A PROCEDURE FOR ISOLATION OF PROPLASTIDS FROM ETIOLATED MAIZE LEAVES

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Proplastids in etiolated leaves develop into mature chloroplasts when the leaves are exposed to light. The structural changes which occur during this process have been described by von Wettstein and coworkers (6, 16, 17). Their electron micrographs show that the organelles undergo extensive development when they are exposed to light. Further study of these organelles should provide information concerning both the metabolic processes and the regulatory mechanisms governing chloroplast development.

Biochemical studies on the development of proplastids have not been extensive. There are a few reports on protein synthesis in these organelles (5, 13), and some work has been done on changes in nucleic acid metabolism during development (4, 18). There is also some information on the development of photosynthetic enzymes in the organelles in the light (1). These studies have all been done with crude preparations, or with preparations stabilized with formaldehyde, because the organelles are unstable and are isolated with difficulty. Such preparations are of limited usefulness for further work, and it has been necessary to develop a better procedure for obtaining the organelles.

This report presents a procedure which has proved successful in the isolation of proplastids from etiolated maize leaves. The procedure was developed by the modification of two published methods (12, 2); however, both the initial homogenization medium and the protocol for differential centrifugation have been changed. The modified procedure, as described below, has made it possible to obtain much larger yields of intact organelles, relatively free of nonplastid contaminants. The preparations have been characterized morphologically with both the light and the electron microscopes; the contaminants have been examined in some detail; and the yield of organelles has been measured at various stages during the isolation procedure. Although the preparations still suffer from a number of limitations, the method represents a substantial improvement over other methods and should be of use to other investigators.

MATERIALS AND METHODS

Proplastids are isolated from etiolated maize leaves (strain WF9 × B37, Illinois Foundation Seeds, Champaign, Ill.) when the first two leaves are fully emerged. The plants are grown at 27°C, and the seedlings are generally 8–10 days old. The leaves are minced and homogenized in a chilled mortar and pestle under a dim green safe-light in a solution containing 0.5 M Tris-HCl (pH 8.0), 0.5 M sucrose, 0.001 M $MgCl_2$, and 0.2% bovine serum albumin. Approximately 3 ml of grinding solution are used for each gram (fresh weight) of leaves. The homogenate is filtered through two layers of Miracloth (Chico-

pee Mills, Inc., New York). The filtrate is centrifuged for 5 min at 40 *g* (500 rpm, Servall GSA rotor) for removal of debris. The resulting supernatant is centrifuged for 15 min at 369 *g* (1500 rpm, Servall GSA rotor) for collection of the first crude plastid pellets. The pellets are dissolved in a small volume of the plastid-free supernatant, and the differential centrifugation is repeated. (The plastid suspension is referred to in the text as crude plastids I.) The resulting plastid pellet is suspended in 10 ml of solution (crude plastids II) and then layered onto a discontinuous sucrose gradient which is prepared by layering 10 ml of a solution, containing 1.3 M sucrose, 0.5 M Tris, 0.001 M $MgCl_2$, and 0.2% bovine serum albumin, over 25 ml of 2 M sucrose in the same solution. The gradients are centrifuged for 30 min in a swinging bucket rotor at 2,500 *g* (Servall HB-4, 4000 rpm). Under these conditions, the plastids accumulate at the interface between the 2 M sucrose and 1.3 M sucrose. The layers are removed from the top of the gradient with a syringe. The number of plastids obtained in each preparation is determined by counting the plastids with a Helber bacteria counter in the light microscope. Bacterial assays are done on Penassay (Difco) agar.

For electron microscopy, the plastids from the sucrose gradient are suspended directly in melted 1.4% agar containing 1.3 M sucrose, and 0.1 M potassium phosphate buffer at pH 7.2. The agar is allowed to harden at 4°C and is then cut into small pieces. The agar cubes are then fixed overnight in a solution containing 5% glutaraldehyde, 0.1 M potassium phosphate buffer, pH 7.2, and 1.3 M sucrose. They are subsequently washed thoroughly in phosphate buffer and fixed again in 2% OsO_4 in 0.1 M potassium phosphate for 4 hr. The agar cubes are dehydrated in a graded series of alcohols and embedded in Epon 812. Leaf segments from etiolated leaves are fixed overnight in 6% glutaraldehyde in 0.15 M potassium phosphate buffer, postfixed in 2% OsO_4, and embedded as above. Sections are stained with 3% uranyl acetate overnight, and poststained with Reynolds' lead citrate stain for 5 min (14). Photographs were taken with a Siemens Elmiskop I.

Two separate experiments were performed for estimating the total number of plastids per leaf: (*a*) The total number of plastids per gram (fresh weight) of leaves was counted directly. For this purpose, a known weight of leaves was fixed in 10% formalin in 0.1 M potassium phosphate buffer, pH 7.4, for 8 hr. This treatment presumably stabilizes all the plastids in the leaf so that they do not rupture during isolation. The fixed leaves were subsequently homogenized with sand in a mortar and pestle, and the number of plastids in the homogenate was counted with a Helber bacteria counter. (*b*) The number of plastids per leaf was estimated indirectly from the amount of protochlorophyll present. For this purpose, it was first necessary to measure the amount of protochlorophyll per plastid. Protochlorophyll was extracted from nonfixed, purified proplastids with 80% acetone and measured according to the method of Anderson and Boardman (1). In the proplastid preparations, 5% of the total chlorophyll is chlorophyll *a*; the remainder is protochlorophyll. Calculations were based on total chlorophyll. Plastid number was determined with the light microscope. The amount of chlorophyll per plastid was 0.018 $\mu\mu$g. Protochlorophyll determinations were then run on whole leaf preparations, and the total number of plastids was estimated by dividing the protochlorophyll values per gram (fresh weight) of leaves by 0.018.

RESULTS AND DISCUSSION

Since the plastid isolation procedure described in this paper was developed primarily for the study of proplastid RNA, it was necessary to obtain preparations in which most of the organelles were intact and free of nonplastid contaminants. The integrity of the isolated organelles was studied by the examination of sectioned organelles with the electron microscope. For purposes of comparison, an electron micrograph of a section through an etiolated maize leaf is presented in Fig. 1. Proplastids in etiolated maize leaves have a well defined structure consisting of an outer membrane, a particulate stroma, and a tightly organized prolamellar body. Similar structures have been described in etiolated leaves of other plants (9, 17).

Early studies of isolated proplastid preparations with the electron microscope were not successful. When the organelles were fixed according to the usual procedures, they appeared to rupture during the initial fixation. Embedding the plastids in agar prior to fixation eliminated this difficulty and made it possible to achieve preparations with good morphological preservation. As shown in Fig. 2, the essential features are preserved in isolated organelles. However, occasional proplastids with disorganized prolamellar bodies are also seen in the sections.

In addition to showing the structures described above, electron micrographs of isolated proplastids show numerous clear areas containing fibrils that have the general morphology described for DNA in chloroplasts (11, 15). As many as five presumptive DNA-containing regions have been counted in some proplastid sections, suggesting that there may be a number of separate units of DNA in the

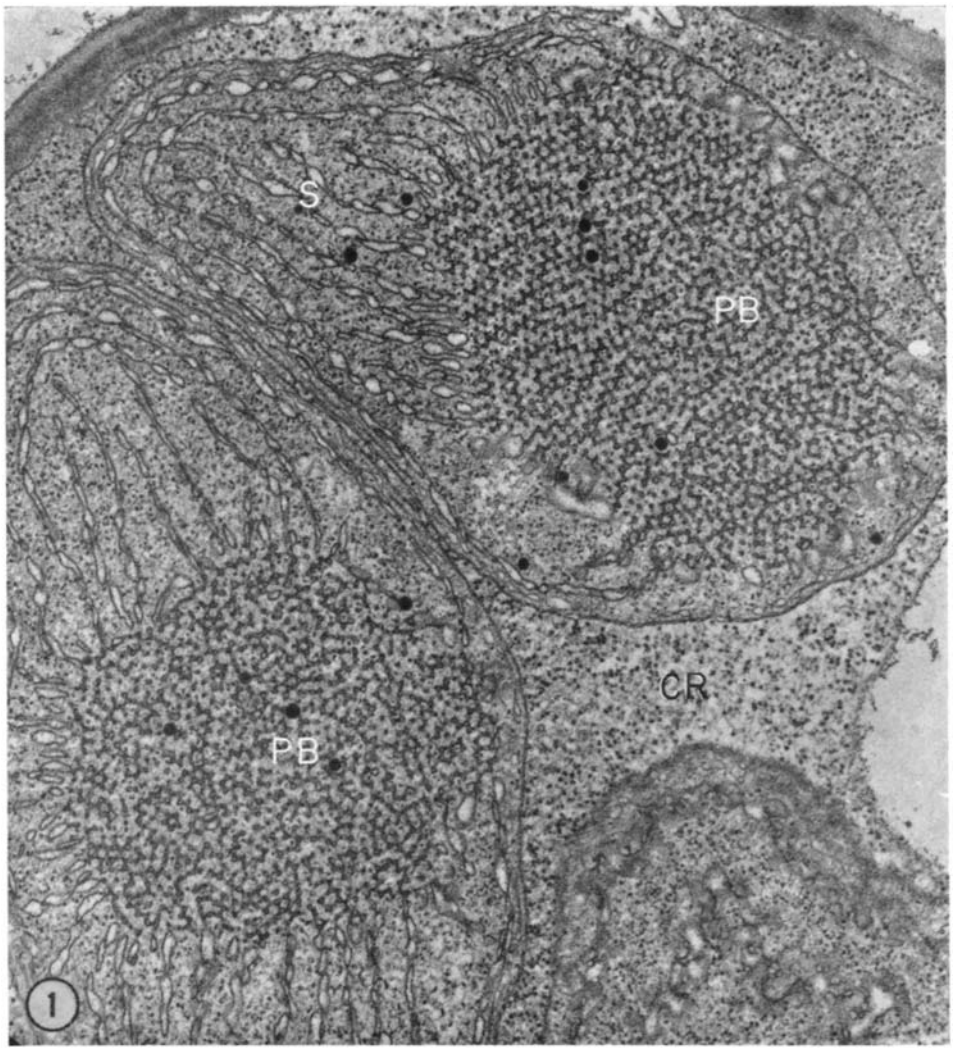

FIGURE 1 Electron micrograph showing small region of leaf cell in etiolated maize. Two proplastids are visible. The prolamellar body (*PB*) has a regular appearance. Plastid ribosomes in the stroma (*S*) are smaller than cytoplasmic ribosomes (*CR*). × 25,500.

organelle. The fibrillar regions sometimes occur at the outer edge of the plastids, and in some sections appear to lie adjacent to the outer membrane. Fig. 3 shows a tangential section through the edge of an isolated proplastid. The fibril-containing region is quite large and is similar in appearance to a bacterial nucleus. Such areas are seen frequently in tangential sections.

Several years ago, RNA was demonstrated cytochemically in proplastids of etiolated maize leaves with the basic dye Azure B (10). The RNA was found to be associated with the particulate material of the plastid stroma. This observation provides a further morphological criterion for evaluating the integrity of the isolated organelle preparations. Since it is thought that material in the stroma of mature chloroplasts is lost during isolation, one might expect this situation to hold true in isclated proplastids as well. When isolated proplastids are stained with Azure B, all the organelles appear basophilic; this basophilia is removed if the organelles are incubated with ribonuclease prior to staining, indicating that a substantial portion of the plastid RNA has been

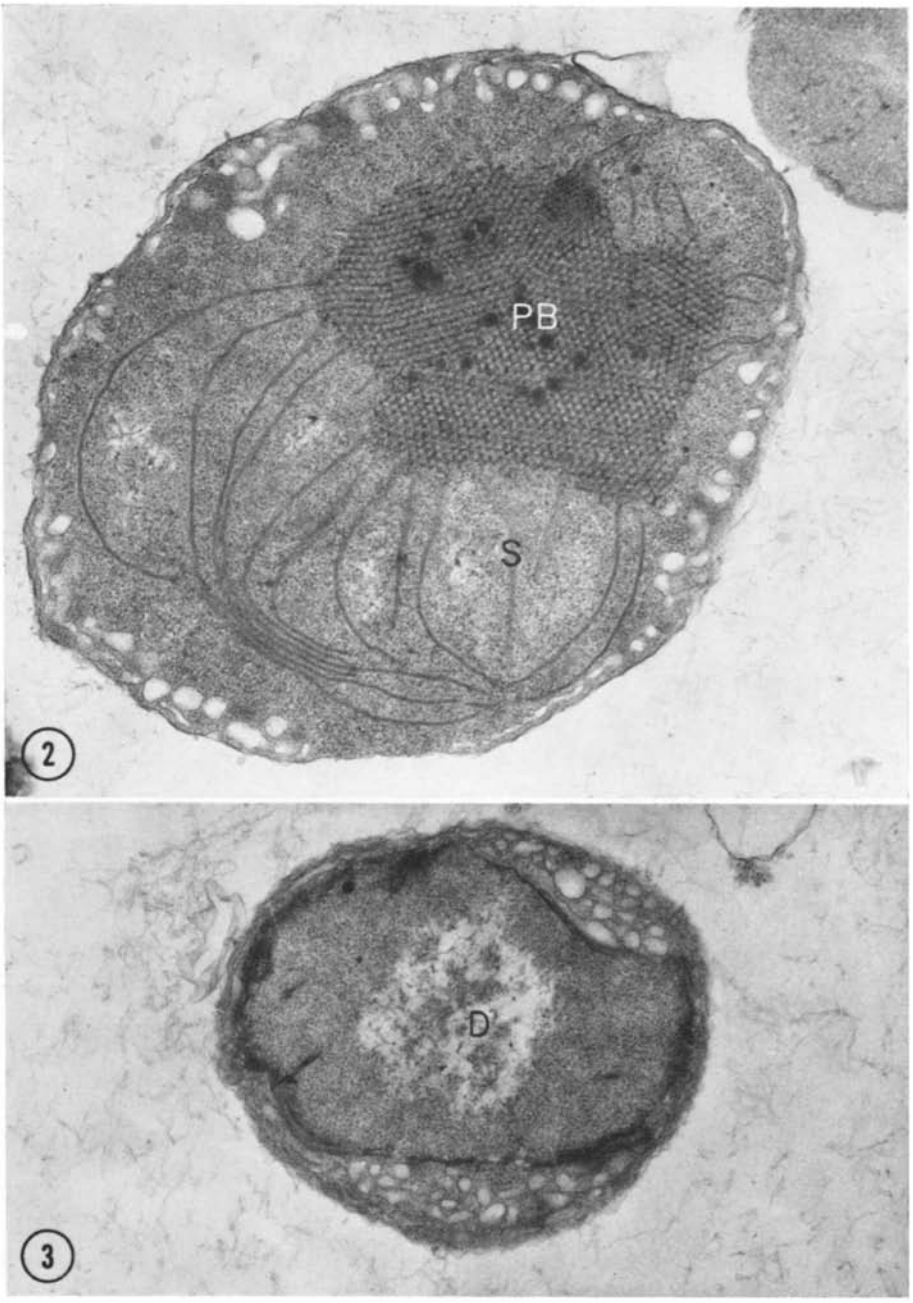

FIGURE 2 *Electron micrograph showing section of an isolated proplastid.* × *26,000.*

FIGURE 3 Electron micrograph of a tangential section through an edge of an isolated proplastid. Central fibrillar region (*D*) resembles DNA. × 24,000.

TABLE I

Loss of Bacteria during a Typical Procedure of Plastid Purification

Initial homogenate prepared from 365 g of etiolated leaves contained roughly 10^{10} plastids. The yield of purified plastids was 5×10^9.

Plastid fraction	Total No. of bacteria	Bacteria remaining
		%
Initial homogenate	1.3×10^9	100
Crude plastids I	2.3×10^8	18
Crude plastids II	8.8×10^7	7
Purified plastids from sucrose gradient	8.3×10^6	0.6

TABLE II

Yield of Proplastids during a Typical Purification Procedure

The per cent of plastids recovered during the various steps of the isolation procedure was calculated with the use of an average value of 2.7×10^{10} plastids as the total number per 100 g (fresh weight) of leaves.

Treatment	Total plastids/ 100 g fresh weight	Plastids recovered
		%
Plastids extracted form formalin-fixed leaves	3×10^{10}	100
Plastids extimated by chlorophyll determinations	2.4×10^{10}	
Plastids obtained in standard isolation		
(1) Homogenate	4.6×10^9	17
(2) Crude plastids I	2.6×10^9	10
(3) Crude plastids II	2.0×10^9	7
(4) Purified plastids	1.6×10^9	6

retained within the organelles. Thus, both electron microscopic and cytochemical observations indicate that the isolated proplastids are well preserved and well suited to the study of RNA and other macromolecules in them, provided the organelles are free of external contaminants.

Two sorts of contaminants have been identified in crude proplastid preparations: subcellular components and bacteria. The subcellular contaminants consist primarily of mitochondria and cytoplasmic fragments. (Nuclei are ruptured during the isolation procedure and are never found in the initial homogenate.) When purified proplastids are examined with the electron microscope, the contaminants are no longer visible. However, electron microscopy is not a satisfactory procedure for estimating contaminants in an organelle preparation, since the sampling is inadequate. In addition, the contaminants must be well preserved under fixation conditions which have been designed to insure the optimal appearance of the organelles being examined. Thus, the proplastid preparations may still contain small numbers of subcellular contaminants which remain undetected with current techniques. As the biochemical properties of these organelles are defined, it will certainly prove possible to design more sensitive assays for the contaminants.

Bacterial contamination, which was tested by plating samples of proplastid preparations on agar, has proved to be a severe problem. Unfortunately, the bacteria are found on the plant leaf surfaces even when plants are germinated and maintained under sterile conditions during growth. So far, it has not been possible to develop a procedure for growing plants free of bacteria, and it has been necessary to remove bacteria from the plastid preparations by differential centrifugation. This procedure is relatively successful, as seen in Table I. In a typical proplastid preparation, which has roughly 10^{10} plastids in the initial homogenate, there are 1.3×10^9 bacteria, or approximately 13 bacteria per 100 plastids. During the process of differential centrifugation, it is possible to reduce this value to 8.3×10^6. Since the total number of plastids has been concomitantly reduced, the contamination is reduced only to two bacteria per thousand plastids. In this process, 99.4% of the bacteria have been removed. Clearly, then, this degree of purity is adequate for some purposes. However, the remaining small number of bacterial cells is sufficient to limit certain studies. In particular, sensitive assays which detect small numbers of molecules may simply measure properties of contaminants.

Recently, several reports have appeared concerning the problem of bacterial contamination in chloroplast studies (7, 8). In particular, there seems to be some question concerning the source of some of the satellite DNA's which have been reported. Crude preparations of chloroplasts from green maize leaves also contain large numbers of contaminating bacteria. Although we have not

devised a procedure for cleaning chloroplast preparations, techniques similar to those described for proplastids should certainly prove effective. For the moment, it seems wise to distrust data on the properties of either proplastids or chloroplasts when the degree of bacterial contamination is not specified.

In addition to examination of the morphology and contaminants of isolated proplastid preparations, the relative yield of plastids per gram of leaf tissue was measured during the different stages of the isolation procedure. In this way, it was possible to evaluate separately the ability of the homogenizing medium to preserve the organelles when they were first isolated, and to measure further losses which occurred during the purification procedure. The yield of proplastids is expressed as the number of plastids isolated compared to the number in the leaf. As indicated in the Methods section, the total number of plastids in the leaves prior to homogenization was measured in two ways. The values obtained with both of these procedures were surprisingly similar (Table II). Table II also shows the yield of proplastids at various stages during the isolation procedure in a typical experiment. In the initial homogenate only 17% of the potential plastids in the leaf was recovered. The major loss of plastids occurs at this step. Further purification steps resulted in some additional losses so that the final preparation contained 6% of the plastids in the leaf. This value has been obtained rather consistently in a large number of preparations. Although it is disappointingly low, this value actually represents a great improvement over that for yields obtained with the previously reported isolation procedures. In particular, the addition of bovine serum albumin to the initial homogenizing medium resulted in a substantial increase in yield. Other components which have been added to the isolating medium in an attempt to improve the yield have proved useless so far. These agents include polyvinylpyrrolidone, Dextran 40, and Ficoll. Improvement of the homogenizing medium will require further experimentation.

The possibility of obtaining from etiolated maize leaves preparations of proplastids with good morphological integrity, relatively good yield, and few contaminants ensures the value of these organelles in future studies of chloroplast development. Very recently it has become possible to further reduce bacterial contamination to negligible amounts in these preparations by using extensive and repeated low-speed differential centrifugation. Though still in progress, these studies suggest that future experiments with proplastids will not be seriously limited by problems of bacterial contamination.

SUMMARY

An improved method for the preparation of proplastids from etiolated maize leaves is described. The integrity of the organelles is monitored with the electron microscope, and the proplastids appear well preserved. Proplastid preparations suffer from severe bacterial contamination unless special precautions are taken during the isolation procedure. Extensive low-speed differential centrifugation is effective in removing most of the bacterial contaminants. The yield of plastids after isolation and purification is 6% of the total number of plastids in the intact leaves.

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