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In vitro Protein Synthesis by Plastids of Phaseolus vulgaris. II. The Probable Relation Between Ribonuclease Insensitive Amino Acid Incorporation and the Presence of Intact Chloroplasts[1, 2]

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Abstract. **Amino acid incorporation into protein by chloroplasts from primary leaves of *Phaseolus vulgaris* L., var. Black Valentine is only partially inhibited by 400 μg/ml ribonuclease. The rate of incorporation, in the presence of ribonuclease, is progressively inhibited with time, and ceases after about half an hour. Preincubation of chloroplasts at 25°, in the absence of ribonuclease, increases the inhibitory effect of ribonuclease on the initial rate of incorporation of amino acid into protein. Examination of electron micrographs of freshly prepared chloroplast suspensions shows that chloroplasts are largely intact. However, after incubation at 25° for 1 hour the chloroplasts are disrupted, as indicated by loss of their stroma contents. It is concluded that the intact chloroplast membrane is relatively impermeable to ribonuclease. Amino acid incorporating activity probably becomes inhibited as the inside of the chloroplast is made accessible to ribonuclease by breakage of membranes during incubation at 25°.**

Amino acid incorporation by ribosomes from chloroplasts (3), or by chloroplast preparations suspended in low osmotic strength media (1, 2, 13, 15) has been reported to be sensitive to ribonuclease. When chloroplasts are suspended in, and incorporation is carried out in high osmotic strength media, the reaction may be fully sensitive (16), partially sensitive (7, 13), or insensitive (5) to ribonuclease. Insensitivity of a portion of incorporating activity to high concentrations of ribonuclease led to the suggestion that the insensitive amino acid incorporation was due to the presence of intact chloroplasts (13). The present work was carried out to investigate this possibility.

Materials and Methods

Biological Materials and Biochemical Determinations. Phaseolus vulgaris L., var. Black Valentine was grown in the dark for 6.5 days, followed by 0.5 day of irradiation with white light (13).

A crude chloroplast preparation was used throughout (13). It was obtained from a leaf mince prepared by chopping leaves in Honda medium (13, 16). In addition to sucrose, tris, mercaptoethanol, and magnesium ion, this medium contains Ficoll and dextran. The incorporation reaction was carried out as described previously (13, 16) using ^{14}C-L-leucine. Each ml of reaction mixture contained 0.8 ml of chloroplasts suspended in Honda medium. In addition the following components were also present in the complete reaction mixture: ATP, pyruvate kinase, PEP, GTP, UTP, CTP, ^{12}C-amino acids other than leucine, magnesium ion, and mercaptoethanol (13). Except where stated, incorporation was determined by scintillation counting by the method of Mans and Novelli (11). In this procedure samples are pipetted onto filter paper disks, and the reaction is stopped immediately after absorption of the samples by dropping the disks into cold trichloroacetic acid-leucine solution. The disks are prepared for counting by a series of extractions including extraction with hot trichloroacetic acid and ethanol:ether.

Ribonuclease A (E.C. 2.7.1.40; from bovine pancreas, 5 × crystallized) was obtained from Sigma Chemical Company, St. Louis, Missouri. It was used without further purification at a final concentration of 400 μg/ml of reaction mixture. Ribonuclease, unless otherwise stated, was added to the otherwise complete reaction mixture at 0°, and within minutes the incorporation reaction was started by warming the reaction mixture rapidly to 25°. Reagents other than ribonuclease were obtained from sources as listed previously (13). The contribution of bacteria to incorporation was determined by measuring incorporation of amino acid into protein insoluble in Triton X-100 (13).

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Electron Microscopy. Small pieces of leaves were fixed in 2% glutaraldehyde solution in 75 mM phosphate buffer (pH 7.0). Penetration was facilitated by vacuum infiltration. The leaf pieces were then rinsed thoroughly with phosphate buffer, and postfixed in 1% OsO_4 in phosphate buffer for 1 hour at room temperature. Plastid pellets were suspended in Honda medium (16) which lacked mercaptoethanol, and contained 50 mM phosphate instead of tris. Fifty percent glutaraldehyde was added to a final concentration of 2%. After 1 hour at 4° chloroplasts were collected by centrifugation at 30,000 × *g* × 0.5 hour, and were embedded in agar (4) to facilitate handling. Small blocks of agar, which contained chloroplasts, were thoroughly washed in phosphate buffer, and were then postfixed in 1% OsO_4 in phosphate buffer, for 1 hour at room temperature. Agar blocks, and tissue pieces were embedded in Epon (9), and sections were stained with uranyl acetate, and lead hydroxide (12).

Results

Ribonuclease progressively inhibits amino acid incorporation when the enzyme is added immediately before, and is present throughout the incorporation assay period (fig 1). Typically, incorporation is partially inhibited during the first 15 minutes of the incubation, and no further incorporation takes place in the interval 45 to 60 minutes. Incorporation may continue, or may completely cease, in the interval 15 to 45 minutes, varying in this aspect from experiment to experiment. Inhibition of incorporation may vary from 20 to 50% during the first 5 minute period of incubation. Without ribonuclease, incorporation usually continues during a 60 minute incubation period.

The incorporation of radioactive amino acid into protein, which is resistant to ribonuclease, is not due to incorporation carried out by bacteria. After various times of incubation with radioactive amino acid, samples were treated with Triton X-100 in order to test for the contribution of bacteria to ribonuclease insensitive incorporation. This detergent makes chloroplasts, but not bacteria, unsedimentable at 6000 × *g* × 20 minutes (13). The radioactivity incorporated into the protein of the sediment, obtained after treatment of the reaction mixture with Triton, was much lower than the radioactivity incorporated into protein in the presence of ribonuclease (table I). Thus, only a small portion of the ribonuclease insensitive incorporation is due to the presence of bacteria in the chloroplast preparation.

Since extremely high concentrations of ribonuclease were used in these experiments, the possibility exists that the inhibitory effect that is observed is due to other enzymes present as contaminants of the ribonuclease preparation. Ribonuclease is an enzyme that is stable to heat (8). Heating the enzyme preparation at 80° for 15

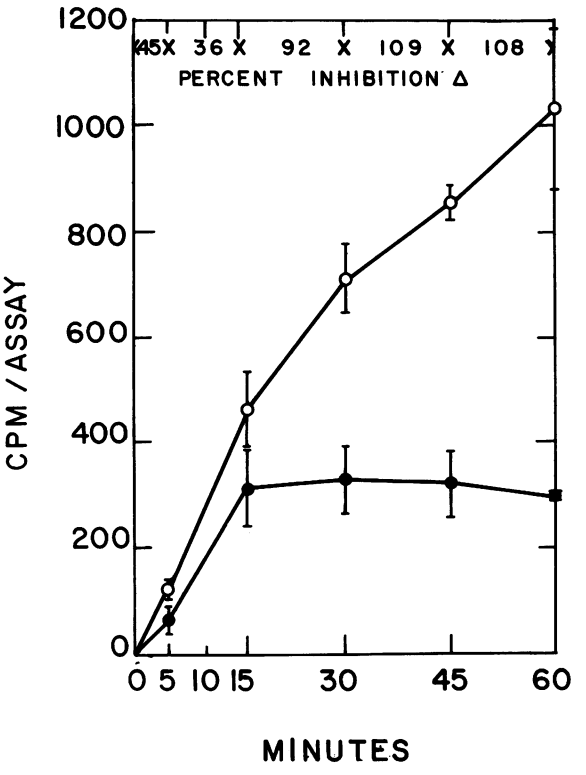

FIG. 1. Effect of ribonuclease on the time-dependence of incorporation: ○, no ribonuclease; ●, with ribonuclease. Enzyme was present from the start of incubation at 25°. Values are for 3 replicate samples, with dispersion given as standard deviation. Values given along the upper abscissa are percent inhibition of incorporation occurring within the time interval indicated by the enclosing brackets.

Table I. *Lack of Relationship Between Incorporation Resistant to Ribonuclease and the Presence of Bacteria*

Radioactivity incorporated into protein, and presented in columns 2 and 3, was determined by the method of Mans and Novelli (11). Radioactivity incorporated into protein that is sedimentable at 6000 × *g* × 20 minutes, after treatment of samples of reaction mixture with Triton X-100, was determined as described by Parenti and Margulies (13), and is presented in column 4. Values obtained by this last method were converted to values that would have been obtained by the method of Mans and Novelli. The conversion factor was determined from replicate samples, half of which were prepared for counting by the first method, and half by the second.

Min at 25°	Incorporation into protein, cpm in Total protein		
	No ribonuclease	With ribonuclease	Protein of bacteria
15	197	119	3
30	315	173	8
60	532	174	24

minutes had no effect on the inhibitory action of the ribonuclease preparation. Thus, the inhibitory effect being studied is probably due to ribonuclease.

The delay in obtaining complete inhibition of incorporation, as described in the first paragraph of this section, suggests the following possibilities. First, the delay might represent the time necessary for ribonuclease to degrade ribonucleic acid which is spatially accessible to it. In this case there would be no membrane barrier, or the chloroplast membrane would be freely permeable to the enzyme. Second, the delay might represent time required for ribonuclease to reach sensitive sites in the chloroplast due to slow penetration of the chloroplast membrane by the enzyme, the membrane remaining intact during this interval. Third, the delay might represent time required for a change to occur in the chloroplast membrane, which is at first impermeable to ribonuclease. In this case, the membrane might be lost, or might remain intact but become freely permeable. If either of the first 2 possibilities were correct, the lag phase in inhibition of incorporation by ribonuclease could be overcome only by preincubation of the chloroplasts with ribonuclease. However, if the third hypothesis were correct, the lag phase could be overcome by preincubation in the absence of ribonuclease.

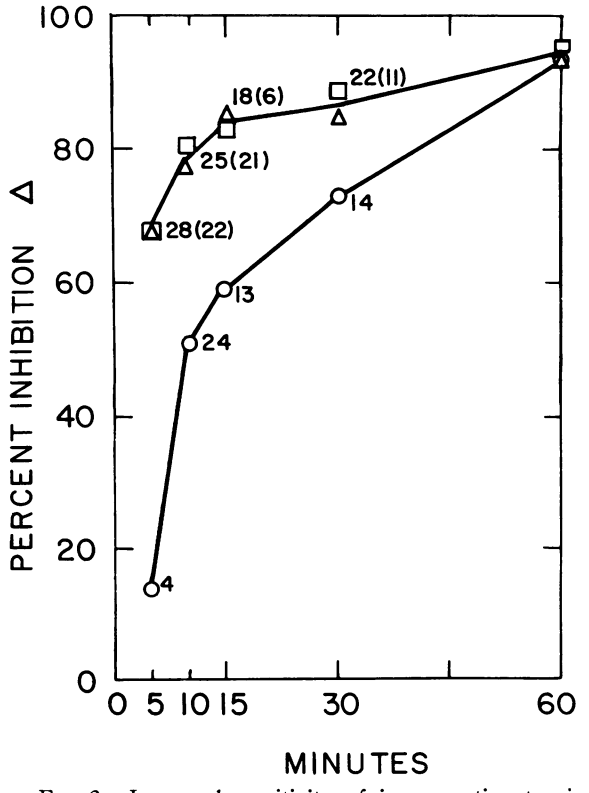

FIG. 2. Increased sensitivity of incorporation to ribonuclease resulting from preincubation of chloroplasts for 30 minutes: ○, preincubation on ice without ribonuclease; △, preincubation at 25° without ribonuclease; □, preincubation at 25° with 400 μg/ml ribonuclease. Points are average values for 4 replicate experiments. Numbers next to the points are standard deviations, those in parenthesis are standard deviations for chloroplasts preincubated at 25° with ribonuclease.

To differentiate between the first 2, and third possibilities, the effect of preincubation of chloroplasts at 25° in the absence of ribonuclease was tested. Chloroplasts were preincubated 30 minutes without ribonuclease, either at 0°, or at the temperature at which incorporation is assayed, 25°. The sample kept at 25° was rechilled to 0°. Ribonuclease was then added to a portion of the chloroplasts preincubated at 0°, and to a portion of those preincubated at 25°. In addition, a chloroplast sample was incubated at 25° for 30 minutes with ribonuclease, and chilled to 0°. The reagents necessary for amino acid incorporation were then added, the reaction mixture warmed to 25°, and samples were taken for determination of radioactivity incorporated into protein as a function of incubation time at 25° in complete reaction mixture. Both chloroplasts preincubated at 0°, and at 25° were later exposed to ribonuclease at 25° for the same lengths of time, and under the same conditions. Preincubation at 25° in the absence of ribonuclease increased the inhibition of the initial rate of amino acid incorporation by ribonuclease (fig 2). No additional inhibition by ribonuclease was observed in those chloroplasts which had been preincubated with ribonuclease at 25°. The preincubation time at 25° which is required to obtain complete

Table II. *Sensitivity to Ribonuclease of Initial Rate of Incorporation as a Function of Time of Preincubation of Chloroplasts at 25°.*

Replicate samples of chloroplasts, in reaction mixture lacking only labeled amino acid and pyruvate kinase, were kept on ice or were incubated at 25°. After each of the specified times, 2 samples were removed to a bath of ice water. After 1 minute, pyruvate kinase, and labeled amino acid were added to both, and ribonuclease to one. Samples were taken for determination of radioactivity incorporated into protein, and the reaction mixtures were placed at 25°. After 5 minutes, additional samples were taken for determination of radioactivity incorporated into protein. Column 2 presents percent inhibition by ribonuclease of the incorporation that occurred in this 5 minute interval. Column 3 presents the values for incorporation that occurred during this interval in the vessels which lacked ribonuclease.

Preincubation	Inhibition by ribonuclease	Radioactivity incorporated in the absence of ribonuclease
Min	%	*cpm*
0	39	252
5	43	234
15	47	128
30	89	96
60	100	41

inhibition by ribonuclease of the initial rate of amino acid incorporation, is about 0.5 hour (table II). This is approximately the time required for incorporation to cease when ribonuclease is present during an assay using chloroplasts which have not been preincubated at 25°. Thus, since preincubation without ribonuclease can overcome the lag in the inhibitory effect of ribonuclease, only the third hypothesis mentioned above seems tenable.

To correlate ribonuclease sensitivity with a change in the chloroplast membrane, electron micrographs of chloroplasts fixed immediately after isolation, and after incubation for 60 minutes at 25° were examined. The majority of the chloroplasts are intact before incubation. Figure 3 shows a typical chloroplast before incubation. It shows the presence of stroma material, and is surrounded by a chloroplast limiting membrane. After incubation for 60 minutes at 25°, almost all of the chloroplasts are broken, as is evident by the absence of the stroma material, and a distinct limiting membrane (fig 4). Except for swollen thylakoids, freshly isolated chloroplasts closely resemble chloroplasts as they appear within leaf cells (fig 5).

Discussion

Inhibition by ribonuclease of ^{14}C-leucine incorporation into protein by bean chloroplasts *in vitro* shows a distinct lag phase. Three possible reasons for this delay in ribonuclease action were considered: A) Time is required for ribonuclease to degrade accessible ribonucleic acid; B) Time is required for ribonuclease to penetrate the chloroplast membrane; C) Time is required for a change in the permeability of the chloroplast membrane to occur. Since the delay in ribonuclease action can be overcome by pretreatment of chloroplasts at 25° in the absence of ribonuclease, possibilities (A) and (B) are ruled out as important factors. The data do not allow one to conclude that the chloroplast membrane is completely impermeable to ribonuclease, but only that impermeability is not an important limiting factor in the case at hand. Under circumstances where the chloroplast membrane would be kept intact during incubation at 25°, a slow rate of penetration might then become the factor limiting the extent of inhibition of amino acid incorporation.

The data support possibility (C). During the time chloroplasts are incubated at 25° their stroma contents are lost. Therefore, it was concluded that rupture of the chloroplast membrane allows ribonuclease accessibility to the sites of amino acid incorporation within the chloroplast. However, since a time dependence curve of membrane breakage was not determined, it remains possible that the membrane becomes permeable to ribonuclease prior to actual rupture. An attempt to determine the time dependence of membrane breakage, using refractility of chloroplasts as a criterion of their intactness (17) was attempted. However, it was not possible to establish 2 distinct classes, refractile and transparent, with these young chloroplasts, as is possible with more well developed chloroplasts (17).

Some inhibition of amino acid incorporation is always observed during the first 5 minutes of incorporation, even with fresh chloroplasts (fig 1). The variation in the extent of this incorporation may be due to variation, from preparation to preparation, in the fraction of chloroplasts with damaged membranes. This suggestion is supported by the observation of Walker (17) that the fraction of broken chloroplasts varies considerably from chloroplast preparation to chloroplast preparation.

It is suggested that differences in sensitivity to ribonuclease of chloroplasts in isotonic media, that have been reported from various laboratories (see Introduction) might be explained in part by differences in the fraction of intact chloroplasts in the preparations. The extent to which membrane integrity is maintained during incorporation assays might also contribute to the degree of ineffectiveness of ribonuclease in inhibiting incorporation.

The reason for the rupture of the chloroplast membranes during the incorporation assay is not known. However, since chloroplasts contain enzymes which free lipid components from themselves (10), it is not unreasonable to assume that the limiting membranes are ruptured as a result of the action of such enzymes contained within the chloroplast.

Mitochondrial amino acid incorporation is not sensitive to the action of ribonuclease *in vitro* (14), or *in vivo* (6), even though ribonuclease is taken up into the cytoplasm of living cells (6). Similarly, it appears that when chloroplasts are intact, amino acid incorporation is not sensitive to ribonuclease. It is felt that ribonuclease resistance of amino acid incorporation can be taken as an indication that the incorporation is occurring within a membrane bounded organelle, once significant contribution by whole cells can be excluded.

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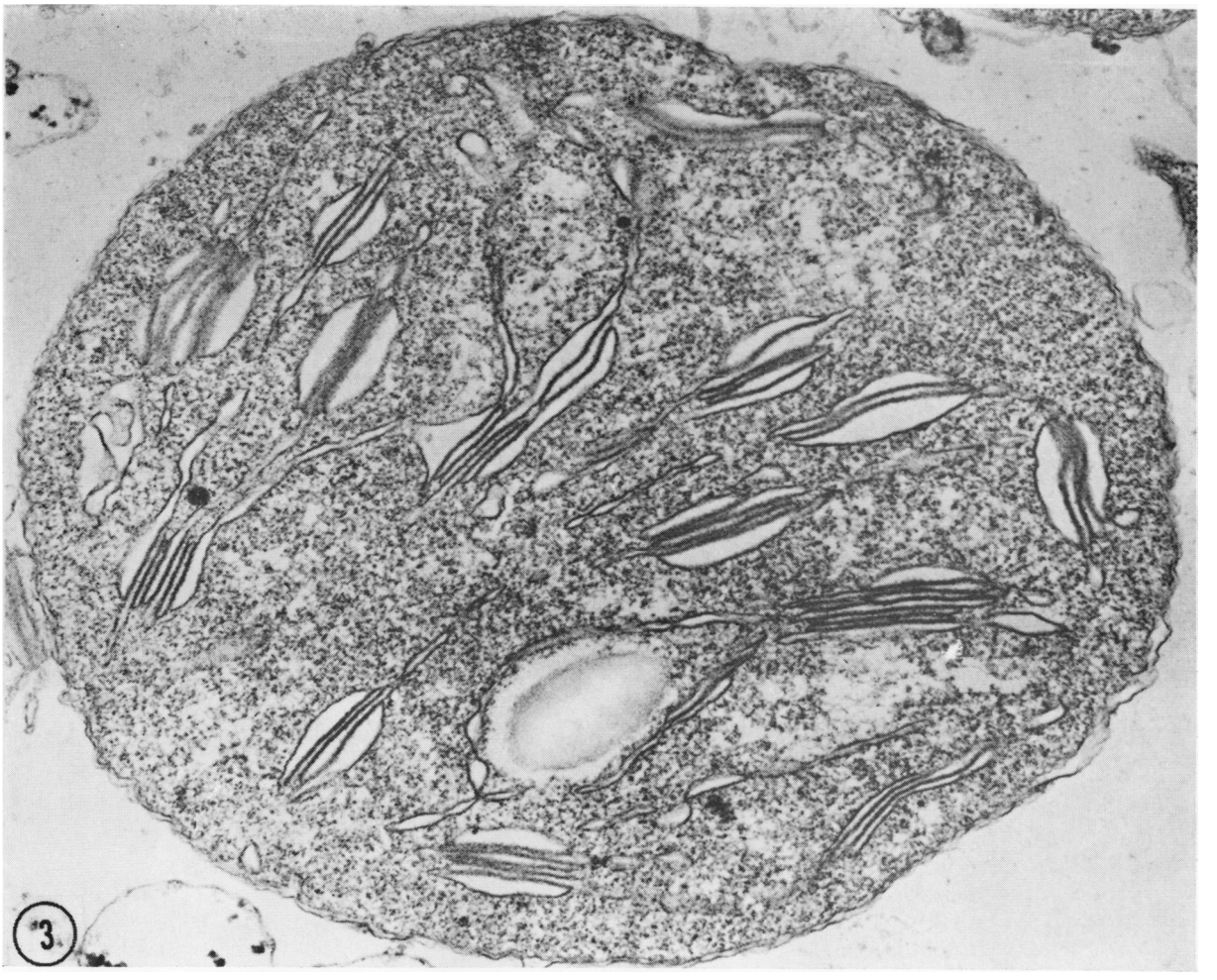

FIG. 3. Chloroplast fixed immediately after isolation. The stroma and limiting membranes are intact, although swelling has occurred. × 35,000.

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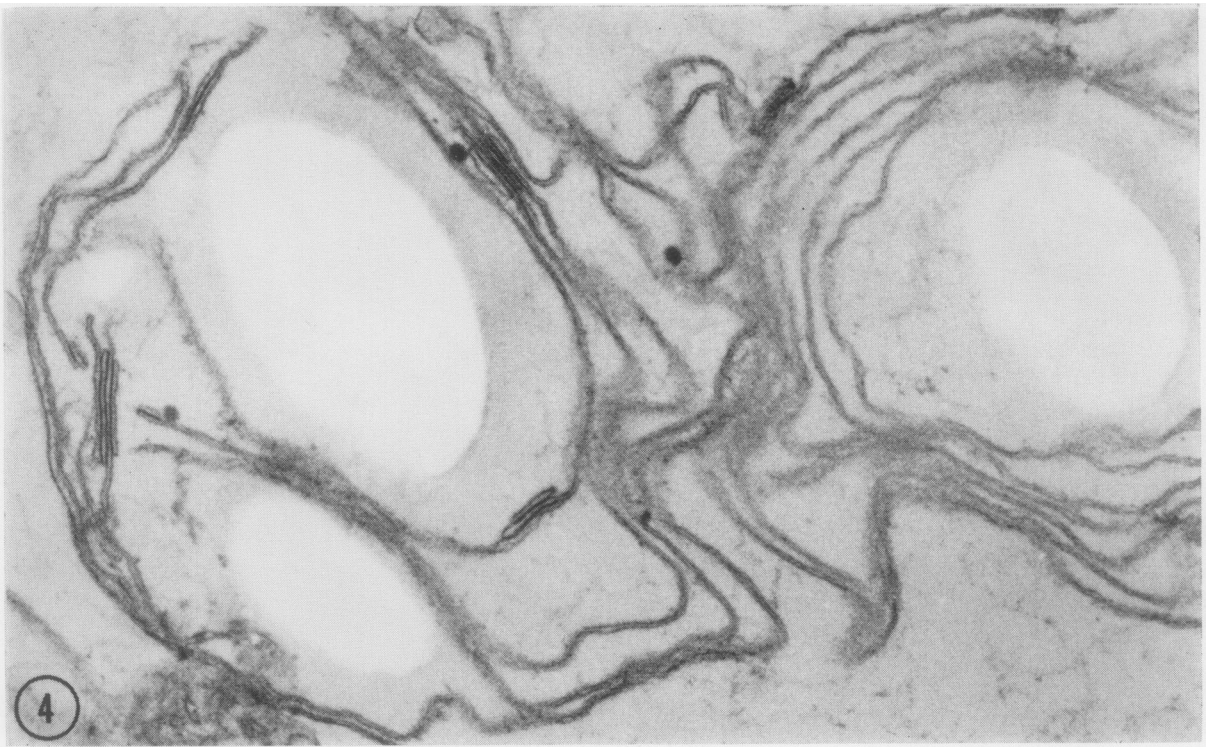

FIG. 4. Chloroplasts fixed after incubation at 25° for 1 hour. The integrity of the chloroplasts is destroyed, but large grains (white) are still present. × 35,000.

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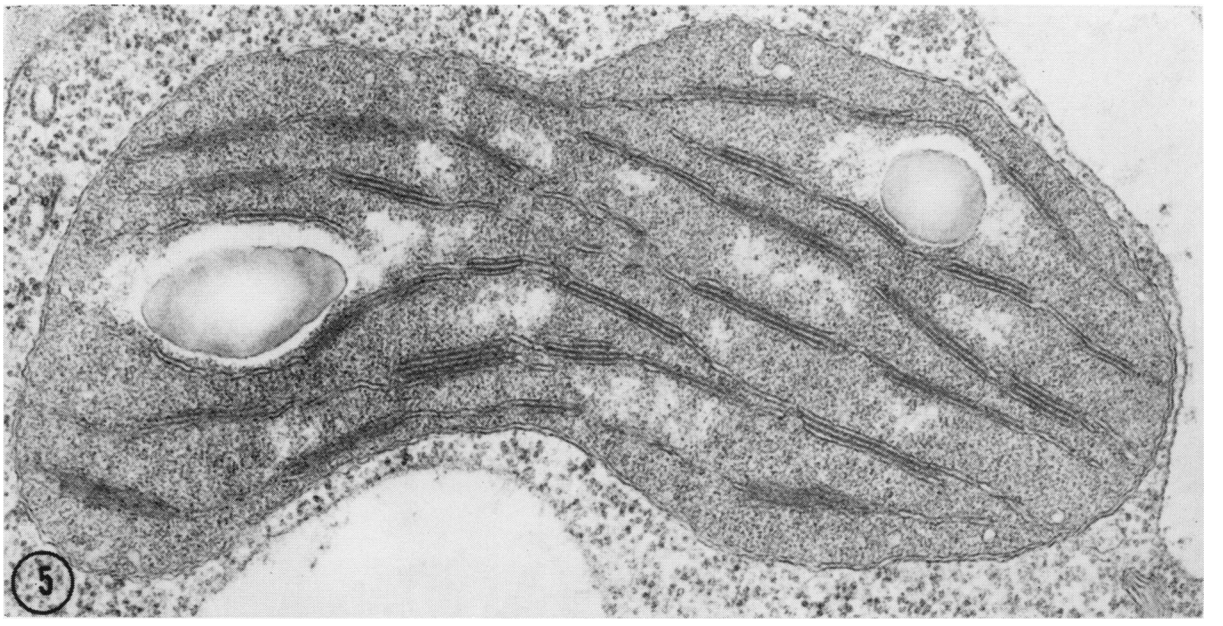

FIG. 5. Chloroplast fixed in leaf cells. The stroma and grana structure is more compact than in isolated chloroplasts. × 35,000.

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