BIOCHEMICAL STUDIES ON THE MEMBRANES OF PEROXISOMES AND GLYOXYSOMES

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ABSTRACT

BIOCHEMICAL STUDIES ON THE MEMBRANES OF PEROXISOMES AND GLYOXYSOMES

By

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Subcellular organelles from spinach leaves, castor bean endosperm, sunflower cotyledons, rat liver and dog kidney were separated using sucrose density gradient centrifugation. Microbodies (peroxisomes or glyoxysomes) were obtained at densities ranging from 1.24 to 1.27 g x cm^{-3}. Etioplasts were found at 1.21 to 1.22, mitochondria at 1.18 to 1.22, chloroplasts at 1.16 to 1.22, microsomes at 1.14 to 1.17, and lysosomes at 1.12 to 1.14 g x cm^{-3}.

In each instance there was a peak of NADH-cytochrome c reductase coincident with the peak activity of the microbody marker, catalase. The NADH-cytochrome c reductase of the plant microbodies was similar to the microsomal enzyme from the same tissue, since it was not inhibited by antimycin A and had a similar pH optimum. The plant mitochondria had an NADH-cytochrome c reductase which was inhibited by antimycin A and had a lower pH optimum than the enzyme in the microbodies and microsomes.

Rat liver and dog kidney peroxisomes also had an antimycin A insensitive NADH-cytochrome c reductase. Further studies on the rat liver peroxisomes indicated that the NADH-cytochrome c reductase was a component of the limiting membrane.

The phospholipid compositions of microbodies from rat liver and castor bean endosperm were found to be very similar to the microsomes. The most abundant phospholipids were phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol.

The presence of the microsomal type NADH-cytochrome c reductase in the microbody membrane and the similarities in phospholipid composition suggest that the microbody membrane is derived from the endoplasmic reticulum.

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ATP	adenosine-5′-triphosphate
EDTA	ethylenediamine tetraacetate
ER	endoplasmic reticulum
DNA	deoxyribonucleic acid
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
Tricine	N-tris (hydroxymethyl) methylglycine
Tris	tris (hydroxymethyl) aminomethane
UDP	uridine diphosphate
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

CHAPTER I

INTRODUCTION -- SUBCELLULAR ORGANELLES

A. Microbodies, Peroxisomes and Glyoxysomes

1. Morphology.

Microbodies were first described by electron microscopists studying kidney and liver cells. They have since been observed in many animal (47) and plant tissues (36, 38). Microbodies have diameters ranging from 0.2 to 1.7 μ and are often spherical, but being pliable they may take other shapes. Their single limiting membrane is about the same thickness (60 to 80 Å) as the membranes of the endoplasmic reticulum (ER) or mitochondria but thinner than the plasma membrane or the lysosome membrane (47). The membrane has the typical three-layered "unit-membrane" structure (67). Microbodies are distinguished from mitochondria by their single membrane and by the absence of internal membranes or ribosomes. The microbody matrix usually has a granular appearance and may contain a dense core or crystalloid structure (36, 47).

2. Biochemical Observations.

Peroxisomes (subcellular particles containing hydrogen peroxide-producing oxidases and catalase) were first isolated from rat liver and dog kidney and thoroughly characterized by de Duve's group (7, 23, 24, 24a, 81). Subsequently similar particles were obtained from other organisms and tissues including protozoa (76), yeast (98), and plant tissues (103-106, 116). Baudhin demonstrated that what the biochemists called peroxisomes were identical to what the electron microscopists called microbodies (7).

Rat liver peroxisomes attain a density of 1.23 g x cm^{-3} in sucrose density gradient centrifugation. This is because the membrane of the peroxisome is freely permeable to sucrose and other small molecules. Also, for this reason rat liver peroxisomes are not sensitive to osmotic shock (24).

Rat liver peroxisomes contain catalase, NADPH-isocitrate dehydrogenase, D-amino acid oxidase, L-α-hydroxy acid oxidase and urate oxidase. The urate oxidase is located in the crystalloid core (24). In addition, they contain serine-glyoxylate aminotransferase and alanine-glyoxylate aminotransferase (85, 108). The compartmentation of the hydrogen peroxide-producing

flavin oxidases with catalase has an internal logic; however the physiological function of these organelles in the liver is not clear (24).

Spinach leaf peroxisomes, which are isolated at a density of 1.26 to 1.27 g x cm^{-3} in sucrose gradients, have all of the enzymes present in the liver particles (103), including urate oxidase (100) but excluding the D-amino acid oxidase (104, 114, 116). The catalase of leaf peroxisomes is often located in the crystalloid core, in contrast to liver peroxisomes (37). In addition, spinach peroxisomes contain malate dehydrogenase, hydroxypyruvate reductase, and four transaminases (85, 114, 116). This amounts to almost complete compartmentation of the glycolate pathway for metabolism which converts the two carbon by-product of photosynthesis -- glycolate -- to glycine, serine, and glycerate (102, 104). The only components of the glycolate pathway absent from the peroxisomes are the glycine decarboxylase and serine hydroxymethyl transferase which are located in the mitochondria (52, 53). This pathway is responsible for the release of CO_2 during photosynthesis (photorespiration), for the production of glycine and serine which are necessary for protein and porphyrin synthesis, and for the supply of C_1 groups needed for cell wall and nucleic acid synthesis (103, 106).

Glyoxysomes, which have been isolated in sucrose gradients from castor bean and other fat storing seeds, are clearly responsible for the mobilization of the lipid stores during seed germination. These microbodies are in close association with the lipid bodies (spherosomes) in situ. Glyoxysomes contain all the enzymes necessary for β-oxidation of fatty acids (21) and all the enzymes of the glyoxylate cycle (14, 20). In addition, they contain several enzymes known to be in plant and animal peroxisomes including urate oxidase (100), L-α-hydroxy-acid oxidase (14), and catalase which is located in the crystaloid (110). Furthermore, Schnarrenberger has recently shown that castor bean glyoxysomal preparations also contain hydroxypyruvate reductase, serine-glyoxylate aminotransferase and glutamate-glyoxylate aminotransferase, enzymes found in spinach leaf peroxisomes (91).

The cell may synthesize and destroy peroxisomes as whole units rather than dealing with the component parts individually. Once rat liver peroxisomes are synthesized they do not acquire additional protein and they do not grow (82). These peroxisomes have a half life of about 3.5 days. They are destroyed at random as wholes (83). Insect fat body microbodies (61) and castor bean glyoxysomes (110) may be destroyed as wholes by autophagic

vacuole formation.

The frequent association of microbodies with gluconeogenic function is interesting but not completely understood. The glycolate pathway in leaf peroxisomes is certainly gluconeogenic as is the glyoxylate cycle of the glyoxysomes. Liver is a gluconeogenic tissue although it is not known how its peroxisomes could be involved in gluconeogenic metabolism.

B. Endoplasmic Reticulum

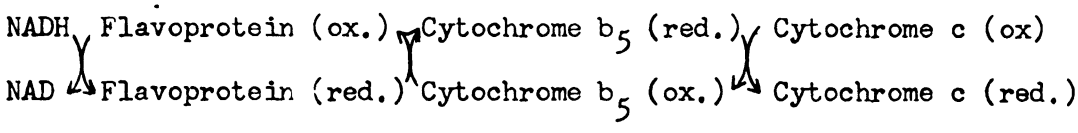
1. Morphology.

This network of membranes is seen throughout most cells. Some parts of the ER are covered with ribosomes; other parts are smooth. The ER appears to be responsible for the production of other organelles such as the Golgi apparatus and microbodies. In meristematic and differentiating cells, microbodies are closely associated with the ER and in some cases the limiting membrane of the microbody appears to be continuous with the ER, usually the rough ER (38, 47, 61, 110). The catalase which can be identified by specific staining techniques is apparently synthesized by ribosomes on the ER and then transferred to the microbodies (45, 58).

2. Biochemical Observations.

Microsomes are small membranous vesicles resulting from the breakage of the endoplasmic reticulum during grinding (19). Because of their small size they sediment more slowly than other organelles and thus can be separated from other components by differential centrifugation. The final step of isolation usually involves pelleting the microsomes at high speed (eg., 100,000 g). Microsomes bearing ribosomes (i.e., rough or heavy microsomes) can be separated from smooth microsomes on sucrose density gradients. Smooth microsomes are found at a density of 1.17 g x cm^{-3}, while rough microsomes are more dense (95).

Microsomes isolated from rat livers have been studied in detail. They are capable of lipid and protein synthesis. They contain a unique electron transport system, the components of which include cytochrome P_{450}, cytochrome b_5, NADH-cytochrome b_5 reductase and NADPH-cytochrome c reductase (91, 113). The cytochrome b_5 and its flavoprotein reductase will function as a NADH-cytochrome c reductase *in vitro* as follows:



However, cytochrome c is not the physiological electron acceptor (96, 97). Glucose-6-phosphatase is also typically associated with rat liver microsomes.

The rough ER is the precursor of the smooth ER. This transition involves a change in phospholipid composition, i.e., the proportion of sphingomyelin increases (54, 70, 112). Although separation and analysis of smooth and rough microsomes revealed subtle differences in enzymatic composition (23), cytochemical studies of the glucose-6-phosphatase in situ revealed no differences between the smooth and rough ER (60). It has been suggested that the smooth microsome fraction contains, in addition, fragments of the Golgi and the plasma membrane (23, 69).

Microsomes have also been obtained from plant tissues such as swiss chard, cauliflower, bean cotyledons, and etiolated wheat (22, 64, 101). These plant microsomes have NADH- and NADPH cytochrome c reductase activities but contain cytochrome b_3 instead of b_5. Microsomes from pea cotyledons or leaves and from Arum spadix have cytochrome b_5 and cytochrome P_{450} (16). Bean cotyledon microsomes have glucose-6-phosphatase (101). As will be shown in part III. B., microsomes may also be isolated from spinach leaves, castor bean endosperm, and sunflower

cotyledons.

C. Mitochondria

Mitochondria have been isolated from plant and animal tissues by differential and density gradient centrifugation. In sucrose density gradients mitochondria from most tissues band at a density of about 1.2 g x cm^{-3}, and thus may be separated from the heavier peroxisomes and the lighter microsomes. The density of rat liver mitochondria slowly increases on exposure to concentrated sucrose so that, after longer centrifugation times, mitochondrial contamination of the peroxisomes becomes greater (8, 39, 81).

The outer membrane of the mitochondria may be selectively ruptured by osmotic treatment, sonication, or digitonin treatment and then separated from the inner membrane plus matrix by differential or density gradient centrifugation (29, 90, 95). The inner membrane plus matrix has a density of 1.21 g x cm^{-3}, while the outer membrane has a density of 1.13 g x cm^{-3}, near that of the microsomes (29).

The inner membranes of the mitochondria bear the components of respiratory electron transport including cytochrome c oxidase and a succinate or NADH-cytochrome c reductase which is distinguished from the microsomal

reductase by its sensitivity to inhibitors such as rotenone and antimycin A. The matrix of the mitochondria contains the enzymes of the citric acid cycle and includes some enzymes or isozymes also found in peroxisomes and glyoxysomes such as malate dehydrogenase and aspartate aminotransferase (29).

The outer mitochondrial membranes have much in common with the microsomes and, therefore, with the ER, including the NADH-cytochrome b_5 reductase and cytochrome b_5. The NADH-cytochrome c reductase of the outer membrane is immunologically similar to that of the microsomes (99). However, the outer membrane lacks the NADPH-cytochrome c reductase, cytochrome P_{450} and glucose-6-phosphatase (29, 90, 95). Similarities in protein composition have also been observed in electrophoretic analysis of the protein components of the two membrane types (89). The phospholipid composition of the outer membrane is similar to the microsomes: phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol are the major species. Compared to the inner mitochondrial membranes, the microsomes and the outer mitochondrial membranes have relatively high amounts of cholesterol. It is for this reason that the outer membrane is sensitive to digitonin. The major phospholipids of the inner membrane are

phosphatidyl choline, phosphatidyl ethanolamine, and cardiolipin (29).

Mitochondria are self replicating; they divide and grow (56). They contain DNA, ribosomes, and are capable of producing some of their own lipids and proteins such as the membrane bound cytochromes. However, other mitochondrial components, such as cytochrome c and some lipids, are synthesized by the ER and transported to the mitochondria. The proteins and lipids of the outer membrane are exclusively imported products, probably coming from the ER though not necessarily in direct continuity with it (54, 56, 88).

D. Chloroplasts and Etioplasts

Chloroplasts, like the mitochondria, are enclosed in a double membrane. The chloroplast contains a network of membranes derived from the inner limiting membrane, the thylakoids, which are arranged in regular stacks, the grana. This membrane system is surrounded by the stroma, which is analogous to the mitochondrial matrix, and enclosed in the double membrane. The thylakoids contain the components of photoelectron transport -- the pigments and cytochromes. Most of the enzymes involved in the photosynthetic carbon dioxide fixation cycle, such

as ribulose-1,5-diphosphate carboxylase, triose phosphate isomerase, and NADP-glyceraldehyde-3-phosphate dehydrogenase are components of the stroma (51).

Like the outer mitochondrial membrane and the peroxisomal membrane, the outer membrane of the chloroplast is freely permeable to sucrose and other low molecular weight species, while the inner membrane is the site of selective transport of certain metabolites such as malate and 3-phosphoglycerate (44).

The lipid composition of chloroplasts is quite unique; galactolipids such as monogalactosyl diglyceride, digalactosyl diglyceride, and the sulfolipid predominate rather than phospholipids. Phosphatidyl glycerol is the major phospholipid (1, 10, 51, 75).

Isolated chloroplasts are usually broken -- devoid of the outer membrane and much of the stroma. In sucrose density gradients the broken chloroplasts reach a density of 1.14 to 1.17 g x cm^{-3}. Whole chloroplasts have a density of 1.21, about the same as mitochondria (87, 92).

Etioplasts are the precursors of chloroplasts and are present mainly in non-green tissues such as castor bean endosperm and etiolated leaves or cotyledons. The internal membranes of this organelle are arranged in a crystalloid structure. Etioplasts have been isolated by

Schnarrenberger, et al (92) on sucrose density gradients at a density of 1.26, very near the peroxisomes and glyoxysomes. Etioplasts contain many of the enzymes known in mature chloroplasts: triose phosphate isomerase, NADPH-glyoxylate reductase, Dopa oxidase, phosphoglycolate phosphatase, and NAD-triose phosphate dehydrogenase. The enzymatic composition of etioplasts may indicate some role for the developing particle in carbohydrate metabolism in the non-green cell (92).

E. The Golgi

The Golgi consist of dictyosomes which are composed of stacks of membranes. These membranes and their enclosed contents may be derived in part from the ER, although dictysomes are capable of dividing and reproducing themselves. The Golgi produce other membranous structures such as lysosomes (25), zymogen granules (70), and secretory vesicles (71). Secretory vesicles may contain cell wall components, for example. As the contents of a secretory vesicle are released to the outside of the cell the membrane becomes a part of the plasma membrane (71). The transition of the ER to a lysosome or plasma membrane via the Golgi is accompanied by a thickening of the membrane from 50 Å in the ER to 70 Å

in the Golgi to almost 100 Å in the plasma membrane (74).

Golgi have been isolated from onion leaves, cauliflower, beef liver, and rat liver (17, 31, 33, 57, 71, 73). They have a density of 1.13 in a sucrose gradient. UDP-galactose-N-acetylglucosamine galactosyl transferase is apparently unique to this organelle, at least in rat liver. Nucleoside diphosphate hydrolases and thiamine pyrophosphorylase are enzymes which have also been detected in some Golgi preparations (18, 31, 71, 73).

The biochemical data support the morphological observations of membrane differentiation. In contrast to microsomes, the Golgi lack cytochrome P_{450}, glucose-6-phosphatase, and have less cytochrome b_5 (33). The transformation of the ER to the plasma membrane also involves a change in lipid composition; phosphatidyl choline becomes less prominent and the proportion of sphingomyelin increases (27, 49, 69, 84).

F. Lysosomes

Lysosomes function in the digestion of endogenously and exogenously derived materials. Exogenous material is brought into the cell enclosed in a bit of the plasma membrane. The resulting vesicle is then engulfed by a lysosome. Lysosomes also engulf cellular organelles

such as microbodies (25, 30). Thus lysosomes may be responsible for the turnover of microbodies (61, 110).

Lysosomes contain a group of acid hydrolases (proteases, lipases, and nucleases) which digest the engulfed materials. The hydrolases are synthesized in the ER, transported to the Golgi, and released in membrane bound vesicles, the lysosomes (8, 25).

The density of rat liver lysosomes is 1.22 g x cm^{-3} in sucrose gradients, intermediate between mitochondria and peroxisomes. However, when rats are injected with Triton WR-1339, the detergent accumulates in the lysosomes and their equilibrium density is reduced to 1.12. Acid phosphatase activity is commonly used to detect lysosomes.

Lysosomal enzymes have been found in isolated spherosomes (lipid bodies) and vacuoles from plants (66).

G. Storage Particles

Lipid storage bodies (spherosomes) are abundant in castor bean endosperm and cotyledons of germinating fatty seeds. They have a very low density and float to the top of a sucrose gradient. The membrane of the lipid body is a single line rather than a three-layered "unit membrane" (107). Two types of lipid vesicles have

been isolated from pea and bean cotyledons. One type is found in adherent groups. The other is larger and singular (2, 72) The larger type contains more triglyceride and phospholipid than the adherent type. Both contain phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol.

Protein storage bodies (alueron grains) also occur in seeds such as castor bean and sunflower (93, 110). These very dense (greater than 1.3 g x cm^{-3}) particles have been isolated from germinating sunflower by Schnarrenberger et al (93) and contain acid protease activity.

H. Nuclear and Plasma Membranes

The nuclear membrane and the plasma membrane appear to be at opposite ends of the spectrum of cellular membranes. The nuclear membrane is similar to the ER and the two membrane systems are continuous in places. The nuclear membranes are richer in protein than the microsomes and therefore have a greater density, 1.20 g x cm^{-3}. They contain NADPH- and NADH-cytochrome c reductases but lack glucose-6-phosphatase (35, 117). Nuclear membranes have a lipid composition similar to microsomes (48, 50, 117).

The isolated plasma membrane has a density of

1.14 to 1.17 in sucrose gradients. It is characterized by a series of phosphate ester-splitting enzymes, including 5′-nucleotidase, acid phosphomonoesterase, glucose-6-phosphatase, and ATPases (23, 28, 55, 69). Compared to the microsomes, the plasma membranes have relatively high amounts of sphingomyelin and cholesterol (23, 27, 70).

CHAPTER II

METHODS AND MATERIALS

A. Isolation of Organelles

1. Spinach

Spinacia oleracea L., Longstanding Bloomsdale, was grown in a controlled environment chamber. Deribbed leaves (200 g) were homogenized for 10 sec in a Waring blendor with 200 ml of a solution of 30% (w/w) sucrose and 0.02 M glycyl-glycine, pH 7.5. The homogenate was squeezed through 8 layers of cheese cloth and centrifuged at 650 g for 5 min.

Zonal centrifugation techniques, as developed by Anderson (3) were used to separate organelles from the spinach homogenate. A discontinuous sucrose gradient was pumped (Cole-Parmer Masterflex pump, model #7014) into an IEC B-30 zonal rotor at 2500 rpm. The gradient was pumped to the edge of the rotor starting with 20 ml of 25% (w/w) sucrose. This was followed by 20 ml each of 33%, 37%, 40%, 40.5%, 41%, 42%, 43%, 44%, 49%, 50%, 51%, 51.5%, 52%, and enough 56% sucrose to fill the rotor to

the center. The capacity of the head was about 560 ml. All sucrose solutions were prepared from Schwarz-Mann "density-gradient grade" sucrose in 0.02 M glycyl-glycine, pH 7.5.

The spinach supernatant, about 230 ml, was pumped into the center of the rotor, displacing an equal amount of the 56% sucrose from the edge. About 50 ml of 56% sucrose remained in the rotor. After centrifugation at 30,000 rpm for 2 hr, the rotor was decelerated to 2500 rpm, and cold water was pumped into the rotor to displace the gradient which was collected in 10 ml fractions beginning with the 56% sucrose. Fractions were collected with a rapid (ca. 50 ml per min) continuous flow. Cross-leakage with the incoming water at the rotating seal assembly was likely if the pump were stopped. The material was kept near 5^{o} throughout the procedure.

Various other grinding techniques and centrifugation procedures were used in the fractionation of spinach leaves, and the fractions obtained were further purified by density gradient flotation (see part III). However, the above procedure was found to give excellent yields and reasonable purity in a short time (ca. 3 hr).

2. Castor Bean Endosperm and Sunflower Cotyledons.

Castor bean (Ricinus communis L.) endosperm was obtained from seedlings after 5 day germination in the dark at 30°. Fifteen grams of endosperm were homogenized for 5 sec in a Sorvall Omni-mixer with 22.5 ml of 24% (w/w) sucrose, 0.165 M Tricine, 10 mM KCl, $MgCl_2$, EDTA, and dithiothreitol (Calbiochem), pH 7.5 (20). About 16 ml of the resulting homogenate was layered on a discontinuous gradient (5 ml of 60%, 2.5 ml each of 56.5%, 53%, 51.5%, 50%, 47.5%, 45%, 42.5%, 40%, 37.5%, 2 ml each 35%, 32.5%, 30%, 27.5%, 25%, 22.5% and 20% w/w sucrose in 10 mM EDTA, pH 7.5). This was centrifuged for 2 hr at 25,000 rpm in a Beckman SW 25.2 rotor. Fractions of 2.5 or 2 ml were collected from the bottom of the tube starting with the most dense sucrose as indicated in Figure 7 (p64).

Sunflower (Helianthus annus L.) cotyledons were homogenized and fractionated using procedures similar to those used for castor bean (91).

3. Animal Tissues.

Livers were obtained from three female Sprague-Dawley rats, 190 to 210 g body weight, which had been injected with 1.5 ml of 10% (w/w) Triton W-1339 (Ruger

Chemical Co., Irvington, N.J.) 3.5 days previous and starved overnight. The livers, 20 to 40 g total, were perfused with grinding medium in situ, minced and homogenized with 7% (w/w) sucrose, 0.02 M glycyl-glycine, pH 7.5, in a Potter-Elvejhem homogenizer with a motor driven teflon pestle (81). The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 2000 rpm for 7 min, including acceleration time. The loaded B-30 zonal rotor contained 295 ml of 3% sucrose, 44 ml of rat liver supernatant, 12 ml each of 15%, 20%, 25%, 30%, 33%, 36%, 38%, 40%, 45%, 46%, 46.5%, 47%, 47.5%, 48%, 49%, and 40 ml of 56% (w/w) sucrose in 0.02 M glycyl-glycine, pH 7.5. This was centrifuged at 35,000 rpm for 35 min. Fractions were collected in 5 ml volumes. The sedimentation distance in this gradient is only about 1 cm which makes the short centrifugation time possible.

To process larger amounts of tissue (eg., 10 livers) it was necessary to pellet the peroxisomes as described by Poole (81). The homogenate was centrifuged at 5000 rpm for 7 min (Beckman 30 rotor, Beckman screw cap tubes) in order to remove cell debris. The resulting pellet was resuspended in about 20 ml of grinding medium and centrifuged as before. The combined 5000 rpm supernatants were centrifuged at 25,000 rpm for 8 min 20 sec

(all centrifugation times include acceleration time). The pellet obtained at 25,000 rpm was resuspended, using a Potter-Elvejhem homogenizer, and recentrifuged at 25,000 rpm. The final pellet was resuspended in about 50 ml and pumped onto the zonal sucrose gradient described above.

Dog kidneys were homogenized and fractionated in the same manner as rat liver. Details are given in Figure 11.

The exact procedures and sucrose gradients used were varied because different types of organelles were encountered in each tissue and because the equilibrium density of a particular organelle varied from tissue to tissue (Table III).

B. Enzyme Assays

All assays were carried out at 25° except where noted. In assays where NADH or NADPH was involved, a change of one A_{340} was equivalent to 161 nmoles in a one ml assay. Components of the enzyme assays that were obtained from Sigma Chemical Co., St. Louis, Mo., were cytochrome c (type II which was usually 75% oxidized), NADH (grade III), NADPH (type II), and antimycin A (type III). Cytochrome b_5 from rat liver microsomes

was generously supplied by Drs. D. Roerig and S. Aust.

1. Catalase.

The rate of disappearance of hydrogen peroxide was measured at 240 nm (12). A change in one A_{240} was equivalent to 71.5 μmoles of hydrogen peroxide in a 3 ml assay. The initial A_{240} of the peroxide was 0.5 to 0.6.

2. Glycolate Oxidase (L-α-hydroxy acid oxidase).

The reduction of the dye, dichlorophenolindophenol, was followed under anaerobic conditions at 600 nm (105). The maximum dye concentration that could be used in this spectrophotometeric assay did not saturate the enzyme so that the rates obtained were about one third of the optimum.

3. Uric Acid Oxidase.

The absorbance decrease of uric acid was recorded at 293 nm; one A_{293} was equivalent to 81.2 nmoles in the 1 ml assay.

4. Aspartate Aminotransferase.

The production of oxaloacetate was coupled to malate dehydrogenase so that the NADH oxidation could be followed at 340 nm (12, 114).

5. Serine-Pyruvate Aminotransferase.

Hydroxypyruvate production was linked to hydroxypyruvate reductase (glycerate dehydrogenase) and NADH oxidation (116).

6. Glutamate-glyoxylate aminotransferase.

The amount of radioactive glycine produced from glyoxylate-1,2-^{14}C during a 15 min incubation at 30° was determined by separating the glycine from the glyoxylate on Dowex 50 H^+ and counting the radioactivity in the glycine fraction (53).

7. NADP-Isocitrate Dehydrogenase.

NADP reduction was measured at 340 nm (116).

8. Malate Dehydrogenase.

With oxaloacetate as substrate NADH oxidation was followed at 340 nm (115).

9. Cytochrome c Oxidase.

After incubating an enzyme aliquot with digitonin for a minute, buffer and reduced cytochrome c were added and the disappearance of reduced cytochrome c was followed at 550 nm. The cytochrome c had been reduced by adding a few crystals of sodium dithionite until the A_{550}/A_{565}

ratio was greater than 6, which was equivalent to 70% reduction. A ratio of 10 was equivalent to 90% reduction (94). The units of activity are described in the NADH-cytochrome c reductase procedure.

10. NADPH-Diaphorase.

The NADPH-dependent reduction of dichlorophenol-indophenol was followed at 600 nm. The assay mixture consisted of 0.5 ml of 0.1 M Tris, pH 7.5, 0.1 ml of the dye (0.25 g per ml), 0.35 ml of enzyme plus water, and 0.05 ml of NADPH (2 mg per ml). A change of one A_{600} was equal to 1.83 nmoles.

11. Triose phosphate isomerase.

NADH oxidation, which could be followed at 340 nm, was obtained by coupling the isomerase to glycerol-phosphate dehydrogenase (9, 92).

12. NADH-Cytochrome c Reductase.

NADH-cytochrome c reductase was assayed by measuring the rate of cytochrome c reduction at 550 nm in a microcuvette containing 0.1 ml of 0.2 M phosphate or glutamate buffer at the indicated pH, 50 µl of oxidized cytochrome c (5 mg/ml), 5 µl of 10 mM KCN (to inhibit any cytochrome c oxidase activity), 70 µl of organelle

suspension plus water (22, 64). Where indicated 2 µl of antimycin A (2 mg/ml in ethanol) was added to the mixture. After obtaining the NADH-independent rate the reaction was initiated with 50 µl of NADH (3 mg/ml). A change of one A_{550} was equivalent to a change of 12.8 nmoles in the 0.27 ml assay. The extinction coefficient for cytochrome c (reduced minus oxidized) is 21.1 $mM^{-1}cm^{-1}$ (109).

13. NADPH-Cytochrome c Reductase.

NADPH-cytochrome c reductase was assayed in a similar way except that 0.15 ml of 0.05 M phosphate, pH 7.3 and 50 µl of organelle suspension plus water were used. Antimycin was included in all assays. The reaction was initiated with 2 µl of NADPH (8.6 mg/ml in 0.1 M bicarbonate, pH 10.4) (113).

14. NADH-Cytochrome b_5 Reductase.

NADH-cytochrome b_5 reductase was assayed by measuring the rate of cytochrome b_5 reduction at 426 nm in a microcuvette containing 0.1 ml of 0.2 M phosphate, pH 8.0, 50 µl of NADH (3 mg/ml). A change of one A_{426} was equivalent to a change in concentration of 2.55 nmoles in the 0.255 ml assay. The extinction coefficient of cytochrome b_5 (reduced minus oxidized) is 100 $mM^{-1}cm^{-1}$

(97).

15. Phosphatases.

Glucose-6-phosphatase was assayed by determining inorganic phosphate released after 10 min, at 35° (79).

Acid phosphatase was assayed using the chromogenic substrate, p-nitrophenyl phosphate. After 10 min incubation at 35°, 0.02 M NaOH was added and the A_{440} read (15).

C. Chlorophyll and Protein Assays

Chlorophyll was determined by the method of Arnon (4).

The Lowry procedure was used to estimate protein (62). The sample volume was kept small enough, eg. 50 µl, so that the sucrose content had little effect on the assay (40).

D. Lipid Determinations.

1. Lipid Extraction.

Fractions of interest from the density gradients were extracted overnight with 10 volumes of 2:1 (v/v) chloroform-methanol. The residue, which usually contained some sucrose crystals, was re-extracted with with 10 volumes chloroform-methanol. The combined extracts were

washed with 0.2 volume of 0.1% $MgCl_2$. The phases were separated by centrifugation, the upper aqueous phase discarded and the wash repeated (34). The extract, now considerably reduced in volume by the wash procedure, was evaporated to dryness in vacuo at about 50°. The lipids were then transferred to a screw cap vial (with teflon lined cap). During the transfer the flask was washed at least 5 times with 1 ml portions of chloroform. The chloroform was removed by a stream of nitrogen and the lipids redissolved in a known amount of chloroform. Aliquots were taken for phosphate determinations and for thin layer chromatography. All solvents were redistilled.

2. Thin Layer Chromatography.

The plates, coated with silica gel F254, 0.25 mm thick (Brinkman) were prewashed by running them in acetone. Aliquots of the lipid samples containing known amounts of phospholipids (600 to 1000 μg phospholipid, assay described below) were taken to dryness, redissolved in a small amount of chloroform and applied to the plate, alternating with standards, about 1.5 cm from the edge. The standards, phosphatidyl inositol, phosphatidyl serine, phosphatidyl ethanolamine and its lyso derivative, phosphatidyl choline and its lyso derivative, phosphatidyl

glycerol, cardiolipin, sphingomyelin, and monogalactosyl diglyceride, were obtained from Applied Science Laboratories, State College, Pa. The plates were run in chloroform, methanol, acetic acid, water (170:25:25:6 v/v) in a chamber lined with filter paper soaked with the solvent (78). The solvent front reached the top edge of the plate in 2 to 3 hr.

3. Lipid Identification.

The plates were dried by heating to 100° for a few min or air dried for a few hours. They were then sprayed with ninhydrin (0.2% in butanol saturated with water) and heated to 100° in an oven containing a pan of water. By comparison with the standards it was possible to identify those lipids containing amino groups, phosphatidyl ethanolamine and its lyso derivative, and phosphatidyl serine.

Following ninhydrin development, the plates were exposed to iodine which gives a temporary yellow-brown color to all lipid spots. Lipids such as phosphatidyl inositol, choline and glycerol, sphingomyelin and cardiolipin, were identified by comparison with the respective standards.

4. Lipid Phosphate Determination.

The micro-modification of the Bartlett phosphate assay (6) was used to quantitate the lipid phosphorus in the original lipid sample or in lipid spots from thin layer plates. The area of the thin layer containing a lipid spot was removed from the plate with a stiff razor blade cut to 1 cm wide and the silica gel transferred, with the aid of a fine brush and glassine paper, to a 12 ml conical centrifuge tube. Then 0.3 ml of 10 N H_2SO_4 was added to each tube and heated to 160° for at least 3 hours, often overnight. The tubes were cooled; 2 drops of phosphate free 30% hydrogen peroxide were added to each sample; each was vortexed to completely stir up the silica gel; and after 1.5 hours at 160° they were inspected. If brown or yellow color remained in any sample the peroxide treatment was repeated in all.

When the digestion process was completed the reagents for color development were added: 0.65 ml water, 0.2 ml of 5% ammonium molybdate, and 0.05 ml of Fiske-Subbarow reagent (0.5 g of 1-amino-2-anaphthol-4-sulfonic acid and 1 g of sodium sulfite, anhydrous, in 200 ml of 15% sodium bisulfite). The tubes were cooked in a boiling water bath for 10 min, centrifuged for about 5 min to pellet the silica gel and the contents read at

830 nm. A series of standards, 5 to 120 nmoles of phosphate, were also carried through the procedure. The background phosphate per unit area in each thin layer plate was determined and taken into account when calculating the amount of phosphorus in each lipid spot. The nmole values obtained were multiplied by the average molecular weight of a phospholipid, 775, to give nano grams of phospholipid.

CHAPTER III

RESULTS -- TISSUE FRACTIONATION

The main purpose of tissue fractionation in these studies was to obtain reasonably pure microbodies in quantities sufficient for analysis of membrane components. The term microbody is used here to include peroxisomes and glyoxysomes. The marker enzymes used to detect microbodies in sucrose density gradient fractions were catalase, glycolate oxidase (L-α-hydroxyacid oxidase), and urate oxidase. The purity of a microbody fraction was estimated from the activities of the markers unique to other organelle fractions. For example, the specific activity of cytochrome c oxidase in the microbody fraction relative to the specific activity in the mitochondria indicated the proportion of mitochondrial contamination in the microbody fraction. Similarly, chloroplast contamination was indicated by chlorophyll or NADPH-diaphorase and etioplasts by triose phosphate isomerase. Acid phosphatase was the marker enzyme used to detect lysosomes. NADH-cytochrome c reductase was used to locate the

microsomes, but it was not unique to this fraction since it was also found in mitochondria and microbodies (see Part IV. A.).

A. The Isolation of Spinach Leaf Peroxisomes

1. Differential Centrifugation.

The first approach to the isolation of cellular components usually involves differential centrifugation. Indeed for many years chloroplasts and plant mitochondria were obtained using only this method (13). In view of recent developments these preparations were impure. For example, catalase, which was thought to reside in the chloroplasts, is actually a component of the peroxisome (104).

The sedimentation of organelles from spinach leaves is shown in Figure 1. The chloroplasts, which are large, sediment faster than the other organelles. The initial steep portion of the chloroplast sedimentation curve probably represents whole chloroplasts while the latter portion corresponds to the broken chloroplasts (87). Spinach peroxisomes, in contrast to liver peroxisomes, sediment more rapidly than the mitochondria. Had the microsomes been assayed for in this experiment they probably would have been least inclined to sediment.

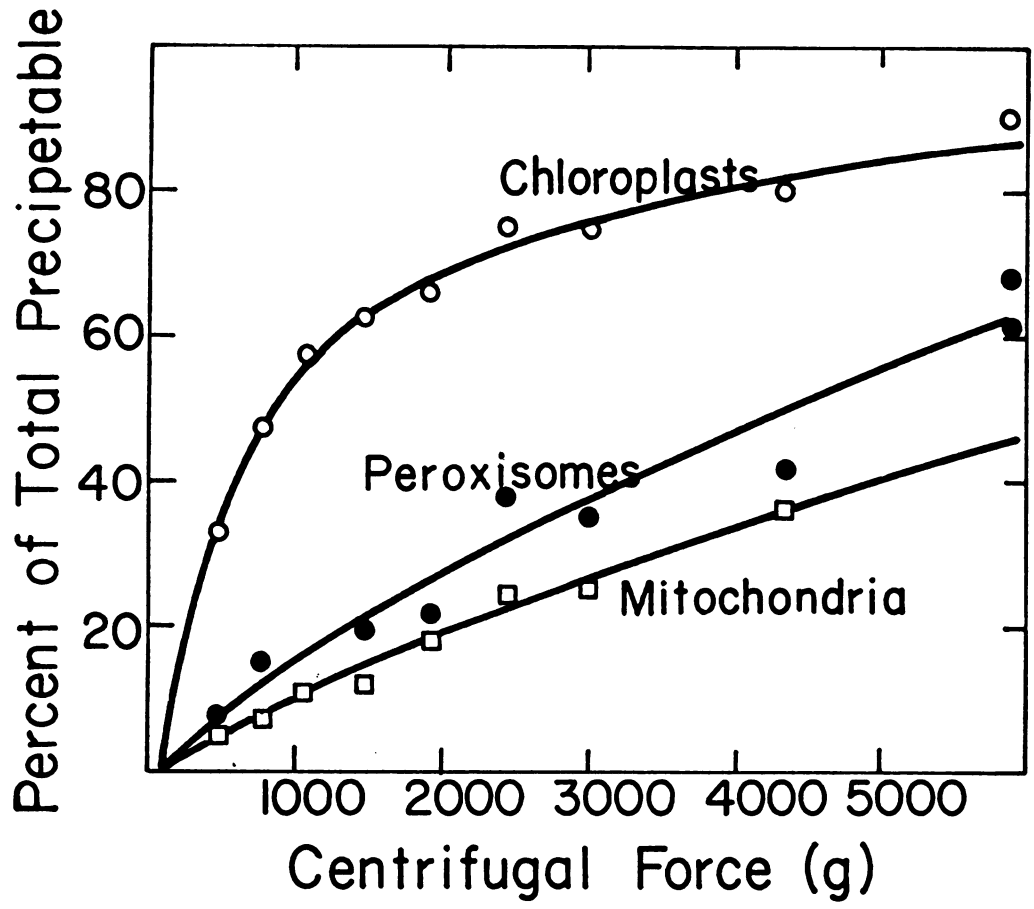


FIGURE 1. Differential centrifugation of spinach homogenate (10 min). Sixty g of spinach were chopped in a parsley grater with 300 ml of 25% (w/w) sucrose, 0.02 M glycyl-glycine, pH 7.5, and filtered through 8 layers of cheese cloth. Ten ml of the grinding medium were placed in the bottom of 45 ml centrifuge tubes; 15 ml of the spinach homogenate was layered on top of each; each tube was centrifuged for 10 min at the force indicated; the pellets were resuspended and assayed for chlorophyll (chloroplasts), glycolate oxidase (peroxisomes) and cytochrome c oxidase (mitochondria). The values are expressed as the percent of the amount of each marker enzyme that had been sedimented at 27,000 g.

The purpose of this experiment was to try to select a centrifugation force which would best discriminate between peroxisomes, chloroplasts and mitochondria. It is clearly not possible to separate the peroxisomes from the mitochondria by this approach. However, one may remove a great deal of chloroplasts from a peroxisomal preparation by differential centrifugation. A force of 1000 g applied for 10 min sedimented more than 50% of the chloroplasts but only 15% of the peroxisomes and 10% of the mitochondria. To minimize the losses of peroxisomes a force of 650 g was selected for routine work.

A second differential centrifugation step has often been employed to concentrate the peroxisomes to be placed on a sucrose gradient. It was found that most of the remaining chloroplasts could be removed by increasing the sucrose concentration to about 40% (w/w) after the first centrifugation and then centrifuging at 27,000 g for 60 min to sediment the peroxisomes. When these peroxisomes were subjected to density gradient centrifugation they were obtained relatively free of chlorophyll but the yields were very low.

2. Grinding Procedures.

An attempt was made to develop grinding procedures which would give better yields of peroxisomes from spinach leaves. Homogenization in the blender results in the breakage of varying amounts (60% to 90%) of the peroxisomes, assuming that the marker enzymes such as catalase and glycolate oxidase are exclusively confined to the peroxisomes in the cell. Spinach leaves were carefully sliced with a french cookery knife, chopped with razor blades, or shredded in a Mouli parsley grater. Using such methods the percentage yields were greatly improved; up to 60% of the peroxisomes could be recovered. However, the absolute yields were no better because fewer cells were being broken, and the chopping procedures were tedious. Thus, the blender remains the method of choice for processing moderate or large amounts of spinach (100 g to 2.5 Kg). Good yields of peroxisomes may also be obtained from small amounts of leaves using a mortar and pestle.

In addition to trying different mechanical techniques, the standard sucrose and glycyl-glycine grinding medium was varied. When the sucrose was replaced by sorbitol in the grinding medium and also in the density gradient, good yields were obtained but the separation of

peroxisomes from chloroplasts and mitochondria was poor. A medium, containing mannitol, EDTA, bovine serum albumin and cysteine, which has been used to isolate mitochondria from plants (13) was tried. Compared to the usual grinding medium, the yields from this more complex medium were poor.

The only medium tested which resulted in better separation and yields was that used by Beever's group to isolate glyoxysomes from castor bean endosperm (20) (Figure 2). This medium contained sucrose, tricine buffer, KCl, $MgCl_2$, EDTA, and dithiothreitol. The separation of the chloroplasts (represented by NADH-diaphorase) (5), from the other organelles was better when this medium was used (Figure 2B). The amount of catalase and glycolate oxidase in the peroxisome fraction was somewhat higher. There was less soluble catalase, but this could have been due to inhibition. The specific activities of the peroxisomal enzymes were also higher while that of the contaminating species, the diaphorase, was less (Table I). However, the amount of soluble glycolate oxidase in this gradient could not be determined because dithiothreitol interfered with the assay. Furthermore, something in the grinding medium destroyed most of the cytochrome c oxidase activity. Excluding the dithiothreitol from the

FIGURE 2. Isolation of spinach leaf organelles from different grinding media. Forty g of spinach were chopped in the parsley grater with 80 ml of 16% (w/w) sucrose, 0.02 M glycyl-glycine, pH 7.5 (Medium A). Another 40 g of spinach were chopped with 80 ml of 16% (w/w) sucrose, 0.165 M Tricine, 0.01 M KCl, 0.01 M $MgCl_2$, 0.01M EDTA, 0.01 M dithiothreitol, pH 7.5 (Medium B). The resulting homogenates were filtered through cheese cloth, the pellets, obtained between 480 g (10 min) and 12,000 g (20 min), were each resuspended in the respective grinding media to make 5 ml and layered on a discontinuous gradient. (5 ml of 56%, 13 ml of 51.5% and 49%, 10 ml of 43%, and 5 ml of 38% w/w sucrose in 0.02 M glycyl-glycine; no EDTA was included in the gradient). The gradients were centrifuged for 3 hr at 25,000 rpm in the SW 25.2 rotor.

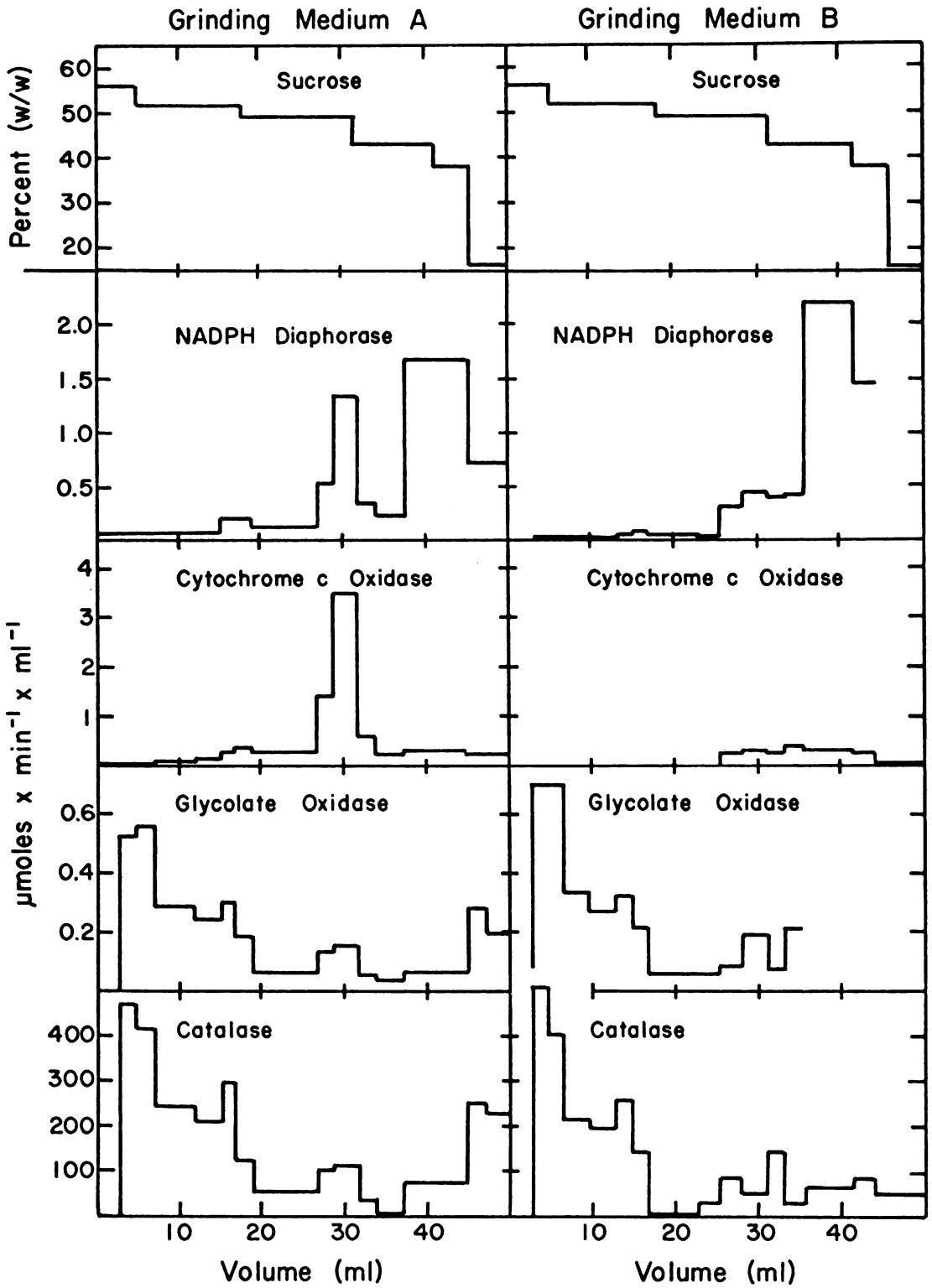

Grinding Medium A
Grinding Medium B
Sucrose
Sucrose
Percent (w/w)
60
50
40
30
20
NADPH Diaphorase
NADPH Diaphorase
2.0
1.5
1.0
0.5
Cytochrome c Oxidase
Cytochrome c Oxidase
4
3
2
1
µmoles x min⁻¹ x ml⁻¹
Glycolate Oxidase
Glycolate Oxidase
0.6
0.4
0.2
Catalase
Catalase
400
300
200
100
10
20
30
40
Volume (ml)
Volume (ml)

TABLE I. Specific activities of marker enzymes in peroxisomes from spinach leaves homogenized in two different media. Peroxisomes were isolated as shown in Figure 2.

Grinding Medium	Catalase	Glycolate Oxidase	NADPH-Diaphorase
	mmole x min^{-1} x mg $protein^{-1}$	nmoles x min^{-1} x mg $protein^{-1}$	
A. Sucrose-glycyl-glycine	1.37	733	184
B. Beever's Medium	1.59	943	123

grinding medium did not result in higher cytochrome c oxidase activity. For these reasons Beever's grinding medium was not used for the routine preparation of organelles from spinach leaves.

3. Density Gradient Flotation of Spinach Peroxisomes and Mitochondria.

Density gradient flotation has been used to further purify glyoxysomes (41) and liver peroxisomes (81). The method was applied to spinach organelles as follows: The peroxisomal fractions and mitochondrial fractions were taken from sucrose density gradients similar to those shown in Figure 2. The sucrose concentration was increased to 55% for the peroxisomal fraction and to 51% for the mitochondrial fraction by adding 66% sucrose. The organelle suspension was then placed in the bottom of an ultracentrifuge tube, a linear gradient was constructed over it, and it was centrifuged at 25,000 rpm overnight. The peroxisomes were recovered (50% to 60% recovery) in a white band just above the 55% sucrose; all of the visible contaminating chlorophyll floated to the top of the gradient. The specific activities of the catalase and glycolate oxidase were consistently increased by at least half. However, the specific activity of cytochrome

c oxidase in the mitochondrial fraction was doubled but much of the chlorophyll remained in the mitochondrial band.

4. Breakage of Spinach Peroxisomes.

Unlike their counterparts from rat liver, spinach peroxisomes from density gradients are easily broken by dilution with water. Dilution with one or two volumes of water (or dialysis, see Table XI) resulted in 80% to 90% breakage of the peroxisomes. Centrifugation of a broken peroxisome suspension at 12,000 g for 10 min sedimented most of the contaminating chlorophyll and cytochrome c oxidase, leaving the soluble peroxisomal enzymes such as catalase and glycolate oxidase in the supernatant. The specific activities of these enzymes were higher in such a supernatant than in the original peroxisome fraction. It is not known to what extent the contaminating mitochondria were broken by this procedure, since a soluble mitochondrial marker was not assayed. If the peroxisomal membranes behave at all like the outer mitochondrial membranes they should have remained in the supernatant of the 12,000 g centrifugation (90). This supernatant was centrifuged at 144,000 g for an hour or more to sediment the membranes and the membranes were analyzed

for enzyme content (Table XI).

B. Zonal Sucrose Density Gradient Centrifugation

The zonal type rotor offered several advantages over the swinging bucket type. Because of the large capacity (the B-30 contains 560 ml; the B-29, 1450 ml) several hundred ml of tissue homogenate could be processed and it was not necessary to pellet the particles. Also, gradients having very short sedimentation distances could be used in the peripheral part of the rotor. This technique was especially successful in the fractionation of rat liver homogenates.

1. Rat Liver.

Usually the low speed supernatant of a homogenate from three rat livers was fractionated on a small (180 ml) gradient in the B-30 rotor (see part II. A. 3.). It was not necessary to pellet the peroxisomes. Because the gradient was only about 1 cm deep the centrifugation time was very short (35 min). This was a rapid procedure, producing reasonably pure particles (Table VII, page 73).

It was found that higher speeds (50,000 rpm) in the zonal rotor and the use of EDTA in the gradient caused the liver peroxisomes to sediment with the mitochondria. Re-isolation of the peroxisomes from a high speed gradient

revealed that at least 50% were broken, whereas 97% of the peroxisomes were recovered when re-isolated from gradients centrifuged at 30,000 rpm. High centrifugal force or hydrostatic pressure probably damaged the liver peroxisomes (23).

2. Castor Bean Endosperm.

Large quantities of castor bean endosperm can be fractionated by zonal centrifugation without prior differential centrifugation. This method was used to prepare quantities of glyoxysomes sufficient for lipid analysis. The homogenate was pumped directly into the rotor containing a suitable gradient. It was not possible to resolve the organelles from this tissue in a 35 min centrifugation time as it was in the case of rat liver. At least two hours were required to obtain equilibrium.

Table II contains the results of zonal sucrose density gradient centrifugation of castor bean endosperm. The results are comparable to those obtained in the swinging bucket rotor (Table VI, page 66). The amount of contaminating mitochondria (cytochrome c oxidase) and etioplasts (triose phosphate isomerase) in the glyoxysomes was slightly higher. No significant amount of particulate acid phosphatase was observed, that is, no lysosomes were

TABLE II. Specific activities of marker enzymes in castor bean endosperm fractions from a zonal sucrose density gradient. The homogenate from 74 g of endosperm (126 ml) was pumped into the zonal rotor containing a gradient of 20 ml each of 15%, 20%, 25%, 30%, 32.5%, 35%, 37.5%, 40%, 42.5%, 44%, 45%, 46%, 47%, 48%, 48.5%, 49%, 49.5%, 50%, 51.5%, and 50 ml of 56% (w/w) sucrose in 10 mM EDTA, pH 7.5. Centrifugation was at 30,000 rpm for 3 hr. Fractions of 10 ml were collected from the edge of the rotor. Ratios are relative to the maximum specific activity for that particular enzyme.

	Catalase		Acid Phosphatase	
Fraction	mmoles x min^{-1} x mg $protein^{-1}$	Ratio	nmoles x min^{-1} x mg $protein^{-1}$	Ratio
Glyoxysomes	7.87	1.000	22	0.066
Etioplasts	1.96	0.250	30	0.090
Mitochondria	0.23	0.029	8	0.024
Microsomes	0.25	0.032	86	0.260
Supernatant	0.13	0.017	335	1.000

	Cytochrome c Oxidase		Triose Phosphate Isomerase	
Fraction	nmoles x min^{-1} x mg $protein^{-1}$	Ratio	nmoles x min^{-1} x mg $protein^{-1}$	Ratio
Glyoxysomes	100	0.024	1180	0.160
Etioplasts	1600	0.390	7200	1.000
Mitochondria	4150	1.000	1420	0.200
Microsomes	35	0.008	1650	0.230
Supernatant	4	0.001	1260	0.180

detected in the gradient.

3. Spinach.

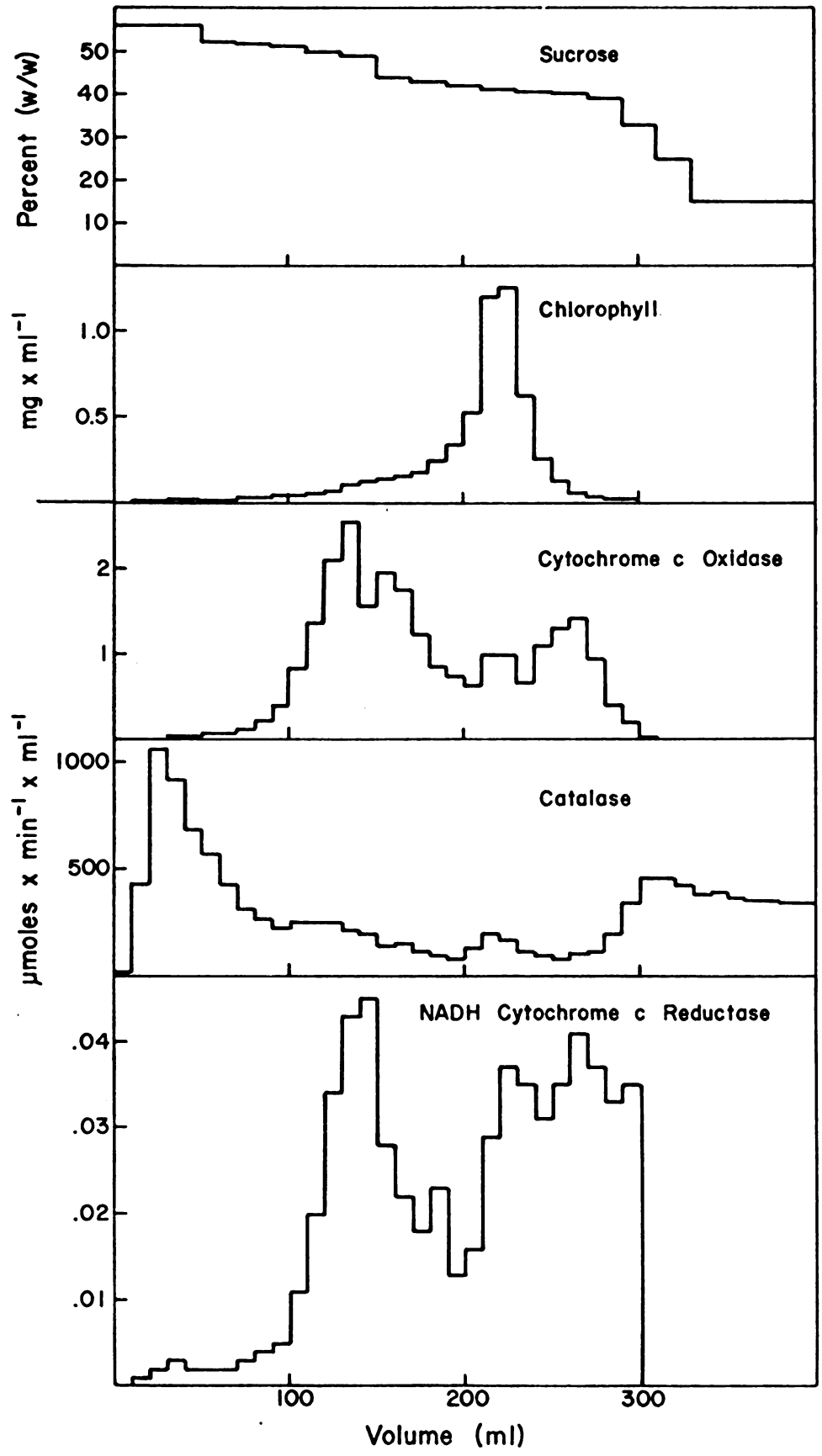
It was especially important to eliminate the necessity of pelleting spinach peroxisomes since they are very fragile and many were broken when pelleted. Often after pelleting in the second step of differential centrifugation only about 10% of the total catalase or glycolate oxidase in the original homogenate was recovered in the peroxisomal fractions from a density gradient. However, 35% recovery was routinely obtained when the peroxisomes were not pelleted. Thus, the zonal rotor was extremely valuable in the isolation of spinach peroxisomes.

It was possible to process large amounts of spinach in the B-29 (1450 ml) rotor. The 650 g supernatant from 630 g of deribbed leaves, which amounted to 950 ml, could be put over a 500 ml gradient in this rotor. After centrifuging long enough to move the peroxisomes into the gradient (about 30 min at 33,000 rpm) the supernatant could be pumped off and a new batch of spinach juice put on. Using this technique, as much as 2.5 kg of spinach have been processed.

Standard peroxisomal preparations were made from

200 g of spinach leaves in the B-30 (560 ml) zonal rotor, as described in the methods section (part II. A. 1.). The typical distribution of marker enzymes in such a zonal sucrose density gradient is shown in Figure 3. Catalase was the peroxisomal marker. About 35% of the total catalase on the gradient was found in a prominant peak in the most dense region of the gradient (56% to 52% sucrose). The rest of the catalase activity was distributed over the gradient with a large amount in the supernatant. Most of the solubilized catalase probably represented broken peroxisomes since other peroxisomal markers such as glycolate oxidase were solubilized to a similar extent. Frederick and Newcomb (37) used a cytochemical stain for catalase to demonstrate that catalase was confined to the peroxisomes of tobacco leaves. The low catalase level in the other organelle fractions such as the small peak of catalase with the chloroplasts was thought to be artifactitious. This may have been the result of some peroxisomes being trapped in the dense chloroplast band. Most of the chlorophyll, probably broken chloroplasts, accumulated between 40% and 41% sucrose. Approximately 60% of the cytochrome c oxidase, the mitochondrial marker, was located in a peak in the 42% to 49% sucrose region of the gradient. The estimated

FIGURE 3. Distribution of subcellular organelles from spinach leaves on a sucrose density gradient. The gradient was constructed as indicated by the sucrose percentages. The organelles detected were the broken chloroplasts (chlorophyll) which peaked at 230 ml, the peroxisomes (catalase) at 30 ml, the mitochondria (cytochrome c oxidase and NADH-cytochrome c reductase) at 140 ml, and the microsomes (NADH-cytochrome c reductase and cytochrome c oxidase) at 270 ml. Above 330 ml was the supernatant which extended to 530 ml. The specific activities of the peak fractions are given in Table IV.


Sucrose
Percent (w/w)
50
40
30
20
10
Chlorophyll
mg x ml⁻¹
1.0
0.5
Cytochrome c Oxidase
2
1
Catalase
µmoles x min⁻¹ x ml⁻¹
1000
500
NADH Cytochrome c Reductase
.04
.03
.02
.01
100
200
300
Volume (ml)

densities of the peak fractions are given in Table III.

The specific activities of the marker enzymes in the peak fractions are given in Table IV, page 51, along with the ratios of the specific activities in the other organelle fractions relative to the maximum specific activity. These specific activity ratios are an indication of the amount of cross contamination (17). For example, the specific activity of the cytochrome c oxidase in the peroxisomes was less than 5% of that in the mitochondria. That is, less than 5% of the protein in the peroxisomal fraction was mitochondrial contamination. However, the actual specific activity of the mitochondrial cytochrome c oxidase was probably higher since the mitochondria were contaminated with chloroplasts. Therefore, the actual mitochondrial contamination in the peroxisomes was probably lower than estimated. The chlorophyll data indicate than more than 30% of the protein in the mitochondrial fraction was whole chloroplasts. Although, only 2% of the total chlorophyll on the gradient was in the peroxisomal fraction, this amounted to 20% contamination on a protein basis.

TABLE III. Densities of subcellular organelles from various tissues in sucrose density gradients. Densities in g x cm^{-3} at 10^{o}. Determined from the refractive indices of the peak fractions from sucrose density gradients.

	Spinach	Castor Bean Endosperm	Sunflower Cotyledon	Rat Liver	Dog Kidney
Microbodies	1.27	1.247	1.270	1.239	1.240
Etioplasts		1.212	1.223		
Mitochondria	1.22	1.191	1.185	1.204	1.187
Chloroplasts, Whole	1.21		1.22		
Chloroplasts, Broken	1.18		1.158		
Microsomes	1.17	1.118	1.150	1.146	1.143
Lysosomes				1.117[a]	1.136

[a]With Triton W-1339.

TABLE IV. Specific activities of marker enzymes in spinach leaf fractions. The overall distribution of the marker enzymes is shown in Figure 3, (Page 48). Ratios are relative to the maximum specific activity for that particular enzyme.

Fraction	Catalase		Cytochrome c Oxidase	
	μmoles x min^{-1} x mg $protein^{-1}$	Ratio	nmoles x min^{-1} x mg $protein^{-1}$	Ratio
Peroxisomes	3040	1.000	85	0.045
Mitochondria	141	0.047	1670	1.000
Chloroplasts	28	0.009	159	0.086
Microsomes	38	0.012	514	0.280

Fraction	Chlorophyll		NADH-Cytochrome c Reductase, pH 7.3		
	μg x x mg $protein^{-1}$	Ratio	nmoles x min^{-1} x mg $protein^{-1}$	Ratio	Antimycin A Inhibition
Peroxisomes	43	0.210	5.9	0.210	0%
Mitochondria	66	0.320	28.0	1.000	71%
Chloroplasts	205	1.000	6.0	0.210	
Microsomes	20	0.098	15.0	0.540	10%

CHAPTER IV

RESULTS -- MEMBRANE BOUND ENZYMES

In view of the observed continuity between the membranes of developing microbodies and the ER and the biochemical similarities of the ER to the membranes of other organelles, the possibility of biochemical similarities between the peroxisomal membranes and microsomes were examined. Part IV presents evidence for such enzymatic similarities; similarities in lipid composition are reported in Part V.

A. NADH-Cytochrome c Reductase in the Various Organelle Fractions

1. Spinach.

The distribution of NADH-cytochrome c reductase on the gradient shown in Figure 3 was similar to that of cytochrome c oxidase. In addition to the mitochondrial peak, a significant amount of both activities was located between 33% and 40% sucrose. These low density particles containing cytochrome c reductase were probably microsomes. The occurrence of cytochrome c oxidase with

these particles was consistently obtained in gradients of spinach particles; however it was not observed in other tissues. This oxidase activity, like that of the mitochondria, was completely inhibited by cyanide.

A small peak of the NADH-cytochrome c reductase was usually found with the peroxisome peak, but there was no corresponding peak of cytochrome c oxidase. The specific activity of cytochrome c reductase in the peroxisomes was more than could be accounted for by mitochondrial contamination. As discussed on page 49, mitochondria represented no more than 5% of the protein in the peroxisome fraction while the specific activity of the NADH-cytochrome c reductase in the peroxisomes was 20% of that in the mitochondria (Table IV, page 51).

The NADH-cytochrome c reductase in the microsome fraction was relatively insensitive to antimycin A (Table IV, page 51) as should be expected (64). The mitochondrial cytochrome c reductase was inhibited 71% or more by antimycin A. The residual activity probably was largely due to that of the outer membrane (29, 90, 95). The mitochondrial NADH-cytochrome c reductase, which was inhibited by antimycin A, was probably the component of the electron transport system located in the mitochondrial inner membrane, while the peroxisomal activity was probably

FIGURE 4. PH optima of the NADH-cytochrome c reductases in spinach peroxisomes and mitochondria. Glutamate buffer was used at pH 8.6 and above; phosphate buffer was used at pH 8.6 and below.

Mitochondria
nmoles x min⁻¹ x ml⁻¹
200
150
MITOCHONDRIA
PEROXISOMES
1.5
1.0
0.5
Peroxisomes
nmoles x min⁻¹ x ml⁻¹
6
7
8
9
pH

similar to that of the microsomes or the outer mitochondrial membrane. The peroxisomal cytochrome c reductase was also not inhibited by antimycin A. The pH optimum of the mitochondrial reductase was pH 6.7 and that of the peroxisomal enzyme was pH 8.6 (Figure 4).

2. Sunflower Cotyledons.

The distribution of NADH-cytochrome c reductase, cytochrome c oxidase, and other markers in density gradient fractions obtained from sunflower cotyledons is shown in Figure 5. The position of the microbody fraction (peroxisomes and glyoxysomes) was established by the catalase activity, by glyoxysomal markers such as isocitrate lyase (not shown) and by other peroxisomal markers such as glycolate oxidase (not shown) (91). The microbodies from sunflower and spinach were somewhat more dense in sucrose gradients than were microbodies from other tissues (Table III, page 50). The sunflower microbody fraction contained no chloroplasts but did contain a large proportion of etioplasts as indicated by the triose phosphate isomerase specific activity (Table V). The mitochondrial fraction also contained a significant amount of etioplasts.

The microbody fraction was usually accompanied by

FIGURE 5. Distribution of subcellular organelles from sunflower cotyledons on a sucrose density gradient. Ten g of cotyledons were obtained from seedlings which had been germinated for four days in the dark and two days in the light. The organelles represented are the microbodies (catalase), etioplasts (triose phosphate isomerase), mitochondria (cytochrome c oxidase), chloroplasts (chlorophyll), and the microsomes (NADH-cytochrome c reductase, pH 7.3). Specific activities are given in Table V.

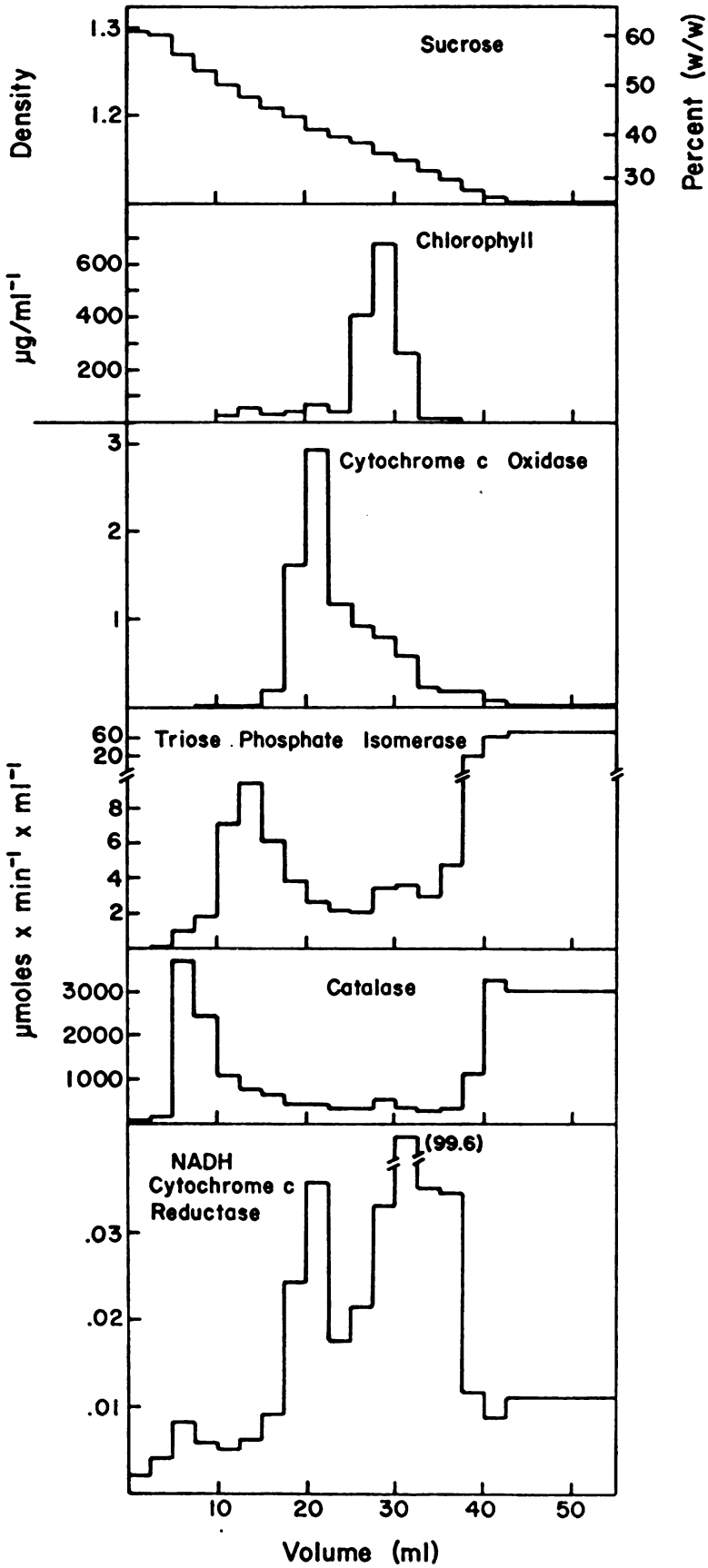

Density
1.3
1.2
Sucrose
60
50
40
30
Percent (w/w)
µg/ml⁻¹
600
400
200
Chlorophyll
3
2
1
Cytochrome c Oxidase
60
20
8
6
4
2
Triose Phosphate Isomerase
µmoles x min⁻¹ x ml⁻¹
3000
2000
1000
Catalase
(99.6)
NADH
Cytochrome c
Reductase
.03
.02
.01
10
20
30
40
50
Volume (ml)

TABLE V. Specific activities of marker enzymes in organelle fractions from sunflower cotyledons. The overall distribution of the marker enzymes is shown in Figure 5. Ratios are relative to the maximum specific activity for that particular enzyme.

Fraction	Catalase: mmoles x min^{-1} x mg protein^{-1}	Catalase: Ratio	Cytochrome c Oxidase: nmoles x min^{-1} x mg protein^{-1}	Cytochrome c Oxidase: Ratio
Microbodies	9.00	1.000	6	0.003
Etioplasts	0.73	0.081	31	0.071
Mitochondria	0.31	0.034	1860	1.000
Chloroplasts	0.09	0.009	670	0.360
Microsomes	0.12	0.015	442	0.240

Fraction	Chlorophyll: µg x x mg protein^{-1}	Chlorophyll: Ratio	Triose Phosphate Isomerase: nmoles x min^{-1} x mg protein^{-1}	Triose Phosphate Isomerase: Ratio
Microbodies	40	0.000	2449	0.320
Etioplasts	37	0.310	7820	1.000
Mitochondria	48	0.400	1920	0.250
Chloroplasts	119	1.000	602	0.077
Microsomes	96	0.800	1290	0.170

Fraction	NADH-Cytochrome c Reductase, pH 7.3: nmoles x min^{-1} x mg protein^{-1}	NADH-Cytochrome c Reductase, pH 7.3: Ratio	Antimycin A[a] Inhibition
Microbodies	20.0	0.560	9.7%
Etioplasts	10.3	0.320	
Mitochondria	24.8	0.690	41.0%
Chloroplasts	5.7	0.160	
Microsomes	35.8	1.000	20.0%

a. From a different experiment.

a peak or shoulder of cytochrome c reductase activity independent of cytochrome c oxidase. The specific activity ratio of the cytochrome c oxidase in the microbody fraction was consistently less than 0.008, indicating a very low level of mitochondrial contamination (Table V). However, the specific activity ratio for the NADH-cytochrome c reductase was always much larger (0.560), indicating that the reductase activity was not the result of mitochondrial contamination. Furthermore, the microbody reductase activity was less sensitive than the mitochondrial enzyme to antimycin A (Table V). The pH optima of the two cytochrome c reductases were also quite different; the microbody reductase functioned best at pH 7.6 while the mitochondrial enzyme was most active at pH 5.5 (Figure 6). Thus, it has been concluded that the microbodies of sunflower cotyledons along with those from spinach leaves contain a NADH-cytochrome c reductase which is distinct from that in the mitochondria.

3. Castor Bean Endosperm.

The sucrose gradient distribution of subcellular components from castor bean endosperm (Figure 7) was similar to the sunflower gradients. In addition to catalase, which is shown, several other glyoxysomal

FIGURE 6. PH optima for microbody and mitochondrial NADH-cytochrome c reductases from sunflower cotyledons. Phosphate buffer was used throughout.

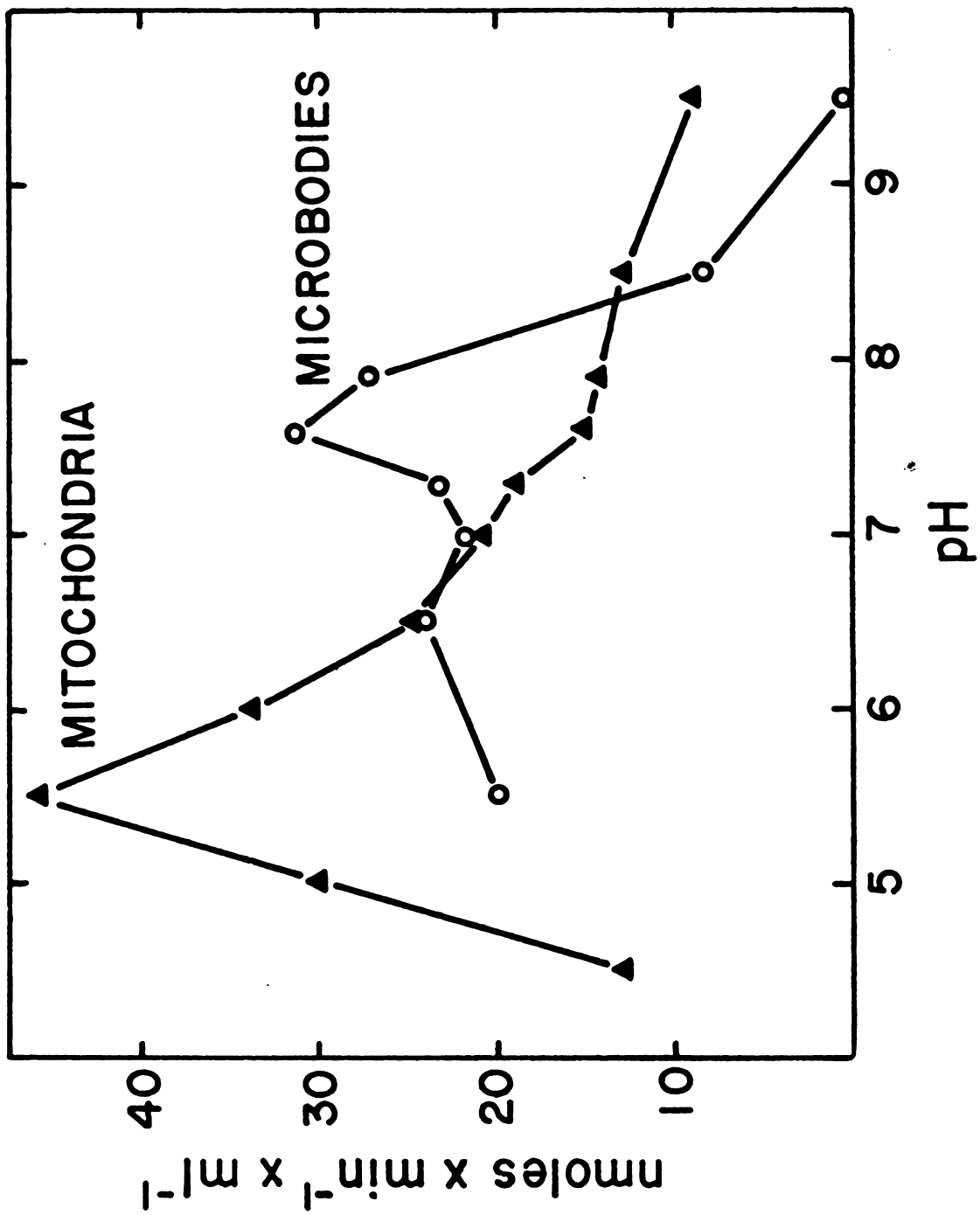
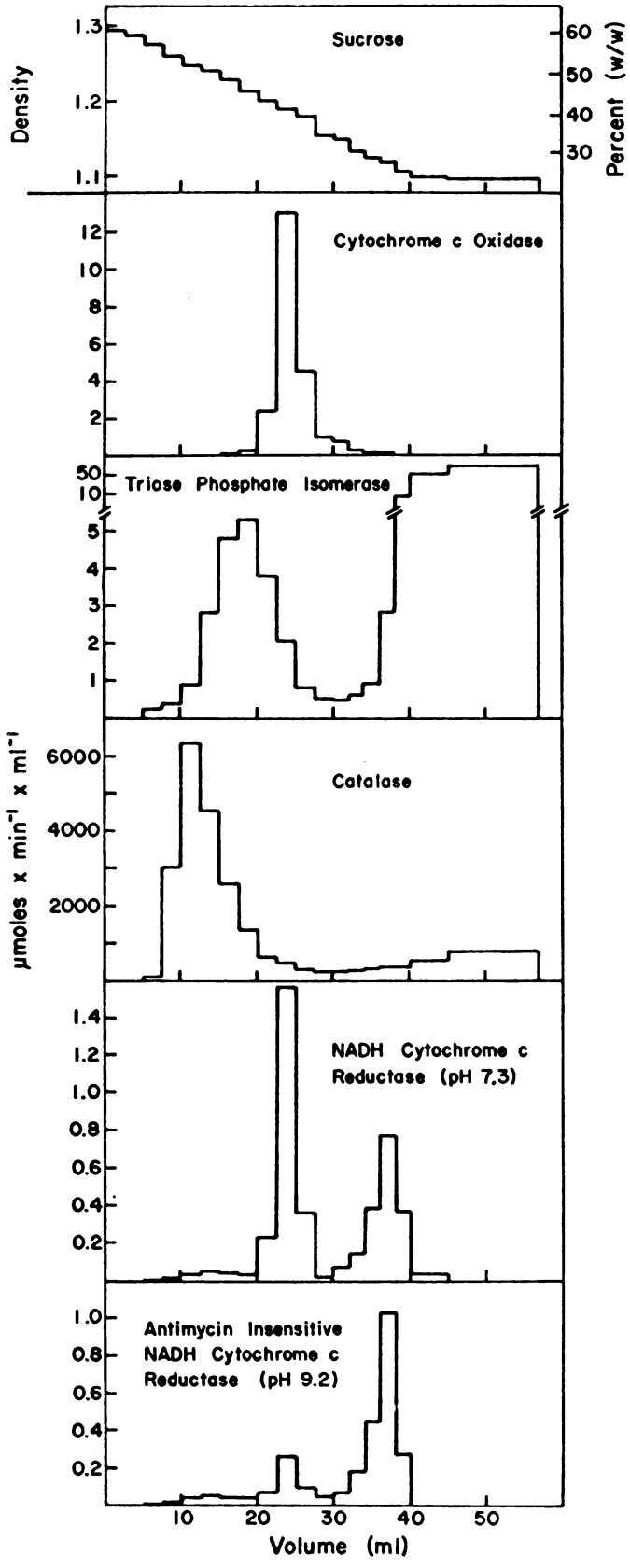

MITOCHONDRIA
MICROBODIES
nmoles x min⁻¹ x ml⁻¹
10
20
30
40
5
6
7
8
9
pH

FIGURE 7. Distribution of subcellular organelles from castor bean endosperm on a sucrose density gradient. The gradient contained 10 mM EDTA. The organelles are glyoxysomes (catalase), etioplasts (triose phosphate isomerase), mitochondria (cytochrome c oxidase and NADH-cytochrome c reductase, pH 7.3), and microsomes (antimycin A insensitive NADH-cytochrome c reductase, pH 9.2) See Table VI for the specific activities of the peak fractions.


Density
Percent (w/w)
Sucrose
Cytochrome c Oxidase
Triose Phosphate Isomerase
Catalase
µmoles x min⁻¹ x ml⁻¹
NADH Cytochrome c Reductase (pH 7.3)
Antimycin Insensitive NADH Cytochrome c Reductase (pH 9.2)
Volume (ml)

marker enzymes such as malate synthetase, citrate synthetase, isocitrate lyase, and glycolate oxidase were assayed and had the same distribution on the gradient (14, 20). Etioplasts, represented by triose phosphate isomerase, were clearly the most serious contaminants of the glyoxysomes. This tissue has no chloroplasts. The specific activity ratio (Table VI) indicates that more than 10% of the protein in the glyoxysomal fraction was etioplast contamination.

While the prominent fraction for the NADH-cytochrome c reductase was normally the mitochondria, when antimycin A was included in the assay the microsomes became the prominent fraction (Figure 7). A small peak of NADH-cytochrome c reductase was associated with the microbodies (the glyoxysomes) as was the case in the spinach and sunflower gradients. Since the specific activity ratio of the glyoxysomal NADH-cytochrome c reductase was much higher than the ratio for the cytochrome c oxidase, the NADH-cytochrome c reductase found in the glyoxysomes could not have been due to mitochondrial contamination (Table VI). The glyoxysomal NADH-cytochrome c reductase was insensitive to antimycin A and had a pH optimum of 9.2 (Figure 8) while the enzyme in the mitochondria was inhibited 87% by antimycin A and

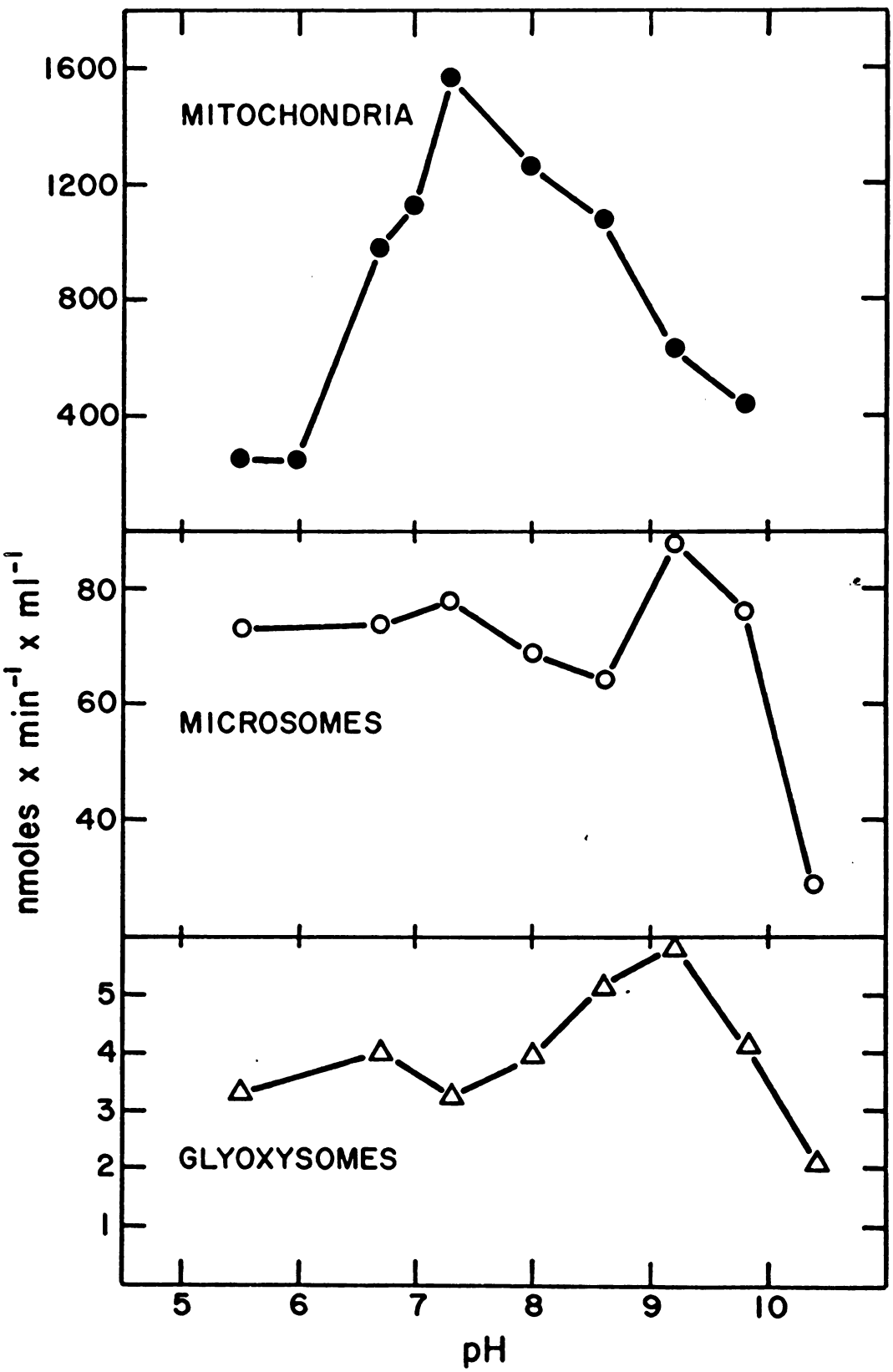
TABLE VI. Specific activities of marker enzymes in organelle fractions from castor bean endosperm. Overall distribution on the sucrose density gradient is shown in Figure 7. Ratios are relative to the maximum specific activity for that particular enzyme.

Fraction	Catalase: mmoles x min^{-1} x mg protein^{-1}	Catalase: Ratio	Cytochrome c Oxidase: nmoles x min^{-1} x mg protein^{-1}	Cytochrome c Oxidase: Ratio
Glyoxysomes	5.10	1.000	27	0.006
Etioplasts	1.56	0.310	250	0.058
Mitochondria	0.16	0.031	4300	1.000
Microsomes	0.11	0.022	53	0.012

Fraction	Triose Phosphate Isomerase: nmoles x min^{-1} x mg protein^{-1}	Triose Phosphate Isomerase: Ratio	NADH-Cytochrome c Reductase, pH 7.3: nmoles x min^{-1} x mg protein^{-1}	NADH-Cytochrome c Reductase, pH 7.3: Ratio	NADH-Cytochrome c Reductase, pH 7.3: Antimycin A Inhibition
Glyoxysomes	735	0.120	26	0.050	0%
Etioplasts	5983	1.000	38	0.073	0%
Mitochondria	680	0.110	520	1.000	87%
Microsomes	881	0.150	244	0.470	0%

Fraction	Antimycin Insensitive NADH-Cytochrome c Reductase, pH 9.2: nmoles x min^{-1} x mg protein^{-1}	Antimycin Insensitive NADH-Cytochrome c Reductase, pH 9.2: Ratio
Glyoxysomes	35	0.110
Etioplasts	49	0.150
Mitochondria	88	0.270
Microsomes	320	1.000

FIGURE 8. PH optima for glyoxysomal, mitochondrial, and microsomal NADH-cytochrome c reductases from castor bean endosperm. Glutamate buffer was used at pH 8.6 and above; phosphate buffer was used at pH 8.6 and below.


MITOCHONDRIA
MICROSOMES
GLYOXYSOMES
nmoles x min⁻¹ x ml⁻¹
pH
1600
1200
800
400
80
60
40
5
4
3
2
1
5
6
7
8
9
10

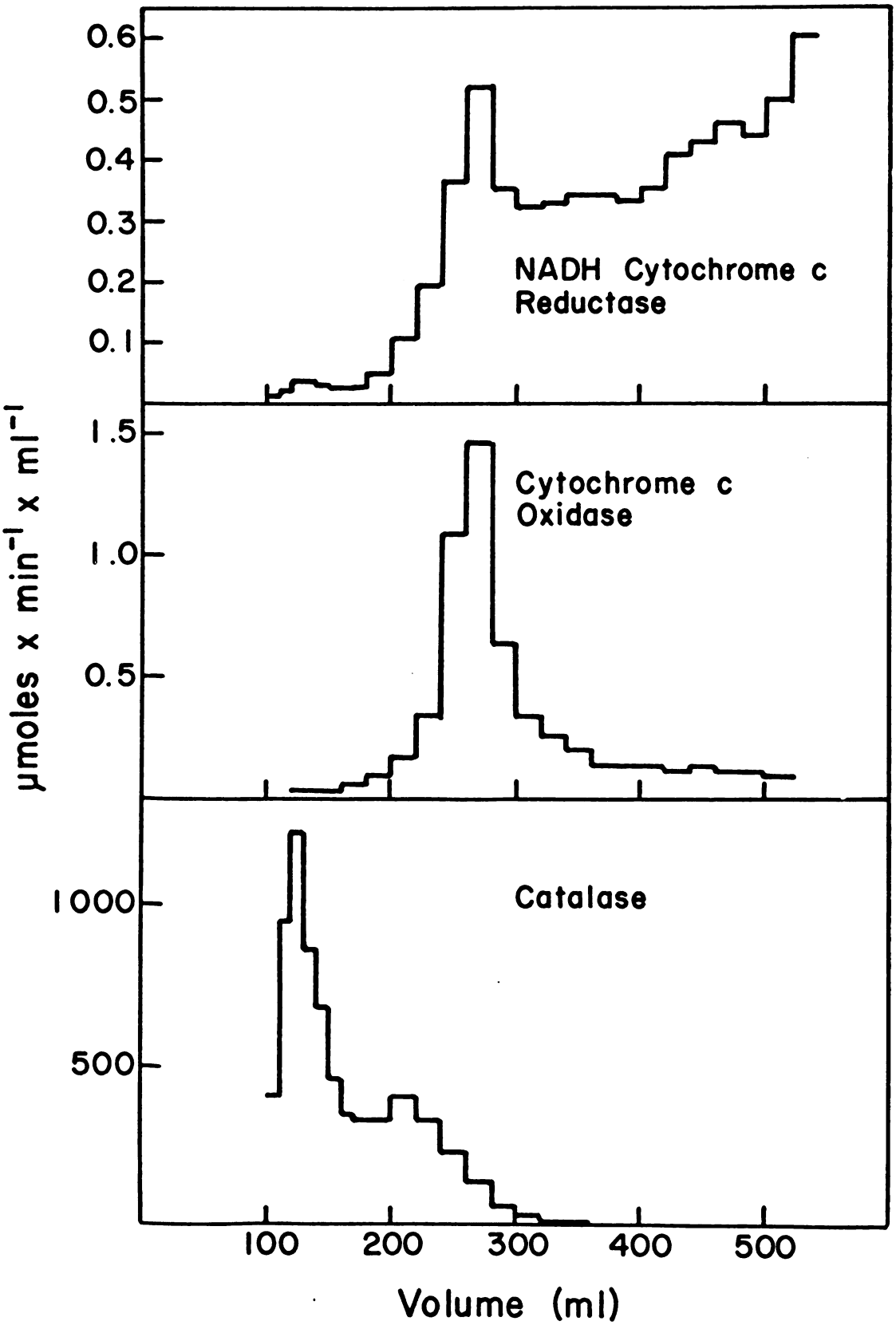
was most active at pH 7.3. The glyoxysomal enzyme was similar to the microsomal activity which was also insensitive to antimycin A and had a pH optimum of 9.2.

It is possible that the NADH-cytochrome c reductase in the glyoxysomes was the result of microsomal contamination. Microsomes with attached ribosomes may be almost as dense as glyoxysomes or microsomes may stick to the glyoxysomal membranes. However, the presence of 10 mM EDTA in the grinding medium and in the gradient should have removed ribosomes from the microsomes and prevented microsomes from adhering to the glyoxysomes.

4. Rat Liver.

The distribution of NADH-cytochrome c reductase among rat liver particles is shown in Figure 9 for an atypical gradient (atypical because it was not centrifuged long enough to reach equilibrium density in the sucrose gradient). Under these conditions a small peak of cytochrome c reductase was observed in coincidence with the catalase peak, the peroxisomes. When centrifuged to equilibrium the cytochrome c reductase peak was obscured by the mitochondrial activity that moved deeper into the gradient. However, the specific activity of the reductase in the peroxisomes was almost as high as in the

FIGURE 9. Distribution of subcellular organelles from rat liver on a zonal sucrose density gradient. The homogenate from two livers was filtered through cheese cloth and centrifuged at 3000 g for 7 min. The 50 ml of supernatant was pumped onto a discontinuous gradient in the B-30 zonal rotor (20 ml each of 20%, 21%, 22%, 23%, 24%, 25%, 29%, 30%, 31%, 32%, 33%, 37%, 38%, 39%, 40%, 46%, 46.5%, 47%, 47.5%, 48%, and 80 ml of 55% w/w sucrose in 0.02 M glycyl-glycine, pH 7.5). After centrifugation at 35,000 rpm for 60 min, the fractions were collected as indicated.


0.6
0.5
0.4
0.3
0.2
0.1
NADH Cytochrome c
Reductase
1.5
1.0
0.5
Cytochrome c
Oxidase
1000
500
Catalase
µmoles x min⁻¹ x ml⁻¹
100
200
300
400
500
Volume (ml)

mitochondria, indicating that it was not mitochondrial contamination (Table VII). The specific activity ratio of cytochrome c oxidase in the peroxisomes was 0.051, indicating that mitochondrial contamination was very low.

The NADH-cytochrome c reductase in the peroxisomes was not inhibited by antimycin A (Table VII) or rotenone. The mitochondrial activity was also insensitive to the inhibitors under normal assay conditions. However, if an aliquot of the mitochondrial fraction was pre-incubated with an equal volume of rotenone solution (0.2% in ethanol) for 1 min at 25°, a much greater inhibition, 56%, was obtained compared to an ethanol control. The peroxisomal cytochrome c reductase was not sensitive to the inhibitors under any conditions.

Although it was possible to distinguish the microbody cytochrome c reductase from the mitochondrial form in plants on the basis of pH optima, there was no distinguishing pH optimum in any of the rat liver fractions. The peroxisomes, mitochondria, and microsomes from rat liver all had nearly constant cytochrome c reductase activity from pH 6.0 to pH 9.2 with no distinct pH optima. Similar results have been obtained with a purified antimycin A insensitive NADH-cytochrome from

TABLE VII. Specific activities of marker enzymes in subcellular fractions from rat liver. Three rat livers were homogenized, and centrifuged at 650 g for 7 min. The supernatant was placed on a small (180 ml) gradient in the B-30 zonal rotor. This was centrifuged at 30,000 rpm for 35 min. The fraction volume was 5 ml. Ratios are relative to the maximum specific activity for that particular enzyme.

	Catalase		Acid Phosphatase	
Fraction	μmoles x min^{-1} x mg $protein^{-1}$	Ratio	nmoles x min^{-1} x mg $protein^{-1}$	Ratio
Peroxisomes	7770	1.000	40	0.190
Mitochondria	2740	0.360	37	0.180
Microsomes	106	0.014	157	0.760
Lysosomes	191	0.025	208	1.000
Supernatant	264	0.034	51	0.250

	Cytochrome c Oxidase		NADH-Cytochrome c Reductase, pH 8.0		
Fraction	nmoles x min^{-1} x mg $protein^{-1}$	Ratio	nmoles x min^{-1} x mg $protein^{-1}$	Ratio	Antimycin A Inhibition
Peroxisomes	128	0.051	308	0.210	0%
Mitochondria	2500	1.000	383	0.211	10%
Microsomes	370	0.150	1470	1.000	0%
Lysosomes	290	0.120	730	0.495	
Supernatant	40	0.016	0	0.000	

rabbit liver microsomes (97).

It was necessary to show that the NADH-cytochrome c reductase activity in the peroxisomes was not merely some flavin oxidase, such as L-α-hydroxy acid oxidase, behaving as a cytochrome reductase. Flavin enzymes such as xanthine oxidase, nitrate reductase, and yeast lactate dehydrogenase are known to exhibit cytochrome c reductase activity (26, 46, 77). Glycolate oxidase and urate oxidase activities were not inhibited or were stimulated when the peroxisomes were treated with digitonin or triton X-100 (Table VIII). On the other hand NADH-cytochrome c reductase of the rat liver peroxisomal fraction was severely inhibited by detergent treatment. These results suggest that the peroxisomal NADH-cytochrome c reductase was a component of the limiting membrane, which was disrupted by the detergents.

To exclude microsomal contamination as the source of NADH-cytochrome c reductase in the liver peroxisomes, attempts were made to separate the peroxisomes from possible contaminating species. The peroxisomal suspension was diluted in either 0.5 volume of water, 0.5 volume of 0.3 M KCl, or 1 volume of 10 mM EDTA, and the peroxisomes re-isolated on density gradients. The peroxisomes diluted in EDTA were reisolated on a gradient containing

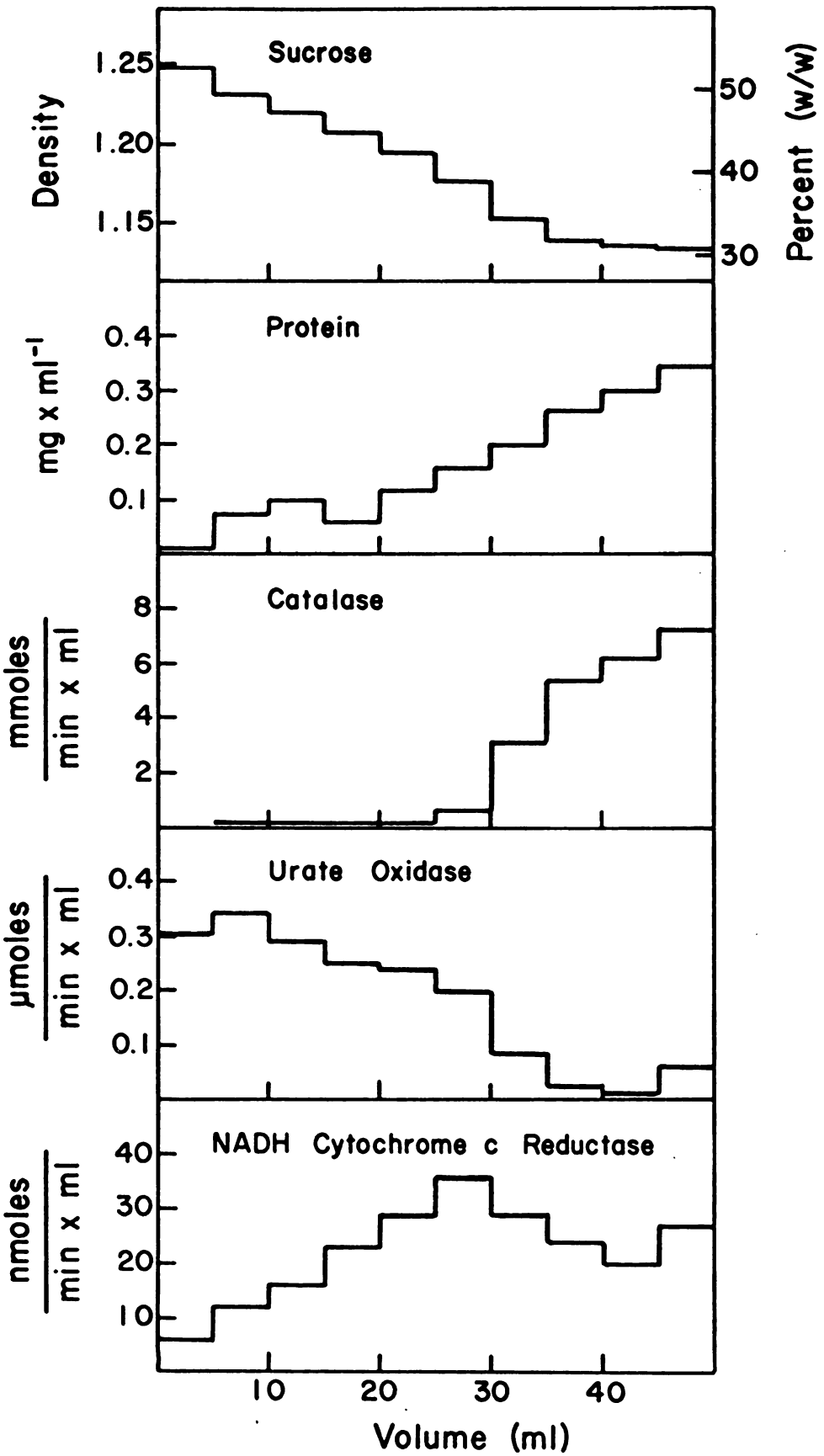
TABLE VIII. The effects of detergents on the NADH-cytochrome c reductase and oxidase activities of rat liver peroxisomes. An aliquot of the peroxisome fraction was incubated with an equal volume of 1% digitonin or 0.5% Triton X-100 for one min at room temperature. Then the usual assay mixture was added.

	Control	Digitonin	Triton
	nmoles x min^{-1} x ml^{-1}		
NADH-Cytochrome c Reductase	40	7	11
Glycolate Oxidase	49	44	55
Urate Oxidase	208	357	325

10 mM EDTA. The KCl or EDTA should have removed any adherent microsomes from the peroxisomes or removed the ribosomes from heavy microsomes. Some of the peroxisomes were broken by the KCl and the EDTA, however the peroxisomes which remained intact and went to the proper density in the gradient were still accompanied by a proportional amount of NADH-cytochrome c reductase. This was a part of the peroxisomes and not the result of microsomal contamination.

If the cytochrome c reductase were located in the limiting membrane of the peroxisome it should be possible to separate it from the other peroxisomal components. This is demonstrated in Figure 10. The peroxisomes were diluted in alkaline pyrophosphate which is known to break rat liver peroxisomes (59, 81). The broken peroxisomes were then fractionated on a sucrose gradient. Most of the catalase and protein was solubilized. The urate oxidase, which is located in the core, went to a density of 1.232. The NADH-cytochrome c reductase went to a density of 1.173, about what would be expected of a membrane. This density was slightly greater than that of rat liver microsomes (Table III, page 50) although it is not known what affect pyrophosphate might have on the density of microsomes. The results support the idea

FIGURE 10. Isolation of rat liver peroxisomal membranes on a sucrose density gradient. One ml of a peroxisome suspension was diluted with one ml of 0.01 M pyrophosphate; the final pH was about 8.5. After being stored overnight at 4°, the suspension of broken peroxisomes was put on a sucrose gradient: 0.5 ml each of 56%, 49%, 47%, 45%, 42%, 38% and 30% sucrose (w/w). This was centrifuged for 60 min at 39,000 rpm in the SW-39 rotor. Fractions of 0.5 ml were collected from the bottom of the tube. Several visible bands were observed.


Sucrose
Density
1.25
1.20
1.15
Percent (w/w)
50
40
30
Protein
mg x ml⁻¹
0.4
0.3
0.2
0.1
Catalase
mmoles / min x ml
8
6
4
2
Urate Oxidase
µmoles / min x ml
0.4
0.3
0.2
0.1
NADH Cytochrome c Reductase
nmoles / min x ml
40
30
20
10
10
20
30
40
Volume (ml)

that NADH-cytochrome c reductase is located in the limiting membrane of peroxisomes.

5. Dog Kidney.

The dogs were not treated with Triton W-1339. Nevertheless, the bulk of the lysosomes were located at a density of 1.136 (Figure 11), which is considerably less than lysosomes from untreated rat liver (81). A significant amount of acid phosphatase was also found at higher densities and amounted to significant contamination in the peroxisomal fraction (Table IX). As previously reported, no urate oxidase activity was found in the kidney peroxisomes (24).

Once again a small peak of NADH-cytochrome c reductase was observed with the peroxisomes. The specific activity ratios of cytochrome c reductase, when compared to cytochrome c oxidase, show that the reductase was not all mitochondrial contamination. The antimycin A inhibition data provoke the same conclusion. The small amount of inhibition in the peroxisomes may have been due to some mitochondrial contamination.

FIGURE 11. Distribution of subcellular organelles from dog kidney on a zonal sucrose density gradient. Twenty-seven g of medulla was homogenized as described for rat liver except that it was first mashed in a mortar with the grinding medium. The zonal rotor contained 87 ml of homogenate, 25 ml each of 15%, 20%, 25%, 30%, 33%, 36%, 38%, 40%, 42%, 44%, 46%, 46.5%, 47%, 47.5%, 48%, and 180 ml of 56% sucrose (w/w). After centrifuging at 30,000 rpm for about 2 hr, 10 ml fractions were collected. Specific activities are given in Table IX.

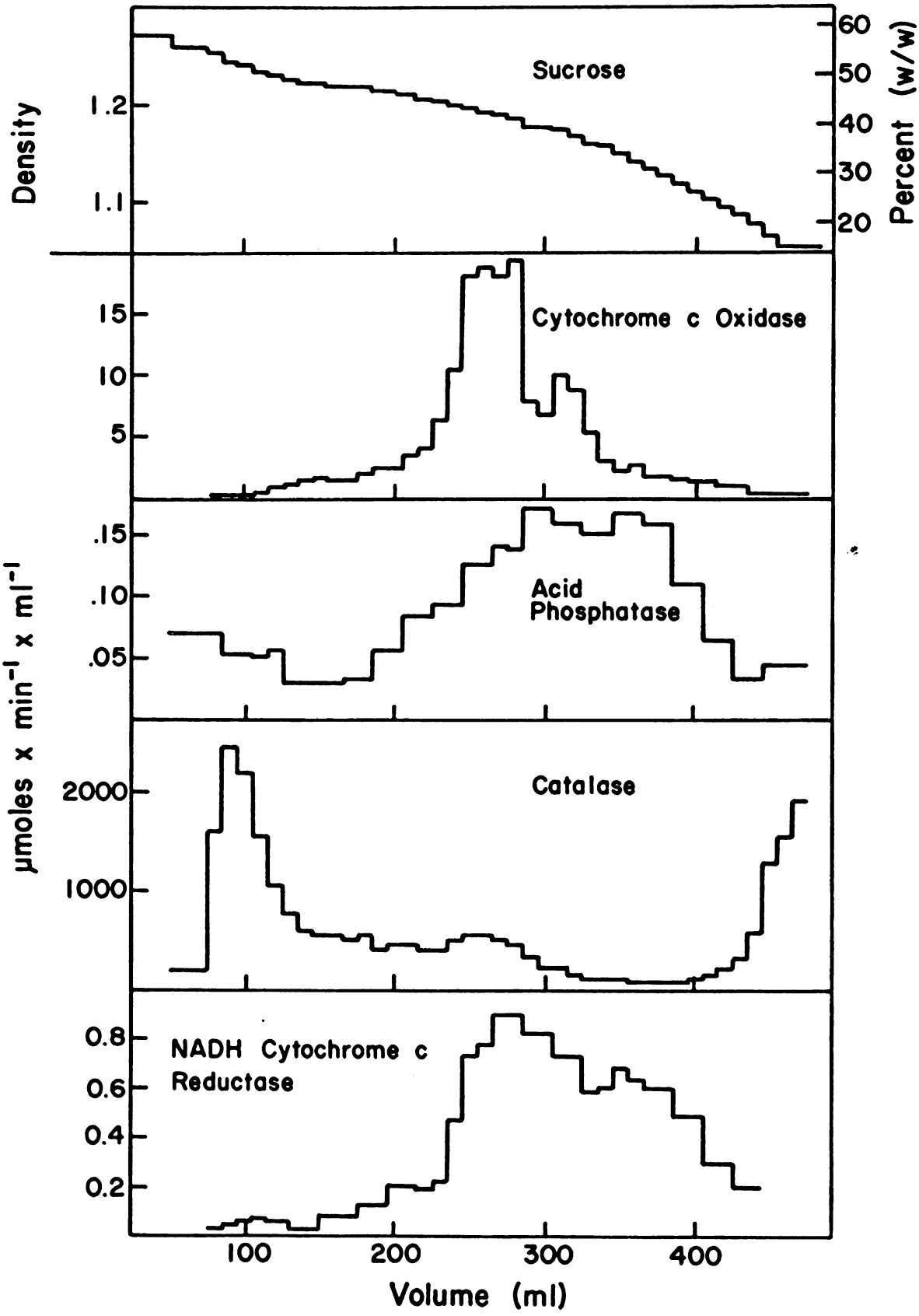

Sucrose
Density
Percent (w/w)
1.2
1.1
60
50
40
30
20
Cytochrome c Oxidase
15
10
5
Acid
Phosphatase
.15
.10
.05
Catalase
2000
1000
NADH Cytochrome c
Reductase
0.8
0.6
0.4
0.2
µmoles x min⁻¹ x ml⁻¹
100
200
300
400
Volume (ml)

TABLE IX. Specific activities of marker enzymes in subcellular fractions from dog kidney. See figure 11 for details. Ratios are relative to the maximum specific activity for that particular enzyme.

Fraction	Catalase: μmoles x min^{-1} x mg protein^{-1}	Catalase: Ratio	Acid Phosphatase: nmoles x min^{-1} x mg protein^{-1}	Acid Phosphatase: Ratio
Peroxisomes	2960	1.00	64	0.66
Mitochondria	78	0.03	24	0.25
Microsomes	53	0.02	80	0.82
Lysosomes	63	0.02	97	1.00
Supernatant	208	0.07	5	0.05

Fraction	Cytochrome c Oxidase: nmoles x min^{-1} x mg protein^{-1}	Cytochrome c Oxidase: Ratio	NADH-Cytochrome c Reductase, pH 8: nmoles x min^{-1} x mg protein^{-1}	NADH-Cytochrome c Reductase, pH 8: Ratio	NADH-Cytochrome c Reductase, pH 8: Antimycin A Inhibition
Peroxisomes	179	0.06	55	0.17	12%
Mitochondria	3260	1.00	155	0.45	66%
Microsomes	1050	0.32	330	1.00	4%
Lysosomes	1210	0.37			
Supernatant	33	0.01	20	0.60	

B. Other Microsomal Enzymes in the Various Organelle Fractions

Since the microsomal enzyme, NADH-cytochrome c reductase, had been found in peroxisomal membranes, other enzymes known to be in microsomes were examined. If the limiting membrane of the peroxisome were analogous to the outer mitochondrial membrane one would expect NADPH-cytochrome c reductase and glucose-6-phosphatase to be absent. On the other hand, if the peroxisomal membrane were derived from the endoplasmic reticulum in a more direct manner than the outer mitochondrial membrane, all of the microsomal components may be present. In any case NADH-cytochrome b_5 reductase should be present since the NADH-cytochrome c reductase activity is probably a manifestation of this enzyme (see the electron transport sequence on page 6).

The specific activities of NADH- and NADPH-cytochrome c reductases, NADH-cytochrome b_5 reductase and glucose-6-phosphatase in the various organelle fractions from rat liver, dog kidney, and castor bean are shown in Table X. The specific activity ratios of the NADPH-cytochrome c reductase in all the microbody fractions were consistently less than the NADH-cytochrome c reductase. In rat liver and castor bean microbodies the differences

TABLE X. Specific activities of microsomal enzymes in subcellular fractions from rat liver, dog kidney, and castor bean endosperm. Specific activity (S.A.) is nmole x min^{-1} x mg $protein^{-1}$. Ratios are relative to the maximum specific activity, designated 1.00 for each series.

	NADH-Cytochrome c Reductase, pH 8.0		NADPH-Cytochrome c Reductase		NADH-Cytochrome b_5 Reductase		Glucose-6-Phosphatase	
	S.A.	Ratio	S.A.	Ratio	S.A.	Ratio	S.A.	Ratio
RAT LIVER								
Peroxisomes	308	0.21	10	0.14	5.2	0.24	39	0.35
Mitochondria	383	0.26	11	0.15	20.8	0.95	48	0.43
Microsomes	1470	1.00	74	1.00	21.8	1.00	112	1.00
Lysosomes	730	0.50	20	0.27			65	0.58
DOG KIDNEY								
Peroxisomes	55	0.17	3.1	0.16	4.5	0.29	36	0.13
Mitochondria	155	0.45	5.6	0.29	11.4	0.75	51	0.19
Microsomes	330	1.00	19.1	1.00	15.2	1.00	270	1.00
Supernatant	20	0.06	1.7	0.09			25	0.09
CASTOR BEAN								
Glyoxysomes	30	0.15	1.7	0.09	1.0	0.18	30	0.40
Etioplasts	43	0.22	3.4	0.19			24	0.32
Mitochondria	260	1.30	3.5	0.19	5.5	1.00	4	0.06
Microsomes	198	1.00	18.0	1.00	0.00	0.00	52	0.70
Supernatant	0	0.00	0.0	0.00			74	1.00

may be significant enough to suggest that these microbody membranes are different from the microsomes.

The specific activities of the NADH-cytochrome b_5 reductase in all fractions were much less than those of the NADH-cytochrome c reductase. One might have expected the cytochrome b_5 reductase activity to be as high as the NADH-cytochrome c reductase activity since presumably they both involve the same flavoprotein. However, the membrane bound flavoprotein, the reductase, may not transfer electrons to the soluble cytochrome b_5 used in the assay system as easily as it does to membrane bound b_5 and thence to cytochrome c.

The assay for glucose-6-phosphatase was not very sensitive. To obtain measurable phosphate release it was necessary to incubate 0.7 ml of the enzymes with the substrate for 10 min. The highest glucose-6-phosphatase activity from castor bean was found in the supernatant, making it difficult to judge the meaning of the activity in the glyoxysomes. The specific activity ratio of this enzyme in the dog kidney peroxisomes was lower than the NADH-cytochrome c reductase while in rat liver peroxisomes it was higher.

In conclusion, the specific activity ratios for the NADH-cytochrome b_5 reductase in the rat liver and

dog kidney peroxisomes suggest that this enzyme is a component of the peroxisomal membrane. NADPH-cytochrome c reductase may not be a component of the peroxisomal membrane. It is difficult to conclude anything about the relationship of the peroxisomal membrane to the microsomes from the glucose-6-phosphatase activities.

C. Subfractionation of Spinach Leaf Peroxisomes

Spinach peroxisomes were subfractionated by osmotic shock and recentrifugation to see if any of the known peroxisomal enzymes were located in the limiting membrane. The technique used to subfractionate spinach peroxisomes has been introduced in Part III. A. 4., page 41, and details are given in Table XI. The low speed pellet consisted mostly of contaminating species, chloroplasts and mitochondria. The high speed pellet contained most of the NADH-cytochrome c reductase, as well as the highest specific activity for this enzyme. Thus, this fraction was thought to have contained the peroxisomal membranes. Most of the peroxisomal enzymes (catalase, glycolate oxidase, hydroxypyruvate reductase, and the three aminotransferases) were completely solubilized and never had significant activity in either pellet, neither in terms of percent nor specific activity. Like the other

TABLE XI. Subfractionation of spinach leaf peroxisomes. The peroxisomal fractions obtained from 630 of spinach on a sucrose gradient were combined and dialyzed against 0.005 M Tris, pH 8.3, and 25% glycerol. The dialysate was centrifuged for 10 min at 12,000 g. The resulting pellet was resuspended in 25% glycerol and recentrifuged at 12,000 g to give the "low speed pellet." The combined supernatants were centrifuged at 144,000 g for 60 min yielding the "high speed supernatant" and the "high speed pellet."

	Distribution			Specific Activity		
	Low Speed Pellet	High Speed Supernatant	High Speed Pellet	Low Speed Pellet	High Speed Supernatant	High Speed Pellet
	Percent of Total Activity			nmoles x min^{-1} x mg $protein^{-1}$		
Protein	20	62	18			
Catalase	13	77	10	2,430,000	4,600,000	2,100,000
Glycolate Oxidase	17	70	13	1,040	1,250	790
Hydroxypyruvate Reductase		96	4		38,000	5,200
Malate Dehydrogenase		96	4		69,000	10,000
Serine-Pyruvate Aminotransferase	2	93	5	15	182	33
Aspartate Aminotransferase	2	93	5	18	228	43
Glutamate-Glyoxylate Aminotransferase	2	95	3	123	2580	279
NADH-Cytochrome c Reductase, pH 7.3	37	0	63	7	0	13

peroxisomal enzymes, NADPH-isocitrate dehydrogenase was completely solubilized in similar experiments. Malate dehydrogenase, under certain conditions (eg., when the peroxisomes were broken by diluting in water), had the highest specific activity in the membrane fraction, the high speed pellet. However this was not reproducible. Malate dehydrogenase, along with aspartate aminotransferase, is known to adhere to membranes under certain conditions (80, 86). Usually all of the peroxisomal enzymes were found in the high speed supernatant; their percentages and their specific activities were highest in this fraction. Thus, it appears that all of the known peroxisomal enzymes except the NADH-cytochrome c reductase are located in the matrix rather than in the limiting membrane. A certain amount of the catalase and glycolate oxidase (10% to 20%) often remained insoluble, even after several washings. This may represent a crystalloid core similar to the urate oxidase core of rat liver peroxisomes. Indeed, histochemical staining for catalase shows that catalase is located in the cores of glyoxysomes and leaf peroxisomes (37, 110).

CHAPTER V

RESULTS -- PHOSPHOLIPID COMPOSITION OF MICROBODIES

A. Rat Liver

The phospholipid compositions of rat liver microsomes and mitochondria determined here (Table XII) were very similar to those reported in the literature (29, 49, 50). The microsomes had the highest proportion of phospholipid. The major phospholipids of the microsomes were phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol. The slight preponderance of phosphatidyl inositol over phosphatidyl ethanolamine is contrary to published data (29). This may have been because the phosphatidyl inositol spot also contained any sphingomyelin present.

The mitochondria were distinguished by the presence of cardiolipin which is known to be a component of the inner membrane. The proportion of phosphatidyl ethanolamine in the mitochondria was somewhat greater than in the microsomes.

The amount of phospholipid in the peroxisomes

TABLE XII. Phospholipid composition of rat liver peroxisomes, mitochondria and microsomes.

	Peroxisomes	Mitochondria	Microsomes
mg phospholipid x mg protein^{-1}	0.086	0.20	0.32
	Percent of Total Phospholipid		
Phosphatidyl choline	55.1	44.5	49.8
Phosphatidyl ethanolamine	16.0	28.1	18.8
Phosphatidyl inositol and Sphingomyelin	19.7	7.1	19.7
Phosphatidyl serine	7.4	1.9	8.5
Cardiolipin	1.6	18.4	3.1

relative to protein was much less than in the microsomes or mitochondria. This is reasonable since the peroxisomal membranes, in contrast to the mitochondrial membranes, consititute only a small proportion of the total peroxisomal protein -- peroxisomes have no internal membranes. The phospholipid distribution in the peroxisomes is strikingly like that of the microsomes. The amount of the mitochondrial phospholipid, cardiolipin, in the peroxisomes was very small.

B. Castor Bean Endosperm

The phospholipid compositions of the castor bean fractions were similar to those of liver, although the microsomes had a lower amount of total phospholipid (Table XIII). As in liver, the major phospholipids of the microsomes were phosphatidyl choline, phosphatidyl inositol and phosphatidyl ethanolamine. These microsomes had somewhat less phosphatidyl serine than the rat liver microsomes.

There was relatively more cardiolipin in the castor bean mitochondria than in the glyoxysomes or microsomes. Cardiolipin has not been previously reported in plant mitochondria although it has been observed among the lipids of whole plants (11, 78) and it has been

TABLE XIII. Phospholipid composition of glyoxysomes, mitochondria and microsomes from castor bean endosperm.

	Glyoxysomes	Mitochondria	Microsomes
mg lipid x (mg lipid + mg protein)$^{-1}$	0.095	0.242	0.181
mg phospholipid x mg protein^{-1}	0.031	0.220	0.126
	Percent of Total Phospholipid		
Phosphatidyl choline	49.0	36.9	50.0
Phosphatidyl ethanolamine	31.4	30.9	26.6
Phosphatidyl inositol and Sphingomyelin	6.1	14.3	18.9
Cardiolipin	2.4	13.7	2.7
Phosphatidyl serine	0.0	4.1	1.8
Unidentified[a]	11.4	0.2	0.0

[a]This spot ran between cardiolipin and monogalactosyl diglyceride standards.

reported in yeast mitochondria (111). Phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol have been reported in plant mitochondria but not quantitated (68, 75). Benson has suggested that plant mitochondria may contain phosphatidyl glycerol rather than cardiolipin (10). No phosphatidyl glycerol was detected in any castor bean fractions.

The phospholipid composition of the glyoxysomes was similar to the microsomes but did not parallel that of the microsomes as closely as it did in rat liver. Both fractions had a large amount of phosphatidyl choline but the glyoxysomes had less phosphatidyl inositol. In addition, the glyoxysomes were distinguished by the presence of an unidentified lipid not present in the other fractions.

C. Spinach

The analysis of spinach phospholipids was complicated by the presence of pigments and by the very limited amounts of peroxisomes obtained even from kilogram quantities of spinach. Also, spinach leaves contain galactolipids which would have been difficult to quantitate in the small amounts of peroxisomes available. Phosphatidyl choline and phosophatidyl ethanolamine were

the most abundant phospholipids in the microsomes and mitochondria. Phosphatidyl glycerol was the major phospholipid in the chloroplasts as has been previously reported (1, 10, 75) although the galactolipids (monogalactosyl diglyceride, digalactosyl diglyceride and the sulfolipid) were probably much more abundant than any of the phospholipids. As in castor bean and animal mitochondria, there was more cardiolipin in the spinach mitochondria than in any of the other organelles. Phosphatidyl serine was usually observed in spinach microsomes but not in the other organelles. The major phospholipids of spinach peroxisomal fractions were phosphatidyl choline and phosphatidyl ethanolamine. Phosphatidyl glycerol and cardiolipin were less prominent components which may have represented chloroplast and mitochondrial contamination, respectively. Galactolipids were observed in all fractions although these may have come from chloroplast contamination.

CHAPTER VI

DISCUSSION

A. NADH-Cytochrome c Reductase in Peroxisomes and Glyoxysomes

The microbodies of every tissue examined contained NADH-cytochrome c reductase. The presence of this enzyme was clearly not the result of mitochondrial contamination nor did it appear to be microsomal contamination. Compared to the amount of contaminating cytochrome c oxidase (the mitochondrial marker), the reductase activity was too high to be mitochondrial. The NADH-cytochrome c reductase in the microbodies was insensitive to antimycin A whereas the mitochondrial activity was not. Furthermore, in plants the pH optimum of the microbody reductase was consistently 2 pH units above the mitochondrial optimum (Figures 4, 6, and 8).

It is possible that the NADH-cytochrome c reductase in the peroxisome fractions was the result of microsomal contamination. However, it is difficult to imagine that all the peroxisome fractions obtained from

the various tissues under various conditions contained coincident microsomal contamination. "Rough microsomes" (that is microsomes bearing ribosomes) should not have sedimented to form a peak at high density in the centrifugation times used for rat liver preparations, 35 to 60 min. Also, the 10 mM EDTA present in the sunflower and castor bean gradients should have removed the ribosomes from the microsomes and prevented microsomes from adhering to the microbodies. Moreover, it was not possible to remove NADH-cytochrome c reductase from rat liver peroxisomes by washing with 5 mM EDTA or 0.2 M KCl. The presence of NADH-cytochrome c reductase in the microbodies was not likely to have been the result of microsomal contamination.

The only way found to remove the NADH-cytochrome c reductase from rat liver peroxisomes was to break them. The reductase then behaved in a sucrose gradient as if it were attached to a membrane (Figure 10). This was probably the limiting membrane of the peroxisomes.

The behavior the NADH-cytochrome c reductase from broken peroxisomes in a gradient indicated that it was not the activity of some flavin oxidase present in the matrix of the peroxisomes. Further proof of this is the fact that the cytochrome c reductase was disrupted by the

action of detergents while the flavin oxidase was not (Table VIII).

NADH-cytochrome c reductase appears to be a component of the limiting membrane of the microbody. The presence of this enzyme in the microbody membrane implies a relationship to the endoplasmic reticulum.

B. Plant Microsomes and Endoplasmic Reticulum

Microsomes from plants have not been previously isolated on sucrose density gradients. Using the antimycin A insensitive NADH-cytochrome c reductase as the marker enzyme, microsomes from spinach leaves, sunflower cotyledons, and castor bean endosperm have been located in sucrose density gradients (Figures 3, 5, and 7). The densities of these particles, which range from 1.12 to 1.17, were similar to animal microsomes (Table IV).

Microsomes have previously been isolated from nonphotosynthetic tissues such as swiss chard midrib and cauliflower by differential centrifugation (22, 64). These microsomes contained antimycin A insensitive NADH-cytochrome c reductase, NADPH-cytochrome c reductase, and cytochrome b_3. Microsomes from animal tissues usually contain cytochrome b_5 instead of b_3.

The enzymatic analysis of castor bean microsomes

reported here can be related to the findings in other plant tissues. No cytochrome b_5 reductase was found in castor bean microsomes because they probably have a cytochrome b_3 reductase instead. Microsomes from castor bean did contain NADPH-cytochrome c reductase. They may have had glucose-6-phosphatase although the specific activity of this enzyme was about 25% higher in the supernatant (Table X). Glucose-6-phosphatase has been reported in microsomes from germinating bean cotyledons, but it becomes more soluble after 2.5 days of germination (101). Similarly much of the glucose-6-phosphatase was soluble in castor bean endosperm which had been germinated for 5 days.

The presence of cytochrome c oxidase in spinach leaf microsomes was an enigma. It is not known whether this represented the inner membranes of broken mitochondria or an actual component of the endoplasmic reticulum. This cytochrome c oxidase was like that in the mitochondria in that it was completely inhibited by cyanide.

The phospholipid composition of plant microsomes was found to be similar to their animal counterparts. Phosphatidyl serine, which is a distinguishing minor component of animal microsomes (29) was observed in spinach microsomes but was almost absent from castor

bean microsomes. Phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol were the major phospholipids of spinach and castor bean microsomes just as they are in animals.

Some of the functions of the endoplasmic reticulum in plant cells can be deduced from morphological observations in plant tissues and by analogies to animals. Protein synthesis clearly takes place on the endoplasmic reticulum in plants since ribosomes and polysomes are associated with certain regions of it. By analogy to animal cells, lipid synthesis probably takes place in the plant ER also. Animal liver microsomes have components, such as cytochrome P-450, which are involved in drug oxidations. These may be unimportant in plants, which are autotrophic. Nevertheless, cytochrome P-450 has been reported in plant microsomes (16).

Morphological observations indicate that the plant ER, like the aminal ER, is involved in the production of organelles such as microbodies.

C. The Relationship of the Microbody Membrane to the Endoplasmic Reticulum

Morphological and biochemical evidence indicate that there are derivative relationships among the various subcellular organelles (see Part I). The likely

relationships are outlined in Figure 12. The nuclear membrane and the outer mitochondrial membranes have much in common with the ER. They have similar lipid compositions and share common enzymatic components. The Golgi membranes may be derived from the ER only in part. The transition involves changes in lipid and enzymatic components. Some of the contents of the lysosomes, zymogen granules, and secretory vesicles may also be produced by the ER. Finally, the membranes of the zymogen granules and secretory vesicles may be incorporated into the plasma membrane (74).

Morphological observations in plant and animal tissues indicate that microbodies are derived directly from the ER. In castor bean endosperm, bodies containing catalase have been seen attached directly to the ER (110). The continuity of microbody membrane with the ER has also been reported in the liver of fetal and adult rats and mice (110, 47). Microbodies also appear to be derived from the rough ER in insect fat body (61). The contents of the microbodies, catalase for example, may also be derived from the ER (45, 58).

The biochemical evidence reported herein also indicates that the microbody membrane is derived directly from the ER. The microbody membrane contains

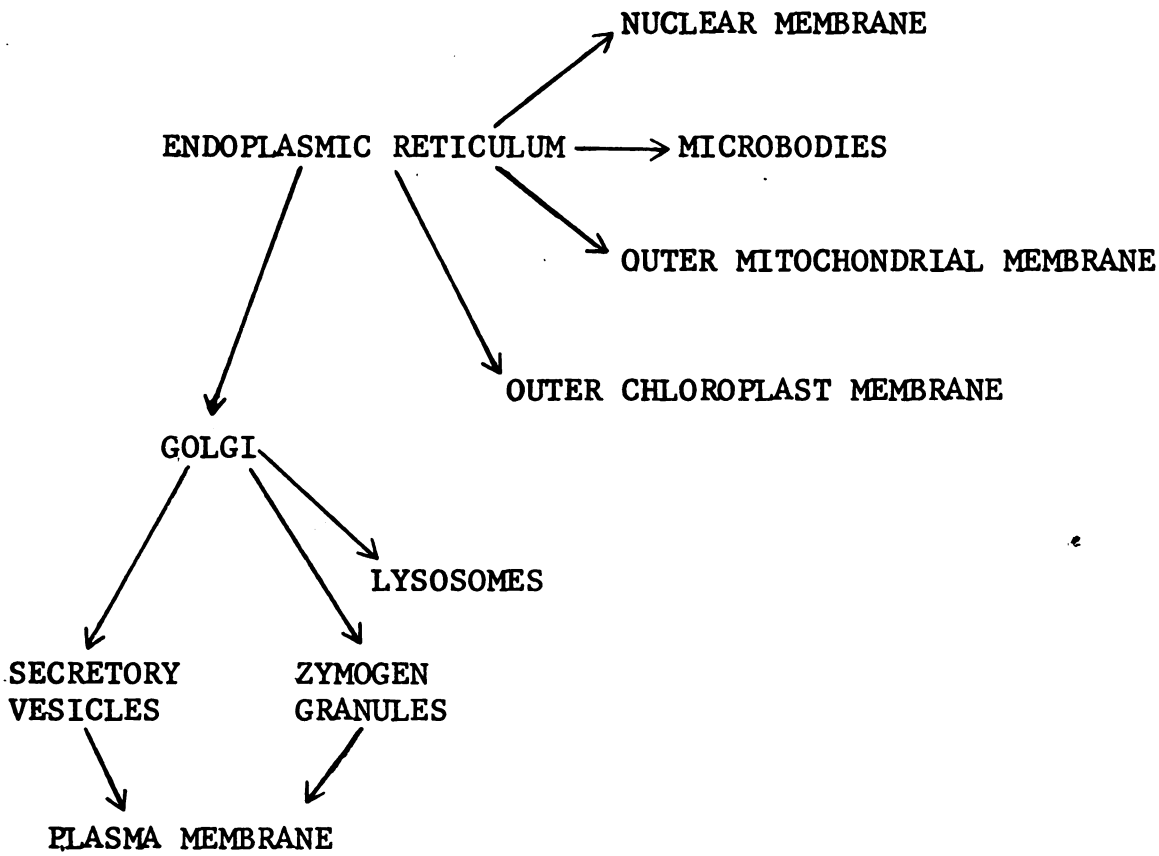


FIGURE 12. Suggested geneology of some subcellular components. See references (25, 74).

NADH-cytochrome c reductase, a microsomal enzyme. Also, the phospholipid composition of the microbody membrane is very similar to that of the ER. Likewise, other membranes such as the outer mitochondrial membrane and the Golgi membrane share common components with the ER. But perhaps in no case is the morphological and biochemical evidence so strongly in favor of direct production from the ER as in the case of the microgody membrane.

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