beans (*Phaseolus vulgaris* var. Cherokee wax or Pinto) were grown in the greenhouse in "Vermiculite" watered with 50% Hoagland solution. Wheat (*Triticum aestivum*), rye (*Secale cereale*), and the interspecific hybrid triticales (*Triticale*) were grown in the greenhouse in soil. Tobacco (*Nicotiana tabacum* var. Hicks Broadleaf) was grown in the greenhouse in steamed soil. *Nicotiana langsdorffii* 2 N, *Nicotiana glauca*, cabbage (*Brassica oleracea*) and radish (*Raphanus sativus*) were grown in soil. *Chlorella vulgaris* was grown with aeration with 5% CO_2 at 20°C on a medium slightly modified from that described by Jorgensen (82) (Table VII).

Purification of Fr I Protein:

(i) Method I: All operations were carried out at 4°C. The leaf tissue was stripped from the midribs, washed in distilled water and blotted dry. The leaves were blended in 0.05 M potassium phosphate buffer, pH 7.0, for 3 min at full speed in an omnimixer. The mixture was filtered through cheesecloth and the filtrate centrifuged at 27,000 g for 30 min. The supernatant was dialysed against distilled water overnight and lyophilized. The dry material was dissolved in a minimal volume of 0.05 M phosphate buffer, pH 7.0, and centrifuged

Table VII

Chlorella Medium Stock Solutions

1. 6 g $NaNO_3$, 400 mg $Ca(NO_3)_2 \cdot 4H_2O$, 500 mg $MgSO_4 \cdot 7H_2O$ and 200 mg K_2HPO_4 in 2 ℓ distilled water.
2. 60 mg $FeSO_4 \cdot 7H_2O$, 20 mg $MnCl_2 \cdot 4H_2O$, 60 mg citric acid and 30 mg boric acid in 200 ml distilled water.
3. 0.440 mg $ZnSO_4 \cdot 7H_2O$, 1.580 mg $CuSO_4 \cdot 5H_2O$, 2.700 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.520 mg $NaVO_3$ and 5.000 mg $EDTA \cdot Na_2$ in 200 ml distilled water.

The stock solutions were autoclaved. For use, 440 ml distilled water were autoclaved and 50 ml "1", 5 ml "2" and 5 ml "3" were added to give the appropriate concentrations of nutrients in the medium.

at 27,000 g for 15 min. The supernatant was centrifuged in a Spinco model L ultracentrifuge in the 50 rotor at 151,000 g for 40 min. The supernatant was dialysed against distilled water overnight and then lyophilized. The material was dissolved in a minimal volume of 0.05 M phosphate buffer, pH 7.0, and chromatographed on a Sephadex G-200 (Pharmacia Ltd.) column (1" x 14") at a flow rate of 0.5 to 1.0 ml per min. The optical density at 280 mμ of each fraction (3 ml) was determined and the fractions containing material which absorbed ultraviolet light at 280 mμ were dialysed against distilled water overnight and lyophilized. One hundred and fifty mg of this material were dissolved in 3 ml of 0.05 M phosphate buffer, pH 7.0, and layered above a linear 5%-20% sucrose density gradient. Centrifugation was conducted in a preparative ultracentrifuge in the SW 25.1 rotor at 63,600 g for 13 hr. The bottom of the tube was pricked and 20 drop fractions were collected. The fractions containing material which absorbed ultraviolet light at 280 mμ were pooled and dialysed against distilled water overnight. The dialysed sample was chromatographed on a Sephadex G-25 (Pharmacia Ltd.) column (1" x 15") and the fractions from the peak tubes were dialysed against distilled water overnight and lyophilized.

Method I is summarized in the accompanying flow sheet (Fig. 1).

(ii) Method II: The second method was essentially that of Paulsen and Lane (16) and is summarized in the accompanying flow sheet (Fig. 2). All operations were carried out at 4°C. Chromatography on DEAE-cellulose (Mann Research Laboratories) and Hypatite C (Clarkson Chemical Co.) were carried out without modification as

Figure 1

Preparation of Fraction I Protein

Method I

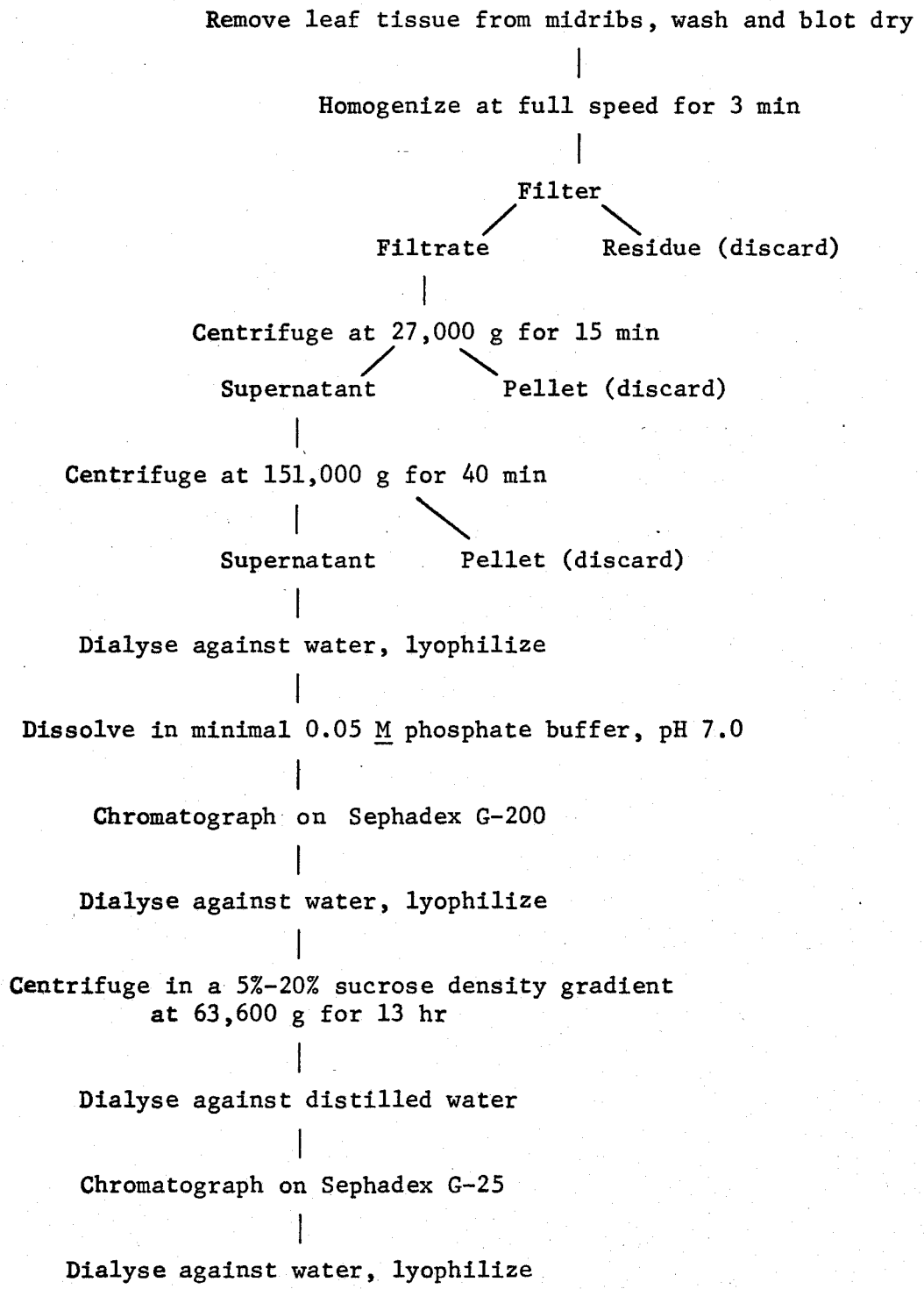
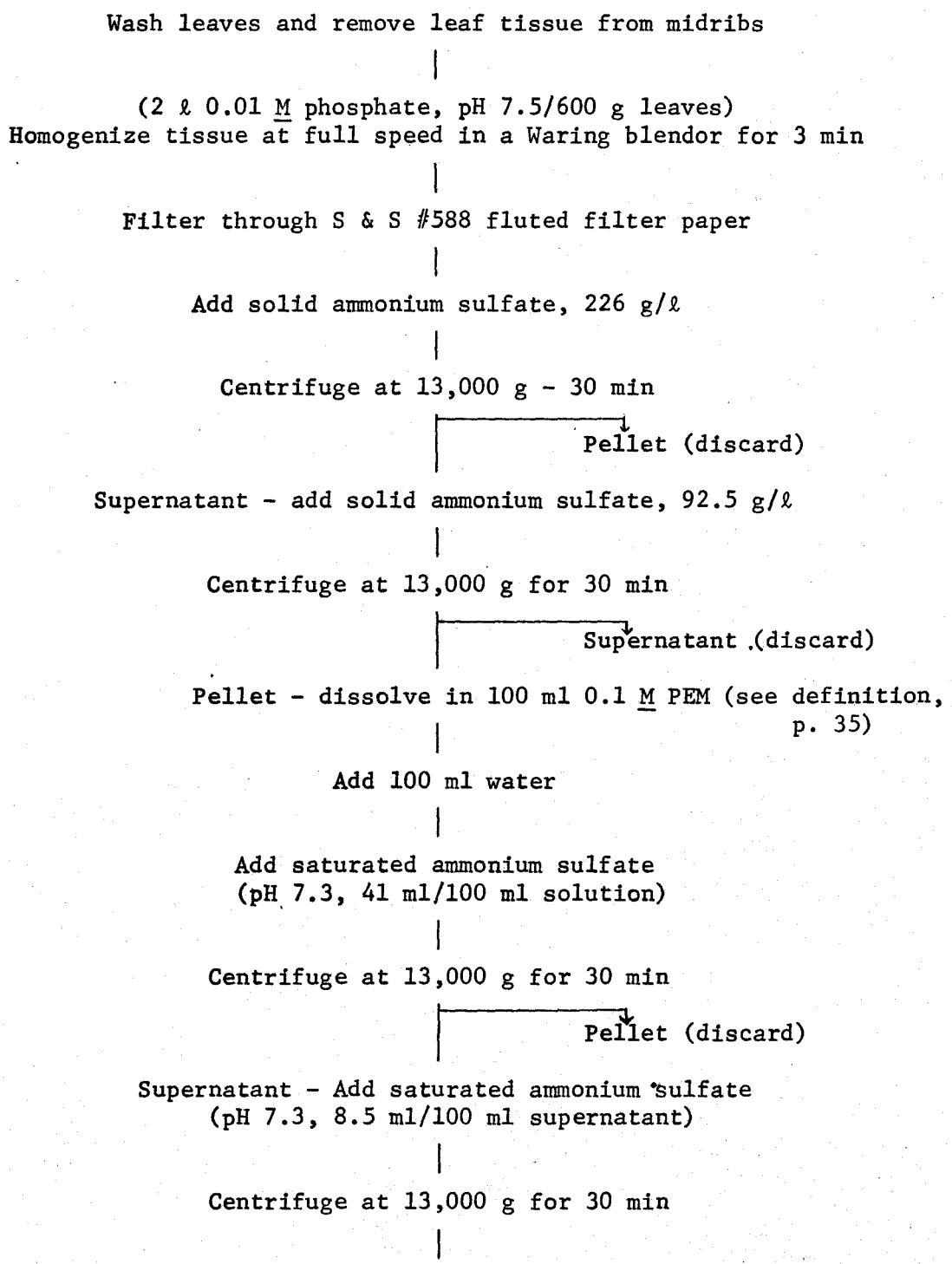


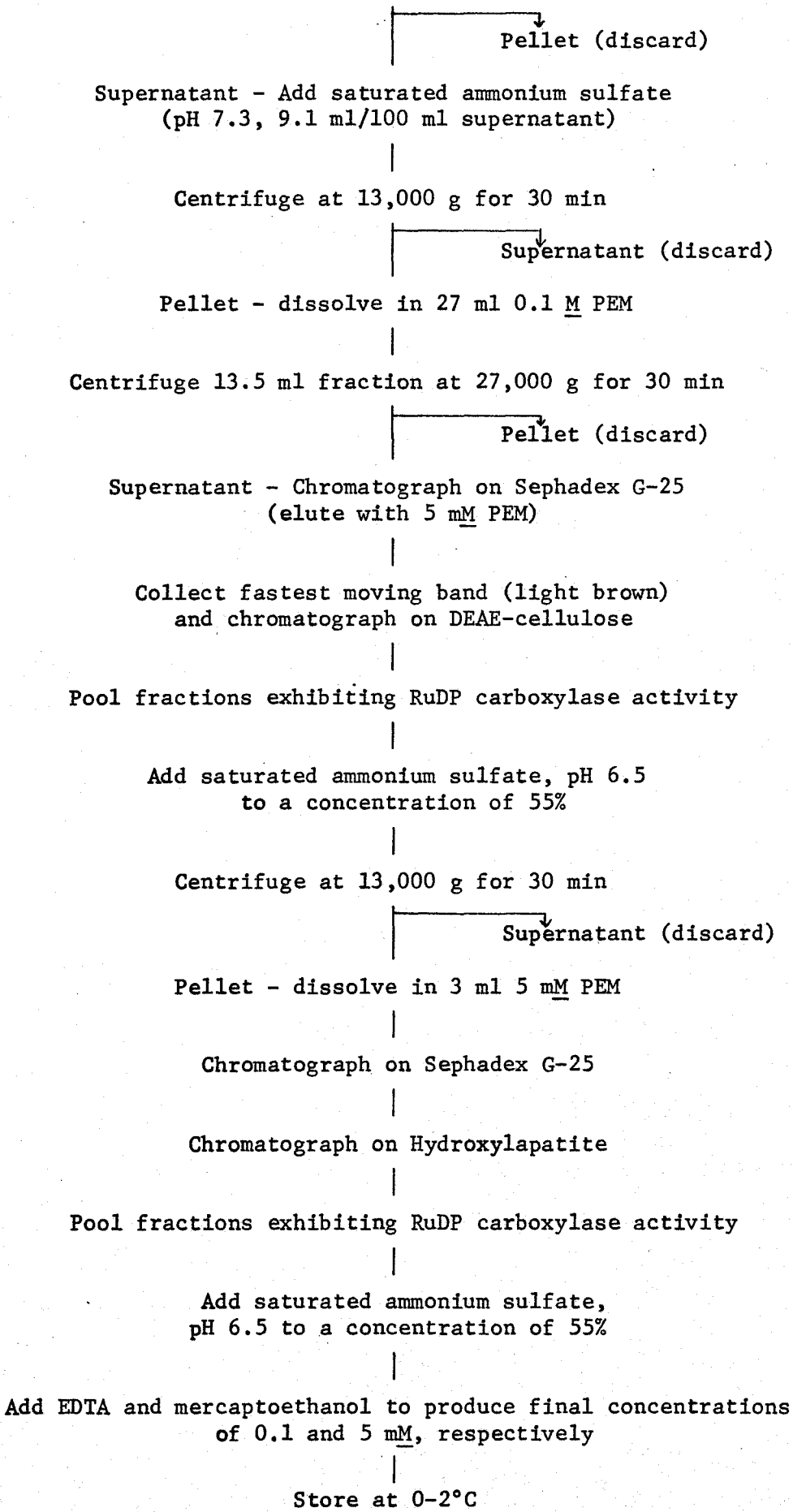
Figure 2

Preparation of Fraction I Protein

Method of Paulsen and Lane (16)

Method II




Pellet (discard)
Supernatant - Add saturated ammonium sulfate (pH 7.3, 9.1 ml/100 ml supernatant)
Centrifuge at 13,000 g for 30 min
Supernatant (discard)
Pellet - dissolve in 27 ml 0.1 M PEM
Centrifuge 13.5 ml fraction at 27,000 g for 30 min
Pellet (discard)
Supernatant - Chromatograph on Sephadex G-25 (elute with 5 mM PEM)
Collect fastest moving band (light brown) and chromatograph on DEAE-cellulose
Pool fractions exhibiting RuDP carboxylase activity
Add saturated ammonium sulfate, pH 6.5 to a concentration of 55%
Centrifuge at 13,000 g for 30 min
Supernatant (discard)
Pellet - dissolve in 3 ml 5 mM PEM
Chromatograph on Sephadex G-25
Chromatograph on Hydroxylapatite
Pool fractions exhibiting RuDP carboxylase activity
Add saturated ammonium sulfate, pH 6.5 to a concentration of 55%
Add EDTA and mercaptoethanol to produce final concentrations of 0.1 and 5 mM, respectively
Store at 0-2°C

described by Paulsen and Lane (16).

(iii) Method III: All operations were carried out at 4°C. Leaves were washed with cold water, stems removed and the blades homogenized in 2 liters of 0.01 M PEM* per 600 g of leaf in a large Waring Blendor for 3 min. After the extract had been filtered through cheesecloth or S & S No. 588 fluted filter paper, the pH of the filtrate was adjusted to 7 with 2 M NH_4OH.

Chlorella cells were harvested, resuspended in 0.01 M PEM, and ruptured in a French pressure cell at 10,000 psi. The pH of the homogenate was then adjusted to 7.0.

The leaves from N. langsorffii or N. glauca were washed with cold water and the stems removed. The leaf tissue was then frozen in liquid nitrogen, powdered and lyophilized. Extracts were later prepared by Method III.

The preparation was centrifuged at 13,000 g for 50 min and the supernatant fractionated with ammonium sulfate (Table IX). After it had stood at 4°C overnight, the suspension was centrifuged at 13,000 g for 30 min, the pellet redissolved in 5 mM PEM, and a small amount of undissolved material removed by centrifugation at 28,000 g for 30 min. The supernatant was centrifuged at 151,000 g for 40 min and the supernatant brought to 55% saturation with ammonium sulfate†.

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* PEM-potassium phosphate, 0.1 mM in EDTA and 5 mM in mercaptoethanol, pH 7.6.

† Ammonium sulfate solution was saturated at room temperature and neutralized with NH_4OH so that its pH at room temperature was 7.3 after five-fold dilution with distilled water. The saturated solution was stored and used at 4°C (100% saturation $\approx$637 g ammonium sulfate per ℓ).

Following centrifugation at 13,000 g for 30 min, the pellet was redissolved in 5 mM PEM and centrifuged at 28,000 g for 30 min to remove any undissolved material. The supernatant was then applied to a column of Sepharose 4B (2.5 x 80 cm) (Pharmacia Ltd.). The column was developed with 5 mM PEM (Fig. 3a) and the percent transmission of the eluant determined continuously at 280 mμ with an LKB "Uvicord II" Absorptiometer. Fraction I protein was identified by electrophoresis on polyacrylamide gel. The contents of tubes containing Fraction I were pooled and the protein again precipitated with 55% ammonium sulfate. The redissolved pellet was centrifuged at 13,000 g for 30 min and the supernatant applied to a Sephadex G-200 column (1.5 x 90 cm). Protein was eluted from the column with 5 mM PEM (Fig. 3b). Contents of tubes containing Fraction I were pooled and stored at 4°C as a precipitate in 55% ammonium sulfate.

Method III is summarized in the accompanying flow sheet (Fig. 4).

Starch Gel Electrophoresis:

The method of starch gel electrophoresis has been described by Smithies (83). Partially hydrolysed starch (Connaught Medical Research Labs, Toronto) was slurried with borate buffer (0.05 M boric acid, 0.02 M sodium hydroxide in the recommended proportions (10.2 gm/100 ml)). The slurry was heated with continual mixing until it became viscous. Suction was then applied to remove gas bubbles from the preparation. The liquid was poured into trays, covered and allowed to set. The electrode vessels were filled with borate

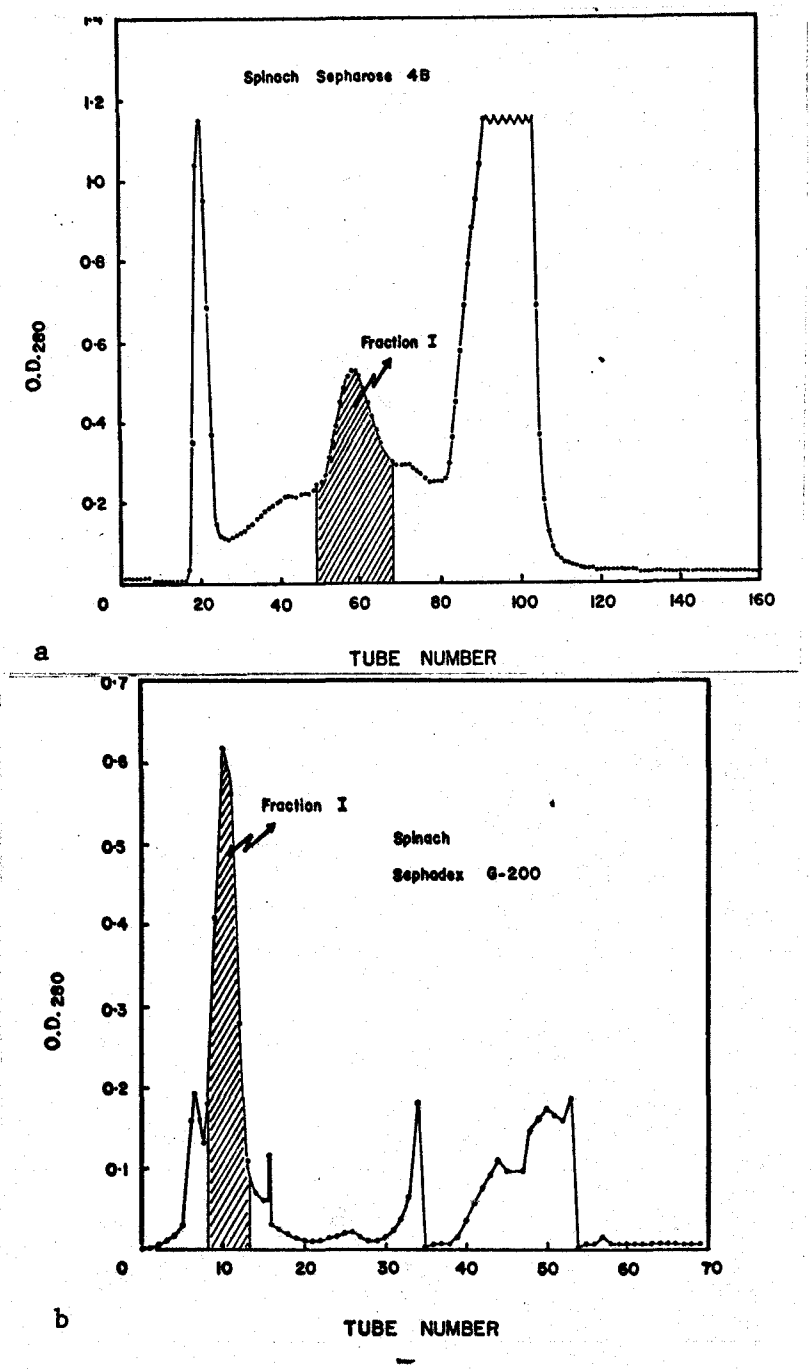


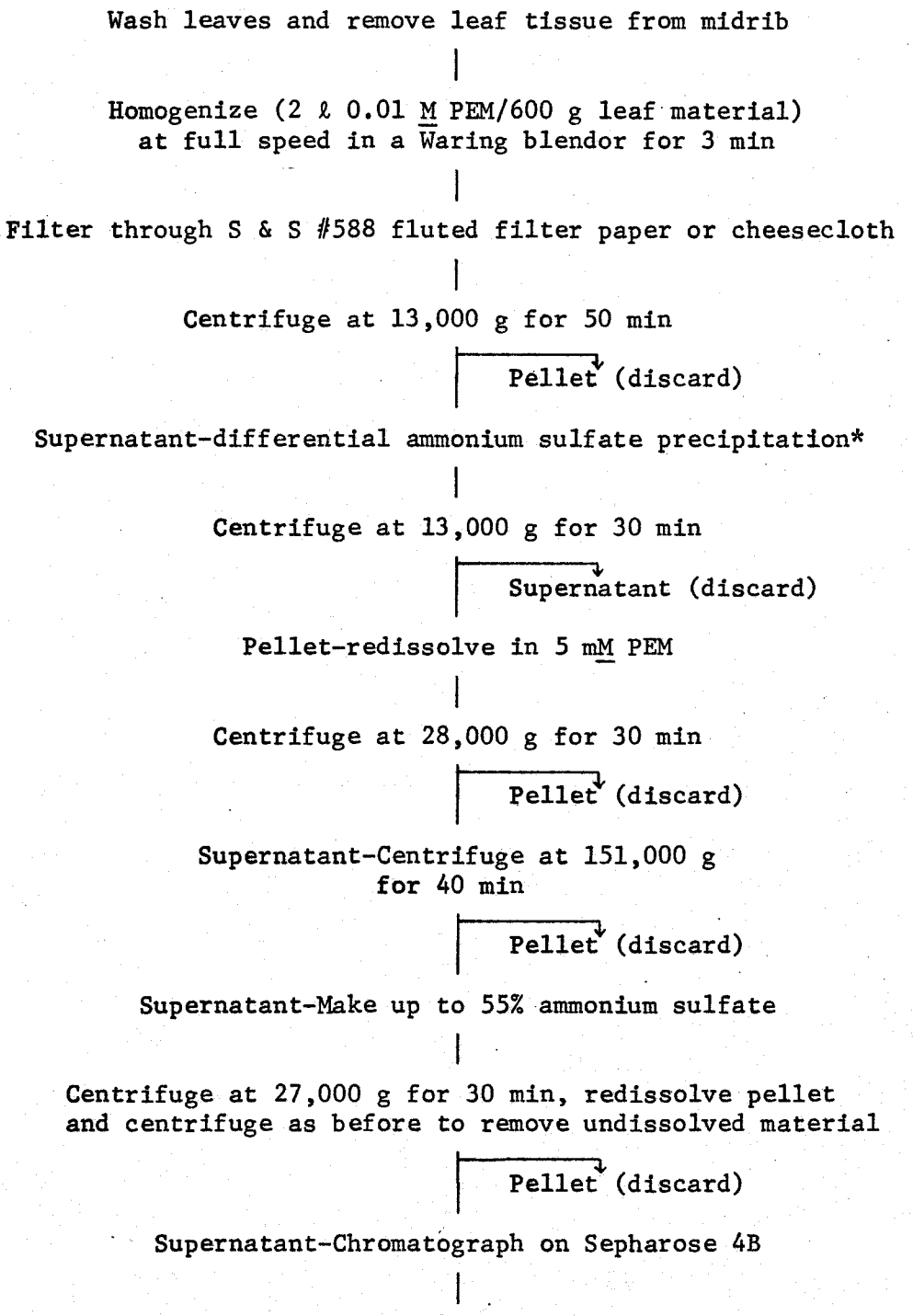
Fig. 3. Chromatography of spinach extract.* (a) Sepharose 4B. Fraction size about 3.5 ml. (b) Sephadex G-200. Fraction size about 4.7 ml. The OD_{260}/OD_{280} ratio of fractions 5, 6 and 7 combined was 1.3 while that of fractions 8 to 13 combined was 0.57. Fr I was identified as the major band on polyacrylamide gel electrophoresis. The purified protein had ribulose diphosphate carboxylase activity and an $S_{w,20}$ of 16.8.

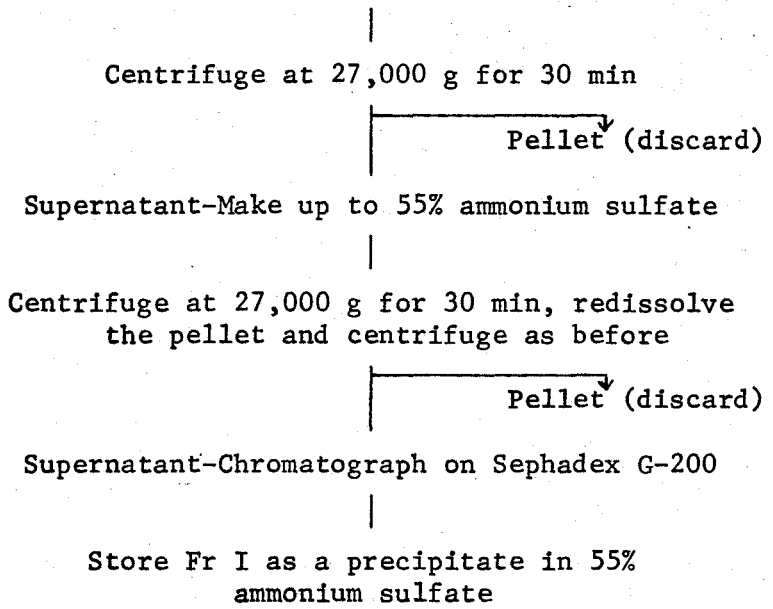
* OD replotted from percent transmission record.

Figure 4

Preparation of Fraction I Protein

Method III





* See Table IX for details.

buffer (18.5 g boric acid, 2.4 g NaOH in 1 ℓ water) and electrophoresis was carried out for 2 hr at 250 V in a horizontal apparatus. Gels were split, stained with 0.1% Amido Black 10 B in wash solution (methanol : acetic acid : water - 40:40:8 v/v/v) and destained by prolonged soaking in wash solution.

Polyacrylamide Gel Electrophoresis:

Electrophoresis was carried out for 3 hr at 300 V in a vertical apparatus (E-C Apparatus Corp., Philadelphia 4, Pa.) in gels prepared from a filtered solution of 10.5 g "Cyanogum 41" (acrylamide) and 0.17 ml N,N,N',N'-tetramethylethylenediamine (E-C Apparatus Corp.) in 150 ml pH 9.2 buffer (40.0 g tris, 4.0 g Na_2·EDTA, 1.52 g boric acid in 4 ℓ water). Ammonium persulfate (0.17 g) was added to polymerize the acrylamide. The buffer chambers were filled with the pH 9.2 buffer described above. Samples were applied in 5 m$\underline{M}$ PEM containing sucrose to increase the density and thus prevent mixing of protein into the buffer chamber. Gels were stained with 0.1% Amido Black 10 B in wash solution and destained by prolonged soaking in wash solution.

Analytical Ultracentrifugation:

Samples were dissolved in 5 m$\underline{M}$ PEM containing 0.1 $\underline{M}$ KCl and centrifuged at 151,000 g for 40 min to remove any suspended material. The solution was then centrifuged in an An-D rotor in a Beckman Model E ultracentrifuge at 44,770 rpm at a temperature of 20°C. The sedimentation constants, calculated from Schlieren photographs, were corrected for the viscosity of the medium.

Degradation of Fraction I:

(1) Performic Acid Oxidation: One-hundred and fifty mg of protein were dissolved in 1.5 ml 97+% formic acid. Thirty percent H_2O_2 (0.038 ml) was added and the reaction carried out with shaking for 40 min at 25°C (84). The reaction was stopped with 75 ml water and the reactants lyophilized.

(2) Reduction and Alkylation: Ten mg protein were dissolved in 1 ml 8 M guanidine-HCl or 8 M urea containing 0.1 M Tris HCl, pH 8.0. One µl mercaptoethanol was added and the reduction was carried out at 38°C for 3 hr. Iodoacetic acid (3.25 mg), or iodoacetamide (3.24 mg), and 1 drop of phenol red were added to the reaction mixture and the pH was maintained at about 8 for 15 min with 0.1 N NaOH. The alkylated protein suspension was then dialysed against water to remove excess reagent and indicator.

(3) Tryptic Digestion: Two methods were used: (a) Modified method of Sanger and Tuppy (85): The protein was suspended in 6 ml 0.2 M ammonium bicarbonate buffer, pH 8.5. Trypsin (0.4 ml, 0.5%) (Worthington Biochemical Corp.) and 1 drop of toluene were added. After shaking for 16 hr at 37°C, the hydrolysis was terminated by addition of 2 drops of 6 N HCl, the digest was centrifuged and the supernatant evaporated to dryness. Distilled water was added and the digest was again evaporated to dryness. This step was repeated once. Distilled water was added, the suspension centrifuged and the supernatant, which represented the Fr I trypsin digest, stored in the freezer. (b) One ml of either water, 1 M urea or 1 M urea containing 0.2 M sodium bicarbonate was added to 10 mg protein. Trypsin (0.1 ml, 0.1%) and 1 drop of phenol red were added. The digest was continued

for 4 hr in a water bath at 37°C with shaking and the pH maintained at about 8 with 0.1 N NaOH. Trypsin (0.1 ml of a 0.1% solution) was added and the digest continued for another 4 hr. In some cases, a third portion of trypsin was added and the digestion continued for a total of 16 hr. Hydrolysis was terminated by adding 6 N HCl to bring the pH below 6.5. The digest was centrifuged and the supernatant was evaporated to dryness. Distilled water was added and the digest was again evaporated to dryness. This step was repeated once. Distilled water was added, the suspension centrifuged and the supernatant, which represented the Fr I trypsin digest, stored in the freezer. When the digest was carried out in 1 M urea, the peptide solution was applied to an IR 120 column at pH 4 and the urea eluted with water. The tryptic peptides were then eluted with 4 N NH_4OH. If the tryptic peptides were to be reacted with dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride, DNS-Cl), column chromatography on IR 120 was omitted.

(4) Chymotryptic Digestion: Chymotryptic digestions were carried out in the same manner as that described for method (a) of the tryptic digestion but using a 0.5% α-chymotrypsin (Sigma Chemical Co., Type III) solution.

(5) Protease Digestion: Protease (Sigma Chemical Co., Bacterial Type VII, subtilisin) digestions were conducted in the same manner as that described for the chymotryptic digestion but using a 0.5% protease solution.

(6) Hydrolysis with HCl: To 1 vol. of protein or peptide solution, in a pyrex test tube, an equal volume of conc. HCl was

added. The tube was sealed and the contents hydrolysed at 110°C for 16 hr. The seal was broken and the hydrolysate evaporated to dryness 3 times, redissolving in a small quantity of water between evaporations.

Fingerprinting:

(1) Method I: A modification of the method of Ingram was used (86). A piece of Whatman #1 chromatography paper was sprayed with pH 6.5 pyridine-acetic acid buffer (25 ml pyridine and 1 ml acetic acid made up to 1 ℓ with water). A spot was partially dried by blotting and the tryptic digest of the Fr I protein applied. Electrophoresis was conducted at 400 V for 1.5 hr. The paper was dried and then ascending chromatography was carried out in butanol-acetic acid-water solvent (4:1:1.8 v/v/v) at right angles to the direction of electrophoresis. The paper was then dried and sprayed with 0.2% ninhydrin in acetone to visualize the peptides.

(2) Method II: Method II was identical to Method I except that electrophoresis was conducted at 3000 V for 50 min on a cooled plate apparatus (Model FP18, Savant Instruments Inc.).

(3) Method III: To a tryptic digest of Fr I protein in 0.6 ml 1 M urea containing 0.2 M sodium bicarbonate, pH 8.0 (2 mg/0.6 ml) was added an equal volume of DNS-Cl (20 mg/ml acetone) (Mann Research Laboratories). The solution was incubated with shaking at room temperature overnight (87). The solution containing the dansylated tryptic peptides was evaporated to dryness and dissolved in 0.5 ml 0.01 N acetic acid, pH 3.5. The solution was applied to a Dowex 50 column (1 x 3 cm) and the dansyl hydroxide (1-dimethylaminonaphthalene-5-sulfonic acid) was displaced with 0.01 N acetic acid, pH 3.5.

The peptides were then displaced with 1 M ammonia in 25% acetone. The process was followed with the aid of an ultraviolet lamp. The acetone eluate was evaporated to dryness and dissolved in 0.3 ml 25% acetone. A silica gel H (E. Merck AG, Dormstadt, Germany) thin layer plate was activated by heating at 110°C for 10 min. The sample (10 µl) was applied approximately 2.5 cm from a corner. Ascending chromatography was conducted in butanol : acetic acid : water (4:1:1.8 v/v/v). The plate was dried at 110°C for 10-15 min and then ascending chromatography was conducted at right angles in methyl acetate : isopropanol : conc. ammonia (45:35:20 v/v/v). The peptides were visualized with UV light.

(4) Differential Spray Reagents: Proline and hydroxyproline were visualized using the isatin-acetic acid reagent of Barrollier et al. (88). This reagent and the isatin-pyridine reagent of Barrollier et al. (88) give a variety of colours with amino acids. The methods of Acher and Crocker (89), Reddi and Kodicek (90), Pauly (91), Sakaguchi (89), and Van Halteren (92) were used to stain for tyrosine, tryptophan, histidine, arginine and glycine, respectively. A method described by Sanger and Tuppy (85) was also used to stain for histidine. Thiol compounds were visualized with sodium azide in ethanol (91) while cysteine was visualized with tetrazolium salts (91).

End-Group Analysis:

(1) Determination of the amino-terminal residues of proteins using 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl).

Described below are modifications of the reaction methods of Gray and Hartley (93) or Gray (94):

(a) Fraction I protein (0.25 mg ≡ 10^{-2} μmoles 25,000 MW protein subunit or 5.8 mg ≡ 10^{-2} μmoles 585,000 MW protein) was dissolved in 0.3 ml 0.1 M sodium bicarbonate. In one preparation, the sample was heated at 90°C for 10 min at this point. An equal volume of DNS-Cl solution (5 mg/ml acetone) was added and the solution was then shaken at room temperature for 3 hr. The sample was evaporated to dryness in vacuo. The dry sample was suspended in 0.5 ml 5 mM PEM and dialysed against water at 4°C with stirring for 24 hr. Following dialysis, the sample was evaporated to dryness in vacuo and 0.2 ml of 6 N HCl were added. The tube was sealed and heated at 105°C for 12-30 hr. The acid was then removed in vacuo.

(b) Fraction I protein (0.25 mg in 0.13 ml 0.1 N NaOH) was diluted to 0.5 ml with 8 M urea containing 0.5 M sodium bicarbonate. An equal volume of DNS-Cl solution (20 mg/ml acetone) was added. After shaking at room temperature overnight, the sample was dialysed against water at 4°C with stirring. Following dialysis, the sample was evaporated to dryness in vacuo and 0.5 ml 6.7 N HCl were added. The tube was sealed and heated at 105°C for 18 hr. The acid was then removed in vacuo. The sample was dissolved in 0.5 ml 25% acetone.

(c) (i) Fraction I protein (5.8 mg) was dissolved in 1 ml 8 M urea containing 0.5 M sodium bicarbonate. An equal volume of DNS-Cl solution (20 mg/ml acetone) was added to the solution which was then shaken at room temperature overnight. A further 20 mg crystalline DNS-Cl was added to the suspension and incubation was continued for 9 hr. The sample was evaporated to dryness in vacuo.

(c) (ii) Fraction I protein (5.8 mg) was dissolved in 1 ml 8 M urea containing 0.5 M sodium bicarbonate. Twenty mg of DNS-Cl were added. The mixture was then shaken at room temperature for 72 hr.

Material prepared as described in section c(i) or (ii) was dissolved in 1 ml 6.7 N HCl. The tube was sealed and heated at 105°C for 18 hr. The acid was then removed *in vacuo*. The sample was dissolved in 2 ml 0.01 N acetic acid, pH 3.5. The solution was applied to a Dowex 50 column (1 x 3 cm) and the dansyl hydroxide displaced with 0.01 N acetic acid. The amino acids were displaced with 1 M ammonia in 25% acetone. This process was followed with the aid of an ultraviolet lamp. The acetone eluate was evaporated to dryness and dissolved in 0.1 ml 25% acetone.

The dansyl amino acids were separated by electrophoresis (93) or thin layer chromatography (95). For electrophoresis, the sample and standards (Sigma Chemical Co.) were applied to a sheet of Whatman 3MM paper. Electrophoresis was conducted at 3500 V for 3 hr on a cooled plate apparatus at pH 4.40 (0.8% acetic acid - 0.4% pyridine). Ascending chromatography was conducted in parallel with markers on a silica gel H thin layer plate which had been activated at 110°C for 10 min. The solvents used were: (1) benzene : pyridine : acetic acid, 80:20:2 (v/v/v), (2) chloroform : *tert*-amyl alcohol : formic acid, 70:30:1 (v/v/v), or (3) chloroform : *tert*-amyl alcohol : acetic acid, 70:30:1/2 (v/v/v). The dansyl amino acids were visualized with UV light.

(2) Edman method (96):

Fraction I protein (5 mg) was suspended in 0.5 ml dioxane : triethylamine : phenylisothiocyanate (Eastman Organic Chemicals) : water, 8.0:0.4:0.1:1.0 (v/v/v/v). Incubation was conducted with shaking at 37°C for 3 hr. The sample was evaporated to dryness under a stream of nitrogen, 0.1 ml water added, and the mixture extracted with three 2 ml portions of benzene to remove excess phenylisothiocyanate, phenylthiourea and residual organic solvent. The mixture was centrifuged and the organic phase was discarded. The aqueous residue was evaporated to dryness under a stream of nitrogen and 0.1 ml water and 0.2 ml acetic acid saturated with HCl (5:1) were added. The mixture was incubated at 40°C for 1 hr and then evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.5 ml water and extracted three times with 0.5 ml portions of ethyl acetate. The organic phase was washed with 0.1 ml water and then evaporated to dryness under a stream of nitrogen. The sample was dissolved in 0.1 ml 90% acetic acid. An Eastman "Chromagram" sheet (Type 6060 - silica gel containing fluorescent indicator was sprayed with 0.1% disodium-EDTA and activated at 110°C for 15 min. Ascending chromatography of the sample (10 µℓ) was conducted in System II (heptane : ethylene chloride : formic acid (75%) - 60:30:5 v/v/v) or System III (heptane : ethylene chloride : formic acid (75%) - 30:60:5 v/v/v) of Sjoquist (97). In some cases, following chromatography in System II, the sheet was dried and ascending chromatography was conducted in System III at right angles to the direction of chromatography in System II. The N-terminal

amino acids were visualized with the aid of a UV lamp.

Amino Acid Analysis:

To 2 ml 5 mM PEM containing Fr I protein (~20 mg) was added an equal volume of 10% TCA (trichloroacetic acid). The suspension was centrifuged and the pellet dissolved in 0.5 ml formic acid (50%). The solution was diluted to 2.5 ml with water, 2.5 ml 10% TCA were added and the suspension was centrifuged. The resulting pellet was extracted with three 5 ml portions of acetone and then with 5 ml ether. The pellet was lyophilized and then dried *in vacuo* over P_2O_5. Protein (5 mg) was dissolved in 2.5 ml 0.1 M NaOH. To a 0.5 ml aliquot in a hydrolysis vial was added an equal volume of 6.1 N HCl. The hydrolysis vials were sealed *in vacuo* and then heated at 110°C for 16, 24, 48 or 72 hr.

Following hydrolysis, the samples were transferred quantitatively to "evapo-mix" tubes. The sample was evaporated to dryness in a Rotary Evapo-mix apparatus (Buchler Instruments, Fort Lee, New Jersey). Water (2 ml) was added to the tube and the sample again evaporated to dryness. This operation was repeated twice. The dry sample was dissolved in 1 ml sample diluting mixture (15% polyethylene glycol 400 (PEG)) and transferred to a test tube. Two 0.5 ml portions of 15% PEG were used to rinse the "evapo-mix" tube. Analyses were carried out by the method Spackman *et al.* (98) with a Spinco model 120C automatic amino acid analyzer adapted for high-sensitivity determinations. The limiting values were used for the determination of the content of leucine, isoleucine and valine. The values obtained from the 24 hr hydrolyses were used for the

determination of the serine and threonine. For all other determinations the average of the values obtained from the 24, 48 and 72 hr hydrolyses was used.

The Determination of Tryptophan:

The molar ratio of tyrosine to tryptophan was determined by the method of Beaven and Holiday (99). The protein was dissolved in 0.1 N NaOH and the optical density of the solution at 280.0, 294.4, 320.0 and 360.0 mμ was determined in a 1 cm quartz cell in a Cary 14 recording spectrophotometer. A line joining the points at 360.0 and 320 mμ was extrapolated as far as 280.0 mμ. This formed the baseline from which optical densities were measured and represented an estimate of absorption due to material other than tyrosine and tryptophan. The molar ratio of tyrosine to tryptophan was calculated using the following formula:

$$\frac{M_{tyr}}{M_{try}} = \frac{0.592\ E_{294.4} - 0.263\ E_{280}}{0.263\ E_{280} - 0.170\ E_{294.4}} \qquad (99)$$

where M_{tyr} and M_{try} are the number of g moles of tyrosine and tryptophan, respectively, in one gram of protein, and $E_{294.4}$ and E_{280} are the extinction values determined at 294.4 and 280 mμ, respectively. The errors involved in this sort of analysis are discussed by Beaven and Holiday (99).

Enzyme Assays:

(1) Ribulose Diphosphate Carboxylase Assay: The method of Paulsen and Lane (16) was used with slight modification.

The complete reaction mixture contained the following components (in micromoles per 0.5 ml, unless otherwise indicated):

Tris (Cl^-) buffer, pH 7.8, 100; D-ribulose diphosphate (RuDP) (Sigma Chemical Co., tetrasodium salt), 0.35; [^{14}C] $NaHCO_3$ (New England Nuclear Corp., 5.0 mc/mM), 2 μc; total $NaHCO_3$, 25; $MgCl_2 \cdot 6\ H_2O$, 5; EDTA, 0.03; mercaptoethanol, 3; and Fr I protein, 0.1-0.7 mg. Controls were included in each assay. In one control, enzyme was omitted from the assay mixture while in the other control the enzyme was heated in assay buffer at 90°C for 10 min before the RuDP and sodium bicarbonate were added. Incubation was conducted at 30°C. At 4, 8 and 12 min, 0.5 ml aliquots were withdrawn from each tube and added to 1 ml 2 N HCl. A 0.5 ml aliquot from each tube was put into a scintillation vial and dried in a forced draft oven for 45 min at 95°C. Water (1 ml) and liquid scintillator (10 ml of a mixture containing 750 ml dioxane, 125 ml 1,2-dimethoxyethane, 125 ml anisole, 12 g PPO (2,5-diphenyloxazole) and 0.5 g POPOP (1,4-bis-2(5-phenyloxazolyl)benzene) was added to each vial. Radioactivity measurements were made in a Nuclear-Chicago Mark I liquid scintillation counter. Disintegrations per min were calculated from the percent efficiencies which were determined with an external standard. One unit of RuDP carboxylase is defined as that amount which catalyzes the fixation of 1.0 μmole of [^{14}C] bicarbonate into an acid-stable form per min under the assay conditions.

(2) Combined "Isomerase-Kinase" Assay:

Again the method of Paulsen and Lane (16) was used with slight modification.

The complete reaction mixture contained the following components

(in micromoles per 0.5 ml, unless otherwise indicated); Tris (Cl^-) buffer, pH 7.8, 100; D-R-5-P(D-ribose-5-phosphate) (Sigma Chemical Co., disodium salt), 2; ATP (adenosine triphosphate, Sigma Chemical Co.), 4; $MgCl_2 \cdot 6\ H_2O$, 5; [^{14}C] $NaHCO_3$, 2 μc; total $NaHCO_3$, 25; mercaptoethanol, 3; EDTA, 0.03; Fraction I protein, 0.07 - 0.25 mg. Again, controls in which enzyme was omitted from the assay mixture or heated in assay buffer at 90°C for 10 min before addition of substrates were carried out. Incubation was conducted at 30°C for 10 or 12 min and the reaction terminated by the addition of 1 ml 2 N HCl to each vessel. Subsequent steps were the same as those described for the RuDP carboxylase assay. One unit of combined enzyme is defined as that amount which catalyzes the fixation of 1.0 μmole of [^{14}C]-bicarbonate into an acid-stable form per min under the assay conditions.

Nitrogen Determination:

The method of Miller and Miller (100) was used for the digestion of the peptides. The sample was dissolved in 1 ml H_2SO_4 diluted 1:1 and heated until white fumes appeared and then for 5 min more. The digestion flask was allowed to cool for 30 sec and 2 drops of H_2O_2 were added down the side of the flask. The sample was heated for 2 min. The steps involving the cooling, addition of H_2O_2 and heating were repeated until the solution was clear and colourless. The flask was then cooled and the sides washed with 5-10 ml water. After 5 min, the determination of nitrogen could be continued.

The solution was placed in a Markham distillation unit. The ammonia was released by the addition of 10 ml saturated NaOH

solution and steam distilled into 10 ml of boric acid-indicator mixture (10 g boric acid and 5 ml indicator mixture [10 ml 0.2% methyl red in 95% ethanol : 5 ml 0.2% methylene blue in water]); the contents of the receiver were titrated with 0.1 N HCl.

Orcinol Test for RNA:

The orcinol test was conducted as suggested by Clark (101). A standard curve was prepared using AMP as the nucleotide standard.

RESULTS AND DISCUSSION

1. The Purification of Fr I Protein:

(a) Method I: Early work was conducted with Fr I protein purified by method I. The effect of the various steps of method I on the purity of the preparation was studied. Horizontal starch gel electrophoresis of crude extract showed an obvious Fr I band upon staining with Amido Black 10 B. One faster-moving and one slower-moving component were also observed. Upon staining with Amido Black 10 B after starch gel electrophoresis of sample taken after purification in Sephadex G-200, a Fr I band was observed and a very small slower-moving band which is believed to represent an aggregate of Fr I protein. Some streaking of slower-moving material was seen. The sample obtained at this stage was dissolved in 0.05 M phosphate buffer, pH 7.0, 0.1 M in KCl to a concentration of 1%. In the analytical centrifuge at 44,770 rpm, a large Fr I component was observed. A small, faster-moving component was also observed. Starch gel electrophoresis of the sample after zone centrifugation showed a strong Fr I band after staining with Amido Black 10 B. Almost no slower-moving material was seen. The sample obtained after zone centrifugation was run immediately in the analytical centrifuge. A large Fr I component and a very small, faster-moving component were observed. The faster-moving component probably consisted of Fr I aggregates.

Electrophoresis on polyacrylamide gel rather than starch gel resulted in an increase in resolving power and sensitivity. Vertical polyacrylamide gel electrophoresis of Fr I prepared by method I indicated the presence of seven components upon staining with Amido Black 10 B. It was thus necessary to obtain an improved method for the purification of Fr I protein.

(b) Method II (Paulsen and Lane (16)) and III: To obtain pure Fr I protein, it was necessary to use a different method of purification. It was decided to try the method of Paulsen and Lane (Method II) (16). Pure spinach Fr I protein was obtained using method II, but the yield was low due to the use of column chromatography on DEAE-cellulose and Hypatite C. In my hands, DEAE-cellulose chromatography did little to improve the purity of the Fr I.

Chromatography of spinach Fr I on Hypatite C with stepwise elution using 5 and 25 m$\underline{M}$ PEM (16) followed by further elution with 500 m$\underline{M}$ PEM gave three UV absorbing fractions (Fig. 5a). Gel electrophoresis showed that all three fractions contained some Fr I protein. The fraction eluted with 5 m$\underline{M}$ PEM had a high $OD_{260:280}$ ratio implying that a considerable amount of nucleic acid was present while the fraction eluted with 25 m$\underline{M}$ PEM appeared to contain pure Fr I protein.

Spinach Fr I prepared by method III had ribulose diphosphate (RuDP) carboxylase activity and gave a single component on polyacrylamide gel electrophoresis (Fig. 6a) and on analytical ultracentrifugation ($S_{w,20}$ = 16.8) (Fig. 7a). The RNA content of this material was less than 0.2% (orcinol test). A second and minor component seen upon polyacrylamide gel electrophoresis probably consists of Fr I dimers (see Introduction, Section 1, Part 3).

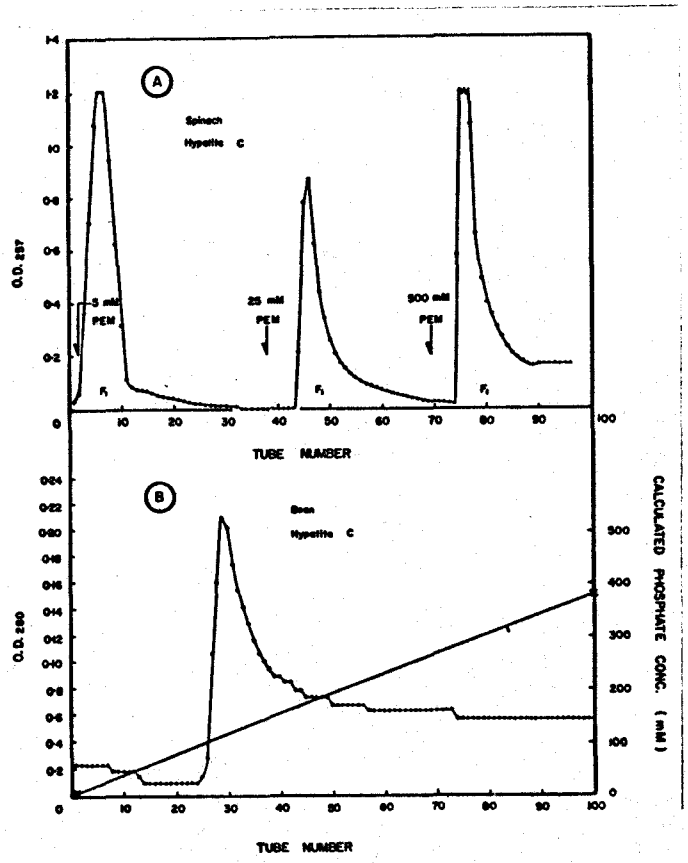


Fig. 5. Chromatography of Fraction I protein on Hypatite C.*
Column: 2.5 x 30 cm. (a) Spinach. Fraction size about 8 ml.
Vertical arrows indicate points of addition of (from left to right)
5, 25 and 500 mM PEM. (b) Bean. Fraction size about 5 ml.

* OD replotted from percent transmission record.

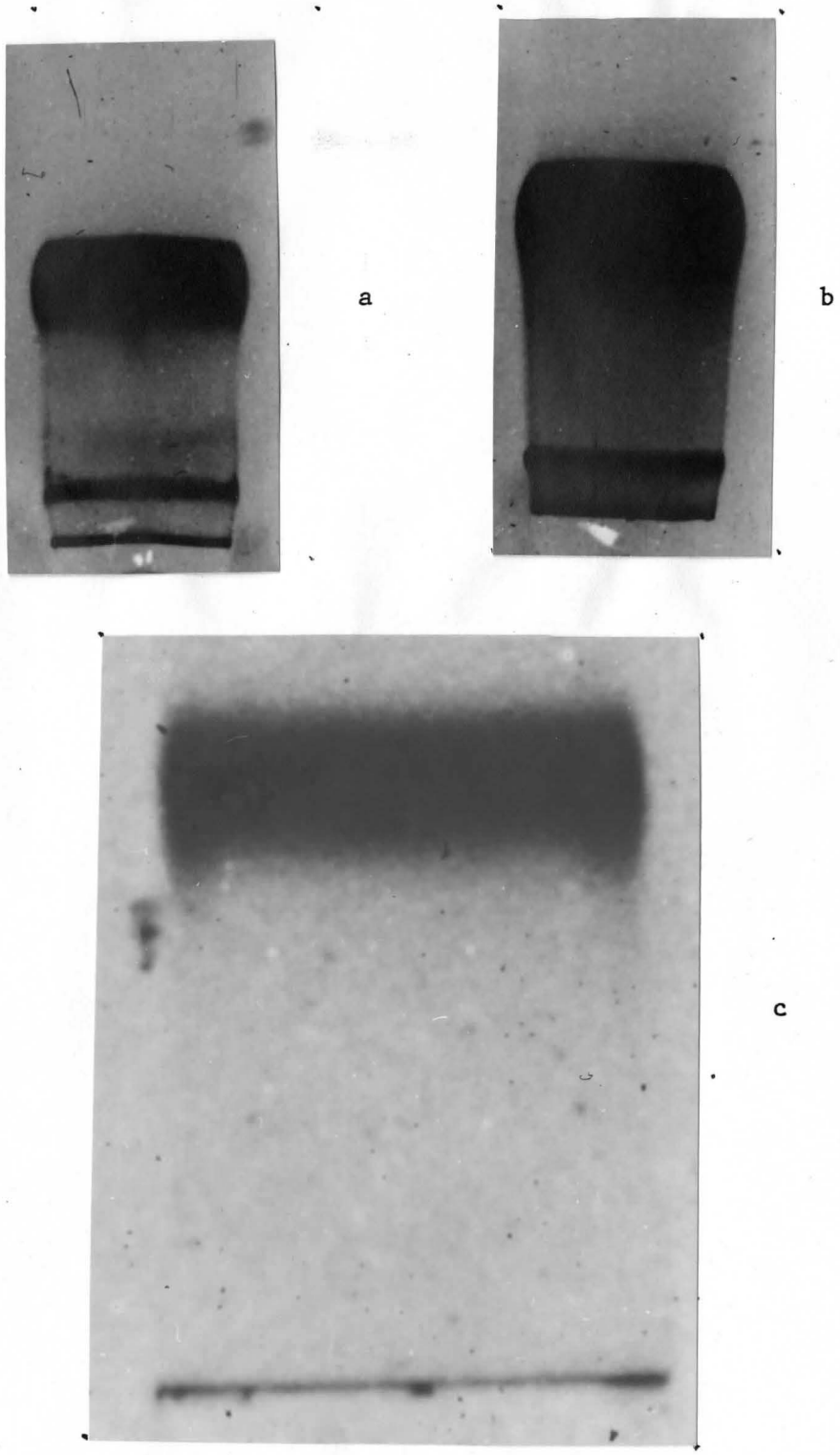


Fig. 6. Polyacrylamide gel electrophoretograms of Fraction I protein. (a) and (b) Spinach and bean, respectively, purified by the new method, (c) Chlorella purified by the new method without the Sepharose 4B step.

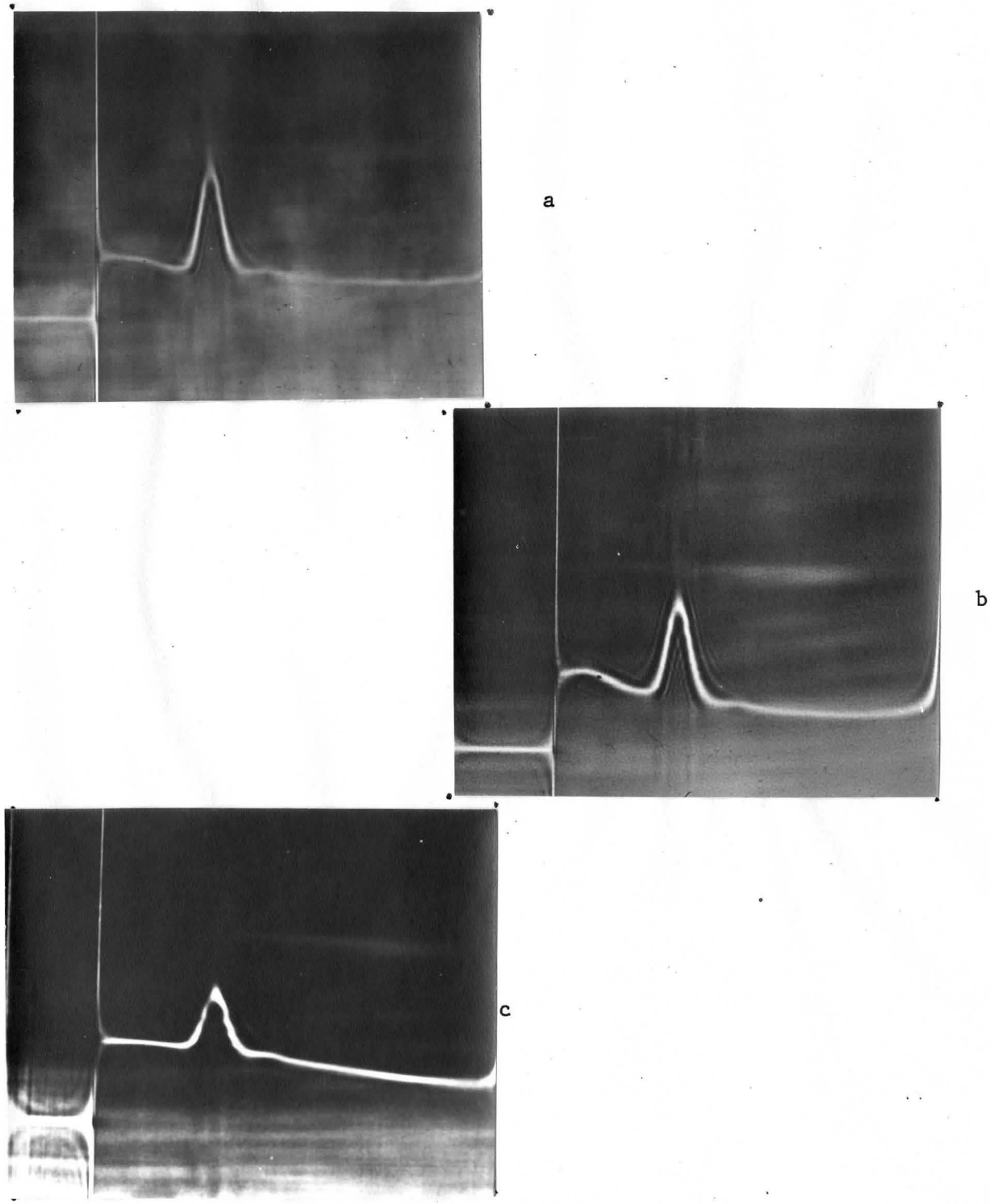


Fig. 7. Sedimentation of purified Fraction I protein. Buffer: 5 mM PEM containing 0.1 M KCl. Speed: 44,770 rpm. Temperature: 20.0°C. Sedimentation from left to right. (a) Spinach, (b) bean and (c) Chlorella.

A very low yield of bean Fr I was obtained by method II. Study of the individual steps of the procedure showed that bean Fr I is salted out at a much lower concentration of ammonium sulfate (Table IX) than that used by Paulsen and Lane (16) and hence is discarded when their method (II) is used without modification. Likewise, bean Fr I is not eluted from Hypatite C columns with the concentration of PEM used for spinach Fr I (Table IX) (Fig. 5b). Bean Fr I prepared by method III contained a single component on polyacrylamide gel electrophoresis (Fig. 6b) and on analytical ultracentrifugation ($S_{w,20}$ = 16.5) (Fig. 7b).

Pea Fr I prepared by method II was, like spinach Fr I, salted out between 226 and 320 g/ℓ of ammonium sulfate and was eluted from Hypatite C by 25 mM PEM. Polyacrylamide gel electrophoresis of pea Fr I prepared by method III gave one major band.

Chlorella Fr I prepared by method II was obtained in reasonable purity but in excessively low yield. A careful study showed that Chlorella Fr I was precipitated by 150 g ammonium sulfate per liter (Table IX) and was eluted from Hypatite C with 100 mM PEM but not with 25 mM PEM. Chlorella Fr I prepared by method III gave a single component on polyacrylamide gel electrophoresis (Fig. 6c) and on analytical ultracentrifugation ($S_{w,20}$ = 19.0) (Fig. 7c).

Nicotiana tabacum Fr I prepared by method III gave a single major component on polyacrylamide gel electrophoresis following chromatography on Sepharose 4B, had RuDP carboxylase activity, and was salted out by 50 g/ℓ of ammonium sulfate.

Fraction I protein prepared from Nicotiana glauca by method III was salted out by 150 g/ℓ ammonium sulfate. Nicotiana langsdorffii prepared by method III was salted out between 226 and 320 g/ℓ of ammonium sulfate. A single major component was seen on polyacrylamide gel electrophoresis of N. langsdorffii.

Fraction I protein prepared from wheat, rye or triticales by method III was salted out between 226 and 320 g/ℓ ammonium sulfate and had RuDP carboxylase activity (see p. 78).

The advantages of the preparative method III over method II are simplicity, high yields of apparently pure protein and adaptability to plants other than spinach. Only the ammonium sulfate concentration must be varied to modify the procedure of method III for the purification of Fr I from the plants studied.

2. Methods for the Enzymatic Hydrolysis of Fr I Protein:

(a) A Comparison of Reduction and Oxidation before Tryptic Hydrolysis: Techniques such as performic acid oxidation or reduction with mercaptoethanol followed by alkylation with iodoacetic acid, or iodoacetamide, were explored. The breakdown of Fr I protein following tryptic hydrolysis was more complete after reduction and alkylation than after oxidation.

(b) Tryptic Hydrolysis: A comparison of the bean Fr I protein and hydrolysate present at various stages of oxidation and hydrolysis (procedure (a)) is shown in Table VIII. Tryptic hydrolysis of oxidized Fr I protein resulted in incomplete digestion of the protein (Table VIII).

Table VIII

Enzymatic Hydrolysis of Oxidized Bean Fr I Protein.
A concentration of 1 mg/ml of native protein corresponds to an optical density of 1.41 at 280 mμ (15)

Sample	Relative OD_{280}
Original Solution	1.00
After Performic Acid Oxidation	0.84*
Solubilized by Trypsin after Performic Acid Oxidation	0.31
Solubilized by Chymotrypsin after Trypsin Treatment	0.20
Residue from Trypsin followed by Chymotrypsin Digestion	0.09

* Decrease may be due to destruction of tryptophan.

Various techniques of tryptic hydrolysis were explored (see Methods, p. 41). The most complete breakdown was obtained by the tryptic hydrolysis of Fr I protein in 1 M urea (procedure (b)). A comparison by the Kjeldahl method (100) of the quantity of peptide present in the hydrolysate with the initial quantity of protein indicated that more than 90% of the Fr I was rendered acid soluble.

(c) Chymotryptic and Protease Hydrolysis: Hydrolysis with chymotrypsin or protease resulted in greater breakdown of the Fr I protein than did hydrolysis with trypsin.

3. The Comparison of Fr I Protein from Different Plants:

(a) Physical Properties: Conditions for ammonium sulfate precipitation of Fr I and for the elution of Fr I protein from Hypatite C have already been discussed and are illustrated in Table IX.

Starch gel electrophoresis of Fr I protein from various species indicated identical mobilities. The greater resolving power obtained with polyacrylamide gel permitted the separation of mixtures of Fr I protein from various plants (Fig. 8). The electrophoretic mobilities on polyacrylamide gel of Fr I from various species are shown in Table IX.

Comparison of the mobilities of the proteins from bean, Chlorella, pea and spinach indicates that Fr I proteins from bean and Chlorella have a higher negative charge at pH 9.2 than do Fr I proteins from pea or spinach. This is consistant with the observation that buffers having different ionic strengths are required to elute

Table IX

Comparison of Fraction I Proteins

	Plant											
	Spinach	Pea	Bean	Chlorella	Wheat	Rye	Triticales	Cabbage	Radish	Nicotiana glauca	Nicotiana langsdorffii	Nicotiana tabacum
Conditions for ammonium sulfate precipitation (grams/liter):												
1. required to begin precipitation	226	226	—*	—*	226	150	226	—	—	—*	>150	—*
2. required to complete precipitation	320	320	150	150	320	320	320	—	—	150	226	50
mM PEM required to elute from Hypatite C	25	25	110	100	—	—	—	—	—	—	—	—
Approximate mobility on polyacrylamide gel electrophoresis relative to the mobility of Fraction I from spinach	1.0	1.0	1.5	1.3	1.2	1.4	1.2	1.2	1.1	1.0	1.2	1.2

* No lower limit established. Some Fraction I precipitate is present in solutions containing less than 50 g ammonium sulfate per liter.

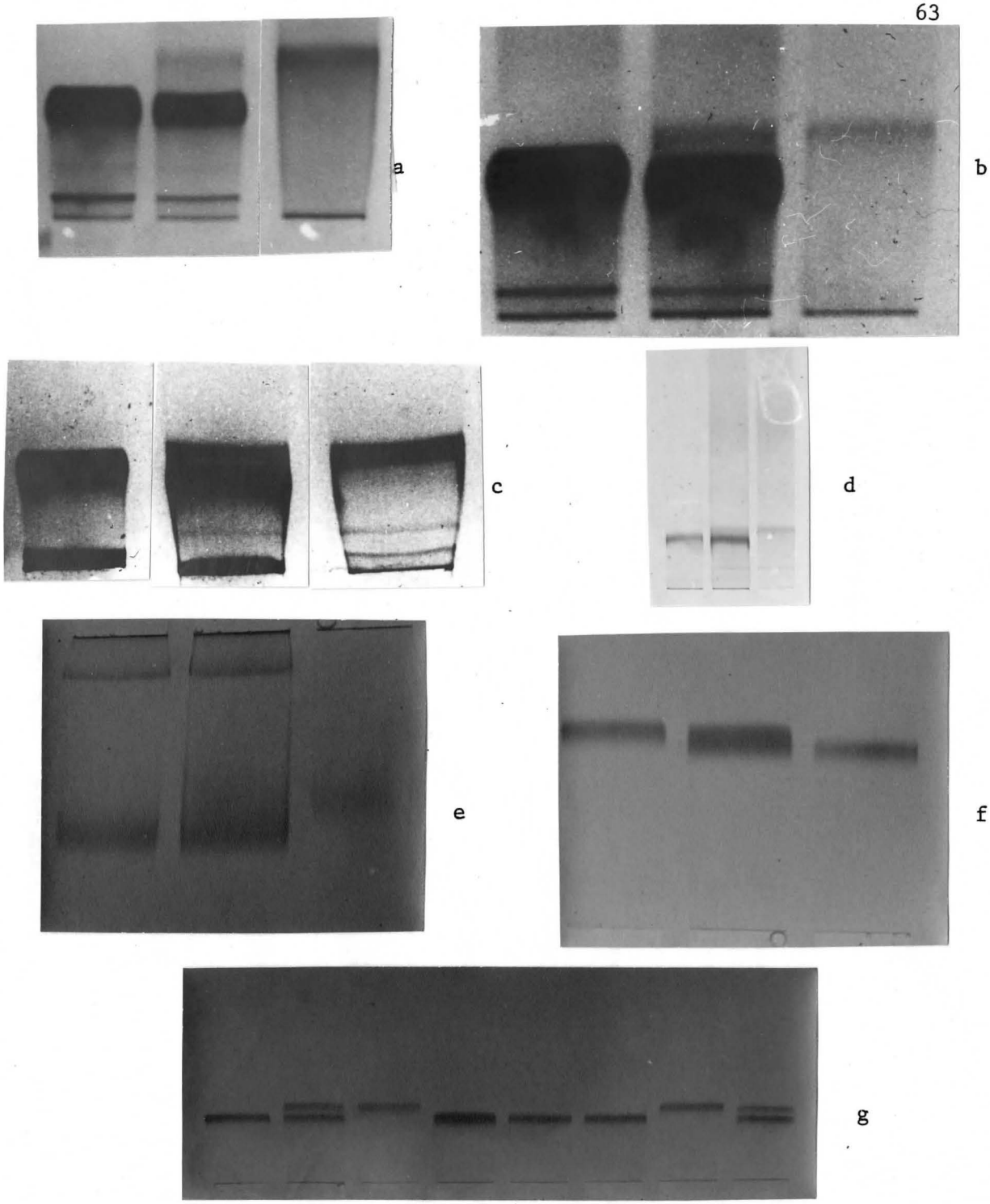


Fig. 8. Polyacrylamide gel electrophoretograms of Fraction I protein mixtures. (a) (from left to right) spinach, mixture of bean + spinach, bean, (b) pea, mixture of pea + bean, bean, (c) spinach, mixture of spinach + Chlorella, Chlorella, (d) Nicotiana glauca, mixture of Nicotiana glauca + Nicotiana langsdorffii, Nicotiana langsdorffii, (e) Nicotiana glauca, mixture of Nicotiana glauca + Nicotiana tabacum, Nicotiana tabacum, (f) cabbage, mixture of cabbage + radish, radish, and (g) wheat, wheat + rye, rye, wheat + triticales, wheat, triticales, rye, rye + triticales.

the Fr I proteins from Hypatite C.

Clear separations were obtained when mixtures of Fr I from the following plants were subjected to polyacrylamide gel electrophoresis: (a) bean and spinach, (b) pea and bean, (c) spinach and Chlorella, (d) N. langsdorffii and N. glauca, (e) N. tabacum and N. glauca, (f) cabbage and radish, (g) rye and triticales, and (h) wheat and rye (Fig. 8). No separations were obtained when mixtures of pea and spinach, N. langsdorffii and N. tabacum, and wheat and triticales were subjected to polyacrylamide gel electrophoresis.

(b) Fingerprints: Early fingerprinting was done on paper using chromatography and low voltage electrophoresis. About twenty-one peptide spots were observed in fingerprints of trypsin digests (oxidation, procedure (a)) of both bean and spinach. Ten peptides in trypsin digests of spinach and nine peptides in trypsin digests of bean were uncharged and were poorly separated on chromatography. There appeared to be a difference between the fingerprints obtained with trypsin digests of spinach and bean but the peptide spots were often diffuse and the fingerprints were not completely reproducible.

Later fingerprinting on paper was done using high voltage electrophoresis and chromatography. When fingerprints were prepared with a tryptic digest of bovine hemoglobin (Sigma Chemical Co., Type 1), the patterns obtained were similar to those obtained by Zuckerkandl et al. (102). More than twenty-four peptide spots were observed in fingerprints of trypsin digests (oxidation, procedure (a)) of both bean and spinach after purification with Sephadex G-200

(Method I). Fr I protein from spinach was seen to differ from that of bean in at least three peptides. This was confirmed by running fingerprints containing both spinach and bean hydrolysates. Fingerprints obtained from a trypsin digest of bean prepared by zone centrifugation (Method I) gave a similar pattern with a few possible differences. Fingerprints obtained from a trypsin digest (reduction, alkylation, procedure (b)) of spinach prepared by method II (chromatography on Sephadex G-200 rather than on DEAE-cellulose) gave a pattern similar to that obtained from a trypsin digest of spinach prepared by method I. The fingerprints were still only fairly reproducible and a ninhydrin positive background was usually observed in the region containing the peptides.

A 100-fold increase in sensitivity was obtained by using dansyl tryptic peptides and fingerprinting on thin layer plates. Fingerprints of dansyl tryptic peptides (reduction, alkylation, procedure (b)) of spinach Fr I protein were prepared by thin layer chromatography (see Methods, p. 43). Of fifty-two to sixty peptide spots observed, three spots were very intense and five were faint. The rest were of moderate and approximately equal intensity. Fingerprints of dansyl tryptic peptides from bean Fr I protein were also prepared by thin layer chromatography and gave thirty-five to thirty-six peptide spots. Control experiments showed that only three spots arise from the peptides produced by autolysis of trypsin. No valid conclusions could be drawn from a comparison of the fingerprints of bean and spinach Fr I. It is possible that the

ninhydrin positive background observed in fingerprints prepared on paper by methods I and II was, in fact, due to the presence of the large number of peptides observed in fingerprints obtained by method III as the use of dansyl peptides and thin layer chromatography increases the sensitivity about one-hundred fold over the use of paper for fingerprinting and ninhydrin to visualize the peptide spots (87).

The data of Ridley et al. (12) indicate that there are 12 lysine residues and 10 arginine residues per minimum molecular weight (24,426.6) of Fr I protein from spinach-beet. Thus, for a molecular weight of 585,000, there would be 288 lysine residues and 240 arginine residues. Assuming that none of the peptides produced by tryptic hydrolysis were identical and that there were no adjacent basic amino acids, 528 peptide spots would be expected on a fingerprint of the tryptic peptides from Fr I. If, as has been suggested, Fr I protein consists of 24 identical subunits (See Introduction, p. 6), a maximum of 22 peptide spots would be expected in fingerprints of tryptic peptides of Fr I protein. More than 22 peptide spots have consistently been observed. Rutner and Lane (32), have suggested that Fr I protein consists of two types of non-identical subunits. If this were the case, a maximum of 44 peptide spots would be expected in fingerprints of tryptic peptides of Fr I protein. Thus, it would appear that Fr I may contain more than two types of subunit as fingerprints of tryptic peptides of Fr I protein contain at least 52 to 60 peptide spots.

Differential spray reagents have also been used on fingerprints (high voltage electrophoresis, chromatography) of spinach and bean trypsin digests (oxidation, procedure (a)) of material purified

with Sephadex G-200 (Method I). No significant difference was indicated in the distribution of various amino acids in the tryptic peptides of bean and spinach. However, the differential spray reagents would only indicate the presence or absence of an amino acid in the peptide and not a change in the quantity of a particular amino acid in the peptide or a change in position within the peptide.

(c) End-Group Analysis: It was first determined that at least 0.1 n$\underline{M}$ of dansyl N-terminal amino acid must be applied to chromatograms in order to have sufficient material to detect. Then, to test the method, egg white lysozyme (10^{-2} $\mu\underline{M}$) (Sigma Chemical Co., Grade I) was reacted with DNS-Cl (1-dimethylaminonaphthalene-5-sulfonyl chloride) using method I(a). Following electrophoresis at pH 4.4, five spots were observed. One spot corresponded to 1-dimethylaminonaphthalene-5-sulfonic acid (DNS-OH), another to 1-dimethylaminonaphthalene-5-sulfonamide (DNS-NH_2) and another to ε-DNS-lysine. Another also occurred in all preparations from spinach and bean Fr I and may correspond to O-DNS-tyrosine. The fifth spot remained at the origin and probably corresponded to di-DNS-lysine as this dansyl amino acid would be expected to remain at the origin. Following ascending thin layer chromatography in benzene : pyridine : acetic acid (80:20:2), two spots were observed. One corresponded to ε-DNS-lysine while the other corresponded to di-DNS-lysine. Thus, the N-terminal amino acid of lysozyme was found to be lysine. This agrees with published results (103).

The first preparation of dansyl spinach Fr I was made by

method I(a) (p. 45). Sufficient material was spotted so that if only one N-terminal amino acid per 585,000 MW of Fr I were present and the dansyl reaction were complete, the spot corresponding to the N-terminal amino acid would be visible following electrophoresis. Three spots were observed. They corresponded to DNS-NH_2, DNS-OH and ε-DNS-lysine. Thus, no N-terminal amino acid was observed for Fr I protein by this method.

The next samples of dansyl bean and spinach Fr I were also prepared by method I(a) but less sample was used. If Fr I consists of three or fewer non-identical subunit types of 24,427 MW and the dansyl reaction were complete, then the amount of material spotted would allow the detection of the N-terminal amino acids following electrophoresis or thin layer chromatography. No material representing N-terminal amino acids could be detected following thin layer chromatography. Following electrophoresis of samples which had been hydrolysed for 12 hr after dansylation, spots corresponding to DNS-NH_2 and DNS-OH were observed. In addition, a streak resulting from large amounts of positively charged material was present. To eliminate the possibility that the hydrolysis was incomplete the hydrolysis time was extended to 29 hr. Patterns observed following electrophoresis of samples hydrolysed for 12 and 29 hr were similar.

At this stage, there appeared to be four possible explanations for the inability to demonstrate the presence of N-terminal amino acids in bean or spinach Fr I: (1) the N-terminal amino acid might be masked, (2) the N-terminal amino acid might be unstable during hydrolysis, (3) more than three different N-terminal amino acids

might be present per 600,000 MW, or, (4) the dansyl reaction may have been incomplete in the time allowed.

The existence of proteins with an acetyl group at the amino end has been demonstrated and has been reviewed by Wieland and Determann (104). Thus, there is a precedent for the possibility that Fr I protein has a masked N-terminal amino acid. However, it was necessary to investigate the other possibilities.

If the reaction was incomplete, one cause might have been inaccessibility of the N-terminal amino acids due to the folding of the protein. In the next preparation, the samples were heated at 90°C for 10 min before the addition of DNS-Cl. This procedure would help to expose any inaccessible N-terminal amino acids in the protein. The amount of material spotted for electrophoresis or chromatography would have allowed the detection of the N-terminal amino acids if there were three or fewer non-identical subunit types and the dansyl reaction was complete. No material corresponding to an N-terminal amino acid could be detected following electrophoresis at pH 4.4 or thin layer chromatography in benzene : pyridine : acetic acid (80:20:2).

Spinach and bean Fr I were exposed to much larger amounts of DNS-Cl in the next preparation (Method I(b)) and the reaction was conducted overnight. Following electrophoresis of the hydrolysate, six spots were present to the cathode side of the origin and three spots and a streak were present to the anode side of the origin. Of the six spots on the cathode side of the origin, one corresponded to DNS-NH_2 and one to ε-DNS-lysine. Of the four remaining spots on the cathode side of the origin, one probably represented O-DNS-tyrosine.

The other three spots on the cathode side of the origin cannot be accounted for. None corresponded to α-DNS-histidine or DNS-arginine. It is possible that the spots represented by-products as two of the spots were also observed in an hydrolysate of dansyl lysozyme subjected to electrophoresis at the same time. Of the spots observed on the anode side of the origin, one corresponded to DNS-OH. The other two had a greater negative charge than DNS-OH but did not correspond to DNS-glutamic acid or DNS-aspartic acid. It is noteworthy that Gray (94) has demonstrated the presence of "by-products" which have a charge similar to that of the unidentified dansyl compounds observed on the anode side of the origin following electrophoresis. The streak observed on the anode side of the origin may contain DNS-amino acids but, once again, it is noteworthy that the hydrolysate of dansylated lysozyme gave a similar streak on the electrophoretogram. Following thin layer chromatography in benzene : pyridine : acetic acid (80:20:2) of the hydrolysates of dansyl bean and spinach Fr I, two major spots were observed. One was at the solvent front and corresponded to DNS-NH_2 while the other was at the origin and probably corresponded to ε-DNS-lysine. A faint streak was observed in the track of the hydrolysate of dansyl spinach Fr I but no specific spot could be discerned. Four spots were observed following thin layer chromatography in chloroform : tert-amyl alcohol : formic acid (70:30:1) of hydrolysates of dansyl spinach and bean Fr I. Of these, one corresponded to ε-DNS-lysine and the others were all present in a sample of DNS-NH_2 which was prepared by method I(a). The spots were by-products of the reaction. Thus, an increase in the

amount of DNS-Cl used in the reaction and in the incubation time did not result in the detection of any N-terminal amino acids. This preparation of the hydrolysates of dansyl spinach and bean Fr I was also submitted to thin layer electrophoresis at pH 4.4 followed by chromatography in chloroform : tert-amyl alcohol : formic acid (70:30:1) at right angles to the direction of electrophoresis. Four spots were observed and they corresponded to DNS-NH_2 (2 spots), DNS-OH and ε-DNS-lysine. Thus, the two-dimensional study did not help in the elucidation of the N-terminal amino acids.

Two further preparations (Methods I(c)(i) and (ii)) were made to ensure that, if an unmasked N-terminal amino acid was present and if the amino acid could react, the N-terminal amino acid would be visible following electrophoresis or chromatography. As a control, dansyl lysozyme was also prepared by these methods. Enough Fr I was used so that, even if there were twenty-four non-identical subunits per 585,000 MW, the dansyl derivative of the N-terminal amino acids would be visible. A large excess of DNS-Cl was used. In one preparation (Method I(c)(i)), a second addition of DNS-Cl was made in case the DNS-Cl hydrolysed before it could react with the N-terminal amino acid. Both preparations were carried out after suspending the protein in 8 M urea containing 0.5 M sodium bicarbonate. One preparation was carried out in an organic solvent (50% acetone) (Method I(c)(i)) while the other was carried out in an aqueous solvent (Method I(c)(ii)). The incubation with DNS-Cl was conducted for a total of 23 hr in the organic solvent and 72 hr in the aqueous solvent. Thus, every step possible was taken to ensure the reaction went to

completion. Following hydrolysis, the DNS-OH (hydrolysed excess reactant) was removed by chromatography on Dowex 50 to minimize the streaking of the DNS-OH during electrophoresis or chromatography. Samples of hydrolysates of dansyl spinach Fr I, bean Fr I or lysozyme prepared by Method I(c)(i) gave the same patterns following electrophoresis or chromatography as did samples prepared by Method I(c)(ii). Thus, the two different methods resulted in the same end products. Following electrophoresis of an hydrolysate of dansyl lysozyme, three spots and a streak were observed on the cathode side of the origin and two spots and a streak were observed on the anode side of the origin. Of the three spots on the cathode side of the origin, one corresponded to ε-DNS-lysine. The other two could not be identified but did not correspond to DNS-arginine or DNS-histidine. Of the two spots observed on the anode side of the origin, one appeared to correspond to di-DNS-lysine, as would be expected, and the other to DNS-OH. Following electrophoresis of hydrolysates of dansyl bean or spinach Fr I prepared by methods I(c)(i) or I(c)(ii), three spots and a streak were observed to the cathode side of the origin and two spots and a streak to the anode side. The three spots on the cathode side of the origin corresponded to those observed following electrophoresis of hydrolysates of dansyl lysozyme. Of the two spots on the anode side of the origin one corresponded to DNS-OH and the other appeared to almost correspond to DNS-phenylalanine or DNS-tyrosine. It is noteworthy that Gray (94) has observed the presence of a "by-product" which would be expected to correspond even more closely with the observed spot than did DNS-phenylalanine or DNS-tyrosine.

It was also suspected that DNS-serine or DNS-proline may have been masked by the spot corresponding to DNS-OH. Thin layer chromatography in chloroform : tert-amyl alcohol : acetic acid (70:30:1/2) of hydrolysates of dansyl spinach or bean Fr I gave streaking and five spots. One spot was at the origin and probably corresponded to DNS-OH and ε-DNS-lysine while two spots were near the solvent front and corresponded to DNS-NH_2. Of the other two spots, one may have corresponded to DNS-proline and the other, which remained very near the origin, may have corresponded to DNS-serine or may have been due to streaking of the sample as a similar spot was observed following chromatography of an hydrolysate of dansyl lysozyme. Thin layer chromatography of an hydrolysate of dansyl lysozyme gave a streak in the above solvent. It was impossible to identify a spot which corresponded to di-DNS-lysine. Thin layer chromatography in another solvent (chloroform : tert-amyl alcohol : formic acid - 70:30:1) did not aid in the elucidation of the N-terminal amino acids of lysozyme, spinach Fr I or bean Fr I.

Attempts to demonstrate an N-terminal amino acid following the dansyl reaction would suggest that if an unmasked N-terminal amino acid is present and reacts with the dansyl reagent, it is either proline, serine (most unlikely) or tryptophan. It is also possible that many different N-terminal amino acids are present in Fr I and that they were not seen because their dansyl derivatives were present in the final mixture in small amounts and could not be identified in the streak observed following electrophoresis or chromatography. When a large excess of DNS-Cl and prolonged incubation periods were used for

the preparation of dansyl lysozyme, it was impossible to identify the presence of a spot corresponding to di-DNS-lysine following thin layer chromatography of an hydrolysate of dansyl lysozyme.

A further attempt was made to identify the N-terminal amino acid of Fr I protein by the Edman reaction. No N-terminal amino acid was observed following thin layer chromatography in either solvent system or following two-dimensional thin layer chromatography of the expected product of the Edman degradation of bean and spinach Fr I. As proline is not destroyed under the conditions used and it is unlikely that tryptophan is destroyed, it would appear that if the N-terminal amino acid was accessible, it was not proline or tryptophan. Thus, it would appear that the N-terminal amino acid(s) of bean and spinach Fr I is masked or inaccessible to the Edman reagents.

Pon (14) attempted to obtain a dinitrophenyl (DNP) derivative of the N-terminal amino acid of Tetragonia Fr I by the method of Sanger (105). No N-terminal amino acid was identified positively and Pon (14) suggested that the N-terminal amino acid might be glycine, proline or cystine as these DNP-amino acids would have been destroyed in his preparations. No other author has published studies of the N-terminal amino acid of Fr I protein.

(d) Amino Acid Analysis: Fraction I protein from spinach and bean was subjected to amino acid analysis as described in the methods. The results obtained are shown in Tables X and XI. Fraction I proteins from spinach and bean have a similar amino acid composition. It is possible that the content of threonine, methionine, isoleucine and phenylalanine are slightly different in bean and spinach Fraction I.

Table X

Amino Acid Analyses of Spinach Fraction I Protein

The figures represent nM amino acid per mg Fraction I. The selected values were obtained on the basis of mean or maximal values (see Methods, p. 48). Selected values (Table XI) for the amino acid analysis of bean Fraction I are included for comparison.

Amino Acid	Time of Hydrolysis (hr)				Selected Values	
	16	24	48	72	Spinach	Bean
lysine	347	377	359	345	360	386
histidine	194	227	214	210	217	206
arginine	390	477	430	424	443	411
aspartic acid	620	644	603	642	630	578
threonine	408	430	383	408	430	382
serine	245	247	202	214	247	258
glutamic acid	795	834	764	858	818	890
proline	324	337	310	369	338	347
glycine	573	614	544	616	591	647
alanine	526	554	502	593	549	559
1/2 cystine					trace	trace
valine	422	461	445	487	487	460
methionine	131	139	129	131	133	112
isoleucine	220	241	249	265	265	320
leucine	585	628	597	625	625	630
tyrosine	316	341	320	332	331	297
phenylalanine	267	290	278	286	284	330

Table XI

Amino Acid Analyses of Bean Fraction I Protein. The figures represent nM amino acid per mg Fraction I. The selected values were obtained on the basis of mean or maximal values (see Methods, p. 48).

Amino Acid	Time of Hydrolysis (hr)				Selected Values
	16	24	48	72	
lysine	454	388	380	392	386
histidine	220	212	200	206	206
arginine	458	432	400	402	411
aspartic acid	568	570	602	564	578
threonine	390	382	384	352	382
serine	268	258	252	222	258
glutamic acid	868	894	928	850	890
proline	350	358	364	320	347
glycine	646	644	668	630	647
alanine	544	562	580	536	559
1/2 cystine					trace
valine	428	452	458	460	460
methionine	110	114	112	110	112
isoleucine	270	288	320	316	320
leucine	614	630	624	614	630
tyrosine	286	292	306	294	297
phenylalanine	314	328	340	324	330

However, the discrepancies in the content of serine and isoleucine in bean and spinach Fraction I may be a result of the hydrolytic conditions. Methionine was not oxidized to the sulphoxide and this may have affected the results for this amino acid. If there is a difference in the amino acid composition of bean and spinach Fr I, it is small.

The values obtained for the amino acid composition of Fr I were converted to molar ratios relative to leucine which was assigned a value of 1.06. These values could then be compared directly to the analyses published by Ridley et al. (12) as their data was published as molar ratios relative to leucine (1.06). Little difference was observed between the two sets of data. Similarly, the values obtained for the amino acid composition of Fr I were converted to molar ratios relative to phenylalanine which was assigned a value of 1.00. The data of Rutner and Lane (32) was published as molar ratios relative to phenylalanine which was assigned a value of 1.00. A comparison with the analyses published by Rutner and Lane (32) indicated that there was little difference between the two sets of values.

The molar ratio of tyrosine to tryptophan in spinach Fr I was determined to be 2.51 by the method of Beaven and Holiday (99). This value agreed with the ratio published by Thornber et al. (10).

(e) Enzyme Activity: The results of an assay for the presence of RuDP carboxylase activity in Fr I protein from spinach, bean, N. tabacum, wheat, rye and triticales is shown in Table XII. The specific activity of the spinach carboxylase is in reasonable agreement with the values published by Pon (11,14), but considerably

Table XII

The Enzymic Character of Fraction I Protein. The reaction time was 12 min for samples 1-3 and 30 min for samples 4-6. All values were obtained from duplicate determinations.

Sample Assayed	dpm		Specific Carboxylase Activity (units/mg)	Specific "Isomerase-Kinase" Activity (units/mg)	Carboxylase/ Isomerase-Kinase Ratio
	Carboxylase Assay	Combined Assay			
1. Spinach	14014	32	0.0455	0.0001	455:1
Denatured Spinach	22	12			
2. Bean	6	5	0.0001	0.0001	
Denatured Bean	0	0			
3. Nicotiana tabacum	1588		0.0075	-	
Denatured Nicotiana tabacum	0				
4. Wheat	7152				
Denatured Wheat	59				
5. Triticales	6185				
Denatured Triticales	62				
6. Rye	6173				
Denatured Rye	13				

lower than those reported by Paulsen and Lane (16).

Nicotiana tabacum Fr I had a low specific activity relative to that of spinach Fr I. The probable explanation is that the N. tabacum Fr I was purified only to the stage preceding chromatography on Sephadex G-200.

No RuDP carboxylase activity was observed for bean Fr I. The criteria used to designate this protein as Fr I were polyacrylamide gel electrophoresis, its sedimentation constant, and its amino acid composition. A possible explanation for the lack of carboxylase activity is suggested by the results obtained in assays for activity in wheat, rye and triticales. Carboxylase activity was present in preparations of wheat, rye and triticales Fr I purified by centrifugation and chromatography on Sephadex G-25 (Table XII). However, when ammonium sulfate fractionation was included in the procedure, activity was greatly decreased in rye Fr I. It is possible that Fr I from rye is more sensitive to ammonium sulfate than is Fr I from wheat or triticales. Thus, bean carboxylase may be inactivated by concentrations of ammonium sulfate which do not totally inhibit the spinach carboxylase. The only reports of carboxylase activity for bean Fr I were for preparations in which ammonium sulfate fractionation was not used (39,106).

Preparations of RuDP carboxylase have often been reported to have phosphoribulokinase and phosphoriboisomerase activity (34,75). The result of a combined assay for the presence of phosphoriboisomerase and phosphoribulokinase activity in spinach Fr I is shown in Table XII. The isomerase-kinase activity in the preparation was

negligible. This finding agrees with the results of other authors (11,13,16,74).

4. Genetic Implications:

Classical genetic studies show that non-chromosomal determinants are involved in chloroplast heredity. The discovery of chloroplast DNA suggests a possible physical basis for such non-chromosomal determinants (107-18). Chloroplast DNA probably codes for chloroplast RNA and at least some chloroplast protein. The fact that, in Euglena, chloroplast ribosomes are relatively rich in the bases adenine and uracil is compatible with this hypothesis since chloroplast DNA is rich in adenine and thymine (111,116,117). Other evidence which supports the hypothesis is the finding that chloroplast preparations carry out DNA-dependent RNA synthesis and incorporate isotopically labelled amino acids into protein (118,120-122). It has been shown that chloroplast DNA codes for the RNA of chloroplast ribosomes (123-4). A comparison of the effects of two inhibitors of protein synthesis, chloramphenicol and cyclohexamide, on Euglena gracilis cells containing developing chloroplasts, implicates chloroplast ribosomes as sites of synthesis of proteins of the photosynthetic electron transfer pathway and of ribulose-1,5-diphosphate carboxylase, NADP-glyceraldehyde-3-phosphate dehydrogenase and Fr I protein (125) provided that the mode of action of chloramphenicol and cyclohexamide is the same in Euglena gracilis and bacterial cells.

If Fr I protein (or any other chloroplast protein) variants could be found within a species, or in two closely related species

which will form interspecific hybrids, it would be possible to ask directly: "Is the gene which determines the structure of the protein chromosomal or non-chromosomal?". Such variants were observed for the Fr I protein of wheat and rye. A clear separation was obtained when a mixture of these proteins was subjected to polyacrylamide gel electrophoresis (Fig. 8g). Fraction I protein was also prepared from triticales, a hybrid obtained from wheat (♀) and rye (♂). The mobilities of the Fr I proteins from wheat and triticales were identical. Ribulose diphosphate carboxylase activity was observed in all three preparations (Table XII).

If chromosomal genes determined the structures of the proteins, the hybrid would be expected to have either a mixture of the two types or else an intermediate type of Fr I. On the other hand, if the gene were non-chromosomal, Fr I of the maternal type should be produced. Since many other chloroplast characteristics are inherited maternally (126), it would be reasonable to wonder if the genetic information for Fr I is encoded in the chloroplast. The Fr I of the hybrid (triticales) was found to correspond to that of the mother (wheat). No Fr I corresponding to the paternal type was observed. This would suggest that the gene coding for Fr I is non-chromosomal.

The karyotypes of wheat and rye suggest that wheat would make an unequal contribution to the hybrid. Wheat, rye and triticales have 21, 7 and 21 + 7 pairs of chromosomes, respectively. The suggestion could be made that, since the wheat-rye hybrid (triticales) would have three maternal genomes for every paternal chromosome, one-fourth of the Fr I might be of the paternal type. This was not

observed. It could also be proposed that, with the large number of subunits in Fr I protein, an intermediate type Fr I might have a very similar electrophoretic mobility to that of the protein from the female parent (wheat), even though it contained some of the characteristics of the male parent. This possibility cannot be eliminated at present but examination of the hybrid from the reciprocal cross (wheat (♂) x rye (♀)) would help to decide the question. The tentative conclusion that the amino acid sequence of Fr I may be determined by chloroplast DNA is in accord with the conclusions of the inhibitor studies mentioned above (125).

In direct contrast to the results obtained by gel electrophoresis of Fr I protein from the parents (wheat, rye) and hybrid (triticales) (Fig. 8g) were those obtained by Johnson and Hall (127) with crude extracts of the seeds of these species. Dissimilar protein "spectra" were obtained for the parental species. The sum of the fractions in the parental "spectra" accounted for all of the protein fractions of the hybrid.

Finally, genetic studies on Fr I can be extended. A hybrid can be obtained from radish and cabbage (128). Fraction I proteins from cabbage and radish have already been shown to have differing electrophoretic mobilities (Fig. 8f). The mobilities of the parental and hybrid types of Fr I could be compared. Similarly, the Fr I from hybrids of various species of Nicotiana can be compared with the parental proteins on gel electrophoretograms.

SUMMARY

In the course of this work with Fr I protein, a new method which uses a combination of ammonium sulfate fractionation, ultracentrifugation and chromatography on Sepharose 4B and Sephadex G-200 for the purification of the protein was developed. This method offers the advantages of simplicity, high yields of apparently pure protein and adaptability to plants other than spinach. Only the ammonium sulfate concentration must be varied to modify the procedure of method III for the purification of Fr I from the plants used by the author.

Clear differences in the electrophoretic mobilities of Fr I from various species were observed. Of particular interest is the observation that mixtures of wheat and rye Fr I and triticales and rye Fr I gave clear separations when subjected to polyacrylamide gel electrophoresis. The electrophoretic mobilities of wheat (maternal parent) and triticales Fr I were identical. These results suggest that a cytoplasmic gene codes for Fr I protein.

The fingerprinting technique could not be applied successfully to demonstrate a definite difference in the tryptic peptides obtained from spinach and bean Fr I. The complex patterns and the large number of peptides observed suggested that three or more non-identical subunit types may constitute the Fr I molecule.

No N-terminal amino acid could be isolated following formation of derivatives with 1-dimethylaminonaphthalene-5-sulfonyl chloride or with phenylisothiocyanate. Either, the N-terminal amino acid of Fr I is masked, or many N-terminal amino acids are present.

There was no significant difference in the amino acid composition of spinach and bean Fr I.

Spinach, N. tabacum, wheat, rye and triticales had considerable RuDP carboxylase activity. Bean Fr I had no RuDP carboxylase activity but this may be a result of the sensitivity of the carboxylase to high concentrations of ammonium sulfate. There was negligible phosphoribulokinase and phosphoriboisomerase in the spinach preparations.

REFERENCES

1. S. G. Wildman and J. Bonner, Arch. Biochem. 14, 381 (1947).
2. S. G. Wildman and A. T. Jagendorf, Ann. Rev. Plant Physiol. 3, 131 (1952).
3. R. W. Dorner, A. Kahn and S. G. Wildman, Biochim. Biophys. Acta 29, 240 (1958).
4. S. G. Wildman, C. C. Cheo and J. Bonner, J. Biol. Chem. 180, 985 (1949).
5. L. Eggman, S. J. Singer and S. G. Wildman, J. Biol. Chem. 205, 969 (1953).
6. J. W. Lyttleton, Biochem. J. 64, 70 (1956).
7. S. J. Singer, L. Eggman, J. M. Campbell and S. G. Wildman, J. Biol. Chem. 197, 233 (1952).
8. D. W. Kupke, J. Biol. Chem. 237, 3287 (1962).
9. J. P. Thornber, S. M. Ridley and J. L. Bailey, Biochem. J. 96, 29C (1965).
10. J. P. Thornber, S. M. Ridley and J. L. Bailey, N.A.T.O. Advanced Study Institute, Biochemistry of Chloroplasts, Aberystwyth, 1965, Vol. 1, Academic Press, London, 275 (1966).
11. N. G. Pon, Arch. Biochem. Biophys. 119, 179 (1967).
12. S. M. Ridley, J. P. Thornber and J. L. Bailey, Biochim. Biophys. Acta 140, 62 (1967).
13. A. Weissbach, B. L. Horecker and J. Hurwitz, J. Biol. Chem. 218, 795 (1956).

14. N. G. Pon, Ph.D. Dissertation, University of California, Berkeley, University of California Radiation Laboratory Report No. 9373 (1960).

15. P. W. Trown, Biochemistry 4, 908 (1965).

16. J. M. Paulsen and M. D. Lane, Biochemistry 5, 2350 (1966).

17. W. J. Archibald, J. Phys. Chem. 51, 1204 (1947).

18. A. Ehrenberg, Acta Chem. Scand. 11, 1257 (1957).

19. I. M. Klotz, Science 150, 697 (1967).

20. J. von Eys, J. Judd, J. Ford and W. B. Womack, Biochemistry 3, 1755 (1965).

21. J. A. Winstead and F. Wold, Biochemistry 3, 791 (1964).

22. J. A. Winstead and F. Wold, J. Biol. Chem. 239, 4212 (1964).

23. L. Kanarek, E. Marler, R. A. Bradshaw, R. E. Fellows and R. L. Hill, J. Biol. Chem. 239, 4207 (1964).

24. K. Weber, H. Sund and K. Wallenfels, Angew. Chem. Intern. Ed. Engl. 2, 481 (1963).

25. E. Marler and C. Tanford, J. Biol. Chem. 239, 4217 (1964).

26. H. Sund, Angew. Chem. Intern. Ed. Engl. 3, 802 (1964).

27. E. Appella and C. L. Markert, Biochem. Biophys. Res. Commun. 6, 171 (1961).

28. C. L. Markert, Science 140, 1329 (1963).

29. Th. Wieland, D. Georgopoulos, H. Kampe and E. D. Wachsmuth, Biochem. Z. 340, 483 (1964).

30. R. Haselkorn, H. Fernández-Moran, F. J. Kieras and E. F. J. van Bruggen, Science 150, 1598 (1965).

31. F. H. C. Crick and J. D. Watson, Nature 177, 473 (1956).

32. A. C. Rutner and M. D. Lane, Biochem. Biophys. Res. Commun. 28, 531 (1967).

33. N. K. Boardman, Biochim. Biophys. Acta 62, 63 (1962).

34. L. Mendiola and T. Akazawa, Biochemistry 3, 179 (1964).

35. J. Mayaudon, Enzymologia 18, 343 (1957).

36. S. G. Wildman, J. M. Campbell and J. Bonner, Arch. Biochem. 24, 9 (1949).

37. S. G. Wildman, J. M. Campbell and J. Bonner, J. Biol. Chem. 180, 273 (1949).

38. J. W. Lyttleton and P. O. P. Ts'o, Arch. Biochem. Biophys. 73, 120 (1958).

39. D. Racusen, M. Foote and J. Collins, Can. J. Botany 42, 960 (1964).

40. A. A. Krasnovsky and L. M. Kosobuskaya, Doklady Akad. Nauk S.S.S.R. 85, 177 (1952).

41. J. H. C. Smith and D. W. Kupke, Nature 178, 751 (1956).

42. R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1961).

43. J. H. C. Smith, Symp. Comp. Biol. Kaiser Found. Res. Inst. 1, 257 (1960).

44. E. L. Smith, J. Gen. Physiol. 24, 565 (1941).

45. E. L. Smith, J. Gen. Physiol. 24, 583 (1941).

46. E. L. Smith and E. G. Pickels, J. Gen. Physiol. 24, 753 (1941).

47. R. S. Criddle, N.A.T.O. Advanced Study Inst., Biochemistry of Chloroplasts, Aberystwyth, 1965, Vol. 1, Academic Press, London, 203 (1966).

48. J. J. Wolken, J. Cellular Comp. Physiol. 48, 349 (1956).

49. Y. Chiba, Arch. Biochem. Biophys. 90, 294 (1960).

50. M. Itoh, S. Izawa and K. Shubata, Biochim. Biophys. Acta 69, 130 (1963).

51. J. S. Kahn, Biochim. Biophys. Acta 79, 234 (1963).

52. S. Takashima, Nature 169, 182 (1952).

53. J. J. Wolken, Brookhaven Symp. Biol. 11, 87 (1958).

54. R. F. Bunting and D. W. Kupke, Federation Proc. 20, 377 (1961).

55. J. J. Wolken, Euglena, Appleton-Century-Crofts, New York, 125 (1967).

56. J. L. Bailey, J. P. Thornber and A. G. Whyborn, N.A.T.O. Advanced Study Inst., Biochemistry of Chloroplasts, Aberystwyth, 1965, Vol. 1, Academic Press, London, 243 (1966).

57. J. H. C. Smith and J. Coomber, Carnegie Inst. Wash. 59, 325 (1960).

58. N. K. Boardman, Biochim. Biophys. Acta 64, 279 (1962).

59. G. C. McCleod and J. Coomber, Carnegie Inst. Wash. 59, 324 (1960).

60. I. Ohad, P. Siekevitz and G. E. Palade, J. Cell Biol. 35, 521 (1967).

61. I. Ohad, P. Siekevitz and G. E. Palade, J. Cell Biol. 35, 553 (1967).

62. T. Akazawa, K. Saio and N. Sugiyama, Biochem. Biophys. Res. Commun. 20, 114 (1965).

63. Z. Dische and E. Borenfreund, J. Biol. Chem. 192, 583 (1951).

64. R. Montgomery, Biochim. Biophys. Acta 48, 591 (1961).

65. U. Heber, N. G. Pon and M. Heber, Plant Physiol. 38, 355 (1963).

66. J. W. Lyttleton, Nature 177, 283 (1956).

67. J. P. Thornber, J. L. Bailey and S. M. Ridley, Biochem. J. 92, 181 (1964).

68. M. Calvin and P. Massini, Experentia 8, 445 (1952).

69. J. R. Quayle, R. C. Fuller, A. A. Benson and M. Calvin, J. Am. Chem. Soc. 76, 3610 (1954).

70. A. Weissbach, P. Z. Smyrniotis and B. L. Horecker, J. Am. Chem. Soc. 76, 3611 (1954).

71. W. B. Jakoby, D. O. Brummond and S. Ochoa, J. Biol. Chem. 218, 811 (1956).

72. J. Mayaudon, A. A. Benson and M. Calvin, Biochim. Biophys. Acta 23, 342 (1957).

73. R. W. Dorner, A. Kahn and S. G. Wildman, J. Biol. Chem. 229, 945 (1957).

74. G. Van Noort and S. G. Wildman, Biochim. Biophys. Acta 90, 309 (1964).

75. G. Van Noort, W. Hudson and S. G. Wildman, Plant Physiol. 36, Suppl. XIX (1961).

76. M. W. Steer, B. E. S. Gunning and D. J. Carr, N.A.T.O. Advanced Study Inst., Biochemistry of Chloroplasts, Aberystwyth, 1965, Vol. 1, Academic Press, London, 285 (1966).

77. R. Cohen, Abstracts, Biophysical Society 9th Annual Meeting, p. 140 (1965).

78. L. Anderson, L. E. Worthen and R. C. Fuller, Federation Proc. Abstr. 26, 725 (1967).

79. M. Santer and W. Vishniac, Biochim. Biophys. Acta 18, 157 (1955).

80. P. A. Trudinger, Biochim. Biophys. Acta 18, 581 (1955).

81. R. C. Fuller and M. Gibbs, Plant Physiol. 34, 324 (1959).

82. E. G. Jorgensen, Physiol. Plantarum 15, 530 (1962).

83. O. Smithies, Advan. Protein Chem. 14, 65 (1959).

84. C. H. W. Hirs, J. Biol. Chem. 219, 611 (1956).

85. F. Sanger and H. Tuppy, Biochem. J. 49, 481 (1957).

86. V. M. Ingram, Biochim. Biophys. Acta 28, 539 (1958).

87. G. Schmer and G. Kreil, J. Chromatogr. 28, 458 (1967).

88. J. Barrollier, J. Heilman and E. Watzke, Hoppe-Seylers Z. Physiol. Chem. 304, 21 (1956).

89. R. Acher and C. Crocker, Biochim. Biophys. Acta 9, 704 (1952).

90. K. K. Reddi and E. Kodicek, Biochem. J. 53, 286 (1953).

91. Quoted in J. L. Bailey, Techniques in Protein Chemistry, Elsevier, New York (1962).

92. M. B. Van Halteren, Nature 168, 1090 (1951).

93. W. R. Gray and B. S. Hartley, Biochem. J. 89, 59P (1963).

94. W. R. Gray, Methods in Enzymology XI, Academic Press, New York, 149 (1967).

95. D. Morse and B. L. Horecker, Anal. Biochem. 14, 429 (1966).

96. J. Boyer and P. Talalay, J. Biol. Chem. 241, 180 (1966).

97. J. Sjoquist, Biochim. Biophys. Acta 41, 20 (1960).

98. D. H. Spackman, W. H. Stein and S. Moore, Anal. Chem. 30, 1190 (1958).

99. G. H. Beaven and E. R. Holiday, Advan. Protein Chem. 7, 319 (1952).

100. G. L. Miller and E. E. Miller, Anal. Chem. 20, 481 (1948).

101. J. M. Clark, Jr., Ed., Experimental Biochemistry, W. H. Freeman and Company, San Francisco (1964).

102. E. Zuckerkandl, R. T. Jones and L. Pauling, Proc. Nat. Acad. Sci. U.S. 46, 1349 (1960).

103. W. A. Schroeder, J. Am. Chem. Soc. 74, 5118 (1952).

104. T. Wieland and H. Determann, Ann. Rev. Biochem. 35, 651 (1961).

105. F. Sanger, Biochem. J. 39, 507 (1945).

106. D. Racusen and M. Foote, Can. J. Botany 43, 817 (1965).

107. A. Gibor and M. Izawa, Proc. Nat. Acad. Sci. U.S. 50, 1164 (1963).

108. E. Baltus and J. Brachet, Biochim. Biophys. Acta 76, 490 (1963).

109. E. H. L. Chun, N. H. Vaughan, Jr. and A. Rich, J. Mol. Biol. 7, 130 (1963).

110. R. Sager and M. R. Ishida, Proc. Nat. Acad. Sci. U.S. 50, 725 (1963).

111. J. Leff, M. Mandel, H. T. Epstein and J. A. Schiff, Biochem. Biophys. Res. Commun. 13, 126 (1963).

112. H. Ris and W. Plaut, J. Cell Biol. 13, 383 (1962).

113. H. Kislev, H. Swift and L. Bogorad, unpublished. Quoted by A. Gibor and S. Granick (ref. 119).

114. A. Gibor and S. Granick, J. Cell Biol. 15, 599 (1962).

115. C. von Wisselingh, Z. Induktive Abstammungs-Vererbungslehre 22, 65 (1920). Quoted by A. Gibor and S. Granick (ref. 119).

116. G. Brawerman, A. O. Pogo and E. Chargaff, Biochim. Biophys. Acta 55, 326 (1962).

117. G. Brawerman, Biochim. Biophys. Acta 72, 317 (1963).

118. N. M. Sissakian, I. I. Filippovich, E. M. Svetailo and K. A. Aliyev, Biochim. Biophys. Acta 95, 474 (1965).

119. A. Gibor and S. Granick, Science 145, 890 (1964).

120. D. Spencer, Arch. Biochem. Biophys. 111, 381 (1965).

121. J. T. O. Kirk, Biochem. Biophys. Res. Commun. 14, 393 (1964).

122. A. Gibor and M. Izawa, unpublished results. Quoted by A. Gibor and S. Granick (ref. 119).

123. N. S. Scott and R. M. Smillie, Biochem. Biophys. Res. Commun. 28, 598 (1967).

124. K. K. Tewari and S. G. Wildman, Proc. Nat. Acad. Sci. U.S. 59, 569 (1968).

125. R. M. Smillie, D. Graham, M. R. Dwyer, A. Grieve and N. F. Tobin, Biochem. Biophys. Res. Commun. 28, 604 (1967).

126. J. L. Jinks, Extrachromosomal Inheritance, Prentice-Hall, Inc., New Jersey (1964).

127. B. L. Johnson and O. Hall, Am. J. Botany 52, 506 (1965).

128. G. D. Karpechenko, Bull. Applied Botany 17, 305 (1927).