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Photosynthetic Carbon Assimilation

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Preface

The photosynthetic fixation of carbon dioxide into organic compounds is mediated by the enzyme ribulose 1,5-bisphosphate (RuBP) carboxylase. The diversity of current research on this protein attests to its central role in biomass productivity, and suggests the importance of a timely and broadly based review. This Symposium was the first devoted exclusively to RuBP carboxylase and was attended by agronomists, plant physiologists, biochemists, molecular biologists, and crystallographers. Special efforts were made to involve young scientists in addition to established investigators.

It is a pleasure to acknowledge financial support provided by the Department of Energy, the United States Department of Agriculture, and the National Science Foundation, and the valued assistance of agency representatives, Drs. Joe Key, Robert Rabson, Elijah Romanoff, and Donald Senich. Thanks are due to Mrs. Margaret Dienes, without whose editorial skills this volume could not have been produced, and to Mrs. Helen Kondratuk as Symposium Coordinator. Finally, we wish to record our indebtedness to Dr. Alexander Hollaender for his tireless efforts in support of all aspects of this Symposium.

Symposium Committee
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FRACTION I PROTEIN AND OTHER PRODUCTS FROM TOBACCO FOR FOOD

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INTRODUCTION

Depending on the point of view, the tobacco plant is either extolled for the solace it brings to those who smoke and/or for the secure economic rewards from its cultivation and manufacture, or branded a weed of unmitigated evil for its effect on health. The latter view now seems to be the more popular. However, there is another possibility. Tobacco plants can be used as a source of high-grade protein for human consumption. The exploitation of this possibility could turn tobacco into an agricultural commodity of undeniable value. Since the idea of using leaf protein is not new, the purpose of this paper is to present reasons for thinking that tobacco plants could be a superior source of supplemental protein in the human diet compared with leaf proteins from other plants.

GROSS PROTEIN COMPOSITION OF TOBACCO LEAVES

Like other succulent leaves such as those of alfalfa, spinach, sugar beets, etc., tobacco leaves contain 80 to 90% water and 10 to 20% solid matter. In a single leaf (Figure 1) that has just reached its maximum growth in area, the accumulated weight of photosynthate (disregarding the polymers in cell walls) consists of about 3/4 protein and 1/4 small molecules (molecular weight $\leq$5000). The leaf proteins represent as direct a conversion of solar energy into protein as is possible in the biological world. About 50% of the leaf proteins are insoluble in water or buffers less alkaline than pH 10. These proteins for the most part comprise thylakoid membranes of chloroplasts and to a lesser degree insoluble proteins

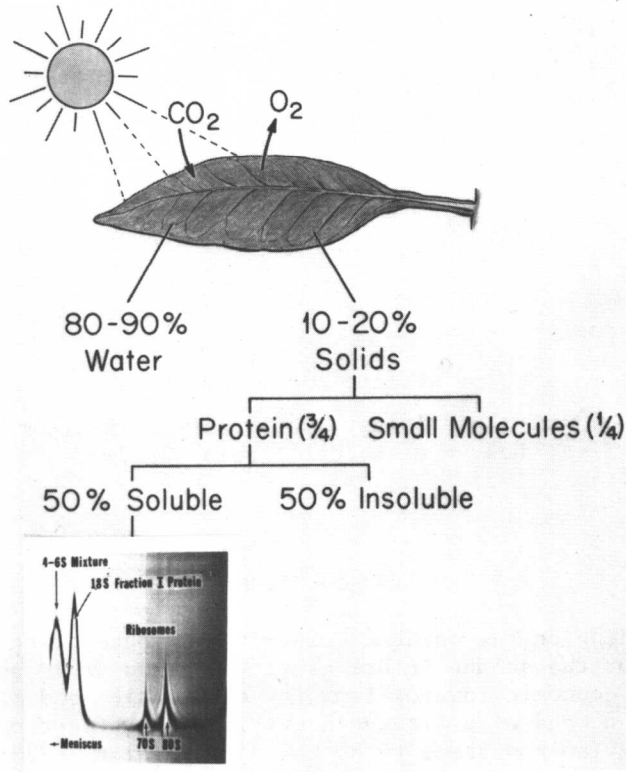

Figure 1. Diagram to show the gross composition of constituents of leaves and the ratio of insoluble to soluble proteins.

constituting the structure of nuclei, mitochondria, and endoplasmic reticulum. Upon isolation, the insoluble proteins are green because chlorophyll and other photosynthetic pigments remain attached to them.

As shown by the analytical centrifuge pattern in Figure 1, the soluble proteins of leaves consist of three classes: (a) the ribosome nucleoprotein class, accounting for about 10%; (b) a mixture of proteins with molecular weights less than 100,000; (c) a single kind of protein with a molecular weight of about 550,000 and having a rather inept name, Fraction I protein (F-I-p), which has been in continuous use since 1947. F-I-p has been shown to be carboxydismutase or ribulose 1,5-bisphosphate carboxylase, the enzyme that catalyzes the first step of carbon assimilation during photosynthesis. Succulent leaves such as tobacco generally contain about 10 mg F-I-p per gram fresh weight of leaves. F-I-p is the most abundant

single protein on earth because it is found in every organism containing chlorophyll a.

F-I-p has been purified to a very high degree from several kinds of plants including spinach, wheat, rice, Chinese cabbage, clover, and tobacco. The extensive knowledge about its structure and function is the product of fundamental research by several groups in different institutions in the United States, Great Britain, Japan, Australia, and New Zealand. Purification of F-I-p was tedious and time-consuming and required expensive reagents and equipment until 1971, when Dr. Nobumaro Kawashima of the Japan Monopoly Corporation, working in our laboratory, succeeded for the first time in crystallizing F-I-p from tobacco leaves (1). This made it possible to study F-I-p in the pure state and thus to uncover unexpected properties that would allow isolation on a scale of several hundred pounds of crystalline F-I-p from an acre of tobacco plants.

PROPERTIES OF CRYSTALLINE F-I-p

Figure 2 shows some properties of F-I-p crystallized from Nicotiana tabacum leaves. Most of these results were obtained with F-I-p from the Turkish Samsun cultivar of N. tabacum, but the results would apply to the more than 30 other cultivars of commercial tobacco we have investigated. Large F-I-p crystals are deposited as dodecahedrons (Figure 2A) and contain 80% water, which results in a relatively huge unit cell (Figure 2B). When dissolved in dilute NaCl solutions, the protein molecules can be seen by electron microscopy (Figure 2C) and have dimensions of about 110 Å. The individual molecules look granular, and all of them have the same appearance. Constant specific enzymatic activity is achieved by the second recrystallization of F-I-p, and no further change occurs with additional recrystallizations (Figure 2D). After the first recrystallization, no heterogeneity is detected when the redissolved protein is subjected to analytical centrifugation (Figure 2E). The molecular weight of F-I-p calculated from its sedimentation constant corresponds closely to the size of the macromolecules seen by electron microscopy. Pure F-I-p consists entirely of amino acids and is water clear in solution when observed by visible light (Figure 2F). When F-I-p crystals are subjected to dialysis against distilled water to remove salt and buffer, they are tasteless and odorless and appear pure white and partially jellified. The pure crystals contain no cations other than one molecule of magnesium per molecule of F-I-p (7).

Two other properties were observed that provided the clue to producing crystalline F-I-p by a simple procedure (2). One is stability to heat (Figure 3). With specific enzymatic activity used as the most sensitive indicator of change in quaternary structure, the data show that F-I-p loses activity at temperatures below 25°C. The diminution is slow, requiring 20 hr to complete

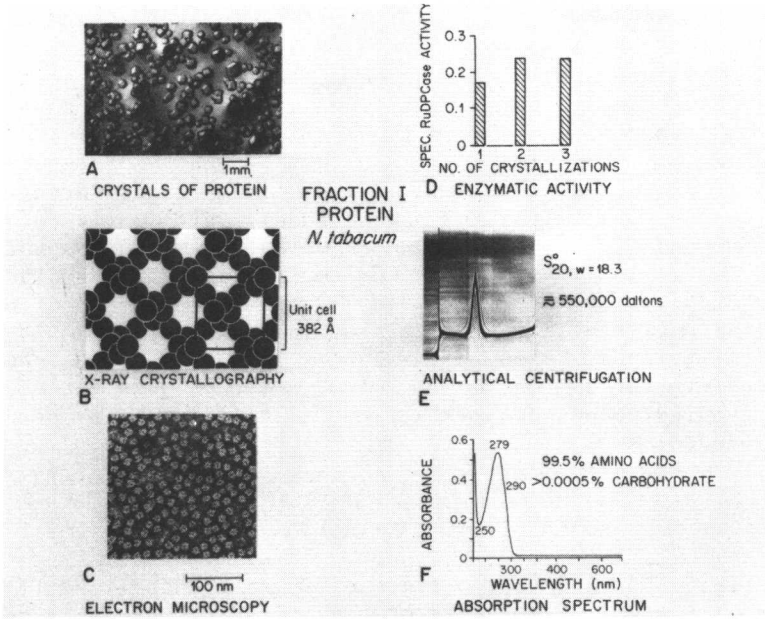

Figure 2. Properties of crystalline Fraction I protein of tobacco leaves. A, after Chan et al. (2); B, C, after Baker et al. (3); D, after Singh and Wildman (4); E, after Sakano and Wildman (5); F, after Sakano et al. (6).

when F-I-p at 25°C is placed in an ice bath at 0°C (Figure 3A). The loss in activity can be reversed by heating the protein to temperatures above 25°C. Heating at 50°C for 20 min caused all of the activity to be regained (Figure 3B) without producing any turbidity in the F-I-p solution. The specific enzymatic activity could be repeatedly diminished by cold treatment and restored by 50° heat.

The second property is solubility. F-I-p catalyzes the combination of carbon dioxide with ribulose 1,5-bisphosphate (RuBP) to form the first product of CO_2 fixation, 3-phosphoglyceric acid (PGA). When crystals of F-I-p were exposed to RuBP, they immediately dissolved, and the protein became enormously soluble (>150 mg protein per ml) in low ionic strength buffer (9). As little as about 8 molecules of RuBP bound to each molecule of protein allows almost complete removal of buffer and ions by dialysis and still keeps the protein soluble. If $NaHCO_3$ is added, F-I-p converts the RuBP to PGA, and the protein becomes highly insoluble, <1 mg protein per ml buffer remaining in low ionic strength solution. However, the protein becomes highly soluble again with addition of cations. At 20 mM

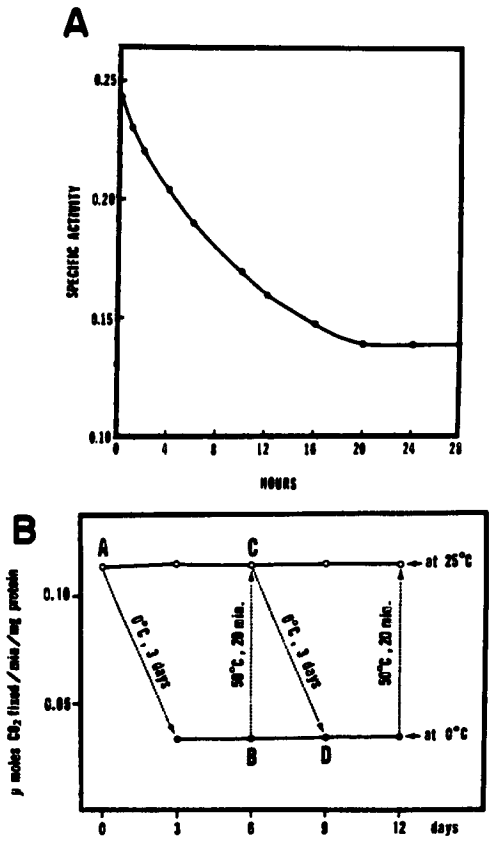

Figure 3. Effect of temperature on the specific enzymatic activity of crystalline Fraction I protein from tobacco leaves. A, after Singh and Wildman (4); B, after Kawashima et al. (8).

or less of the salts shown in Figure 4, F-I-p is virtually insoluble (<0.025 mg/ml), but it dissolves as the concentrations of the salts are increased. It dissolves to a much greater extent in the presence of Na^+ than K^+, to some extent in the presence of NH_4^+, and to a very limited extent in the presence of Li^+. As seen in Figure 4, 80 mM NaCl was sufficient to dissolve nearly 1 mg of F-I-p per ml. Since tobacco leaves contain about 10 mg F-I-p per gram leaf or roughly 12 mg F-I-p per ml of H_2O in the leaf sap, it became apparent that the cation content of the leaf itself was probably sufficient to maintain all of the F-I-p in solution during extraction. This finding, coupled with the observation that cold conditions were not required to maintain integrity of the F-I-p molecules, led to a

simple procedure for preparing crystalline F-I-p not only from N. tabacum but also from more than 60 other species of Nicotiana.

ESSENTIAL AMINO ACID CONTENT OF TOBACCO F-I-p

Tobacco F-I-p is composed of 18 amino acids accounting for 99.8% of its mass. In Table 1, the amounts of the 9 amino acids essential for human nutrition found in F-I-p are listed and compared with the amounts in other proteins of animal and plant origin and in the provisional pattern of essential amino acids deemed optimal for human nutrition by the UN Food and Agricultural Organization. Except for methionine, the amounts of essential amino acids in tobacco F-I-p either meet or exceed the standards of the provi-

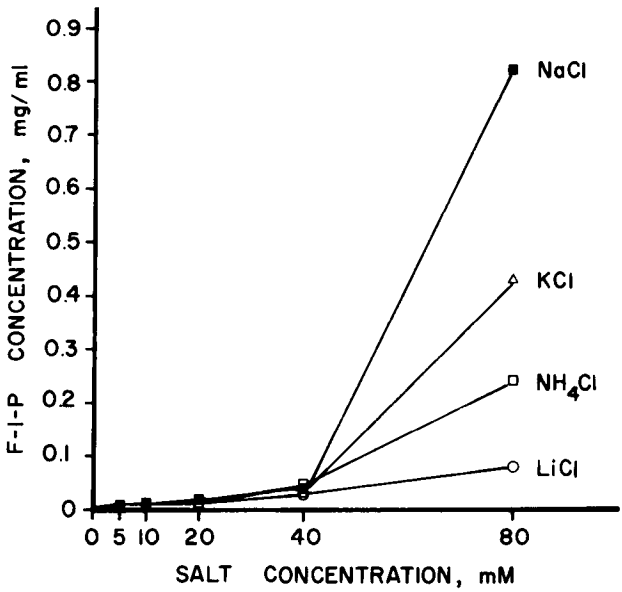

Figure 4. Solubility of Fraction I protein as a function of kind and concentration of cations. Experiment was done by suspending crystals of F-I-p in a fixed volume of salt solution, allowing 16 hr for complete solvation, and then removing undissolved protein by low speed centrifugation before determining amount of F-I-p in solution.

Table 1

Essential Amino Acids in Fraction I protein From Tobacco Compared With Other Plant Proteins, Casein, and FAO Provisional Pattern (Grams per 100 grams protein)

Amino acids	Prov. pat.(10)	Ca-sein(11)	Soy prot.(12)	Wheat prot.(11)	Corn prot.(11)	Rice prot.(11)	F-I-p*
Ile	4.2	7.5	5.8	4.0	6.4	5.2	4.2
Leu	4.8	10.0	7.6	7.0	15.0	8.2	8.8
Lys	4.2	8.5	6.6	2.7	2.3	3.2	5.8
Phe	2.8	6.3	4.8	5.1	5.0	5.0	4.4
Tyr	2.8	6.4	3.2	4.0	6.0	5.7	4.9
Met	2.2	3.5	1.1	2.5	3.1	3.0	1.6
Thr	2.8	4.5	3.9	3.3	3.7	3.8	5.2
Try	1.4	1.3	1.2	1.2	0.6	1.3	1.5
Val	4.2	7.7	5.2	4.3	5.3	6.2	7.2

*Calculated from amino acid analyses (13) for F-I-p from tobacco.

sional pattern. Also, tobacco F-I-p is superior in its essential amino acid content to soy and grain proteins.

When treated with the enzymes of the digestive gut, F-I-p breaks down into 80 to 100 small peptides. The absence of disulfide bonds aids in its digestion. These properites of tobacco F-I-p together with its amino acid composition and absence of taste and odor persuaded what was then the RANN division of the National Science Foundation to support a project to prepare 0.6 kg of crystalline F-I-p for biological evaluation.

CRYSTALLINE F-I-p PRODUCTION ON A 6 TO 12 g/day SCALE

Production of 0.6 kg of F-I-p crystals required for an animal feeding test was greatly facilitated by Dr. Richie Lowe of the University of Kentucky informing us prior to publication (14) of his discovery that F-I-p would crystallize when clarified tobacco leaf extracts were subjected to Sephadex chromatography.

Turkish tobacco plants were grown in a greenhouse at a spacing of one plant per 0.25 ft^2 until they attained a height of 18 to 21 in. The leaves were detached from the stems. The blades of a gallon size Waring blender were covered with 400 ml of water and 10 ml of β-mercaptoethanol. Then 2 kg of fresh leaves were homogenized in this solution. The homogenate was filtered through two layers of fine cheesecloth supported on a 32 mesh screen. The cheesecloth was twisted by hand to press out the green juice, which had a volume

of about 1900 ml and a pH ranging from 5.7 to 6.2 for different extractions. By comparing the amount of chlorophyll retained in the filter cake with the amount in the green juice, it was estimated that about 70% of the leaf cells had been ruptured by blending and had released their contents as cell-free green juice.

In 400- to 500-ml batches, the green juice was heated in a 50° water bath for 10 min. After it was removed from the bath, 10 ml of 50 times concentrated Tris-HCl-EDTA buffer was added to produce a pH of 7.6 to 7.8 and a final concentration of 0.025 M Tris and 0.0005 M EDTA. When the juice was left standing at room temperature, partial precipitation of a green sediment from a brown juice could be seen. The neutralized juice was centrifuged at 5000 g for 15 min. The brown juice (total volume, ca. 1700 ml) was collected and passed through a G-25 Sephadex column equilibrated with Tris-HCl-EDTA buffer. As the buffer passed through, the total soluble proteins of the tobacco leaves eluted in the void volume of the column. Frequently crystals of F-I-p appeared during collection of the eluate or shortly thereafter. The eluate was allowed to stand for 2 to 3 days at 8° to allow more F-I-p crystals to form and settle into a thin layer at the bottom of the flask. Then the proteins that remained dissolved in the mother liquor were decanted so that the F-I-p crystals could be collected and washed with distilled water. The crystals were redissolved in 0.1 M NaCl solution, which was then centrifuged to remove slight amounts of undissolved material. The water-clear supernatant solution was dialyzed against distilled water to cause recrystallization of F-I-p. The recrystallized protein was transferred to flasks, and the salt-free protein was lyophilized to produce a white nonhygroscopic powder which was tasteless. The yields of recrystallized F-I-p were consistently between 3 and 4 g dry protein per kg fresh tobacco leaves during the fall, winter, and spring of 1976-77.

PROTEIN EFFICIENCY RATIO OF CRYSTALLINE F-I-p COMPARED WITH CASEIN

As reported by Ershoff et al. (15), when rats were fed on a diet in which crystalline tobacco F-I-p was the sole source of protein, they grew faster during a 28-day period than did control rats whose sole source of protein was casein. This shows that pure F-I-p is an outstanding protein from the standpoint of nutritional value even though it had appeared to be deficient because it contains less methionine than does casein.

Because crystalline F-I-p from tobacco leaves is composed entirely of amino acids and contains no carbohydrates, purines, pyrimidines, pigments, or minerals (except for the sulfur in some amino acids) and because of its high nutritional value and absence of odor and taste, it has the potential of being developed into important therapeutic products for the treatment of a number of medical conditions. It should be particularly valuable in feeding patients with various types of renal disease whose sodium and potassium intake

must be rigorously controlled, and it might well be incorporated in special diets that could reduce the required frequency of hemodialysis for patients suffering from renal failure. A number of other medical uses have been envisaged (15).

ECONOMIC POTENTIAL OF TOBACCO GROWN PRIMARILY AS A SOURCE OF PROTEIN FOR HUMAN NUTRITION

Tobacco plants are remarkably efficient converters of solar energy into plant biomass solids. The seeds of the plants are very small (ca. 14,000 seeds/g) and retain a germination capacity close to 100% for several years.

Starting from a dry seed weighing ca. 7×10^{-5} g, a plant containing >150 g of dry solids in its aerial portion will develop in 4 months under favorable environmental conditions. Given enough space, the plant will grow taller than a man. Casual inspection of a row of tobacco plants growing alongside a row of corn plants gives the impression that the biomass per m^2 of space is greater for tobacco than for corn.

Current tobacco culture, e.g., for producing Burley tobacco in Kentucky, is based on growing 8000 plants/acre, which gives a harvest of 900 to 1100 dry kg/acre or, at 85% moisture, roughly 13,600 kg of fresh plants. Compared with forage crops such as alfalfa, which have been extensively studied for leaf protein production (16, 17), tobacco grown in the conventional manner does not have an impressive biomass yield. However, tobacco can be grown as a forage crop like alfalfa. In California we have been growing tobacco at a density of one plant per 0.25 ft^2 outdoors in the San Fernando Valley. During July and August 1977, plants grown for 6 weeks and cut off 4 in. above soil level had an average fresh weight of 0.125 kg each, which is equivalent to >20,000 kg fresh weight tobacco plants per acre. At 15% dry matter, a single harvest would produce about 3000 kg dry matter per acre. With harvested plants left to regenerate new shoots for a second harvest followed by replanting and two more harvests, yields approaching 60 metric tons of fresh tobacco plants per acre per year can be envisaged by conservative reckoning.

Sixty metric tons of fresh tobacco plants can be processed into 130 kg of dried crystals of F-I-p. In addition the same plants would yield two other valuable kinds of proteins and three other products, as indicated by the flow sheet in Figure 5. From the mother liquor remaining after removal of F-I-p crystals, 530 kg of dry protein can be recovered which is as nutritious as casein and nearly tasteless and odorless, and could be added to less nutritious foods to upgrade them. Extraction of the green precipitate with organic solvents yields 540 kg of a dry mixture of protein, nucleic acid, and starch having a slight but pleasant odor and a barely sweet taste. Distillation of the solvent leaves 360 kg of dry solids consisting of fats, lipids, chlorophyll, carotene, etc. Washing the Sephadex columns with water after elution of soluble proteins produces a