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BIOCHEMICAL EFFECTS OF TOBACCO ETCH VIRUS INFECTION ON TOBACCO LEAF TISSUE—I.

PROTEIN SYNTHESIS BY ISOLATED CHLOROPLASTS

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Abstract—Intact chloroplasts isolated from leaf tissue of *Nicotiana tabacum* L. systemically infected with tobacco etch virus incorporated glycine-1,2-^{14}C into a TCA insoluble product at the same rate as did chloroplasts from healthy plants. Tissue from infected plants, however, contained only about two thirds as many chloroplasts on a fresh weight basis as tissue from healthy plants. Total chlorophyll and protein per chloroplast were unaltered by infection.

INTRODUCTION

TOBACCO ETCH, incited by tobacco etch virus, is a common disease of tobacco and is characterized by an initial chlorosis of tissue adjacent to veins, followed by mottling, leaf distortion, and stunting. The symptoms are, in most respects, those of a typical "mosaic" disease.

Virus replication in host cells involves the synthesis of virus specific protein, presumably at the expense of normal host protein.[1] Thus, reports on alterations in nitrogen metabolism of virus infected plants are numerous.[2] In plants which contain distinguishable chloroplasts, two protein synthesizing sites are known. In addition to the nucleus-endoplasmic reticulum system, chloroplasts contain an apparently complete protein synthesizing system including DNA, sRNA, activating enzymes, and ribosomes.[3–5]

The investigation reported here was undertaken to determine the possible effects of tobacco etch virus infection on the protein synthesizing system of tobacco chloroplasts. The usual methods of obtaining intact chloroplasts, such as grinding tissue with sand or homogenizing with the Waring blendor, did not prove satisfactory, and a method of extraction that would give a high proportion of intact chloroplasts was developed.

RESULTS AND DISCUSSION

Isolation of Chloroplasts

Homogenization of tobacco leaf tissue by grinding with sand in buffered isotonic sucrose or homogenization in a Waring blendor, even for periods as short as 10 sec, ruptured almost all of the chloroplasts. Examination, by phase contrast microscopy, of such a homogenate

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[1] S. G. WILDMAN, C. C. CHEO and J. BONNER, *J. Biol. Chem.* **180**, 985 (1949).
[2] T. O. DIENER, *Ann. Rev. Phytopath.* **1**, 197 (1963).
[3] J. BOVE and I. D. RAACKE, *Arch. Biochem. Biophys.* **85**, 521 (1959).
[4] D. SPENCER and S. G. WILDMAN, *Biochemistry* **3**, 954 (1964).
[5] A. GOFFEAU and J. BRACHET, *Biochem. Biophys. Acta* **95**, 302 (1965).

or the 1000 × *g* fraction made from it revealed mainly a large number of chloroplast fragments. A more gentle method of disrupting the cells was obviously required, and several methods were tested. The one which proved most satisfactory was grinding the tissue in a mortar with buffered isotonic sucrose and 4-mm diameter glass beads (see Experimental). As shown in Table 1, this extract contained only about 17–18 per cent of the total chlorophyll present in the tissue. However, 80 per cent of the chlorophyll was in the 500 × *g* and 1000 × *g* fractions which contained intact chloroplasts. The remaining 20 per cent was in the 10,000 × *g* fraction, which contained chloroplast fragments.

TABLE 1. EFFICIENCY OF CHLOROPLAST ISOLATION FROM HEALTHY TOBACCO LEAF TISSUE

Fraction	Mg Chlorophyll
Whole tissue	61·0
Homogenate	10·4
500 × *g* Pellet	3·2
1000 × *g* Pellet	5·3
12,000 × *g* Pellet	2·0
12,000 × *g* Supernatant	0·4

Microscopic examination of fractions obtained by differential centrifugation revealed intact chloroplasts, starch, a few nuclei, and cellular debris in the 500 × *g* fraction. This fraction is usually discarded in tissue fractionation procedures but, as shown in Table 1, it contains about 30 per cent of the chlorophyll extracted from the tissue. The 1000 × *g* fraction contained numerous intact chloroplasts (50 per cent of the total chlorophyll extracted), some starch, a few particles of the size range of mitochondria, and some chloroplast fragments. The 12,000 × *g* fraction contained numerous mitochondria-size particles and chloroplast fragments (20 per cent of the total chlorophyll extracted). The supernatant from this fraction (12,000 × *g* supernatant) contained no particles visable with the light microscope. The 1000 × *g* fraction could be further purified by sucrose density gradient centrifugation. Two bands, both containing intact chloroplasts, resulted from density gradient centrifugation. No differences between the chloroplasts from the two bands could be detected by phase contrast microscopy. Isolation of the more prominent band and recentrifugation on a sucrose gradient did not resolve this band further (Fig. 1). Figure 2 shows the microscopic appearance of the 1000 × *g* fraction and the chloroplast band isolated by density gradient centrifugation of the 1000 × *g* fraction. The greater homogeneity of the preparation and the higher ratio of chloroplasts to smaller particles after density gradient centrifugation is apparent. The most obvious effect of density gradient centrifugation was the removal of starch and chloroplast fragments, both of which sedimented to the bottom of the tube.

Effect of Infection on Protein Content of Chloroplasts

Chloroplasts were isolated from equal weights of etch infected and healthy leaf tissue and brought to the same volume in buffered sucrose. Total chlorophyll and protein were determined. Healthy and etch infected plants contain unequal numbers of chloroplasts but equal amounts of chlorophyll per chloroplast,[6] so chlorophyll concentration can be used as an index of the number of chloroplasts. As shown in Table 2, chlorophyll (and therefore the

[6] T. G. NYE and R. E. HAMPTON, *Phytochem.* 5 1187 (1966).

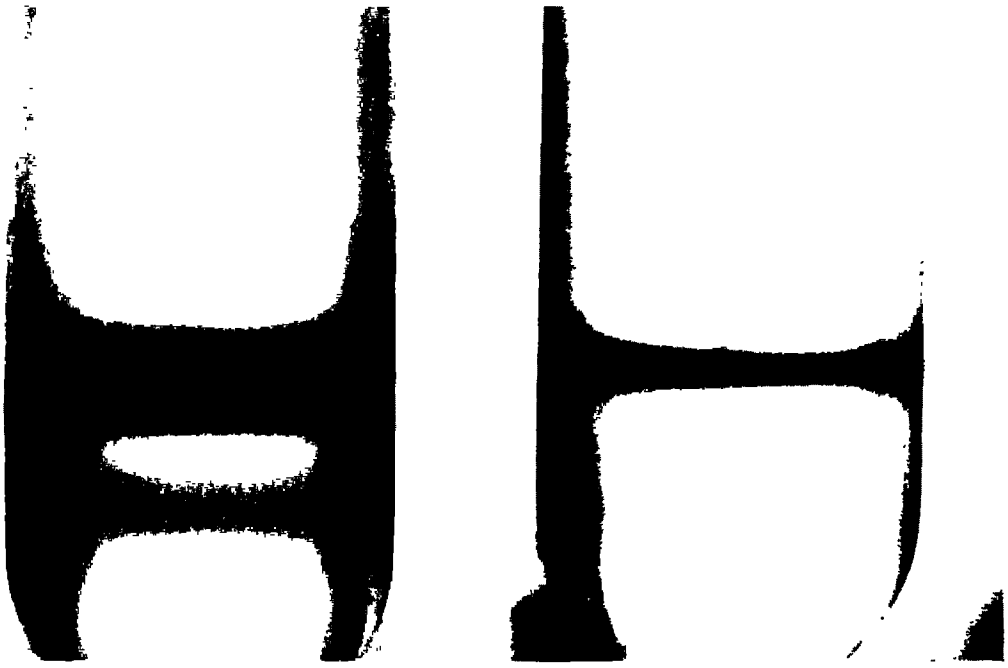

FIG. 1. CHLOROPLAST BANDS PRODUCED ON SUCROSE GRADIENT TUBES.

Photographed with red back-lighting. The tube on the right shows the result of recentrifuging the major (upper) band isolated from the tube on the left.

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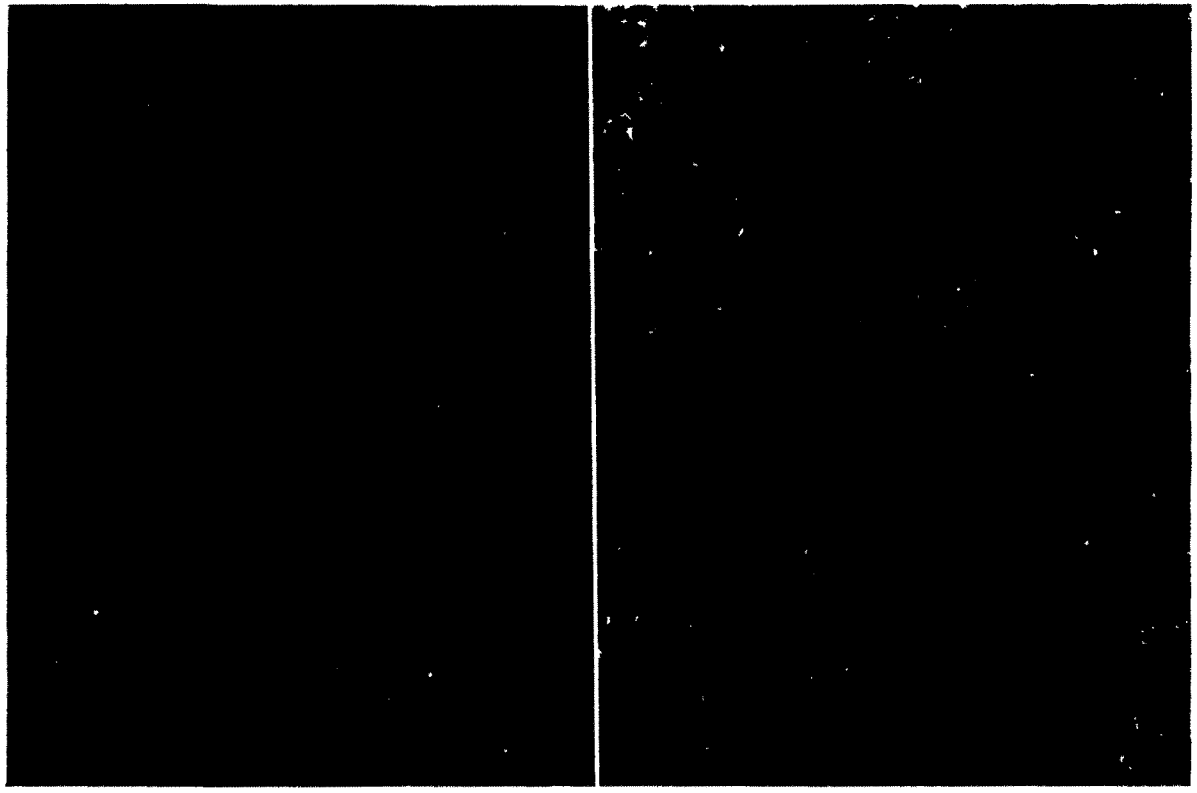

FIG. 2. PHASE CONTRAST PHOTOMICROGRAPHS OF THE $1000 \times g$ FRACTION (LEFT) AND OF THE CHLOROPLAST BAND FROM SUCROSE GRADIENT CENTRIFUGATION OF $1000 \times g$ FRACTION (RIGHT).

number of chloroplasts) was decreased about 35 per cent in infected plants. However, the protein concentration per mg chlorophyll was unchanged.

TABLE 2. TOTAL CHLOROPHYLL AND PROTEIN CONTENT OF THE 1000 × *g* FRACTION FROM HEALTHY AND ETCH INFECTED PLANTS

Source	Total chlorophyll	Protein	mg Protein/mg chlorophyll
Healthy	246 μg/ml	2·03 mg/ml	8·3
Infected	160 μg/ml	1·35 mg/ml	8·4

The decrease in chloroplast numbers in virus infections of the mosaic type is well known, but whether this is a result of destruction of chloroplasts or a failure of proplastids to develop is not clear.[7] In either event, it is unlikely that the smaller numbers of chloroplasts extracted from infected plants resulted from a greater fragility of such chloroplasts.

Amino Acid Incorporation by Chloroplasts

The ability of the total 1000 × *g* fraction to incorporate amino acids was compared to that of a chloroplast fraction obtained by density gradient centrifugation. This was done in order to determine if further purification of the chloroplast fraction would result in a reduction of amino acid incorporation. The results are summarized in Table 3. A small but

TABLE 3. GLYCINE-^{14}C INCORPORATION BY TOBACCO CHLOROPLASTS ISOLATED BY DENSITY GRADIENT CENTRIFUGATION AND BY THE 1000 × *g* FRACTION FROM HEALTHY TOBACCO LEAVES

Preparation	μg Chlorophyll	Net* cpm
Density gradient centrifugation	70	94†
1000 × *g* Fraction	70	81†

* Incubated 120 min with 10 μc glycine-1,2-^{14}C (see Experimental).
† Difference not significant.

significant incorporation occurred and no difference between the two could be detected. When the 1000 × *g* fractions from healthy and etch infected plants were diluted to contain equal numbers of chloroplasts and compared with respect to their ability to incorporate glycine-1,2^{14}C, no consistent difference was observed. Table 4 summarizes the results of three experiments. Incubation of the chloroplasts for periods of longer than 60 min resulted in little additional incorporation, indicating that the incorporation observed was not due to bacterial contamination. The total incorporation was very low, and this is probably because of the small number of chloroplasts used (100 μg chlorophyll). From these data it was concluded that infection does not consistently affect the protein synthesizing system of chloroplasts but, by reducing the number of chloroplasts, reduces the total amount of protein synthesized by these organelles in the tissue.

[7] KATHERINE ESAU, *Botan. Rev.* **14**, 413 (1948).

TABLE 4. GLYCINE-1,2-^{14}C INCORPORATION BY CHLOROPLASTS FROM HEALTHY AND ETCH INFECTED TOBACCO LEAVES

Source	Cpm, experiment No.*		
	I	II	III
Infected	62	68	63
Control	54	47	76

* Incubated 60 min with 10 μc glycine-1,2-^{14}C (see Experimental).

EXPERIMENTAL

Plants of *Nicotiana tabacum* (Var. Ky 26) were grown and inoculated as described .[6] The plants were inoculated 25 days after the seed was sown and the three top, systematically infected leaves were harvested 19–20 days after inoculation. Control plants were grown under the same conditions but were not inoculated, and the top three leaves were used.

Tissue Fractionation

Leaves from which the midveins had been removed were surface sterilized in a 5 per cent solution of commercial sodium hypochlorite ("chlorox") for 5 min, rinsed with deionized water, and chilled to 3–5°. 20 g of tissue were then ground in a prechilled mortar in 30 ml cold 0·36 M sucrose in 0·05 M tris-HCl buffer at pH 7·5 containing 20 g of 4 mm diameter glass beads. The resulting slurry was pressed through a double layer of cheesecloth to remove

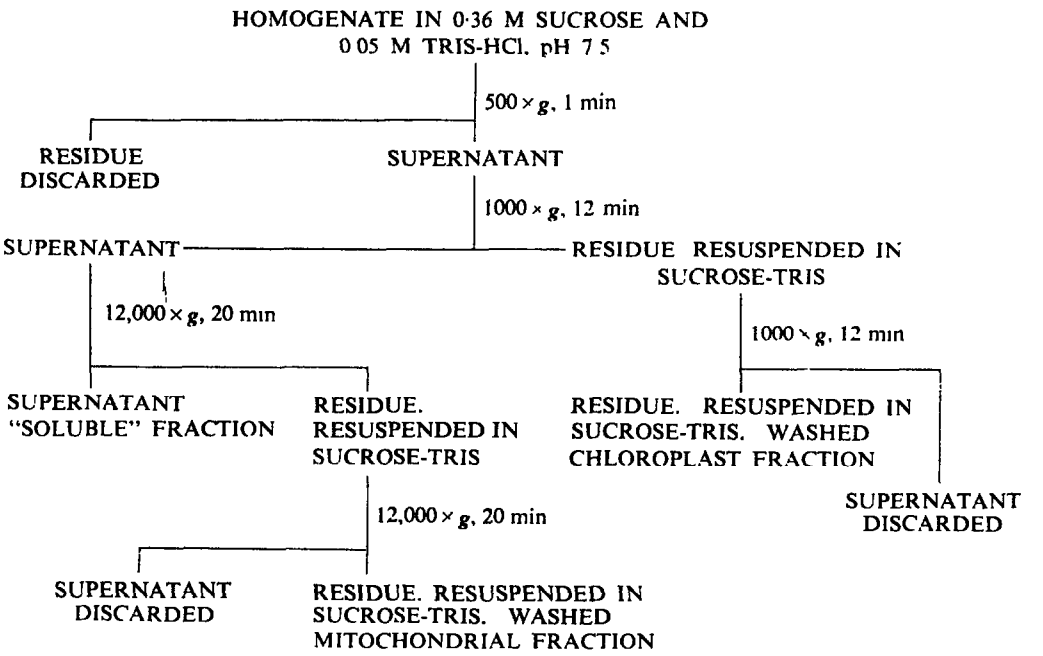

FIG. 3. FRACTIONATION PROCEDURE USED FOR TOBACCO LEAF HOMOGENATES.

the glass beads and larger debris. The homogenate was fractionated by differential centrifugation as shown in Fig. 3 by use of a refrigerated centrifuge. All steps were carried out at near 0°.

The $1000 \times g$ fraction was further purified by centrifugation on sucrose gradients at $58000 \times g$ for 30 min. The gradients were prepared by successively layering 5 ml each of 1·76 M, 1·40 M, 1·10 M, 0·80 M, 0·50 M, and 0·37 M sucrose solutions in 0·05 M tris-HCl at pH 7·5. The tubes were allowed to stand overnight in the refrigerator to form a smooth gradient. Two ml of the concentrated $1000 \times g$ fraction was layered on top of the gradient just prior to centrifuging in the SW 25·1 head of the Spinco ultracentrifuge.[8]

Amino acid incorporation was measured by adding to 1 ml of chloroplasts containing 100 μg chlorophyll, 10 μc of glycine-1,2-^{14}C (1 mc/m mole specific activity); 20 μg pyruvate kinase; and 0·1 ml of a mixture of ^{12}C-amino acids, nucleotide triphosphates, ATP, phosphoenolpyruvate, and mercaptoethanol as described by Spencer and Wildman.[4] The reaction mixture was kept at 0–1° until all additions were made and then quickly brought to 23° by immersing in a water bath. Each sample was run in triplicate tubes and three checks, to which the isotope was added at the end of the incubation period, were included. At the end of the incubation period, 1 ml of 0·05 M glycine-^{12}C was added to each tube followed immediately by 8 ml of cold 7 per cent trichloroacetic acid. After 10–15 min in an ice bath, the tubes were centrifuged for 15 min at $1800 \times g$, the supernatant was discarded, and the precipitate was resuspended and washed a second time with 10 ml of 7 per cent TCA. The precipitate was dissolved in 1 ml hot 0·5 N NaOH, transferred to 1 in. stainless steel planchets, and dried at 120°. The samples were counted to a preset count of 10,000, using a thin end-window gas flow system adjusted to count in the Geiger region. Net count rate was obtained by subtracting background and check counts from the gross count rate.

Protein was estimated by the method of Lowry *et al.*,[9] and total chlorophyll by the method of Arnon.[10]

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