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LITERATURE CITED

Bechdel, S. E., Clyde, A. W., Cromer, C. O., Williams, P. S., Pennsylvania State College, *Agric. Exp. Sta. Bull.* **396**, 12 (1940).
Dorn, C., Klopfenstein, T., Ogden, R. L., Kehr, W. R., *Feedstuffs*, **48**, **A4**, Feb 9 (1976).
Hoglund, C. R., *Mich. Agric. Exp. Econ-Rep.*, 947 (1964).
Kohler, G. O., Palter, R., *Cereal Chem.* **44**, 512 (1967).
Livingston, A. L., Allis, M. E., Kohler, G. O., *J. Agric. Food Chem.* **19**, 947 (1971a).
Livingston, A. L., Knowles, R. E., Amella, A., Kohler, G. O., Arnold, W. N., Smith, K. V., *Feedstuffs*, **48**, **A8** Feb 9 (1976).
Livingston, A. L., Knowles, R. E., Kohler, G. O., *J. Assoc. Off. Anal. Chem.* **54**, 981 (1971b).
Livingston, A. L., Knowles, R. E., Kohler, G. O., *ARS Tech. Bull.*, 1414 (1976).
Livingston, A. L., Knowles, R. E., Nelson, J. W., Kohler, G. O., *J. Agric. Food Chem.* **16**, 84 (1968).
Livingston, A. L., Nelson, J. W., Kohler, G. O., *J. Assoc. Off. Anal. Chem.* **52**, 617 (1969).
McCready, R. M., Guggolz, J., Silviera, V., Owens, H. S., *Anal. Chem.* **22**, 1156 (1950).
McGilliard, A. D., *J. Am. Oil Chem. Soc.* **49**, 57 (1972).
Oser, B. L., "Hawk's Physiological Chemistry", 14th ed, The Blakiston Co., Toronto, 1965, p 1029.
Potter, A. L., Ducay, E. D., McCready, R. M., *J. Assoc. Off. Anal. Chem.* **51**, 748 (1968).

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Solubility Properties of Fraction I Proteins of Maize, Cotton, Spinach, and Tobacco

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The solubility characteristics of the major leaf protein, fraction I protein, of four plant species (tobacco, spinach, cotton, maize) have been determined and compared. The solubility in ammonium sulfate solutions was identical for all four proteins. Furthermore, all four proteins underwent isoelectric precipitation at about pH 4.5. The proteins of tobacco, spinach, and cotton (C_3 species) were precipitated only above 60 °C, but that of maize (a C_4 species) was partially precipitated at temperatures as low as 43 °C. Tobacco fraction I protein crystallized in low-salt media only when treated with $MgCl_2$ and $NaHCO_3$ and dialyzed at pH values around 7.5. At higher pH the protein remained soluble; at lower pH noncrystalline precipitates were obtained. Temperature had little effect on the crystallization. Crystallization could be obtained by (1) treatment with $MgCl_2$ and $NaHCO_3$ in the presence of salt, followed by dialysis in salt-free buffers, or by (2) direct addition of $MgCl_2$ and $NaHCO_3$ to protein in salt-free buffer. $CaCl_2$ and $MnCl_2$ replaced $MgCl_2$ effectively. The fraction I proteins of spinach, cotton, and maize did not precipitate or crystallize in low-salt media under a wide range of conditions of temperature and pH.

Interest in leaf protein concentrates for animal and human consumption has increased in recent years as a result of an anticipated world-wide need for alternate protein sources and a desire to derive maximum usefulness from agricultural crops. With leaf protein concentrates intended for human use, it is desirable to separate the soluble protein fraction from the pigmented chloroplast membranes as well as from other flavor-producing components to give a protein concentrate of neutral flavor. One successful purification procedure has been developed which utilizes selective heat precipitation of the pigmented membrane and soluble protein fractions of alfalfa (Edwards et al., 1975).

Fraction I protein is the predominant soluble component of leaf homogenates and thus is a major fraction of most colorless leaf protein concentrates (Kawashima and Wildman, 1970; Sarkar et al., 1975). The name "fraction I" arises from the observation that soluble leaf proteins are separated by ultracentrifugation or gel chromatography into two molecular weight classes, fractions I and II (Wildman and Bonner, 1947; Sarkar et al., 1975). The higher molecular weight class, about 500 000 to 600 000, is fraction I protein. In tobacco leaves, up to 50% of the soluble protein is fraction I protein (Kawashima and Wildman, 1970).

In those plants whose primary photosynthetic product is 3-phosphoglyceric acid, the C_3 plants, fraction I protein consists almost entirely of a single protein, ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) (Kawashima and Wildman, 1970; Siegel et al., 1972; Jensen and Bahr, 1977), which is responsible for the incorporation of CO_2 during photosynthesis. The ribulose-1,5-bisphosphate carboxylases of spinach and tobacco have been extensively studied. The molecular weight of ribulose-1,5-bisphosphate carboxylase is 560 000 (Paulsen and Lane, 1966; Trown, 1965; Pon, 1967; Ridley et al., 1967). SDS–polyacrylamide gel electrophoresis reveals two sizes of subunits: 55 000 and 12 000 daltons, respectively. The native protein probably consists of eight subunits of each size (Rutner and Lane, 1967; Baker et al., 1975).

The fraction I protein of tobacco and several related species can be crystallized by dialysis of crude leaf extracts against low ionic strength buffers. Following a centrifugation step to remove particulate matter and a chromatography step to remove phenolic compounds, fraction

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I protein crystals are obtained from the crude extract in a day or less. These crystals give a constant specific activity of ribulosebisphosphate carboxylase after two recrystallizations and are free of carbohydrate, lipid, and nucleic acid (Chan et al., 1972; Sakano et al., 1973). Crystallization of fraction I protein offers a particularly simple method which could be applied to processing leaf proteins from crop plants, since crystallization gives pure white proteins. However, when fraction I proteins from a number of crop species have been subjected to conditions under which tobacco fraction I crystallizes, crystals have not been obtained (Sakano et al., 1974; Kawashima and Wildman, 1971). The basis for this difference between tobacco fraction I and those of almost all other species tested is not known.

In light of the renewed interest in leaf protein concentrates as nutritional supplements and the need to develop alternate methods for their preparation, we have explored some basic solubility properties of the fraction I protein component of tobacco and compared these properties with those of fraction I proteins from spinach, cotton, and maize.

PROCEDURES

Purification of Fraction I Protein. Fraction I proteins were prepared from freshly harvested, washed, and chilled leaves of 7-day-old maize (*Zea mays* L. var. Golden Cross Bantam (*N); 4- to 6-week-old cotton (*Gossypium hirsutum* L. var. Stoneville 502 glandless); 4- to 6-week-old spinach (*Spinacia oleraceae* L. var. Viroflay); 8- to 10-week-old tobacco (*Nicotiana tabacum* L. var. Turkish Samsun). All procedures were conducted at 4 °C. The leaves, 0.3–0.5 g of fresh weight per milliliter of medium, were homogenized with a Waring blender in 0.2 M NaCl, 50 mM mercaptoethanol, 25 mM $Na_2S_2O_5$, 1% soluble polyvinylpyrrolidone, 50 mM Tris, 0.5 mM EDTA, adjusted to pH 7.4 with HCl. Sufficient solid ammonium sulfate was added to give 30% saturation at 25 °C, and the precipitate was discarded. Addition of further ammonium sulfate to the supernatant (to 60% saturation) yielded a precipitate which was redissolved in a minimal amount of 0.2 M NaCl, in phosphate buffer (25 mM phosphate, 0.5 mM EDTA, 0.2 mM dithioerythritol (DTE), and 3.0 mM NaN_3 at pH 7.4). The protein was then chromatographed on a Bio-Gel A-5m column (4 × 55 cm) equilibrated with the same buffer. The major peak of protein corresponding to the molecular weight range 300 000–700 000 was collected. This fraction was dialyzed against 5 mM phosphate, 0.5 mM EDTA, 0.2 mM DTE, and 3.0 mM NaN_3 at pH 7.4 and then applied to a DEAE-cellulose column (25 × 40 cm) equilibrated with this same buffer. The fraction I protein was eluted with 0.0 to 200 mM linear NaCl gradient. A single peak was obtained which had a trailing edge of protein with reduced ribulose-1,5-bisphosphate carboxylase specific activity. The major fractions of highest carboxylase specific activity were pooled as fraction I protein.

The 280 nm/260 nm absorbance ratio of the DEAE-purified preparations was 1.6–1.7. Except as described below for maize, the stained gels of fraction I protein preparations exhibited one major polypeptide band after nondenaturing polyacrylamide gel electrophoresis. Despite the inclusion of phenol oxidase inhibitors during the initial extraction and the use of low temperature, fraction I proteins from all sources, except spinach, declined markedly in ribulose-1,5-bisphosphate carboxylase activity during the purification. On the first day the purification was completed through the Bio-Gel A-5m chromatography step in order to separate the protein from phenolic compounds as rapidly as possible. Partially or completely purified fraction I protein was stored as a 60% ammonium sulfate precipitate at 4 °C. The ammonium sulfate was removed prior to further use of the protein by Sephadex G-25 chromatography. The concentration of fraction I proteins in solution was calculated from the observed absorbance at 280 nm and the extinction coefficient of Sakano and Wildman (1974) for tobacco or the coefficient of Pon (1967) for spinach. The tobacco extinction coefficient was used for maize and cotton.

Crystalline fraction I protein from tobacco was prepared by the standard method of Chan et al. (1972). No difference in any solubility properties, including ability to crystallize, was noted between tobacco fraction I proteins prepared by the above chromatographic method or the crystallization method of Chan et al. (1972).

RuBP Carboxylase Assay. Ribulose 1,5-bisphosphate (RuBP) was synthesized from ribose 5-phosphate and creatine phosphate in the presence of a CO_2-free spinach leaf enzyme extract, phosphocreatinekinase, and ADP at pH 7.8–8.0. The RuBP was purified by activated charcoal filtration, barium precipitation, and ion-exchange chromatography on Dowex 50W-X8. All other reagents were obtained commercially.

The assay mixture for RuBP carboxylase activity contained the following components: 50 μmol of Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] adjusted to pH 8.3 with NaOH; 4 μmol of $MgCl_2$; 3 μmol of $NaHCO_3$ (3.75 Ci/μM); 25–35 μL of sample; 0.09 μmol of RuBP. The total reaction volume was 315 μL, and the assay temperature was 25 °C. The enzyme was incubated for 5 min with all components of the assay medium except RuBP. After the addition of RuBP, incubation proceeded for another minute. The reaction was terminated with 0.2 mL of formic acid. Excess $^{14}CO_2$ was driven off by evaporating the sample to dryness on a planchet, and the sample was counted in a gas flow counter.

PEP Carboxylase Assay. The phosphoenolpyruvate carboxylase assay (Maruyama et al., 1966) contained the following components: 80 μmol of Tris–HCl, pH 7.8; 10 μmol of $KH^{14}CO_3$ (0.44 Ci/μM); 2 μmol of phosphoenolpyruvate; 2 μmol of $MgCl_2$; 5 μmol of glutathione; 5 μmol of NADH; 14 units of malate dehydrogenase; and the sample to be assayed. The total reaction volume was 1 mL. The assay was terminated with 0.25 mL of formic acid after 15 min of incubation at room temperature. Aliquots of 0.5 mL were dried on a planchet and counted in a gas flow counter.

Solubility Experiments. The fraction I proteins were treated with RuBP or with $MgCl_2$ plus $NaHCO_3$ prior to use in most experiments. These treatments consisted of incubating the protein at about 10 mg/mL for 2 h at 37 °C in 10 mM Tris–HCl, 0.2 M NaCl, 0.1 mM DTE, and 0.5 mM EDTA, at pH 7.4. Either 0.3 mM RuBP or 25 mM $MgCl_2$ and 25 mM $NaHCO_3$ were present during the incubation.

RESULTS

Crystallization in Low-Salt Media. Previous studies (Chan et al., 1972; Sakano et al., 1973; Kwok et al., 1971; Sakano et al., 1974; Kawashima and Ayabe, 1972) have shown that fraction I protein from tobacco leaves can be crystallized. Crystals were obtained by taking crude protein extracts, prepared in 25 mM Tris–HCl, pH 7.4, 0.2 M NaCl and incubated with 3 mM $MgCl_2$ and 20 mM $NaHCO_3$, and dialyzing them into 25 mM Tris–HCl, pH 7.4, which lacked NaCl (Chan et al., 1972). Crystals so obtained could be redissolved in buffer containing NaCl. Treatment of the protein with RuBP, instead of $MgCl_2$

Table I. Solubility of Tobacco Fraction I Protein at Low-Ionic Strength[a]

	Solubility of tobacco fraction I protein, mg/mL		
	$MgCl_2$, $NaHCO_3$ treated		RuBP treated
pH	4 °C	37 °C	4 °C
5.5-5.6	0.08	0.09	5.3
6.5-6.6	0.16	0.42	>6.8
7.4-7.5	0.27	0.74	>6.8
8.4-8.5	3.4	9.0	>7.3
9.3-9.4	7.3	8.0	>6.3

[a] Tobacco fraction I protein in 10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1 mM DTE, and 0.5 mM EDTA was treated with RuBP or with $MgCl_2$ and $NaHCO_3$ for 2 h at 37 °C and then dialyzed against 100 volumes of 10 mM buffer, 0.1 mM DTE, and 0.5 mM EDTA, 3 mM NaN_3 for 16-24 h. Buffers were Mes (2-(*N*-morpholino)ethanesulfonic acid), adjusted with NaOH, at pH 5.5-5.6 and 6.5-6.6 and Tris-HCl at pH 7.4-7.5 and higher. Dialyses were performed at 4 and 37 °C. Initial protein concentrations were 7-9 mg/mL; solubility was determined from A_{280} remaining in solution after dialysis. A solubility of "greater than" the quoted figure means that no precipitate was visible.

plus $NaHCO_3$, prevented crystallization; RuBP also caused fraction I protein crystals to redissolve in the absence of salt (Kwok et al., 1971).

We have measured the effects of temperature, pH, and substrate pretreatment on the crystallization and precipitation of tobacco fraction I protein and compared these effects with the solubility behavior of fraction I protein of spinach, cotton, and maize. Tobacco fraction I protein, prepared by the standard crystallization method (Chan et al., 1972), was dissolved in buffer and treated with 25 mM $MgCl_2$ plus 25 mM $NaHCO_3$ or with 0.3 mM RuBP at 37 °C for 2 h. These solutions were then dialyzed at 4 °C for 16–24 h at several pH values between 5.5 and 9.5. The $MgCl_2$ plus $NaHCO_3$-treated fraction I protein was also dialyzed at 37 °C. At pH values above 7.4–7.5 the solubility of tobacco fraction I protein increased rapidly so that few or no crystals were obtained (Table I). At the lower pH values, the precipitated protein consisted of largely spherical structures with diameters of about 5 μm, rather than the dodecahedral 50–100 μm crystals obtained at pH 7.4–7.5. No difference in crystal or precipitate form was observed when the dialysis was done at 37 °C. The $MgCl_2$ plus $NaHCO_3$-treated form of tobacco fraction I protein was slightly more soluble at 37 °C than 4 °C. The crystals and precipitates from $MgCl_2$ plus $NaHCO_3$-treated fraction I protein were redissolved by the addition of 0.3 mM RuBP or 0.2 M NaCl, regardless of the pH at which they were produced. At pH 6.5–6.6 and 7.4–7.5 resolubilization was complete in a few seconds, at pH 5.5–5.6 the resolubilization required 1 to 2 h and was never complete. In contrast to the crystallization after pretreatment with $MgCl_2$ plus $NaHCO_3$, when the tobacco fraction I protein was treated with RuBP prior to dialysis, no precipitation or crystallization occurred, except at pH 5.5–5.6 where a fine amorphous precipitate was noted.

In order to determine whether fraction I protein from other plants had solubility properties similar to tobacco, identical dialysis experiments were performed using $MgCl_2$ plus $NaHCO_3$-treated and RuBP-treated fraction I proteins from spinach, cotton, and maize (Table II). The $MgCl_2$ plus $NaHCO_3$-treated proteins were dialyzed at 4 and 37 °C; the RuBP-treated proteins were dialyzed at 4 °C only. Spinach fraction I protein was dialyzed at a concentration of 7.5–10 mg/mL. Amorphous precipitates were obtained at pH 6.2–6.3 and 5.6–5.7, and no precipitates were observed at higher pH values. The possibility that even lower ionic strength buffer would result in precipitation or crystallization of spinach fraction I protein was examined by continuing the dialysis of the protein at pH 6.5–6.6 and 7.4–7.5 an additional 48 h at 4 °C against a one-tenth dilution of the buffer used in Table II. No precipitate or crystals were observed.

The solubility of fraction I protein from cotton, dialyzed at 4–5 mg/mL, was similar to spinach fraction I protein. Maize fraction I protein, dialyzed at 2–3 mg/mL, gave results similar to those obtained with cotton and spinach when dialyzed at 4 °C. However, at 37 °C maize fraction I protein yielded precipitates at all pH values except 8.3–8.4. None of the precipitates obtained from dialysis of spinach (pH 6.2–6.3 and 5.6–5.7), cotton (pH 5.6–5.7), or maize (37 °C) fraction I proteins were resolubilized upon addition of 0.2 M NaCl or 0.3 mM RuBP.

These results confirm and extend those of Chan et al. (1972), Sakano et al. (1973), Kwok et al. (1971), and Sakano et al. (1974) on the requirements for crystallization of tobacco fraction I protein. Only the $MgCl_2$ and $NaHCO_3$-treated protein crystallized; the crystals were redissolved by addition of either NaCl or RuBP. Temperature had little effect on crystallization or solubility. Large dodecahedral crystals were obtained only at pH 7.4; lower pH gave spherical structures, at higher pH the protein remained soluble. The extension of the conditions of crystallization to a wider range of pH, temperature, and ionic strength than previously reported failed to yield crystals of the spinach, maize, or cotton fraction I proteins, for reasons which remain unknown.

Precipitation by Divalent Metal Ions. The RuBP-treated fraction I protein from tobacco has been reported to be precipitated by Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} in low ionic strength media (Kawashima and Ayabe, 1972). In order to assess whether this characteristic of

Table II. Solubility of Spinach, Cotton, and Maize Fraction I Protein at Low-Ionic Strength[a]

	Solubility of fraction I protein, mg/mL								
	Spinach			Cotton			Maize		
	$MgCl_2$-$NaHCO_3$ treated		RuBP treated	$MgCl_2$-$NaHCO_3$ treated		RuBP treated	$MgCl_2$-$NaHCO_3$ treated		RuBP treated
pH	4 °C	37 °C	4 °C	4 °C	37 °C	4 °C	4 °C	37 °C	4 °C
5.6-5.7	5.7	2.2		0.5	0.05	0.24	0.34	0.05	0.43
6.2-6.3	7.2	6.4	6.4	>4.2	1.7	>4.2	2.3	0.15	>2.5
6.6-6.7	>9.5	>9.1	>8.3	>4.2	>3.8	>4.2	>2.8	0.29	>2.5
7.4-7.5	>9.5	>9.8	>8.3	>4.5	>4.7	>4.4	>2.9	2.7	>2.9
8.3-8.4	>9.8	>9.5	>8.0	>4.2	>4.5	>4.2	>3.1	>3.3	>2.7

[a] Fraction I proteins from spinach, cotton, and maize were treated with RuBP or with $MgCl_2$ and $NaHCO_3$ and dialyzed at various pH values as described for tobacco (Legend, Table I). Dialysis time was 16-24 h. Initial concentrations (mg/mL) were: spinach, 7.5-10; cotton, 4-5; and maize, 2-3.

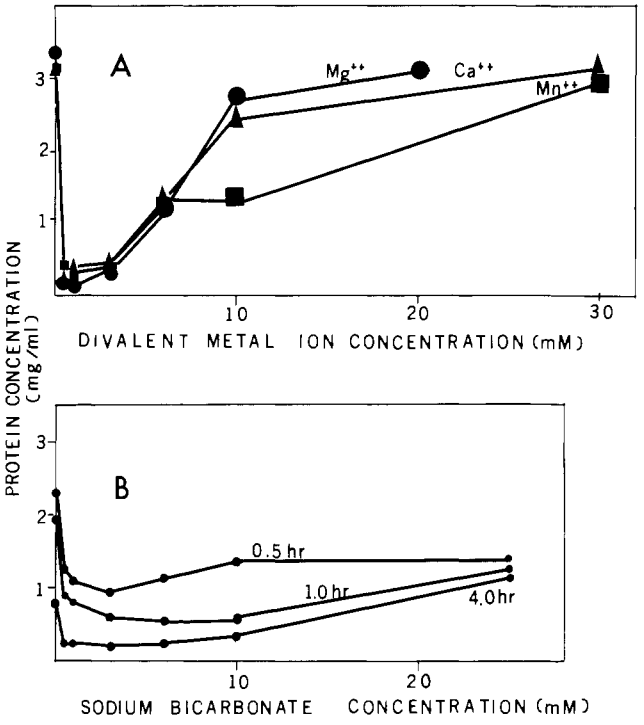

Figure 1. Solubility of RuBP-treated tobacco fraction I protein. Aliquots of RuBP-treated protein (10 mg/mL) were diluted threefold with 10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 3.0 mM NaN_3, and the indicated concentrations of divalent metal chloride and $NaHCO_3$. After centrifugation (18000g × 3 min), the protein remaining in solution was estimated by reading optical density at 280 nm. (A) Effect of metal ion concentration on solubility in the presence of 2 mM $NaHCO_3$ after 5 h. (B) Effect of $NaHCO_3$ concentration in the presence of 2 mM $MgCl_2$ at 0.5, 1.0, and 4.0 h. To exclude atmospheric CO_2, the buffers were freshly prepared with boiled water in sealed, N_2-flushed vials.

tobacco fraction I protein might facilitate its isolation from leaf protein concentrates, the conditions required for metal ion precipitation of RuBP-treated tobacco and spinach fraction I proteins were examined. The RuBP-treated proteins were dialyzed against 500 volumes of buffer for 16 to 24 h to lower the ionic strength and remove excess RuBP. Aliquots of these proteins were then added to buffered solutions containing the desired concentrations of $MgCl_2$, $CaCl_2$, or $MnCl_2$. In most experiments $NaHCO_3$ was also included.

RuBP-treated tobacco fraction I protein was precipitated by Mg^{2+}, Ca^{2+}, and Mn^{2+} ions in buffer which also contained 2 mM $NaHCO_3$ (Figure 1A). Precipitation occurred at the lowest divalent metal ion concentration tested, 0.5 mM, but became less as the divalent metal ion concentration increased above 10 mM. At 0.5 and 1.0 mM Mg^{2+}, Ca^{2+} and Mn^{2+}, and 3 mM Mg^{2+} the precipitate was entirely crystalline, with dimensions of 10 to 50 μm. At 3 and 6 mM Ca^{2+} and 6 mM Mg^{2+} the crystals were mixed with fairly large quantities of a fine amorphous precipitate, and the precipitates at 10 mM Mg^{2+} and Ca^{2+} were entirely amorphous. The precipitates at 3, 6, and 10 mM Mn^{2+} were also entirely amorphous. A tenfold dilution with water of the protein-containing supernatants at 20 and 30 mM metal ion caused a rapid precipitation (both amorphous and crystalline).

The role of HCO_3^- in precipitation of RuBP-treated tobacco fraction I protein was studied by incubating the protein with 2 mM $MgCl_2$ and varying HCO_3^- concentrations, followed by measurement of the protein still in solution at 0.5, 1, and 4 h (Figure 1B). Since it is difficult to remove all CO_2 from the buffers, some precipitate was observed in the absence of added HCO_3^-, but there was always more when HCO_3^- was present. At the highest concentration of HCO_3^- tested (25 mM), the solubility of the protein had increased, presumably because of the increased ionic strength. The precipitates at 4 h were entirely crystalline (10–50 μm), except at no added HCO_3^- and 0.5 mM HCO_3^-, where mixtures of crystalline and amorphous precipitate were observed.

Identical experiments were conducted with RuBP-treated spinach fraction I protein. In contrast to the behavior of tobacco fraction I protein, no precipitation or loss of 280 nm absorbance in the supernatants were observed at any of the conditions tested. These conditions were: concentrations of $MgCl_2$, $CaCl_2$, or $MnCl_2$ between 0 and 15 mM at 2 mM HCO_3^-, and concentrations of HCO_3^- between 0 and 25 mM at 2 mM $MgCl_2$. A separate sample of spinach fraction I protein was incubated at 3 mM $NaHCO_3$ and 2 mM Mg^{2+} for up to 96 h without appearance of precipitate.

Isoelectric Precipitation. The fraction I proteins of tobacco, spinach, cotton, and maize were examined for differences in isoelectric precipitation behavior. Both RuBP-treated and $MgCl_2$ plus $NaHCO_3$-treated proteins were studied. After an incubation of 30 minutes, at 25 °C, the final pH and protein remaining in solution were determined. The fraction I proteins of all four species underwent isoelectric precipitation at similar pH values (Table III). The apparent isoelectric points were 4.4–4.7. No differences were observed between the RuBP-treated and $MgCl_2$ plus $NaHCO_3$-treated forms of a given fraction I protein.

Since the treatment of maize fraction I protein at 37 °C with RuBP or $MgCl_2$ plus $NaHCO_3$ caused partial precipitation of the preparation, untreated maize fraction I protein was also studied. The same isoelectric precipitation behavior was observed, except that substantial protein was precipitated at the lowest pH value tested (pH 3.3). In the treated maize fraction I protein samples no protein was precipitated at this pH.

Ammonium Sulfate Precipitation. The fraction I proteins of tobacco, spinach, and maize were precipitated by similar concentrations of ammonium sulfate (Table IV). Fraction I protein from cotton precipitated at slightly higher concentrations. No major effects of temperature or pH were observed. Pretreatment of tobacco and spinach

Table III. Isoelectric Precipitation of Fraction I Proteins at 25 °C[a]

Fraction I protein source	Treatment	pH range of precipitation	Estimated isoelectric point
Tobacco	$MgCl_2$-$NaHCO_3$	4.0–4.9	4.4
	RuBP	4.1–4.9	4.4
Spinach	$MgCl_2$-$NaHCO_3$	4.2–5.2	4.6
	RuBP	4.3–5.2	4.7
Cotton	$MgCl_2$-$NaHCO_3$	4.2–4.9	4.6
	RuBP	4.2–5.0	4.6
Maize	$MgCl_2$-$NaHCO_3$	3.8–5.0	4.4
	RuBP	3.8–4.9	4.4
	Untreated	<3.3–4.9	

[a] Fraction I proteins were incubated in 0.05 M acetic acid-acetate-0.2 M NaCl at several pH values and the absorbance at 280 nm of the supernatants determined after a 30-min incubation and centrifugation. The pH range of precipitation was defined as that range of pH values at which the solubility was less than 0.5 mg/mL. The estimated isoelectric point was the pH of minimal solubility.

Table IV. Precipitation of Fraction I Proteins by Ammonium Sulfate[a]

Fraction I protein source	Treatment	Concentration required to reduce solubility to 2 mg/mL: pH 7.3, 4 °C	pH 7.3, 25 °C	pH 8.3, 25 °C	pH 6.3, 25 °C
Tobacco	$MgCl_2$-$NaHCO_3$	39	38	37	32
	RuBP		38		
Spinach	None	32	38	38	33
	$MgCl_2$-$NaHCO_3$		36		
	RuBP		36		
Cotton	None	46	44	47	44
	RuBP		46		
Maize	None	37	33	34	31

[a] Aliquots of fraction I protein preparations were added to media containing 50 mM Tris–HCl, 50 mM Mes (adjusted with NaOH), 1 mM EDTA, and various concentrations of ammonium sulfate. After a 2-h incubation, the protein solutions were centrifuged and the protein remaining in solution calculated from the absorbance at 280 nm. Incubations were done at 4 and 25 °C, with the media adjusted to pH 6.3, 7.3, or 8.3. The protein concentration was initially 4 to 6 mg/mL. Ammonium sulfate concentrations are expressed as percent of saturation at 25 °C.

fraction I proteins with either RuBP or $MgCl_2$ plus $NaHCO_3$ did not alter their solubility at high ammonium sulfate concentrations.

Thermal Precipitation. Since a method for the production of alfalfa leaf protein (Edwards et al., 1975) is based on the thermal denaturation of the leaf proteins, the solubility of the fraction I proteins as a function of temperature was determined. Protein solutions in 10 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 0.1 mM DTE, and 0.2 mM EDTA were heated in a water bath for 5 min. The precipitate, if any, was sedimented and an aliquot of supernatant used to determine protein still in solution. The solubilities of spinach and maize fraction I proteins as a function of temperature were quite different (Figure 2). Spinach fraction I protein began to precipitate only above 60 °C, whereas maize fraction I protein precipitated at temperatures as low as 43 °C. Tobacco and cotton fraction I proteins behaved the same as spinach fraction I protein.

The basis for the difference in thermal stability of fraction I proteins of spinach and maize appeared to be the presence of proteins other than RuBP carboxylase in maize fraction I. Fraction I proteins from spinach and maize were analyzed by SDS–polyacrylamide gel electrophoresis (Figure 3A,B). Both fraction I protein preparations contained polypeptides of about 55 000 and 12 000 daltons, corresponding to the reported subunits of RuBP carboxylase (Rutner and Lane, 1967; Baker et al., 1975). Maize fraction I protein preparations contained significant amounts of additional polypeptides: a major band at 120 000 daltons, and a small band at 40 000 daltons.

When a solution of maize fraction I protein (38 mg/mL) was heated for 10 min at 50 °C, approximately 25% of the protein was denatured. Analysis of the polypeptide pattern of the supernatant and precipitate by SDS–polyacrylamide gel electrophoresis revealed that both fractions contained the 55 000 and 12 000 dalton polypeptides. The supernatant, however, was almost completely free of the 120 000 and 40 000 dalton components, while the precipitate was enriched in these same polypeptides (Figure 3C,D). Polypeptide bands, not visible in either the untreated sample nor in the heated supernatant, appear in the heat-precipitated sample. These are polypeptides with

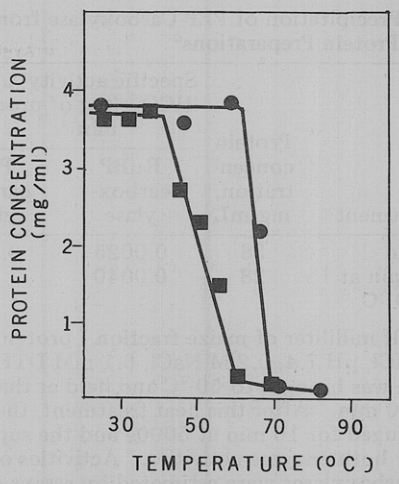

Figure 2. Thermal precipitation of spinach and maize fraction I proteins. Spinach and maize fraction I proteins were dissolved in 10 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 0.1 mM DTE, and 0.2 mM EDTA at concentrations of 3.8 and 3.7 mg/mL, respectively. Each sample was held at indicated temperature for 5 min and then centrifuged. The protein remaining in solution was calculated from the absorbance at 280 nm. The tube containing the protein solution was then subjected to a 5-min incubation at the next highest temperature. Neither of the fraction I proteins was treated with RuBP or $MgCl_2$ plus $NaHCO_3$ prior to this experiment (●, spinach; ■, maize).

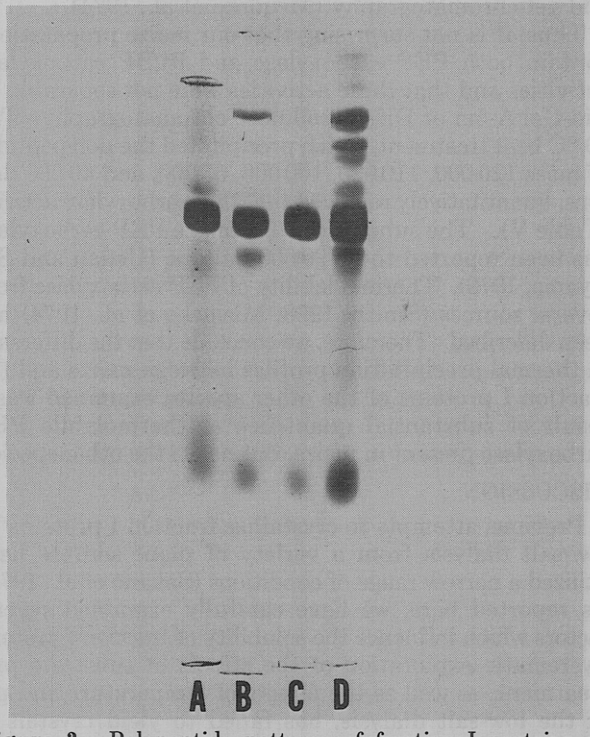

Figure 3. Polypeptide patterns of fraction I proteins on SDS–polyacrylamide gel electrophoresis. (A) 10 μg of spinach fraction I protein. (B) 25 μg of maize fraction I protein. (C) 25 μg of maize fraction I protein, from supernatant after 10 min at 50 °C. (D) 35 μg of maize fraction I protein, from precipitate after 10 min at 50 °C. Protein samples were purified through DEAE-cellulose (see Procedures section). Electrophoresis was performed by the method of Weber and Osborn (1969). Gels contained 7.5% acrylamide and 0.1% sodium dodecyl sulfate.

approximate masses of 110 000, 100 000, and 65 000 daltons.

It is likely that one or more of the polypeptides precipitated by heat were components of phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31). Maize is a C_4 plant in which primary CO_2 fixation occurs via PEP carboxylase localized in the cytoplasm of the mesophyll

Table V. Precipitation of PEP Carboxylase from Maize Fraction I Protein Preparations[a]

Treatment	Protein concentration, mg/mL	Specific activity, μmol of HCO_3^- (mg of protein)$^{-1}$ min^{-1}	
		RuBP carboxylase	PEP carboxylase
None	38	0.0025	0.162
10 min at 50 °C	28	0.0040	

[a] One-half milliliter of maize fraction I protein in 10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 0.1 mM DTE, and 0.2 mM EDTA was brought to 50 °C and held at that temperature for 10 min. After this heat treatment, the solution was centrifuged for 10 min at 5000*g* and the supernatant assayed for both enzyme activities. Activities of RuBP- and PEP-carboxylases were estimated by assays described in the Procedures section.

cells (Slack et al., 1969; Huber et al., 1976). PEP carboxylase from maize has recently been purified and characterized (Uedan and Sugiyama, 1976). It has a mass of 400 000 daltons. Other PEP carboxylases are also of high molecular weight (Utter and Kohlenbrander, 1972; Ting and Osmond, 1973; Miziorko et al., 1974). In addition, the very small amounts of PEP carboxylase present in spinach copurified with RuBP carboxylase through DEAE-cellulose and gel chromatography (Miziorko et al., 1974).

Thus, it is not surprising that our maize preparations contain both PEP carboxylase and RuBP carboxylase activities and that these activities were not separable by Bio-Gel A-5m or DEAE-cellulose chromatography. The 50 °C heat treatment, which precipitated the polypeptides of mass 120 000, 110 000, 100 000, 65 000, and 40 000 daltons, quantitatively removed the PEP carboxylase activity (Table V). The subunit size for maize PEP carboxylase has been reported to be 99 000 daltons (Uedan and Sugiyama, 1976). Thermal lability of PEP carboxylase from several sources (Smith, 1968; Miziorko et al., 1974) has been described. Therefore, we conclude that the difference in thermal precipitation profiles between maize and the fraction I proteins of the other species examined was a result of substantial quantities of thermolabile PEP carboxylase present in maize, but not in the other species.

DISCUSSION

Previous attempts to crystallize fraction I proteins by low-salt dialysis from a variety of plant sources have utilized a narrow range of conditions (Sakano et al., 1974). As reported here, we have carefully examined several factors which influence the solubility of fraction I protein. Systematic exploration of the effects of substrate pretreatment, as well as the effects of temperature and pH of the low-salt dialysis, has failed to yield crystals of spinach, cotton, or maize fraction I proteins. Only at pH values near 5.5 were precipitates obtained; these appeared to be due to irreversible isoelectric precipitation, since they were not redissolved by adding NaCl or RuBP. It is unlikely that the inability of chromatographically purified spinach fraction I protein to crystallize from leaf extracts during low-salt dialysis is due to some special property of crude tobacco leaf extracts. Tobacco fraction I protein was readily crystallized from both crude leaf extracts and from preparations which had been purified chromatographically.

Apparently, sufficient differences exist in the three-dimensional structure of the fraction I proteins of tobacco and other species, such that crystal formation is not thermodynamically favored under low-salt conditions for spinach, cotton, or maize proteins. The hypothesis that the inability to obtain crystals from these fraction I proteins is due to thermodynamic, rather than kinetic, considerations is supported by the observation that tobacco fraction I protein gave crystals within 2 to 3 min when the salt-free, RuBP-treated protein was added to a buffered solution containing $MgCl_2$ and $NaHCO_3$ (Figure 1B). In contrast, the identically treated spinach fraction I protein did not give crystals or precipitate even after 96 h. Although spinach fraction I protein did not crystallize under low-salt conditions, layering of concentrated protein solution over an equal volume of saturated ammonium sulfate produced spherical structures similar to those obtained with low ionic strength dialysis of tobacco fraction I protein at pH 6.5.

The results obtained with tobacco fraction I protein reemphasize that a precise set of conditions is required for crystal formation. Pretreatment with $MgCl_2$ and $NaHCO_3$ was essential, the RuBP-treated (or untreated) protein remaining soluble in low-salt buffer. The pH of the low-salt dialysis was also a critical factor for crystallization to occur. The solubility of tobacco fraction I protein rapidly increased as the pH was raised above 7.4–7.5, although at high initial protein concentrations, crystals could still be obtained. As the pH was reduced below 7.4, the solubility of the protein decreased and the size and morphology of the precipitates were altered.

With regard to the parameters we have measured, the conditions required for crystal formation by tobacco fraction I protein parallel those required for maximal activation of its RuBP carboxylase activity (Bahr et al., 1977; Jensen and Bahr, 1977; Lorimer et al., 1976). Both spinach and tobacco fraction I proteins are maximally activated by preincubation with Mg^{2+} and CO_2; both are inactivated by preincubation with RuBP. These observations suggest that the protein conformation associated with enzyme activation is different from that required for crystallization.

Crystalline bodies have been noted in the chloroplasts of both tobacco and spinach leaves which were subjected to osmotic stress (Murakami, 1974). These crystalline bodies are similar in structure to those of crystalline tobacco fraction I protein formed in vitro by low-salt dialysis. This suggests the intriguing possibility that there may be a set of conditions under which both spinach and tobacco fraction I may crystallize in vitro. The clue to defining these conditions may be found by studying induction of crystallization of fraction I protein in intact, isolated chloroplasts.

Our results suggest techniques which might be used to produce edible fraction I protein from tobacco leaf material either from leaves arising as second growth after harvest or from by-products of normal processing of tobacco. For example, in addition to crystallization by dialysis of $MgCl_2$- and $NaHCO_3$-treated protein against low-salt media, simply adding $MgCl_2$ and $NaHCO_3$ to protein already freed of salt will yield crystalline fraction I protein. Furthermore, crystallization might also be used in combination with the existing methods for processing alfalfa leaf protein, by modifying the heat precipitation protocol (de Fremery et al., 1975). However, these proposed crystallization procedures are apparently not readily applicable for preparing fraction I from other species. Crystallization at low ionic strength remains a property of fraction I from species of *Nicotiana* and one or two other species of the Solanaceae (Sakano et al., 1974). Further studies of additional major crops, especially forage crops, are required.

In the event that it is not possible to determine suitable conditions for crystallization of fraction I protein from

these other species, the selective heat precipitation procedures used in alfalfa processing may be useful in obtaining soluble, fraction I enriched, proteins from them. However, since pigments and membranes are removed by a 55 °C heat step in this procedure, it will not be useful for obtaining soluble protein fractions from maize leaves. Our results showed substantial amounts of soluble maize protein are precipitated by even short incubations at 50 °C. This thermolability of the major fraction of maize soluble leaf protein may be characteristic of all C_4 plants. Thus, it will be necessary to explore other techniques (for example, isoelectric precipitation) for purifying leaf protein from C_4 forage crops.

From the results presented here on the solubility properties of maize, cotton, spinach, and tobacco fraction I protein, we conclude the following.

(1) Existing procedures for crystallizing tobacco fraction I under low-salt conditions might be adapted for large-scale purification of the tobacco protein.

(2) The solubility properties of fraction I from maize, cotton, and spinach in low-salt media are different from those of tobacco. Thus, there are no procedures as yet available for large-scale crystallization of these proteins. That the Mg-HCO_3 treatment, which activates enzymatic activity in both spinach and tobacco, leads only to crystallizations in tobacco suggests that conformational changes associated with enzyme activation are not the same as those required for crystallization.

(3) Since the composition of fraction I proteins of maize and other C_4 plants differ from fraction I proteins of C_3 plants, it is probable that the development of crystallization and/or purification procedures for C_4 fraction I protein will necessitate a different approach from those for C_3 plants.

LITERATURE CITED

Bahr, J. T., Bourque, D. P., Capel, M., in preparation (1971).
Baker, T. S., Eisenberg, D., Eiserling, F. A., Weissman, L., *J. Mol. Biol.* **91**, 391 (1975).
Chan, P. H., Sakano, K., Singh, S., Wildman, S. G., *Science* **176**, 1145 (1972).
de Fremery, D., Miller, R. E., Knuckles, B. E., Bickoff, E. M., Kohler, G. O., Proceedings of the 12th Technical Alfalfa Conference, American Dehydrators Association, Mission, Kansas, 1975, p 104.
Edwards, R. H., Miller, R. E., de Fremery, D., Knuckles, B. E., Bickoff, E. M., Kohler, G. O., *J. Agric. Food Chem.* **23**, 620 (1975).
Huber, S. C., Hall, T. C., Edwards, G. E., *Plant Physiol.* **57**, 730 (1976).
Jensen, R. G., Bahr, J. T., *Annu. Rev. Plant Physiol.*, in press (1977).
Kawashima, N., Ayabe, T., *Plant Cell Physiol.* **13**, 523 (1972).
Kawahima, N., Wildman, S. G., *Annu. Rev. Plant Physiol.* **21**, 325 (1970).
Kawashima, N., Wildman, S. G., *Biochim. Biophys. Acta* **229**, 749 (1971).
Kwok, S. Y., Kawashima, N., Wildman, S. G., *Biochim. Biophys. Acta* **234**, 293 (1971).
Lorimer, G. H., Badger, M. R., Andrews, T. J., *Biochemistry* **15**, 529 (1976).
Maruyama, H., Easterday, R. L., Chang, H. C., Lane, M. D., *J. Biol. Chem.* **241**, 2405 (1966).
Miziorko, H. M., Nowak, T., Mildvan, A. S., *Arch. Biochem. Biophys.* **163**, 378 (1974).
Murakami, S., in Proceedings of the Third International Congress on Photosynthesis, Vol. III, Avron, M., Ed., Elsevier, Amsterdam, 1974, p 2073.
Paulsen, J. M., Lane, M. D., *Biochemistry* **5**, 2350 (1966).
Pon, N. G., *Arch. Biochem. Biophys.* 119, 179 (1967).
Ridley, S. M., Thornber, J. P., Bailey, J. L., *Biochim. Biophys. Acta* **140**, 62 (1967).
Rutner, A. C., Lane, M. D., *Biochem. Biophys. Res. Commun.* **28**, 531 (1967).
Sakano, K., Kung, S. D., Wildman, S. G., *Plant Cell Physiol.* **15**, 611 (1974).
Sakano, K., Partridge, J. E., Shannon, L. M., *Biochim. Biophys. Acta* **329**, 339 (1973).
Sakano, K., Wildman, S. G., *Plant Sci. Lett.* **2**, 273 (1974).
Sarkar, S. K., Howarth, R. E., Hirichi, M., McArthur, J. M., *J. Agric. Food Chem.* **23**, 626 (1975).
Siegel, M. I., Wishnick, M., Lane, M. D., *Enzymes, 3rd Ed.*, **6**, 169 (1972).
Slack, C. R., Hatch, M. D., Goodchild, D. J., *Biochem. J.* **114**, 489 (1969).
Smith, T. E., *Arch. Biochem. Biophys.* **125**, 178 (1968).
Ting, I. P., Osmond, C. B., *Plant Physiol.* **51**, 439 (1973).
Trown, P. W., *Biochemistry* **4**, 908 (1965).
Uedan, K., Sugiyama, T., *Plant Physiol.* **57**, 906 (1976).
Utter, M. F., Kolenbrander, H. M., *Enzymes, 3rd Ed.*, **6**, 117 (1972).
Weber, K., Osborn, M., *J. Biol. Chem.* **244**, 4406 (1969).
Wildman, S. G., Bonner, J., *Arch. Biochem.* **14**, 381 (1947).

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