ULTRASTRUCTURE OF CHLOROPLASTS ISOLATED BY NONAQUEOUS EXTRACTION

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INTRODUCTION

Since the early introduction of the nonaqueous method of isolating cell organelles (2) and its modification for use in isolating chloroplasts (3, 5, 13), it has been used extensively in the study of the intracellular distribution of water-soluble enzymes, minerals, and photosynthetic reaction products (4, 6–16).

Tests of the purity of isolated chloroplasts have been carried out with anthocyanin as a marker of vacuolar material and with various enzymes as markers of mitochondrial and hyaloplasmic components (6, 7, 13). Except in a few instances, visual observations of the purity of these preparations and the structure of these plastids have been confined to light microscopy (16). Where electron microscopy has been used, the isolated plastids generally have been prepared for study

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by treating them with an aqueous solution (8) of permanganate or osmium tetroxide followed by the usual dehydration series prior to the embedding of the sample in plastic (1, 4). It is evident that during this fixation procedure the nonaqueously isolated plastids are subjected to the stresses of rapid rehydration, and that the associated swelling and washing of material from the surface of the plastid and from the stroma distorts the plastid and makes it extremely difficult visually to detect possible cytoplasmic contamination in these preparations.

The present study was undertaken in an attempt to develop a method of sample preparation for electron microscopy that could be used routinely in the study of nonaqueously isolated cell organelles and that would permit detailed electron microscope observations of these preparations without the changes induced by fixation in an aqueous medium.

MATERIALS AND METHODS

Young, vigorously growing broad bean plants (*Vicia faba* L.) were kept in the dark for 24 hr immediately before they were used. On the day of the freeze drying, the plants were placed in the light for 1½ hr. The leaves then were cut into 1-cm strips and the major veins removed. The strips were frozen in a flask kept at about −70°C by a mixture of dry ice and acetone. In other experiments, the strips were frozen in liquid nitrogen or in hexane or isopentane cooled to near liquid-nitrogen temperatures. After freezing, the leaf strips were dried under vacuum at −25°C. This operation usually took 48 hr. The frozen-dried leaf strips then were stored under vacuum over P_2O_5 at −25°C.

Chloroplasts were isolated by a modification of Stocking's (13) method in which 0.4 g of dry leaf material was ground at −25°C in 30 ml of hexane-carbon tetrachloride mixture with a density of 1.26 at 20°C. The grinding was carried out for 15 sec in a Virtis homogenizer at full speed with 8 g of acid-washed glass homogenizing beads in a grinding vessel.

After the grinding, the preparation was filtered through four thicknesses of cheese-cloth, and a 5-ml aliquot was layered on top of 22 ml of a linear density gradient in a 30-ml swinging bucket centrifuge tube. The gradient, prepared from hexane and carbon tetrachloride, varied in density (measured at room temperature) from 1.27 at the top of the tube to 1.49 at the bottom of the tube. The preparation wsa centrifuged for 2½ hr at 20,000 rpm (35,000 *g*) in a swinging bucket head, Spinco SW 25.1. The resulting bands in the sample were collected by means of a gradient-sectioning device similar to the one described by Anderson (1).

The upper green band contained the most pure plastids, and for this study the plastids were not further purified. This band was mixed with ice-cold hexane and centrifuged at 4,000 *g* for 5 min. The clear supernatant was discarded and the pelleted plastids were dried under vacuum without washing them. They were stored over P_2O_5 under vacuum at −25°C. All operations were carried out at 0 to −25°C.

A series of solutions containing 3% glutaraldehyde buffered at pH 7.1 with 0.1 M phosphate buffer and ranging in 5% steps from 0 to 90% acetone was prepared. These solutions were kept at ice temperatures or below, and the dry samples of nonaqueously isolated plastids were fixed in each of the solutions for 50 min.

After fixation, each sample was washed with an acetone buffer solution containing the same concentration of acetone as that present in the fixation solution. The samples were then treated for 2½ hr with 1% osmium tetroxide dissolved in the same concentration of acetone as that present in the fixation solution.

The osmium tetroxide treatment was followed by another acetone wash of the same concentration, and then by dehydration in increasingly more concentrated acetone solutions. The dehydrated samples were transferred to 100% acetone, to acetone: propylene oxide, to pure propylene oxide, to propylene oxide:plastic series, and finally to pure plastic. The entire operation took about 6 hr. During the osmium tetroxide treatment and the subsequent steps, the temperature of the solutions was maintained at 0°C or below, depending on the solution. Ice temperature was maintained for solutions containing 50% acetone or less. For solutions above 50% acetone to 70% acetone, the temperature was −20°C; above 70–100% acetone and to the 50% propylene oxide:50% plastic step, the temperature was −70°C. The temperature was increased to −20°C for the 25% propylene oxide : 75% plastic step, and then the samples were transferred to pure plastic at room temperature. Polymerization was carried out at 60°C in a vacuum oven for approximately 12 hr.

In addition, plastid samples were treated directly with 1% osmium tetroxide dissolved in 95% acetone, with 1% osmium tetroxide dissolved in 100% acetone, and with 1% osmium tetroxide dissolved in 100% hexane without previous glutaraldehyde treatment.

RESULTS

The graded series from fixation in buffer to fixation in dry hexane shows that, at the more polar end of the series, Fig. 1 *a–c*, excessive swelling

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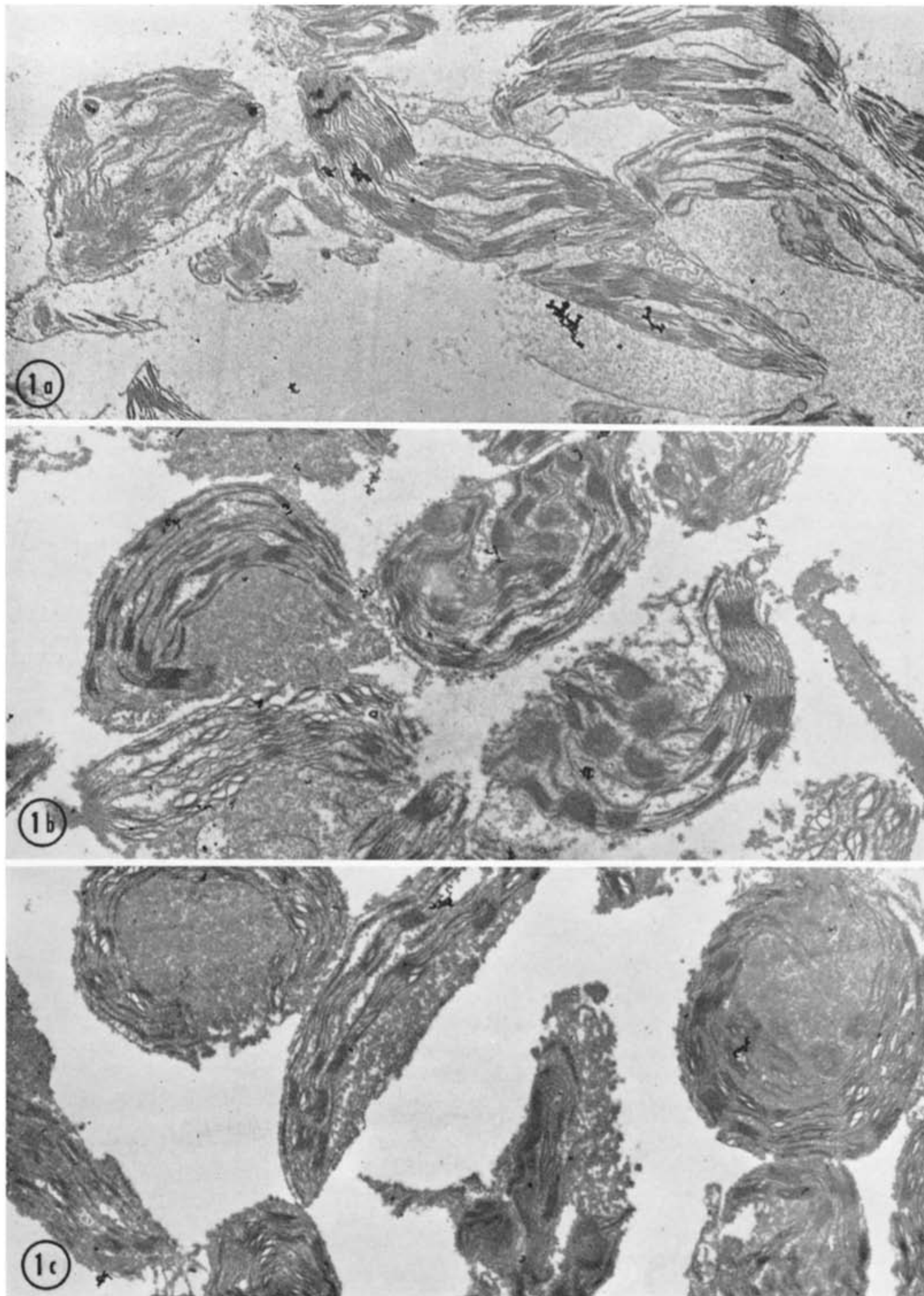

FIGURE 1 Effect of fixing nonaqueously isolated chloroplasts in decreasingly less polar solutions of 3% glutaraldehyde. Fig. 1 *a*. Plastids fixed in buffer. × 10,000. Fig. 1 *b*. Plastids fixed in 25% acetone. × 10,000. Fig. 1 *c*. Plastids fixed in 30% acetone. × 10,000.

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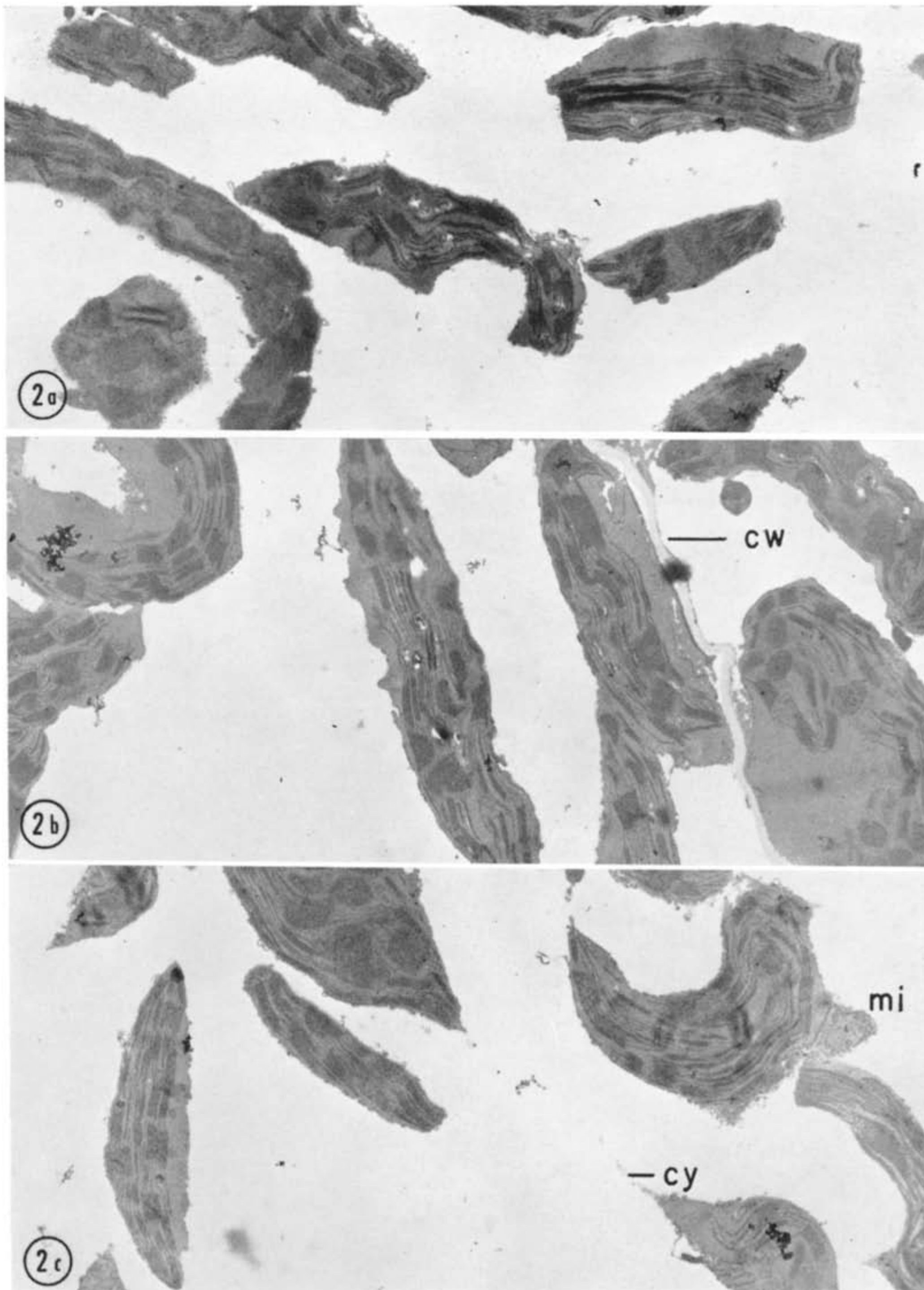

FIGURE 2 Effect of fixing nonaqueously isolated chloroplasts in decreasingly less polar solutions of 3% glutaraldehyde. Fig. 2 *a*. Plastids fixed in 45% acetone. × 10,000. Fig. 2 *b*. Plastids fixed in 50% acetone. Part of a cell wall (*cw*) is visible. × 10,000. Fig. 2 *c*. Plastids fixed in 60% acetone. A mitochondrion (*mi*) and some cytoplasm (*cy*) are visible. × 10,000.

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and disruption of the plastids occurred. This was accompanied by loss of stromal material into the surrounding medium. As the proportion of water in the original fixation solution was reduced, the extent of swelling and leaching decreased. The reader should compare the plastids fixed in buffered glutaraldehyde (Fig. 1 *a*) with plastids fixed in glutaraldehyde in 30% acetone (Fig. 1 *c*). When the fixation was done in 45–60% acetone, the stroma stained heavily and remained in place, associated with the internal membrane system (Fig. 2 *a–c*).

At the other end of the series, plastids treated in the less polar solutions were poorly stained and details of internal structure or of contaminating cytoplasmic components were much less visible (Fig. 3 *a*, *b*).

It should be noted that these preparations were not washed and, consequently, contamination of the plastids by certain cellular components can be identified. In the samples either from the low polarity (Fig. 3 *a*, *b*) or from the high polarity (Fig. 1 *a*, *b*) ends of the series, such contamination is not easily identified. However, the preparations fixed in 45–50% acetone rehydrated sufficiently but not excessively, and good staining did occur. In these samples, a remnant of a cell wall is visible (Fig. 2 *b*), an occasional wisp of cytoplasm

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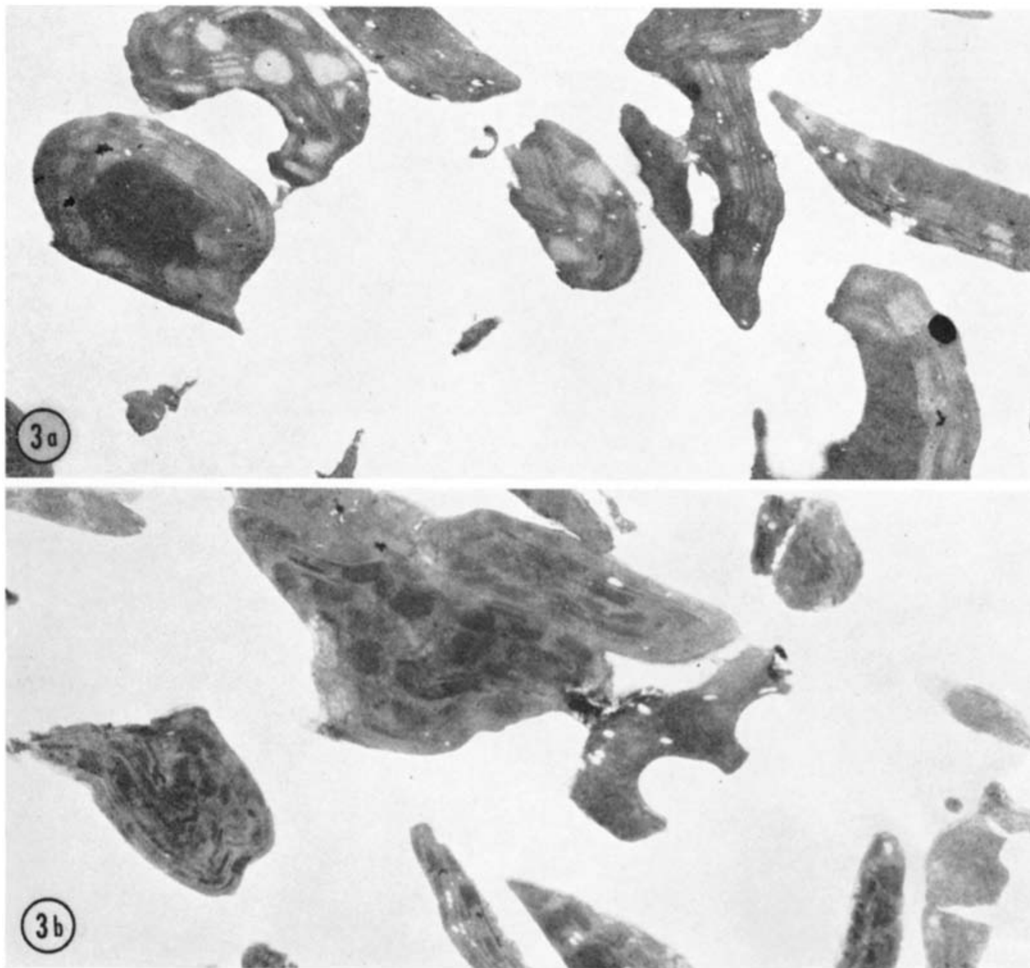

FIGURE 3 a Nonaqueously isolated chloroplasts fixed in 3% glutaraldehyde in 80% acetone. × 10,000. Fig. 3 *b*. Nonaqueously isolated chloroplasts treated directly with 1% osmium tetroxide dissolved in hexane. × 10,000.

may be seen attached to a plastid (Fig. 2 *c*), and a mitochondrion may be identified (Fig. 2 *c*).

In spite of the fact that these plastids were not purified after separation on the density gradient, relatively little contaminating material is evident in the preparations. The success of the nonaqueous method appears to be related to the nature of the plastid envelope that acts as a point of weakness in the presence of the cold nonaqueous solvents. The cytoplasm that, during the freeze-drying process, dried onto the plastids appears to be stripped away from the plastids when the dried leaves are ground in cold nonaqueous solvent. This process leaves the irregular plastid surface relatively free of adhering cytoplasm (Fig. 2 *b*). Occasionally, a portion of the envelope remains on the plastid, but, in general, in the presence of the nonpolar grinding medium (carbon tetrachloride:hexane), the plastid envelope weakens and allows a separation of the adhering cytoplasm from the plastid.

The method described here of fixing nonaqueously isolated plastids in a buffered glutaraldehyde-acetone mixture is suitable for routine monitoring and study of nonaqueous preparations. It is evident from this study that nonaqueous chloroplasts may be prepared relatively free of adhering cytoplasm and that such plastids, in addition to having internal membrane systems, contain dense stroma areas presumably still with the naturally occurring, water-soluble components.

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