Lipid fixation during preparation of chloroplasts for electron microscopy

ALPASLAN ONGUN, W. W. THOMSON, and J. B. MUDD

Department of Biochemistry, Department of Life Sciences, and Statewide Air Pollution Research Center, University of California, Riverside, California 92502

ABSTRACT Reaction of osmium tetroxide with isolated spinach chloroplasts fixed completely the glycolipids, phosphatidyl glycerol, and phosphatidyl choline. Under the same reaction conditions only 30% of the chlorophyll was fixed.

Reaction of potassium permanganate with isolated spinach chloroplasts fixed more than 90% of the glycolipids, phosphatidyl glycerol, and phosphatidyl choline, provided the reaction period was long enough. Potassium permanganate also fixed the chlorophyll.

Reaction of osmium tetroxide and potassium permanganate with isolated ^{14}C-lipids from *Chlorella pyrenoidosa* fixed 59% and 66% of the radioactivity, respectively. The lipids that were not fixed included sterols and pigments.

Electron micrographs show that chloroplasts extracted with chloroform–methanol after fixation in osmium tetroxide or potassium permanganate differ from those dehydrated with acetone mainly in that in the former, osmiophilic globules have been removed and there seems to be some fusion of the boundary membranes and grana membranes. These effects may be due to the extraction of unfixed, neutral lipids such as sterols and quinones.

KEY WORDS chloroplasts · lipids · extraction · fixation · osmium tetroxide · potassium permanganate · electron microscopy · osmiophilic globules · membranes

THIS STUDY WAS UNDERTAKEN in an attempt to evaluate the reactivity of osmium tetroxide and potassium permanganate with lipid components of the chloroplast membrane system.

Osmium tetroxide and potassium permanganate are two of the most commonly used fixatives in electron microscopy. Although these fixatives are used widely, there is some controversy concerning the chemistry of their interaction with cellular structures. As far as osmium tetroxide is concerned, the two major points of controversy are, firstly, whether the initial reaction with the double bonds of fatty acid residues is followed by migration of the osmium to the polar part of the lipid molecule, and secondly, whether there is a direct reaction of osmium tetroxide with the polar groups (1–4). Potassium permanganate certainly reacts with the double bonds of the fatty acid residues, but there is some question whether there are additional reactions with pigments (5) and with proteins (6).

In addition to these uncertainties, the quantities of lipid fixed by osmium tetroxide and potassium permanganate are not well defined. Korn and Weisman have measured the amount of lipid extractable from *Acanthamoeba* sp. after fixation with osmium tetroxide and potassium permanganate, and found that most of the neutral lipids and considerable amounts of phospholipids were extractable after fixation (7). On the other hand, Ashworth, Leonard, Eigenbrodt, and Wrightsman found that the lipids of rat liver were extracted only to the extent of 0.7–7.2% after fixation in osmium tetroxide (8).

In the study described here we investigated whether the glycolipids and phospholipids of chloroplasts are quantitatively fixed during preparation for electron microscopy.

EXPERIMENTAL PROCEDURE

Greenhouse-grown spinach plants (*Spinacia oleracea*, var. Bloomsdale) were used. Leaves were harvested from 45–50-day old plants, washed in distilled water, and chilled at 4°C for 1 hr. After the midribs had been removed, the leaves were ground in 0.5 M ice-cold sucrose solution in a Waring Blendor for three 30-sec periods punctuated by 10-sec pauses. All containers were cooled before use. The homogenate was filtered through four layers of cheesecloth and the chloroplasts were isolated by centrifugation of the filtrate at 1000 *g* for 7 min after a

preliminary centrifugation at 200 *g* for 2 min to remove the cellular debris.

Chlorophyll was determined according to Arnon (9). Lipids were extracted in chloroform–methanol according to Bligh and Dyer (10).

In the control experiments, the lipids were extracted before fixation of the chloroplasts. In other experiments, we fixed chloroplasts before extracting the lipids by suspending the chloroplasts in either 1% OsO_4 buffered at pH 7.0 with phosphate, or 2.5% $KMnO_4$. Fixation time was varied as indicated in Results. Some of the chloroplasts were then dehydrated immediately in a series of water–acetone mixtures up to 100% acetone and these dehydration solvents were combined for lipid analysis; lipids were first extracted from other fixed chloroplasts in chloroform–methanol and then the chloroplasts were dehydrated. The extraction solvents were evaporated to dryness under vacuum and the lipids were dissolved in chloroform.

The leaf sections were fixed either for 20 min in 2.5% $KMnO_4$ or for 24 hr in 1% osmium tetroxide buffered at pH 7.0 with phosphate. All material was embedded in a Maraglas mixture containing, by volume, 7 parts Maraglas 655 (The Marblette Corp., Long Island City, N.Y.), 2.8 parts Cardolite NC 513 (Minnesota Mining & Manufacturing Co., Newark, N.J.), and 0.2 parts *N*,*N*-dimethylbenzylamine. Thin sections were cut on a Porter–Blum MT 2 ultramicrotome. All sections were stained first with uranyl acetate for 1 hr and then by lead citrate for 10 min, and examined with a Hitachi Hu 11 electron microscope.

Lipids were chromatographed on thin layers (0.25 mm) of Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.) on Pyrex plates (20 × 20 cm), the gel being activated at 120°C for 30 min before chromatography. The plates were developed in chloroform–methanol–7 N NH_4OH 65:30:4 in the first direction and in chloroform–methanol–acetic acid–water 170:25:25:6 in the second direction (11). Developed plates were allowed to dry in the air for 20 min and the spots were made visible under UV light after the plates had been sprayed with a 0.2% solution of 2′,7′-dichlorofluorescein in 95% ethanol.

To obtain enough lipid for the analysis of phosphorus and sugar, we developed four plates of each sample, scraped the spots off the plates, and combined them. Lipids were extracted from silica gel with successive 3-ml portions of chloroform–methanol 1:1, chloroform–methanol 1:2, and methanol. Phospholipid extracts were evaporated to dryness under a stream of nitrogen at room temperature in digestion flasks and the amounts of P were determined after digestion of the lipids with H_2SO_4–HNO_3 (12). Glycolipids were hydrolyzed in equal volumes of methanol and 2 N HCl at 100°C for 30 min, dichlorofluorescein and fatty acids were removed with ether, and sugars were determined according to Dubois, Gilles, Hamilton, Rebers, and Smith (13). Amounts of intact lipids were calculated from the phosphorus or sugar determination, using calculated molecular weights for the various glycerolipids.

RESULTS

Electron Microscopy

The degree of disorganization induced in the chloroplasts by the procedures used to isolate them can be assessed by comparison with the control tissues fixed with permanganate and osmium in situ (Figs. 1 and 2). These electron micrographs resemble those published many times before. (Fig. 3 will be referred to later.)

Fig. 4 is an electron micrograph of isolated chloroplasts from which the lipid was extracted prior to fixation. No evidence for the presence of membranes could be seen in sections of the extracted pellet in either osmium- or permanganate-fixed material.

Figs. 5 and 6 are micrographs of isolated chloroplasts, fixed with permanganate and osmium tetroxide, respectively, and then dehydrated through a series of acetone–water mixtures that is commonly used to prepare isolated chloroplasts for electron microscopy. The ultrastructure of these chloroplasts was generally similar to that in Figs. 1 and 2. The grana and intergranal connections were well preserved, and in many instances the limiting membrane of the chloroplasts was intact. Where the limiting membrane was severely ruptured or had been completely stripped off, most of the stroma had also been leached out. It should be noted at this point, however, that small osmiophilic globules were present in these isolated and osmium-fixed chloroplasts (Fig. 6, arrows) as well as in the chloroplasts fixed with osmium in situ (Fig. 2, arrows).

The chloroplasts that had been fixed with permanganate or osmium tetroxide and subsequently extracted with methanol–chloroform (Figs. 7 and 8) were similar in form and ultrastructure to those that had only been dehydrated in acetone (Figs. 5 and 6). As with the acetone-dehydrated chloroplasts, many of them still possessed a limiting membrane and a considerable amount of stromal material.

Although the fixed and methanol–chloroform-extracted chloroplasts were similar in ultrastructure to the acetone-dehydrated chloroplasts and to chloroplasts in situ, two differences in the methanol–chloroform-extracted chloroplasts were apparent at low magnifications. The most obvious of these was the absence of osmiophilic globules. These structures are easily recognized and have been reported frequently in the literature (14). The

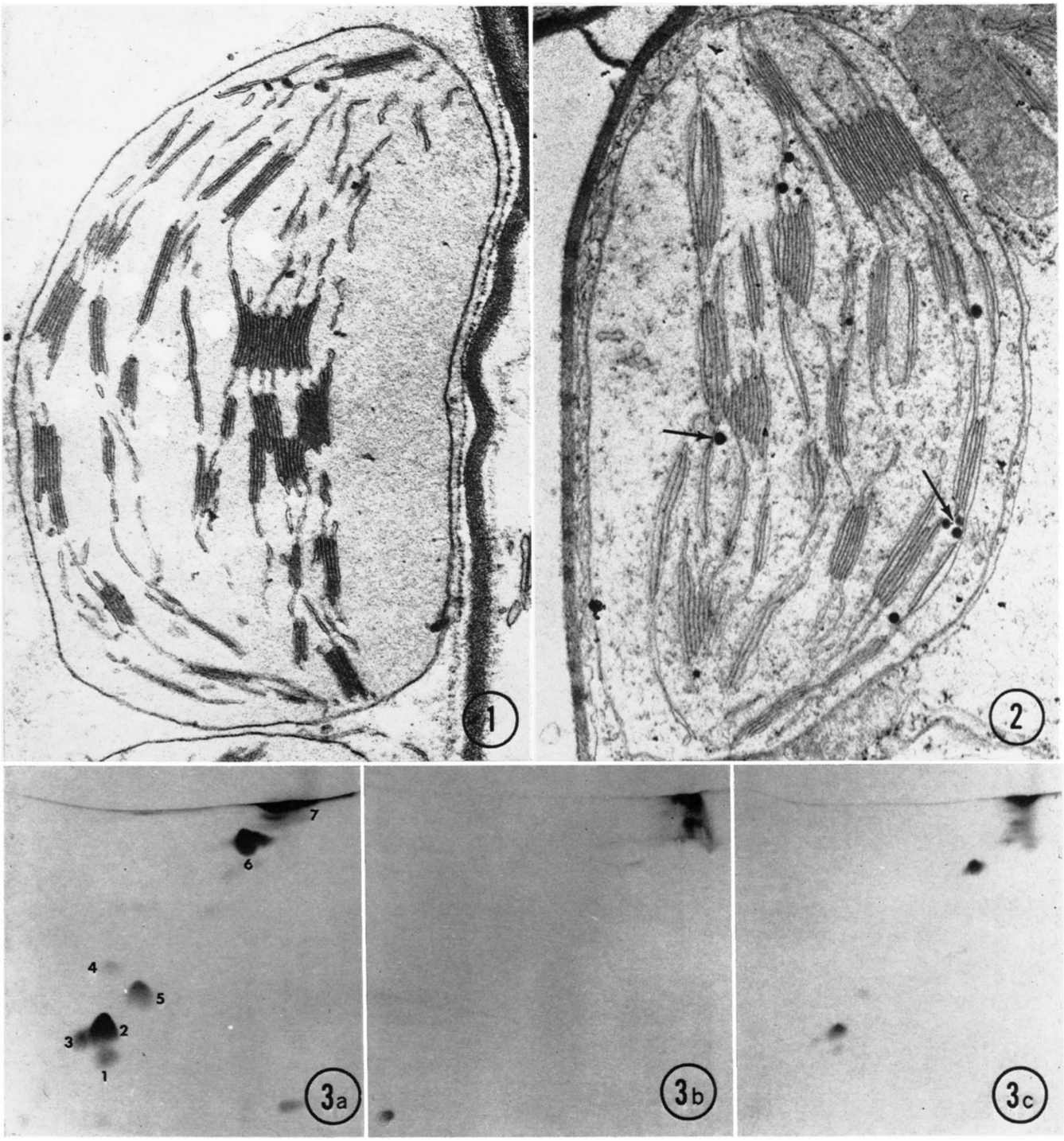


FIG. 1. Chloroplast fixed in situ with potassium permanganate. ×24,000.
FIG. 2. Chloroplast fixed in situ with osmium tetroxide. The arrows indicate osmiophilic globules. ×24,000.
FIG. 3. Two-dimensional thin-layer chromatography of chloroplast lipids extractable after (*a*) no fixation; (*b*) osmium tetroxide fixation, 15 min; (*c*) potassium permanganate fixation, 3 min. The origin is at the bottom left of the plates. The first solvent moved from left to right and the second solvent moved from bottom to top. Identification of compounds: *1*, sulfoquinovosyl diglyceride; *2*, digalactosyl diglyceride; *3*, phosphatidyl choline; *4*, phosphatidyl ethanolamine; *5*, phosphatidyl glycerol; *6*, monogalactosyl diglyceride; *7*, pigments and neutral lipids.

second difference was that the chloroplasts that were completely or partially bounded by a membrane were closely appressed; in many instances the boundary membranes of the chloroplasts appeared to have fused together (Fig. 7). This apparent fusion of the chloroplast membrane was never observed in tissue preparations and rarely observed in the isolated chloroplasts dehydrated in acetone after fixation.

After permanganate fixation of chloroplasts in situ the grana consist of many compartments, and each compartment is separated from the next by a partition (Fig. 9, arrow). This partition is composed of two electron-opaque layers separated by an electron-transparent region. This general arrangement of the granal membrane was also observed in the isolated and permanganate-fixed chloroplasts after either acetone dehydration (Fig. 10) or methanol–chloroform extraction (Fig. 11).

After osmium tetroxide fixation of chloroplasts in situ, the resolution of grana membranes into two electron-opaque layers separated by an electron-transparent space was only occasionally observed (Fig. 12, arrow). This resolution was also seldom observed in the isolated, fixed, and acetone-dehydrated preparation (Fig. 13). After osmium fixation and methanol–chloroform extraction a more drastic change in the ultrastructure of the granal organization was noted in that the partitions appeared as single dense lines and frequently several partitions had fused into a single dense band (Fig. 14, arrow).

Lipid Analyses of Chloroplasts Before and After Fixation With Osmium Tetroxide or Potassium Permanganate

Table 1 shows the composition of the lipid extractable from chloroplasts before and after a 10 min exposure to osmium tetroxide. Two of the less abundant phospholipids, phosphatidyl ethanolamine and phosphatidyl inositol, were not measured in these experiments. It is apparent that the glycolipids and phospholipids are not completely fixed in the 10 min reaction period. After fixation for 10 min the galactosyl diglycerides were more readily removed by chloroform–methanol than by acetone. The other lipids were extracted equally well by the two solvents.

Extension of the time of reaction with osmium tetroxide, combined with more thorough mixing of the chloroplasts with the reagent, resulted in complete fixation of the glycolipids and phospholipids, at least of those that were determined (Table 2). Chlorophyll was readily extracted. After fixation with potassium permanganate for 3 min, a significant amount of glycolipid, phospholipid, and chlorophyll was extractable (Table 2). Acetone and chloroform–methanol extracted these lipids equally well. Chromatographic separation of the lipids from this experiment is shown in Fig. 3.

It was found that longer reaction times with permanganate fix more lipid, but up to 10% of the lipid was still extractable even after 15 min (Table 3). The virtual absence of chlorophyll from the extracts of permanganate-fixed chloroplasts is in contrast to the considerable amount extractable after fixation with osmium tetroxide.

Reaction of Osmium Tetroxide and Potassium Permanganate With Isolated Lipid

Radioactive lipid was prepared by Mr. S. Sumida by incubation of *Chlorella pyrenoidosa* with acetate-^{14}C in the light. Lipids were extracted with chloroform and washed free of water-soluble compounds. The radioactive lipid (300,000 dpm) in 1.0 ml of chloroform was shaken for 1 min with 3.0 ml of either 1% osmium tetroxide or 2.5% potassium permanganate in a conical glass tube at room temperature. The aqueous layer was withdrawn and the remainder washed with water; these aqueous fractions were combined and an aliquot was taken for counting. The chloroform layer was withdrawn from the insoluble residue, which was then washed with chloroform; the chloroform extracts were combined and an aliquot was taken for counting. Because the residue stuck to the wall of the tube and was intractable, an accurate determination could not be made directly, but only by difference. In the osmium tetroxide experiment less than 1% of the radioactivity was found in the water-soluble fraction and

TABLE 1 Effect of Osmium Tetroxide Fixation on Chloroplast Lipid Extractability

Fixation	Extraction	Amount of Lipid Extracted									
		MG		DG		SL		PG		PC	
		μg	%	μg	%	μg	%	μg	%	μg	%
None	Chloroform–methanol	7500	100	5320	100	1770	100	2070	100	850	100
OsO_4, 10 min	Acetone*	630	8	810	15	220	12	475	23	260	31
OsO_4, 10 min	Chloroform–methanol	1630	22	1240	24	230	13	443	21	260	31

Chloroplasts were prepared from 80 g of deveined spinach leaves. The lipids of one aliquot were extracted and assayed as described in Experimental Procedure. A second aliquot was exposed to osmium tetroxide and the chloroplast pellet was then taken through the normal dehydration procedure. The lipid in the combined acetone washings was analyzed. A third aliquot was exposed to osmium tetroxide and the chloroplast pellet was then extracted with chloroform–methanol.

Abbreviations: MG, monogalactosyl diglyceride; DG, digalactosyl diglyceride; SL, sulfoquinovosyl diglyceride; PG, phosphatidyl glycerol; PC, phosphatidyl choline.

* This is the pool of acetone solutions used in regular dehydration.

TABLE 2 EFFECT OF POTASSIUM PERMANGANATE AND LONGER OSMIUM TETROXIDE FIXATION ON CHLOROPLAST LIPID EXTRACTABILITY

Fixation	Extraction	Amount of Lipid Extracted											
		MG		DG		SL		PG		PC		Chlorophyll	
		μg	%	μg	%	μg	%	μg	%	μg	%	μg	%
None	Chloroform–methanol	3280	100	2270	100	610	100	1860	100	1000	100	6280	100
$KMnO_4$, 3 min	Acetone*	570	17	365	16	170	28	371	20	226	23	2540	40
$KMnO_4$, 3 min	Chloroform–methanol	530	16	395	17	150	25	360	19	237	24	2340	37
OsO_4, 15 min	Acetone*	0	0	0	0	0	0	0	0	0	0	4360	69
OsO_4, 15 min	Chloroform–methanol	0	0	0	0	0	0	0	0	0	0	4240	68

Chloroplasts were prepared from 100 g of deveined spinach leaves. Five equal aliquots were obtained. The lipids of one aliquot were extracted without any fixation. Two aliquots were exposed to $KMnO_4$ and two to OsO_4. The treated chloroplasts were either taken through the normal dehydration procedure or extracted with chloroform–methanol. Electron micrographs were prepared from the chloroplasts of this experiment.

See Table 1 for abbreviations.

* Pool of acetone solutions used in regular dehydration.

TABLE 3 EFFECT OF LONGER REACTION WITH POTASSIUM PERMANGANATE ON CHLOROPLAST LIPID EXTRACTABILITY

Fixation	Extraction	Amount of Lipid Extracted											
		MG		DG		SL		PG		PC		Chlorophyll	
		μg	%	μg	%	μg	%	μg	%	μg	%	μg	%
None	Chloroform–methanol	8000	100	5000	100	1450	100	1990	100	840	100	9720	100
$KMnO_4$, 15 min	Chloroform–methanol	477	6	233	5	130	9	110	6	48	6	750	8

Chloroplasts were prepared from 50 g of deveined spinach leaves. Two equal aliquots were treated according to procedures described in Experimental Procedure.

See Table 1 for abbreviations.

40% of the radioactivity in the chloroform fraction, leaving 59% in the residue. In the potassium permanganate experiment 5% of the radioactivity was found in the water-soluble fraction and 29% in the chloroform fraction, leaving 66% in the residue. The chloroform extracts from both experiments were separated by one-dimensional thin-layer chromatography in a system suitable for the separation of neutral lipids (15). In this system the phospholipids and glycolipids stay at the origin. In the untreated lipid sample this area contained 66% of the radioactivity, which is close to the amount of lipid which reacted with the fixatives to form an insoluble residue. The remaining radioactivity was found in areas corresponding to pigments, sterols, and sterol glycosides. Two-dimensional thin-layer chromatography of the chloroform extract from osmium tetroxide-treated lipids indicated complete absence of monogalactosyl diglyceride, digalactosyl diglyceride, sulfolipid, phosphatidyl glycerol, phosphatidyl choline, and phosphatidyl ethanolamine. Galactolipids and sulfolipid were also absent from the chloroform extract of potassium permanganate-treated lipids; phospholipids were present, but their quantities in the treated sample were only small fractions of those in the untreated sample. It is apparent that the lipids which react with potassium permanganate and osmium tetroxide include mainly glycolipids and phospholipids. Other lipid components are still extractable by chloroform–methanol or in the dehydration procedure (Fig. 3). Their removal may result in changes in the dimensions of ultrastructural features such as the widths of the grana membranes in chloroplasts.

DISCUSSION

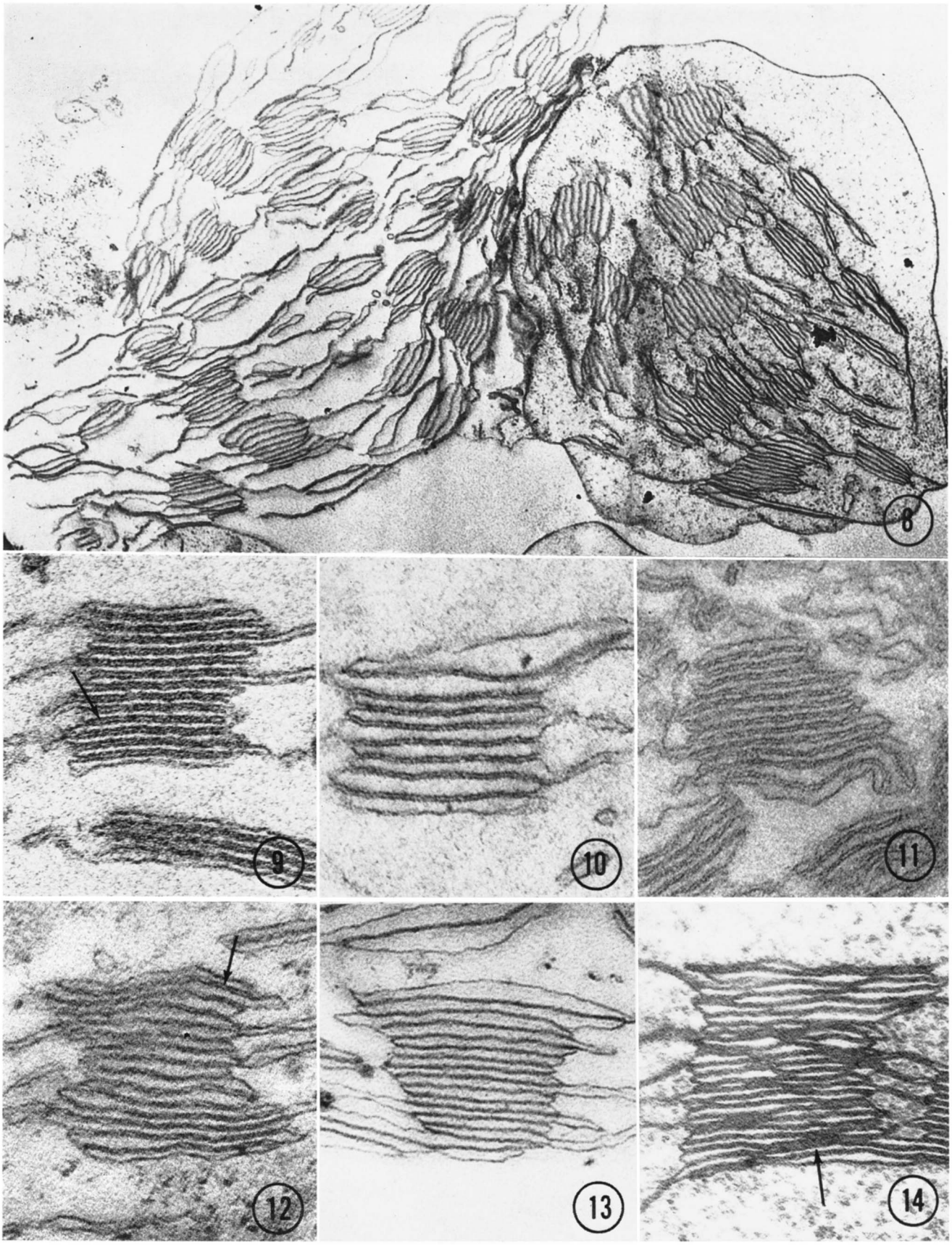
Fleischer, Fleischer, and Stoeckenius have reported (16) that bovine heart mitochondria depleted of 95% of their

FIG. 4. Micrograph of chloroplast material after extraction of lipid with chloroform–methanol. ×71,000.

FIG. 5. Isolated chloroplast fixed with potassium permanganate for 3 min and dehydrated with acetone. ×18,000.

FIG. 6. Isolated chloroplasts fixed with osmium tetroxide for 15 min and dehydrated with acetone. The arrows indicate the osmiophilic globules. ×24,000.

FIG. 7. Isolated chloroplast fixed with potassium permanganate for 3 min and extracted with chloroform–methanol. ×22,000.



lipid still maintained the unit membrane structure. The experiments reported in this paper show that when the lipids of chloroplasts are extracted with chloroform–methanol, structural features can no longer be demonstrated. The technique of extraction is physically harsh, but the chloroplast preparations which have been previously treated with osmium tetroxide and potassium permanganate go through the same harsh extraction procedure with very good retention of features of ultrastructure. After potassium permanganate fixation, micrographs of chloroplast preparations from which 20% of the glycolipids and phospholipids and 40% of the chlorophyll were extractable showed characteristic structural features. In chloroplasts, then, some lipid fixation is necessary for retention of ultrastructure, but we have not yet determined how much.

Provided that the time of reaction of chloroplasts with osmium tetroxide and potassium permanganate is long enough, complete fixation of glycolipids and phospholipids can be achieved. This result is in agreement with the observations of Ashworth et al. on fixation of the lipids of rat liver tissue by osmium tetroxide (8). It should be emphasized that in our experiments we concentrated attention on the glycolipids and phospholipids. The experiments of Korn and Weisman (7) show that the lipid extractable from *Acanthamoeba* sp. after fixation with osmium tetroxide and potassium permanganate is mainly neutral lipid. Our experiments with radioactive *Chlorella* lipid indicate that the lipid that does not react with osmium tetroxide or potassium permanganate includes sterols, sterol glycosides and esters, and pigments. Glycerolipids with saturated fatty acid residues may not be reactive; however, the content of saturated fatty acids in the photosynthetic tissue of plants is very low so that the amount of glycolipids which do not react with the fixatives should be negligible. The quantitative reaction of osmium tetroxide and potassium permanganate with the glycolipids and phospholipids of chloroplasts is in contrast to the incomplete reactions with *Acanthamoeba* lipids as reported by Korn and Weisman (7). This quality of the chloroplast lipids should make possible the quantitative radioautographic studies of lipid metabolism envisaged by Korn (2).

The major difference in the chemical analysis of lipid components extractable after fixation of chloroplasts with either osmium tetroxide or potassium permanganate is the fixation of chlorophyll by the permanganate. This difference is correlated with the presence of an electron-transparent region in the grana structure of the potassium permanganate-fixed chloroplasts. Weier and Benson have proposed that this electron-transparent region contains chlorophyll, quinones, and pigments (5). Our results are in agreement with their proposal. It is relevant to this point that Magree, Henninger, and Crane (17) have reported that the extraction of quinones from chloroplasts with heptane reduced the width of single membranes from 100–135 A to 50–80 A. The elimination of the electron-transparent space after osmium tetroxide fixation may represent a similar preferential extraction of a lipid component.

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FIG. 8. Isolated chloroplasts fixed with osmium tetroxide for 15 min and extracted with chloroform–methanol. ×26,000.
FIG. 9. High magnification view of a granum from a chloroplast fixed in situ with potassium permanganate. The arrow indicates a partition of the granum. ×100,000.
FIG. 10. High magnification view of granum fixed with potassium permanganate for 3 min and dehydrated with acetone. ×68,000.
FIG. 11. High magnification view of a granum from an isolated chloroplast fixed in potassium permanganate for 3 min and extracted with methanol–chloroform. ×64,000.
FIG. 12. High magnification view of a granum from a chloroplast fixed with osmium in situ. The arrow indicates a partition of the granum. ×100,000.
FIG. 13. High magnification view of a granum from an isolated chloroplast fixed in osmium tetroxide for 15 min and dehydrated in acetone. ×64,000.
FIG. 14. High magnification view of a granum from isolated chloroplasts fixed in osmium tetroxide for 15 min and extracted with chloroform–methanol. The arrow indicates a site of fusion of granal partitions. ×82,000.

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