Partial Purification and the Enzymatic Nature of Fraction I Protein of Rice Leaves*

LETICIA MENDIOLA AND T. AKAZAWA

From the International Rice Research Institute, Los Baños, Laguna, Philippines†

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The major protein component of rice leaves was partially purified by extraction of the leaf proteins and gel filtration of the extracts on Sephadex columns. This protein was characterized by starch-gel electrophoresis as the predominant protein band, and in the purified preparation was the only protein component discernible. The isolated protein preparation is believed to be identical to fraction I protein as studied by others. The fixation of $^{14}CO_2$, using ribose 5-phosphate as substrate in the system, was definitely catalyzed by the isolated protein fraction. The main product of $^{14}CO_2$ fixation was 3-phosphoglyceric acid. The three enzymatic activities involved in the system, namely, phosphoriboisomerase, phosphoribulokinase, and ribulose 1,5-diphosphate carboxylase, are believed to be associated with the fraction I protein of rice leaves.

Wildman and Bonner (1947) first described the presence of a single, electrophoretically homogeneous protein, designated as fraction I, which constituted about 75% of the total proteins of spinach-leaf cytoplasm. Several studies have since confirmed the presence of a major protein component which constitutes a large portion of the total soluble proteins in green plant leaves (Wildman and Jagendorf, 1952; Wildman and Cohen, 1955). This protein has been characterized mainly in the analytical ultracentrifuge, having a sedimentation constant of about 18 Svedberg units. Its molecular weight has been estimated at about 375,000 (Eggman *et al.*, 1953). Although previously believed to be a cytoplasmic protein, fraction I was later shown by Lyttleton and Ts'o (1958) to be associated mostly with the chloroplasts in spinach leaves.

Since fraction I comprises a large part of the leaf proteins, its isolation and chemical and biological characterization have been the subject of much study. But its enzymatic nature still is not definitely known. Dorner *et al.* (1957) first observed the similarity in physical properties between fraction I protein and the carboxylation enzyme isolated from spinach leaves by Horecker's group (1956), stating that it was extremely probable that the carboxylation enzyme in spinach is fraction I protein. Later, Lyttleton and Ts'o's preparation of fraction I protein from spinach chloroplasts exhibited some RuDP carboxylase activity.[1] More recently, Park and Pon (1961) observed that about 90% of the total RuDP carboxylase activity in chloroplasts was associated with a preparation which was largely fraction I protein. Thus, this protein may be implicated in the photosynthetic carbon dioxide fixation.

In some work preliminary to the study of protein metabolism in rice plants, we observed the presence of a major protein band on starch-gel electrophoresis, and wondered if this major component might be fraction I. As others have used dextran gels to separate the various components of a mixture by virtue of their difference in molecular size, we attempted to separate the high-molecular-weight protein from the other leaf proteins by gel filtration on Sephadex columns (Flodin, 1962). The present work describes the partial purification of the major protein fraction of rice leaves by gel filtration on Sephadex columns and subsequent studies of its enzymatic nature with respect to photosynthetic carbon dioxide fixation.

Materials and Methods

Rice plants of the Taichung No. 1 variety, *indica* type, were grown in the greenhouse. The plants were harvested at the 3.5- to 4-leaf stage. The third top leaf of each plant was taken, pooled, and used as starting material.

Preparation of Soluble Proteins from Leaves.—The buffer used was that of Lyttleton (1962) for the preparation of ribosomes from chloroplasts and consisted of 0.025 M Tris buffer, *p*H 7.5, containing 0.001 M $MgSO_4$, 0.0005 M $CaCl_2$, and 0.0002 M $MnCl_2$. The extraction medium consisted of the Tris-Mg-Ca-Mn buffer with 0.02% merthiolate and 12.5% glucose added. Glucose was included as sugars have been found to stabilize leaf proteins (Heitefuss *et al.*, 1959). Centrifugations were carried out in a Beckman-Spinco Model L preparative ultracentrifuge. Other operations were carried out in a cold room at 4°.

The leaves were weighed and minced with scissors into a mortar containing three volumes of the extraction medium. The mixture was ground to a fine pulp with an equal weight of fine sand, and the resulting slurry was pressed through several layers of cheesecloth. The dark green liquid was centrifuged to remove the sand and cellular debris. The rather turbid supernatant was spun at 35,000 × *g* for 20 minutes. The dark green pellet, consisting mainly of broken chloroplasts and mitochondria, was discarded. The clarified extract was then centrifuged at 105,000 × *g* for 2 hours. The final supernatant was a clear, amber-colored fluid.

For the isolation of the ribosomes, the pellet from the last centrifugation was resuspended in the extraction medium and centrifuged as before. The washed pellet was resuspended, this time in Tris-Mg-Ca-Mn buffer and spun at 27,000 × *g* for 20 minutes to remove the dark green residue which persisted up to this point. The protein extracts and ribosomal suspension were then stored at −20°.

RNA was determined by the method of Littlefield *et al.* (1955). The protein residues were then subjected to Kjeldahl digestion and nitrogen determined by Nessler's reagent. The relative amounts of protein

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† Mailing address: The International Rice Research Institute, Manila Hotel, Manila, Philippines.

[1] The following abbreviations are used: R-5-P, ribose 5-phosphate; RuDP, ribulose 1,5-diphosphate; 3-PGA, 3-phosphoglyceric acid; TPNH, reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; DEAE-, diethylaminoethyl-.

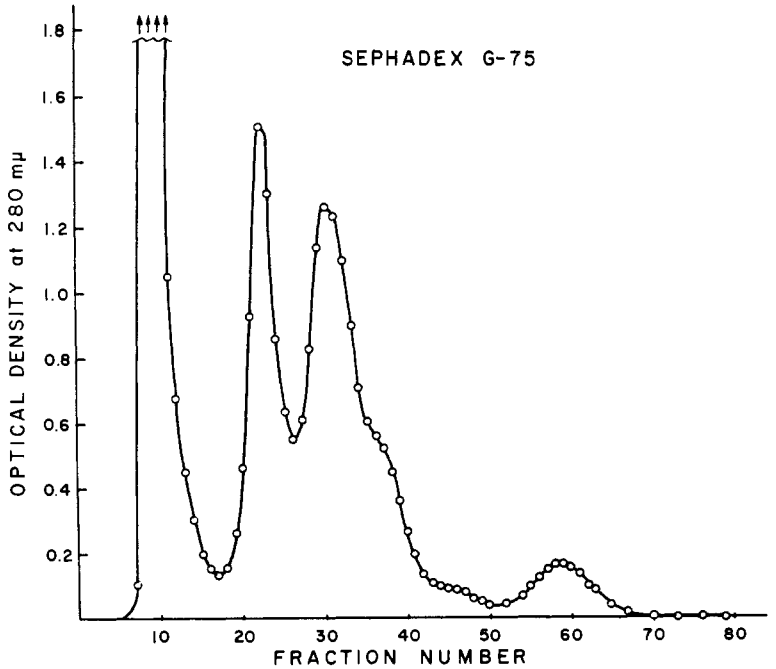

FIG. 1.—Gel filtration of 105,000 × *g* final supernatant on Sephadex G-75 (1.8 × 40 cm column). A larger column and more sample was actually used for the protein fractionations (see text).

and nucleic acid were also directly determined by the spectrophotometric method (Warburg and Christian, 1941).

Gel Filtration on Sephadex Columns.—The dextran gels Sephadex G-75 and G-200 were used and prepared for gel filtration on columns, similar to the procedure described by Flodin (1962).

Sephadex G-75 (medium) was employed for the preliminary separation of leaf proteins. The gel, suspended in Tris-Mg-Ca-Mn buffer, was poured onto a column (2.6 × 45 cm). After the gel had settled the column was placed in the cold room and washed overnight with the same buffer. Twenty ml (about 125 mg protein) of the final supernatant, prepared as described above, was carefully layered on top of the gel bed underneath the buffer by means of a thin polyethylene tubing connected to a syringe. When all of the material had passed below the top layer of the gel bed, the column was connected to a buffer reservoir and elution carried out with a pressure head of about 1 m. The eluate was collected in 5-ml fractions and the optical density at 280 mμ was measured for each fraction. Fractions corresponding to each peak were pooled and concentrated with Carbowax 6000. Glucose, up to a concentration of 12.5%, and 0.02% merthiolate were then added and the solutions stored at −20°.

The further separation of protein components was carried out on a Sephadex G-200 (200–400 mesh) column (3.2 × 43 cm). This was equilibrated and eluted with Tris-Mg-Ca-Mn buffer containing 0.1 M NaCl. The procedure was as outlined above, except that the optical density of each fraction was measured at 260 mμ as well.

Starch-Gel Electrophoresis.—Electrophoretic separations were carried out by vertical-gel electrophoresis (Buchler Instruments, Inc., N.J.). The preparation of the gel and buffers was as described by Smithies (1955), except that the discontinuous buffer system of Poulik (1957) was employed. The gel was made up of 65 g hydrolyzed starch in 500 ml Tris-citrate buffer (0.08 M Tris adjusted to *p*H 8.5 with citric acid). The molten gel was poured into a Lucite mold (12.3 × 30.5 × 0.6 cm). The electrode compartments contained 0.3 M sodium borate buffer, *p*H 8.0. Electrophoresis was carried out in the cold room for 5–6 hours, with a starting potential of 200 v across the gel length. The brownish boundary would then be about 7–8 cm from the sample origin. The gel was sliced for staining. Proteins were stained with Amido Black 10B. Acid phosphatase was determined according to Levinthal *et al.* (1962), except that the reaction was carried out in 0.1 M sodium acetate buffer (*p*H 5.0). The gel slice was left in the staining solution for several hours, after which the stained gel was fixed and washed in methanol–acetic acid–water (5:1:5). A positive reaction resulted in reddish-pink stains. Protein bands not giving this reaction gave a dark yellow stain.

Experiments on CO_2 Fixation.—The assay mixture was essentially similar to the carboxylation system of Jakoby *et al.* (1956), with purified phosphoribulokinase and carboxylase enzymes and R-5-P as substrate. The assays were carried out in Warburg flasks with the following components (in μmoles) in a final volume of 1.5 ml: Tris buffer (*p*H 7.4), 100; glutathione, 10; $MgCl_2$, 5; ATP, 5; aliquots of the protein preparation up to about 1 mg protein; in the side arm, $KHCO_3$, 20; R-5-P, 5; and $NaH^{14}CO_3$, 4 μc (about 3.2 × 10^5 cpm). Control flasks where either ATP or R-5-P was omitted were run simultaneously. The flasks were gassed with a mixture of 95% N_2 and 5% CO_2 and equilibrated at 25°. After tipping, the incubation was continued for 40 minutes with shaking. The reaction was stopped by adding 0.05 ml glacial acetic acid. The whole mixture was transferred to test tubes immersed in a boiling water bath for 1.5–2 minutes, and centrifuged. Aliquots of the samples (usually 0.2 ml) were plated on planchets. Radioactivity was determined in a Nuclear Chicago gas-flow counter.

To determine the products of CO_2 fixation, another incubation experiment was run with about 1.3 mg of protein and 200 μc (about 4.3 × 10^7 cpm) of $NaH^{14}C$ C The experimental conditions were otherwise identical to those outlined above. An aliquot was then applied on a Dowex 1-Cl column (0.8 cm^2 × 8 cm) and the components fractionated according to the method of

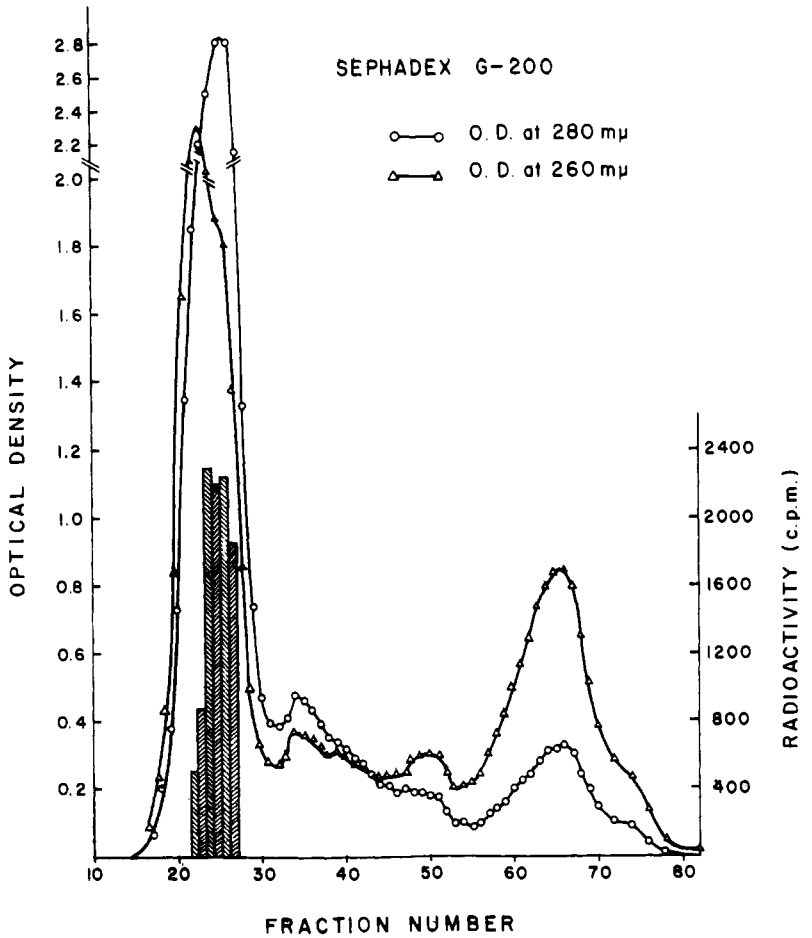

FIG. 2.—Gel filtration on Sephadex G-200 (3.2 × 45 cm column). Sample: 17 ml of pooled, concentrated fraction 13-20 (*ca.* 60 mg of protein) from Sephadex G-75. The shaded areas represent the fixed radioactivities when aliquots of the fractions were incubated with the assay system for $^{14}CO_2$ fixation. Details in the text.

Mori *et al.* (1960) for the fractionation of sugar phosphates and nucleotides. The fractions were assayed for phosphorus, sugars, nucleotide (OD at 260 mμ), and radioactivity. In the latter case, 0.2-ml aliquots were counted in a Packard Tri-Carb liquid scintillation spectrometer. Other aliquots were taken for paper chromatography and subsequent radioautography, following the method of Benson *et al.* (1950).

Miscellaneous.—R-5-P, ATP, ADP, and α-naphthyl acid phosphate (sodium salt) were obtained from Sigma Chemical Co. $NaH^{14}CO_3$ (12 mc/mmole) and 3-PGA (tricyclohexyl ammonium salt) were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Hydrolyzed starch was purchased from Connaught Medical Research Laboratories, Toronto, Canada, and Sephadex G-75 from Pharmacia, Uppsala, Sweden.

RESULTS

Extraction of Soluble Proteins from Leaves.—The extraction procedure employed was severe enough to release the chloroplast proteins as soluble material. Preliminary experiments with milder methods of extraction showed by microscopic observation that the chloroplasts are easily broken. The final supernatant (105,000 × *g*) revealed the same protein components as the clarified extract on starch-gel electrophoresis. Thus, the soluble proteins remain in the supernatant. Furthermore, the RNA-protein ratio of the pellet was much higher than that of the final supernatant. The absorption spectrum of the resuspended pellet showed a maximum at 260 mμ, indicating that ribosomes were contained in this fraction.

Separation of Proteins by Gel Filtration.—The pattern resulting from the gel filtration of the final supernatant on Sephadex G-75 is illustrated in Figure 1. For convenience, the pattern shown is that of a column smaller than the one actually used. This pattern is reproducible with repeated runs. Several peaks are easily distinguishable, although only the first peak appeared to contain protein. The fractions corresponding to about the first two-thirds of this peak were rather turbid and accounted for at least 90% of the protein N of the original sample applied on the column. The remainder of the peaks were presumably nonprotein impurities and not precipitable with trichloroacetic acid. Thus some were flavonoids which exhibited blue and yellow fluorescence.

A portion of the pooled concentrated fractions corresponding to the first two-thirds of the protein peak from the Sephadex G-75 gel filtration was then applied on a Sephadex G-200 column. The resulting pattern is illustrated in Figure 2. Here, the material which came off in a single peak from the previous column has been resolved into four or five discernible peaks. It will be seen later on that the proteins came off in all but the last two peaks, the latter having higher absorbancies at 260 mμ than at 280 mμ. The smaller of the last two peaks exhibited maximum absorption at 260 mμ in the ultraviolet region and may be a nucleotide. The nature of the last peak is not known. The absorption spectrum was rather diffuse. Another experiment employing higher buffer concentration, i.e., 0.1 M Tris-HCl,

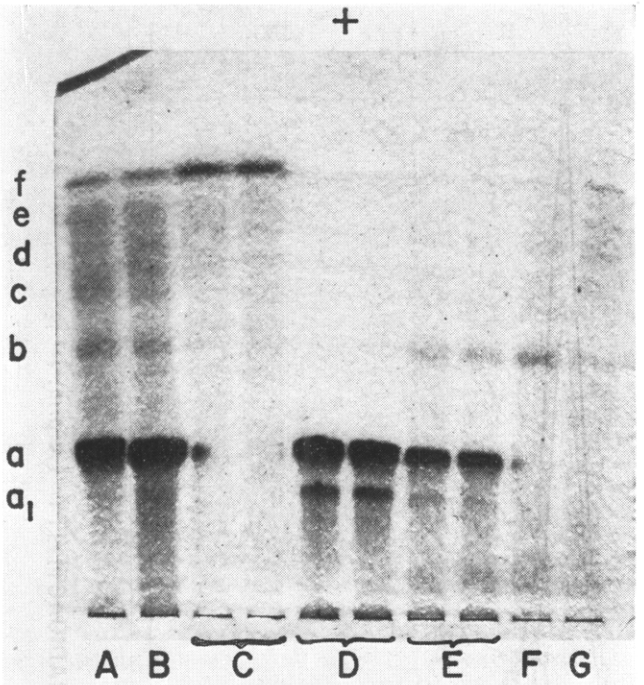

FIG. 3.—Starch-gel electrophoretic pattern of protein fractions (stained with Amido Black 10B). Conditions of the run are described in the text. (A) 105,000 × *g* final supernatant; (B) Fr. 13–20 and (C) Fr. 21–29 from Sephadex G-75 column; (D) Fr. 22–27, (E) Fr. 28–31, (F) Fr. 32–37, and (G) Fr. 38–46 from Sephadex G-200 column. All pooled fractions were concentrated before electrophoresis.

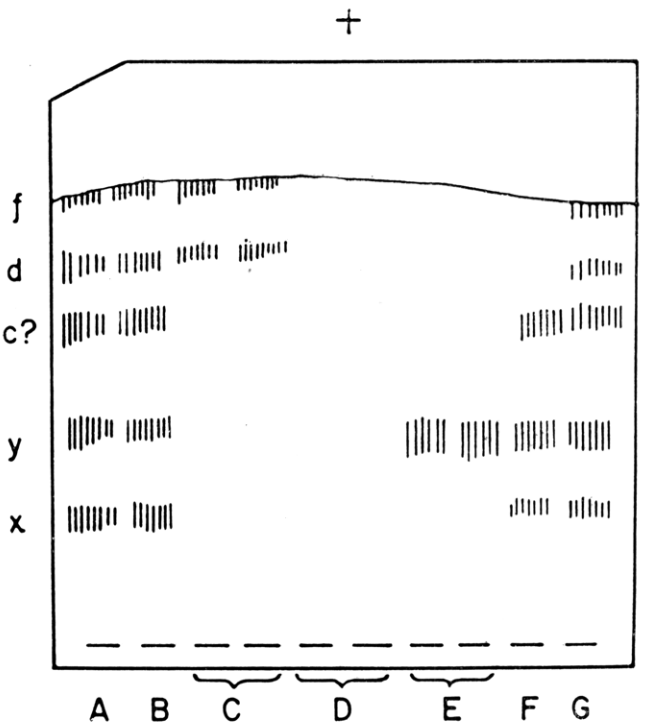

FIG. 4.—Acid phosphatase pattern of protein fractions after starch-gel electrophoresis. A through G, as explained in Fig. 1. Staining conditions are described in the text.

*p*H 8.0, containing 1 M NaCl, increased the retention of the material on the column but did not improve the resolution of the major protein from the other components. Preliminary attempts to fractionate the proteins on DEAE-Sephadex A-50 by stepwise elution with increasing concentrations of sodium chloride in the buffer have been without success.

Characterization of the Protein Components by Starch-Gel Electrophoresis.—The protein components of the samples obtained during the fractionation procedure are shown in Figure 3. Another slice from the same gel was stained for acid phosphatase and the tracing of the pattern is illustrated in Figure 4. One may readily observe (Fig. 3) the major protein band *a* which is quite separate from the other faster-moving minor proteins. Most of the latter gave a positive acid phosphatase reaction. Insofar as the proteins discernible on starch gel are concerned, all the components originally present in the supernatant (A) were recovered in the first peak from the gel filtration on Sephadex G-75 (B and C). Almost all of the major protein *a* was contained in the first two-thirds or so of the peak (B), whereas the minor proteins came off in most of the fractions of the peak. The fractionation of the proteins after gel filtration through Sephadex G-200 are represented in D through G. Fractions 22–27 (D) were found to contain only the major protein band *a* and a slower moving component a_1 which is believed to be an aggregation product of the former, as will be discussed later. These fractions did not give any acid phosphatase reaction, in agreement with the absence of minor protein bands. The latter fractions of the main peak, however, revealed the appearance of some other components as well, mostly *b*. The rest of the small peaks were poorly resolved and contained the minor proteins.

The absorption spectrum of the fractions containing only the major protein band (fractions 22–27) was typically that of a protein. The ratio of the optical densities at 280–260 mμ, however, indicated the presence of less than 2% nucleic acid. Actual analyses for protein and RNA gave an RNA-protein ratio of 0.019, in close agreement with the spectrophotometric determination.

CO_2 *Fixation by the Isolated Major Protein Component.*—The fractions from the main peak of the Sephadex G-200 gel filtration were tested for their ability to catalyze the fixation of CO_2, using ribose-5-phosphate as substrate. Fractions 22–27 were individually tested and the activities, expressed as fixed radioactivity, coincided with the pattern traced by the optical density at 280 mμ (see Fig. 2). These fractions were then pooled and concentrated (2.6 mg protein/ml) and used for another experiment to determine the fixation of CO_2 with varying amounts of the protein in the incubation mixture. The amount of CO_2 incorporated, expressed as fixed radioactivity, was more or less proportional to the protein concentration. An almost negligible amount of radioactivity amounting to only about 46 cpm/0.5 mg protein was detected when either R-5-P or ATP was omitted from the incubation mixtures.

The main product of CO_2 fixation was found to be 3-PGA, as confirmed by ion-exchange chromatography of the incubation mixture (Fig. 5). Furthermore, all the radioactivity initially present in the sample applied on the column was recovered in this peak. The results of paper chromatography and radioautography revealed one main spot, although rather diffuse, in the region where phosphoglyceric acid is expected to appear.

DISCUSSION

Fraction I protein has been found in the leaf extracts of various plants (Singer *et al.*, 1952) and is widely distributed in the plant kingdom (Dorner *et al.*, 1958).

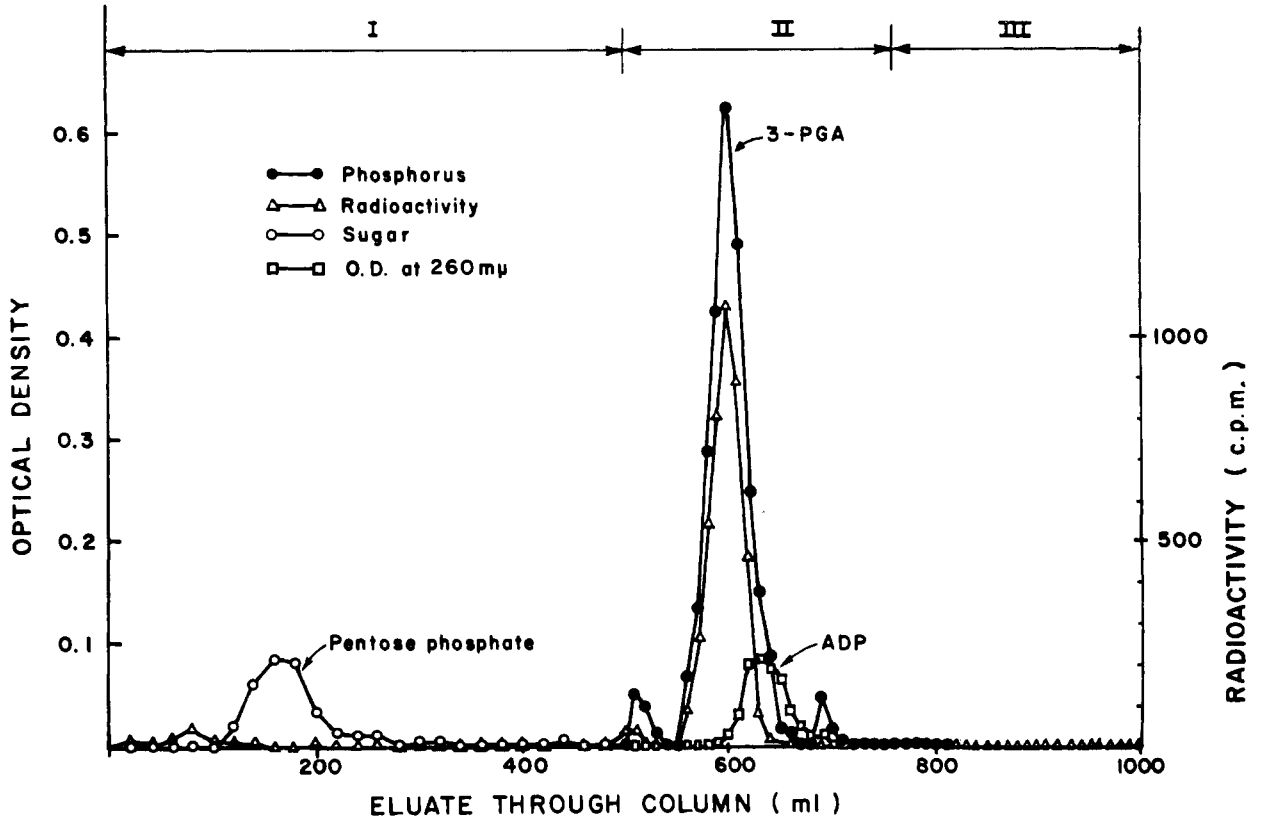

FIG. 5.—Ion-exchange-column chromatography of the reaction products of the incubation mixture ($^{14}CO_2$ fixation experiment) described in the text. The eluting solutions used were I, 0.03 N NH_4Cl; II, 0.01 N HCl, 0.01 N NaCl; and III, 0.01 N HCl, 0.03 N NaCl. Twenty-ml fractions were collected for I and 10-ml fractions for II and III. Aliquots (0.2 ml) were mixed with a scintillator and radioactivity was counted in glass vials.

Thus, it is not at all surprising to find that the major protein component isolated from the rice leaf extracts is fraction I. This protein behaves as a high-molecular-weight substance, as also observed by previous workers. It is one of the slower-moving components among the leaf proteins on starch-gel electrophoresis, indicating its large molecular size (Smithies, 1959). The early appearance of the protein from the Sephadex columns also suggests this property.

The starch-gel electrophoretic pattern of the isolated fraction reveals that the major protein band is accompanied by a slower-moving component designated as a_1 in Figure 3 (D). This component seems to increase during the purification procedure (compare A, B, and D) and also upon concentration and storage in the deep freeze. This may well be an aggregation product of fraction I protein, similar to the one previous workers have observed to separate out as a heavier component, usually accompanying preparations of fraction I protein in the analytical ultracentrifuge. That the main protein fraction isolated here has been fairly well separated from the other minor proteins also is indicated by the absence of any acid phosphatase staining bands (see Fig. 4, D-G) which seem to characterize the latter proteins (Lyttleton, 1956; Wildman and Cohen, 1955). Recently, zone electrophoresis on polyacrylamide gel, using, Tris–disodium ethylenediamine tetraacetate–borate buffer at *p*H 9.0, still showed that the main protein fraction from Sephadex G-200 consisted only of the major band along with the slower-moving aggregation product, although the other fractions revealed some additional minor bands.

The presence of nucleic acid in the purified protein fraction is difficult to explain. The possible contamination by ribosomal nucleoprotein particles cannot be ruled out entirely, although these were mostly deposited in the pellet from the 105,000 × *g* centrifugation. The elution pattern of the main peak (Fig. 2) shows that the nucleic acid forms the leading edge of the peak and does not exactly fit the protein pattern. The abilities of the individual fractions to fix CO_2 in the presence of R-5-P, expressed as fixed radioactivities, seem to coincide more with the optical densities at 280 mμ than with those at 260 mμ. Thus it seems unlikely that the nucleic acid is bound to the major protein component as nucleoprotein. In fact, Lyttleton (1956) was able to prepare from whole-leaf extracts fraction I protein practically free from RNA. Later, a similar preparation was obtained from chloroplast extracts of spinach leaves (Lyttleton and Ts'o, 1958).

The fixation of CO_2 into a stable 3-carbon compound, 3-PGA, catalyzed by RuDP carboxylase, occupies a key position in the photosynthetic carbon cycle. The fact that the isolated protein fraction was able to catalyze the fixation of CO_2 into 3-PGA with R-5-P as substrate merits serious consideration. In the present study, at least three enzymes are involved, namely, phosphoriboisomerase, phosphoribulokinase, and RuDP carboxylase. Smillie (1963) showed the latter two enzymes to be confined mainly to the chloroplasts in *Euglena*. At about the same time Heber *et al.* (1963) found that TPNH-dependent triosephosphate dehydrogenase, along with the carboxylase, is present almost entirely in the chloroplasts of some mature leaves. Fuller and Gibbs (1959) also demonstrated the close association between the carboxylase enzyme and the chloroplasts in spinach. The presence of these enzymes in the chloroplasts is further supported in the experiments of Trebst *et al.* (1959), who demonstrated the fixation of CO_2 into 3-PGA and sugar phosphates, using isolated chloroplasts with R-5-P as substrate, coupled to photosynthetic phosphoryla-

tion. The above considerations, along with the location of fraction I protein in the chloroplasts, led us to speculate with Park and Pon (1961) that this major protein fraction may contain several enzymatic sites for the photosynthetic carbon cycle, although the adsorption of these enzymes cannot be ruled out completely. It would not be economical, as Kupke (1962) comments, for such a major protein component as fraction I to be concerned with only one enzyme activity. Although the carboxylase activity previously has been associated with fraction I preparations, it is highly probable that the other two enzyme activities involved in this study also can be attributed to fraction I protein. We did not try to find out whether the partially purified protein fraction could catalyze the reactions beyond 3-PGA upon the addition of the necessary cofactors, TPNH, for instance. Elucidation of this question, along with a more rigorous investigation into the presence of the three enzymatic activities on the same individual molecule, await further work.

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Added in Proof

We have recently come across a report by Wildman *et al.* (1961) dealing with preparations of Fraction I protein from spinach and tobacco leaves by differential centrifugation and also by Sephadex G-75 and DEAE-cellulose chromatography. These workers have similarly found Fraction I protein to contain the three enzymatic activities, namely, phosphoriboisomerase, phosphoribulokinase, and ribulose diphosphate carboxylase.

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