ENZYMOLOGICAL AND FINE STRUCTURAL STUDIES ON MICROBODIES AND MITOCHONDRIA OF *EUGLENA GRACILIS* STRAIN Z GROWN UNDER VARIOUS ENVIRONMENTAL CONDITIONS

by

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ABSTRACT

Enzymological And Fine Structural Studies On Microbodies And Mitochondria Of Euglena Gracilis Strain Z Grown Under Various Environmental Conditions

The observation that growth or treatment of various photosynthetic organisms with exogenous fatty acids brought about several modifications in fine structure, led to enzymological and further fine structure studies on the microbodies and mitochondria of Euglena gracilis strain Z. Aerated, acetate-supplemented dark-grown and continuously light-grown Euglena gracilis strain Z possess ≃ 300 and 600 microbodies/cell, respectively. Microbody catalase is demonstrated cytochemically with 3,3' diaminobenzidine (DAB)/H_2O_2. Between 12 - 30 hours of greening, some microbodies undergo a unique type of "multilobed" division. In such microbodies, long (≃ 0.5 μm), thin (30-50 A^o) fibrils are evident; coupled with photometric finding of nucleic acids in sucrose gradient isolated microbodies, DNA is suspect.

Euglena microbodies were compared with higher plant microbodies, using endosperm, leaf mesophyll and guard cells; morphologically all are similar and give positive DAB reactivity.

Aeration with CO_2-depleted air results in undetectable levels of catalase; since re-aeration with CO_2-containing air results in its restoration, it appears that this gas regulates catalase activity in acetate-supplemented Euglena.

In 72-hour greening Euglena, compared to dark-grown cells; a) catalase activity in cell-free fractions increases ≃ 2-fold on a per mg protein basis, and ≃ 2-fold on a per cell basis-whereas catalase activity in microbodies isolated from discontinuous sucrose gradients

increase 1.4-fold, both on the bases of specific activity and per cell; b) specific activities of glyoxysomal marker enzymes (isocitrate lyase and malate synthase) remain constant both with cell-free fractions and microbodies isolated from sucrose gradients; on a per cell basis, glyoxysomal marker activity doubles; c) specific activities of peroxisomal marker enzymes (glycolate dehydrogenase and hydroxypyruvate reductase) increase ≈ 6-fold in both cell-free fractions and microbodies isolated on sucrose gradients;activities increase ≈ 12-fold on a per cell basis.

To correlate microbody data, in regard to number, fine structure, protein content and enzyme activity, two models are offered. The first involves a binary fission type of division of each pre-existing microbody in dark-grown cells; the predicted consequences of such a division do not correlate well with some of the enzyme and fine structure data. The second model involves a multi-lobed type of division of peroxisomal microbodies (assumed to represent 1/12 to 1/10 the total number of microbodies in dark-grown cells); the predicted consequences of such a division correlate well with observations.

An enzyme is demonstrated cytochemically in mitochondrial matrices of aerated, acetate-supplemented Euglena gracilis strain Z, under conditions of DAB incubation other than those considered optimal for cytochrome oxidase. Activity of this enzyme is transitory, light-induced, and possibly photosynthesis-dependent.

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EXPLANATION OF ABBREVIATIONS USED IN FIGURES

BB	basal body	MU	mucous body
C	chloroplast	N	nucleus
CM	cytoplasmic membrane	NM	nuclear membrane
CW	cell wall	NP	nuclear pore
EM	electron micrograph	NR	nuclear region
ER	endoplasmic reticulum	OG	osmiophilic granule
F	flagellum	P	plastid
G	granum	PEL	pellicle
GB	Golgi body	R	ribosome
IS	intracellular space	RES	reservoir
L	lysosome	RM	reservoir microtubule
LM	lamellum	S	stromal region
M	mitochondrion	TP	tonoplast
Mb	microbody	V	vacuole
MT	microtubule		

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INTRODUCTION

I. Rationale of Work

In a short communication, Brody et al. (1969) reported that a naturally-occurring factor, protein-like in nature, found in the leaves of Ricinus communis, was capable of modifying many of the parameters of photosynthesis, both in isolated chloroplasts of Ricinus and in cross-reaction systems (i.e. chloroplasts from other plants suspended in aqueous extracts of Ricinus leaves). Among the effects of this protein were an increase in low temperature fluorescence at 698 nm and a decrease at 685 nm and 735 nm; associated with these emission changes were changes in low temperature fluorescence excitation. In the aforementioned paper, the following statement was made: "Preliminary studies with the electron microscope (in conjunction with Mr. James E. White), and experiments with oxygen evolution suggest that the observed steady-state fluorescence changes are associated with disruption of both normal lamellar structure and electron transport" .

In 1969 I collaborated in a continuation of this work (Cohen et al., 1969) in which it was demonstrated that certain long-chain (C-18) unsaturated fatty acids (linolenic, linoleic, oleic or ricoleic) had similar effects and could serve as good model systems for the action of Ricinus leaf extract[1, 2] on isolated chloro-

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[1] Benson (1964) reported that the lamellae of spinach chloroplasts were rich in galactolipids, which may contain $\lesssim$ 96 % of their lipids in the form of C-18 unsaturated fatty acids.

[2] A proposed mode of action of this Ricinus protein was that it possessed lipolytic action and was therefore liberating fatty acids from the chloroplast (Cohen et al., 1969 ; Nathanson and Brody, 1970).

plasts of higher plants and algae. Both the protein factor of the Ricinus leaf extract (also to be referred to as " RLE ") and exogenous C_{18} unsaturated fatty acids were observed to have photosynthesis-associated effects additional to those cited by Brody et al. (1969) . Among these were modifications of : a) fluorescence induction, b) fluorescence emission and excitation, c) light-induced absorption by chlorophylls a_{II} and a_{I}, d) Hill activity, and e) system II and system I- associated electron flow. In addition, high concentrations of exogenously-added unsaturated fatty acids brought about changes in chloroplast ultrastructure; the latter findings constituted my contribution to the study. These configurational changes (i.e. loss of chloroplast envelope, swelling of stromal and then granal thylakoids) were assumed by us to result from some conformational change in chloroplast lamellar protein.

Earlier workers had also suspected that conformational changes were brought about in chloroplast lamellae under the influence of exogenous fatty acids. Light-scattering changes, resulting from the addition of fatty acids, have been interpreted as arising from the swelling of chloroplasts (Siegenthaler and Packer, 1965; Pedersen et al., 1966; Molotkovsky and Zheskova, 1966). Murarkami and Nobel (1967) demonstrated by packed cell volumes and electron microscopy that exogenously-added fatty acids cause (light-dependent) swelling in isolated spinach chloroplasts.

Brody and Brody had much earlier (1961) postulated that monomers and dimers of chlorophyll were attached periodically to protein helices in chloroplast lamellae, and that conformational changes in protein could cause changes in proportions of these types of chlorophyll. Just recently, Brody and Nathanson (1972), in experiments

utilizing circular dichroism, have demonstrated that the deaggregation of chlorophyll-containing systems by exogenous fatty acids results not only from direct effects on chromophores, but also by indirect effects on the proteins to which the chlorophylls are attached.

These early observations (Brody et al. 1969; Cohen et al., 1969) with isolated chloroplasts led me to inquire into the possible role of fatty acids in the development and control of form and function of chloroplasts in situ. To this end, fine structure studies were undertaken to determine the influence of exogenous fatty acids on a blue-green alga, a red alga, a green alga, and an euglenoid. These experiments soon indicated, that in addition to chloroplasts, other organelles in situ (e.g. mitochondria and microbodies) were also being affected.

The effects of exogenous fatty acids were most extensively investigated with Euglena gracilis. Since, when grown heterotrophically in the dark, it lacks chlorophyll and chloroplasts - converting to photosynthetic metabolism upon exposure to light - it seemed an ideal organism for such a study.

Mitochondria as well as chloroplasts seemed to be affected by exogenous fatty acids. I wished to determine whether yet another organelle - the microbody - would be similarly affected. Although microbodies are difficult to visualize by standard techniques of electron microscopy, the presence of catalase in these organelles makes it possible to utilize the cytochemical identifier 3,3' - diaminobenzidine (DAB) . Utilization of DAB in the cases of dark-

grown, greening and continuously light-grown Euglena gracilis (cultured on media supplemented with glucose plus linolenic acid or just linolenic acid as sole carbon and energy source) gave positive cytochemical results. Just after obtaining these results, I read with surprise the papers of Lord and Merrett (1971) and Graves et al. (1971 a) stating that microbodies of Euglena do not contain catalase.

Earlier work (Reeves et al., 1962; Haigh and Beevers, 1964) had indicated that acetate (C_2) supplementation of Euglena induces enzymes of the glyoxylate cycle. Since β-oxidation has been reported to occur in microbodies (Cooper and Beevers, 1969) and since the end products of such β-oxidation produce 2-carbon fragments similar to acetate, I performed further experiments to determine whether DAB-mediated polymerization could be observed with cells grown on acetate-supplemented media, because there exists an extensive literature on the physiology of such acetate-supplemented Euglena (Smillie, 1968), while there is an absolute lack of literature on fatty acid-supplemented cells. These early investigations led to work which became a major part of my dissertation effort. One of the first questions I attempted to answer was why my results were positive in regard to catalase in Euglena, whereas other workers were unable to detect this enzyme.

During experiments utilizing catalase-mediated DAB polymerization to localize microbodies in Euglena, another DAB-reactive enzyme was noted, by electron microscopy, in some mitochondria of 20 - 72 hour greening cells. Experiments were then performed in an effort to learn more about this mitochondrial enzyme from the points of view of its nature and factors controlling its development.

II. History and Background to Work

A. *Euglena* Plastids: Changes With Greening

Dark-grown *Euglena* contain about 10 ellipsoidal-shaped proplastids, having a mean length of 1.6 μm, a mean width of 0.6 μm and bounded by a double membrane (Klein *et al.*, 1972). Upon exposure to continuous light, the proplastids of *Euglena* develop into ≈ 10 disc-shaped chloroplasts at least 7 μm in length (Klein *et al.*,1972). Moreover, the conversion from a proplastid structure to a mature chloroplast in *Euglena gracilis* var. *bacillaris* commences after the sixth hour of greening (Schiff, 1970, Klein *et al.*, 1972).

After 24 hours of development, the mean plastid length is 2.4 μm (Klein *et al.*, 1972) ; however, the number and orientation of thylakoids show wide variation even in the plastids of the same cell. By 24 hours of greening, the mean number of thylakoids per plastid is 7-8 in *Euglena gracilis* var. *bacillaris* (Klein *et al.*, 1972). At this stage of development, most plastids still contain both girdle and straight thylakoids. As the plastid develops during the first 24 hours of greening, prolamellar bodies are less frequently observed; they are rarely seen at all after 24 hours of greening (Schiff, 1970; Klein *et al.*, 1972).

From 24 hours of greening to the achievement of the mature chloroplast (72 - 96 hours of greening) development proceeds essentially as described by Ben-Shaul *et al.* (1964) - that is: a) the number of lamellae (each containing 4-6 thylakoids) increases to about 13 in a fashion essentially linear with respect to time, b) the number of unfused thylakoids decreases, so that very few unfused thylakoids are

apparent by 72 hours of greening, and c) the length of the plastid remains uniform during this period.

Accompanying morphological development of the chloroplast and chlorophyll formation is the synthesis of carotene, proteins and enzymes associated with photosynthesis (Schiff and Zeldin, 1968). Photosynthetic competence is accompanied by the appearance of lipids characteristic of light-grown cells (Krinsky and Goldsmith, 1960; Erwin and Bloch, 1963 b; Rosenberg and Pecker, 1964; Krinsky et al., 1964).

Changes in type and composition of fatty acids occur with greening in Euglena (see Table 1). Euglena gracilis strain Z grown in the light on organic medium has large amounts of α-linolenic acid (mainly in the glycolipids of the chloroplasts), while Euglena grown in the dark has C_{20}, C_{22}, and C_{24} polyenoic fatty acids of the δ-linolenic type (mainly in the phospholipid portions of their membranes)[3], [4]. Double labeling experiments have demonstrated the existence of two pathways of polyenoic acid biosynthesis in this organism (Hulanicka et al., 1964); and, therefore, two categories of polyunsaturated fatty acids may be found (Erwin and Bloch, 1962, 1963 b; Rosenberg, 1963; Korn, 1964; Hulanicka et al., 1964). These two categories arise because the desaturation of oleic acid in Euglena can proceed in two different directions (see Figure 1). In the α-linolenate pathway (or " plant " pathway), oleate is progressively desaturated toward

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[3] α-linolenic acids (9,12,15-C_{18}) occur widely in photosynthetic microorganisms and higher plants, while δ-linolenic acids (6,9,12-C_{18}) are found in protozoa and metazoa (Erwin, 1968).

[4] Polyenoic fatty acids are those which contain more than one double bond (West and Todd, 1971). A few examples are given in Table 3.

Table 1. Fatty Acid Composition Of Plastid Fractions From Green And Dark-Grown Euglena Gracilis Strain Z.

(according to Rosenberg et al., 1965)

Carbon atoms	Fatty acid Number of double bonds	% by weight of total fatty acid	
		Chloroplast	Proplastid
13	0	1.5	3.0
14	0	4.0	3.5
15	0	2.0	3.0
16	0	8.0	6.0
16	1	5.5	1.5
16	2	7.0	-
16	3	14.5	-
17	0	2.0	1.5
17	1	5.5	2.5
18	0	-	1.0
18	1	2.5	2.5
18	2	8.0	0.5
18	3	12.5	1.0
19	1	1.5	-
19	2	-	1.5
19	4 and more	-	5.0
20	2	3.5	3.0
20	4 and more	14.0	48.0
21	4 and more	-	2.0
22	4 and more	-	10.5

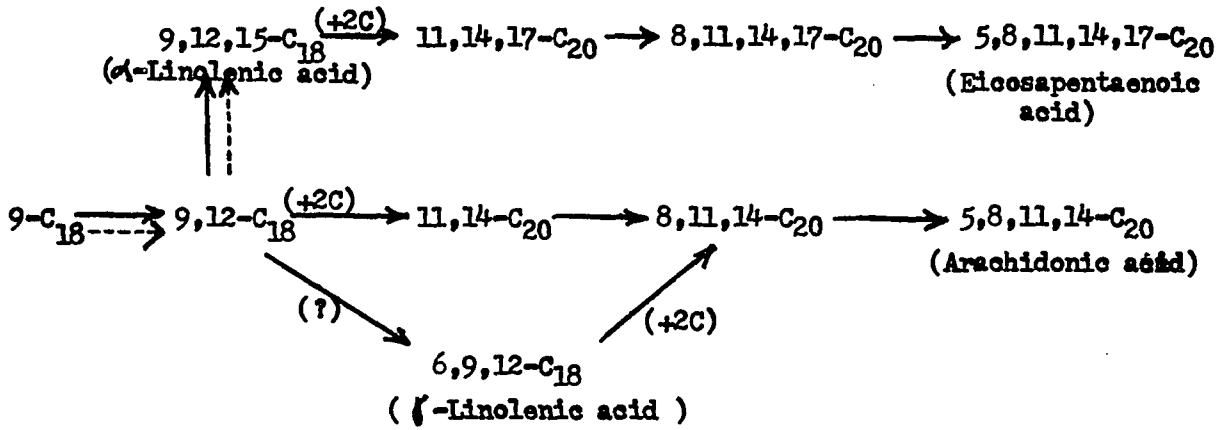

Figure 1. Major pathways of polyunsaturated C_{18} and C_{20} fatty acids in Euglena. Arrows with dotted lines indicate major pathways in photoauxotrophic, and those in solid lines indicate major pathways in heterotrophic cells.
(according to Erwin, 1968)

the methyl end of the molecule, forming linoleic and α-linolenic acids. In the δ-linolenate pathway (or "animal" pathway) after the formation of linolenic acid, double bonds are formed between the 9,10 position and the carboxyl end of the molecule; chain elongation by this pathway results in the synthesis of arachidonic acid and other C-20 and C-22 polyunsaturated fatty acids (see review by Erwin and Bloch , 1964) .

Several new proteins appear during chloroplast development , as has been demonstrated by chloroplast antigen studies (Lewis et al.,1965) and chloroplast enzyme studies (Smillie, 1963,1968). Among these newly synthesized enzymes are those of the carbon dioxide fixation cycle [TPN-triosedoheptulose phosphate dehydrogenase (Fuller and Gibbs, 1959; Brawerman and Konigsberg, 1960; Hudock and Fuller, 1965) and ribulose diphosphate carboxylase (Fuller and Gibbs, 1969)] , enzymes connected with photosynthetic electron transport [TPN- transhydrogenase (Lazzarini and San Pietro, 1963; Lazzarini and Woodruff, 1964) and cytochrome 552 oxidase (Nishimura , 1959; Wolken and Gross, 1963; Perini et al., 1964 a, b)] and enzymes involved in photorespiration [transketolase (Peterkofsky and Racker, 1961; Smillie, 1963)] . For a list of chloroplast-localized enzymes see Smillie (1963, 1968) .

Photosynthetic carbon dioxide fixation is completely inhibited if chloroplast development proceeds in the presence of DCMU [3 (3,4-dichlorophenyl)-1,1-dimethyl urea] a specific inhibitor of photosynthesis, which blocks the flow of electrons from Photosystem II (Schiff et al., 1967; Schiff, 1970) . However, Euglena allowed to green in DCMU, develop normal size chloroplasts, with more than the usual number of thylakoids per lamella; pyrenoids are seldom observed in DCMU-treated cells (Schiff, 1970). Therefore, DCMU experiments indicate

that the developing chloroplast of Euglena is capable of forming membranes and pigments in the absence of photosynthesis.

B. Euglena Mitochondria

Benda (1898) first coined the term "mitochondrion" , derived from two Greek words - "mitos" (a thread) and "chondros" (a grain). Causey (1926) estimated by light microscopy an average of only 16 spherical mitochondria per Euglena gracilis Klebs cell and speculated that there was a definite ratio between cell volume and number of mitochondria. Baker (1933) believed to have observed by light microscopy, division of mitochondria in Euglena; however, most workers agree with Buetow (1968) that division has not yet been demonstrated. Recent work has shown that the mitochondria in Euglena can vary in number, shape, size and structure (Wolken and Palade, 1953; Gibbs, 1960; Siegesmund et al., 1962; Brandes et al., 1964; Malkoff and Buetow, 1964; Leedale et al., 1965; Buetow, 1968).

Gibbs (1960) reported that numerous spherical rod-shaped mitochondria are found throughout the cytoplasm of Euglena cells, most abundantly immediately beneath the pellicle. This confluence of mitochondria below the pellicle has been reported in dark-grown Euglena gracilis (Wolken and Palade, 1953; Leedale et al., 1965) and streptomycin-bleached Euglena gracilis (Brandes et al., 1964; Malkoff and Buetow, 1964; Leedale et al., 1965). Moreover, Leedale et al. (1965) have proposed that an irregular surface is characteristic of euglenoid mitochondria.

In Euglena gracilis, the mitochondrion is bounded by two 60 A° membranes separated by a 60 A° intermembrane space (Gibbs, 1960) . The inner mitochondrial membrane invaginates, forming short cristae which extend 33-50 % of the way across the mitochondrion

(Wolken and Palade, 1953; Gibbs, 1960; Brandes et al., 1964) . Furthermore, the Euglena cristae possess characteristic constrictions in the basal region (Leedale et al., 1965) .

Occasionally the mitochondria of Euglena gracilis contain relatively dense granules of unknown significance (Buetow, 1968)[5] ; acid phosphatase has been cytochemically demonstrated in the mitochondrial matrix and cristae (Brandes et al., 1964) . Krawiec and Eisenstadt (1970) and Lord and Merrett (1971) were unable to detect cytochrome oxidase in Euglena mitochondria by photometric assays.

The mitochondria of Euglena appear to be rather pliable and pleomorphic (Buetow, 1968) . Leedale et al.(1965) reported by fine structure studies that the mitochondria of dark-grown Euglena spirogyra were most often oval or elongated (0.5 μm wide and up to 10 μm long) and dispersed throughout the cytoplasmic matrix; in light-grown cells, the mitochondria most frequently were located between the chloroplast and the pellicle. However, Leedale et al. (1965) indicate that at any specific time in greening, all gradations between the above types of mitochondria may be found in any particular culture of Euglena, with one state predominating. Therefore, in living Euglena the mitochondria are in a state of "flux, continually move and change configurations, fuse, branch and change their shapes" (Buetow, 1968) .

Often the number of mitochondria in Euglena increase under conditions which reduce the chloroplast content, e.g. , dark-grown Euglena gracilis (Wolken and Palade, 1953) , heat-produced colorless Euglena spirogyra (Leedale, 1966) and numerous mutants (Stern et

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[5] Possibly cation storage granules (Brown and Bertke, 1969) .

al., 1964; Schiff and Epstein, 1968) . Furthermore, Schiff and Epstein (1968) have suggested, on the bases of fine structure and gas exchange, that an active chloroplast may control the structural development and respiratory activity of Euglena mitochondria.

Under conditions of carbon (acetate) starvation , many mitochondria of Euglena gracilis var. bacillaris (strain SM-L1) become encapsulated in membrane-bound cavities (Brandes et al., 1964) similar to the cytolysomes (autophagic vacuoles) described in mammalian cells (deDuve and Wattiaux, 1966) . These encapsulated mitochondria are subsequently digested away (Brandes et al., 1964) and most of the remaining mitochondria appear normal (Brandes et al., 1964; Buetow , 1968) . Brandes et al. (1964) have reported a few examples in which very long mitochondria are located in the periphery of Euglena after 7 days of carbon starvation.

C. Euglena Microbodies

1. Introduction and Definition of Terms

The term "microbody" was intoduced by electron microscopists (Rhodin, 1954; Gänsler and Rouiller, 1956) for the morphological description of single-membrane bound organelles (approximately 1 μm) observed in kidney and liver cells. Similar organelles have also been disclosed in a variety of plant cells. Among these are endosperm (Breidenbach and Beevers, 1967; Breidenbach et al., 1968 ; Cooper and Beevers, 1969; Frederick and Newcomb, 1969 a; Ching, 1970; Gruber et al., 1970; Longo and Longo, 1970; Trelease et al., 1971) , seedlings of other than fat-storing plants(Cronshaw, 1964; Frederick and Newcomb, 1971 ;Feierabend and Beevers, 1972 a,b) , mesophyll

leaf cells of both C_3 and C_4 plants [6] (Frederick et al., 1968; Frederick and Newcomb, 1969 a,b; Gruber et al., 1970; Frederick and Newcomb,1971; Bjorkman et al., 1971) , tobacco stem and callus tissue (Frederick et al., 1968), oat coleoptile (Thornton and Thimann, 1964; Frederick et al.,1968; Rocha and Ting, 1970) .

Microbodies have also been detected in yeast (Avers and Federman, 1958; Szabo and Avers, 1969), molds (Kobr et al., 1969), and reported in brown algae (Bouck, 1965) . Fine structure studies have substantiated the presence of microbodies in a variety of microorganisms (Müller, 1969; Graves et al., 1971 a,b; Gerhardt and Berger, 1971; Hanzely et al., 1971; Gergis, 1971; Brody and White, 1972, 1973 ; White and Brody, 1973 a,b) .

DeDuve (1965) and deDuve and Baudhuin (1966) recognized the biological significance of catalase in microbodies. Microbodies are respiratory organelles containing hydrogen peroxide-generating oxidases and large amounts of catalase. Catalase reduces hydrogen peroxide to water either peroxidatively (requiring an electron donor other than H_2O_2) or catalytically (in which a second molecule of H_2O_2 serves as the electron donor) . As shown in Figure 2, oxygen is reduced to water in microbodies by a two-step mechanism involving hydrogen peroxide as an intermediate. Depending on cell type, the electron donors (R_1H) in the first step include uric acid, D-amino acids, L-amino acids, glycolic acid and L-α-hydroxy acids. The electron donors in the second

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[6] In plants which fix carbon dioxide by the Calvin cycle, the CO_2 acceptor molecule is 5-C ribulose diphosphate; phosphoglyceric acid (3-C) is the first detectable product of such fixation. In plants which fix carbon dioxide by the Hatch-Slack mechanism, the CO_2 acceptor molecule is phosphoenolpyruvate; the first detectable products of such fixation are 4-C compounds such as malate and aspartate.

step are either another molecule of hydrogen peroxide or a substrate (R_2H), such as formaldehyde, methanol, ethanol, phenol or nitrate ions. (For a complete list of substrates, see Nicholls and Schonbaum, 1963.) According to deDuve and Baudhuin (1966), peroxidative disposal of hydrogen peroxide (path X in Fig. 2) is favored by high concentrations of catalase and R_2H or low concentrations of H_2O_2; converse conditions favor the catalytic reaction (path Y in Fig. 2).

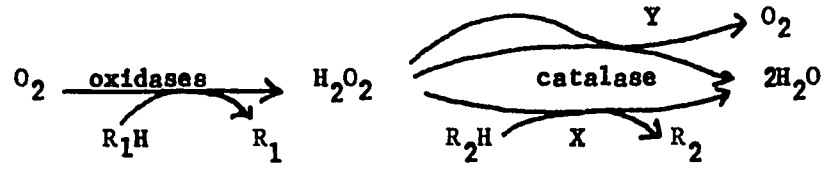

Figure 2. Enzymic reactions in microbodies.
(from deDuve and Baudhuin, 1966)

2. Fine Structure

a) Morphology

Microbodies are characterized morphologically as being single-membrane bounded organelles, with diameters ranging from 0.2 μm to 1.5 μm, and possessing granular matrices which often contain dense cores of crystalline inclusions (Hruban and Rechcigl, 1969; Tolbert, 1971) . Microbodies are heterogeneous in shape and frequently appear pliable (Frederick and Newcomb, 1969 a; Gruber et al., 1970), and their membranes often seem to be continuous with the smooth endoplasmic reticulum by means of contoured sleeve-like projections (Hagiwara et al., 1961; Graves et al., 1971 a) . During greening, cytoplasmic invaginations into microbodies of cucumber cotyledons have been noted (Trelease et al., 1971),

suggesting a mechanism by which the organelle may change its enzymic complement.

b) Cytochemical Stain

Since microbodies contain catalase, 3,3'-diaminobenzidine (DAB) has been used for its cytochemical localization. DAB has been employed to localize various hemoproteins, and its action depends upon the oxidation of 3,3' diaminobenzidine to a polymeric form that can interact with osmium tetroxide to form electron-opaque osmium black (Seligman et al., 1968) . This DAB oxidation may be catalyzed by several hemoprotein compounds, including myoglobin (Goldfischer, 1967), cytochrome oxidase (Seligman et al., 1968), and peroxidase (Karnovsky, 1965; Graham and Karnovsky, 1966; Friend and Farquar, 1967). The peroxidative action of catalase with DAB was first demonstrated for beef liver catalase by Keilin and Hartree (1936) . DAB cytochemical techniques used in the present work follow Novikoff and Goldfischer's (1968) modification of Graham and Karnovsky's (1966) original DAB procedure for peroxidase. Seligman et al. (1968) have proposed a hypothetical formation of the oxidative polymerization of DAB to an indamine polymer (Figure 3) .

Conditions of DAB incubation may be varied to favor the reactivity of catalase (pH 9.0, 37° C) or cytochrome oxidase (pH 7.0, 25° C) with diaminobenzidine (Novikoff and Goldfischer, 1969; Gerhardt and Berger, 1971) . Evidence that accumulation of the DAB reaction product in microbodies is mediated primarily by catalase rather than peroxidase has been provided in part by experiments employing aminotriazole, a specific inhibitor of catalase (Heim et al., 1956; Margoliash and Novo-

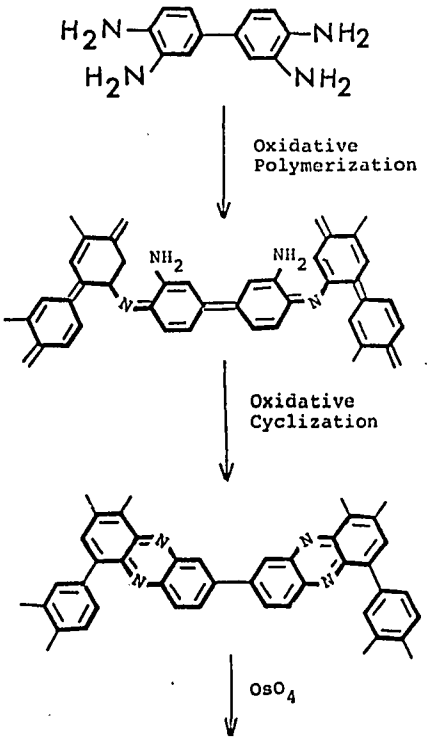

Figure 3. Hypothetical formulation of the oxidative polymerization of DAB to an indamine polymer.
(from Seligman *et al.*, 1968)

grodsky, 1958, 1960) . Moreover, detection of peroxidase has usually been accomplished at a lower pH (7.4) than that used to detect catalase [9.0 (Graham and Karnovsky, 1966, Seligman et al., 1968)] . The inhibitory effect of aminotriazole on catalase without also inhibiting peroxidase has not been well documented in plants (Frederick and Newcomb, 1969 b) . However, this selective specificity has been shown in animal leucocyte cells, where catalase activity was inhibited 96 % by a concentration of aminotriazole that did not affect peroxidase (Rechcigl and Evans, 1963).

Additional evidence that DAB reactivity of leaf microbodies is catalase-mediated, rather than peroxidase-mediated, is based on enzyme studies of cell-free fractions (Tolbert et al., 1971) . In this study it was found that most of the catalase activity was located in the particulate fraction which contained single-membrane bound organelles (microbodies) ; peroxidase activity was detected mainly in the soluble fraction, with a small proportion in chloroplast and mitochondrial fractions.

3. Isolation

Various methods have been utilized for the isolation of microbodies (Breidenbach et al., 1968; Tolbert et al., 1968; Beevers, 1969; Szabo and Avers, 1969; Tolbert et al., 1970; Yamakazi and Tolbert, 1970; Tolbert, 1971; Graves et al., 1972) . Essentially, these isolation procedures consist of three main steps : 1) grinding the tissue in a sucrose medium, 2) differential centrifugation for particulate enrichment, 3) final separation by discontinuous or continuous sucrose density gradients. Successful separation of Euglena microbodies is accomplished by sucrose gradient centrifugation because microbodies increase

in specific densities in concentrated sucrose and ultimately sediment to a specific density of $\lesssim$ 1.20 gm/cm^3 , mitochondria sediment to a specific density of $\lesssim$ 1.17 gm/cm^3 , and the two chloroplast bands sediment to specific densities of $<$ 1.00 gm/cm^3 (Graves et al., 1972).

4. Physiology

a) Enzymes

"Catalase is probably the only enzyme found in all microbodies" (Brown and Chatiopadhyay, 1971); however, its activity was until recently thought to be associated with mitochondria. Thomson and Klipfel (1957) first observed from gradient centrifugations that catalase sedimented in non-mitochondrial fractions. Isozymes of catalase have been demonstrated by electrophoresis (King and Gutman, 1964) . The extensive literature on catalase has been reviewed (Higashi and Peter, 1963 a,b ; Rechcigl and Price, 1968) .

Subdivision of microbodies into glyoxysomes and peroxisomes has been made on the basis of the former containing the enzymes involved in the glyoxylate path or cycle (Breidenbach and Beevers, 1967) and the latter containing the enzymes of the glycolate cycle (Frederick and Newcomb, 1969 b) [which in the case of leaf peroxisomes, function in photorespiration[7] (Tolbert et al., 1968; Kisaki and Tolbert, 1969)] . Not only has it become evident that microbodies are heterogeneous in their specific enzymic composition, but also functions ascribed exclusively to these organelles may be shared by others. Müller (1969) and Hogg (1969) have shown that in

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[7] For discussion of photorespiration see pages 23 and 24.

Tetrahymena pyriformis three of the enzymes of the glyoxylate cycle (citrate synthase, aconitase, and malate dehydrogenase) are found in the mitochondria and two (malate synthase and isocitrate lyase) in the peroxisomes.

The glyoxylate cycle (originally) explained the growth of several microorganisms on 2-carbon units as sole carbon sources (Kornberg and Krebs, 1957) . The energy requirements for these microorganisms could be supplied by the oxidation of acetate through the Krebs cycle; however , the Krebs cycle could not provide a net synthesis of intermediates. The glyoxylate cycle plays a pivotal role in the conversion of fats into sugars and, as shown in Figure 4, is a modified Krebs cycle. By this cycle, fatty acids are broken down by β-oxidation into acetyl-CoA , which is then converted into citric acid and isocitric acid. Isocitrate lyase splits isocitric acid into succinate and glyoxylate; therefore, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (enzymes of the Krebs cycle) are by-passed. The succinate is converted into fumarate by succinic dehydrogenase. The fumarate is then converted into malate by fumarase, and malate is converted into oxaloacetate by malic dehydrogenase according to the Krebs cycle. However, glyoxylate combines with a molecule of acetyl Co-A in the presence of malate synthase to produce malate. The resulting malate may be converted to oxaloacetate, which would start the cycle over again by combining with another molecule of acetyl Co-A. However, much of the oxaloacetate is decarboxylated, producing phosphoenolpyruvic acid which can be converted into sugars by reversal of glycolysis (gluconeogenesis) .

b) Physiological Roles

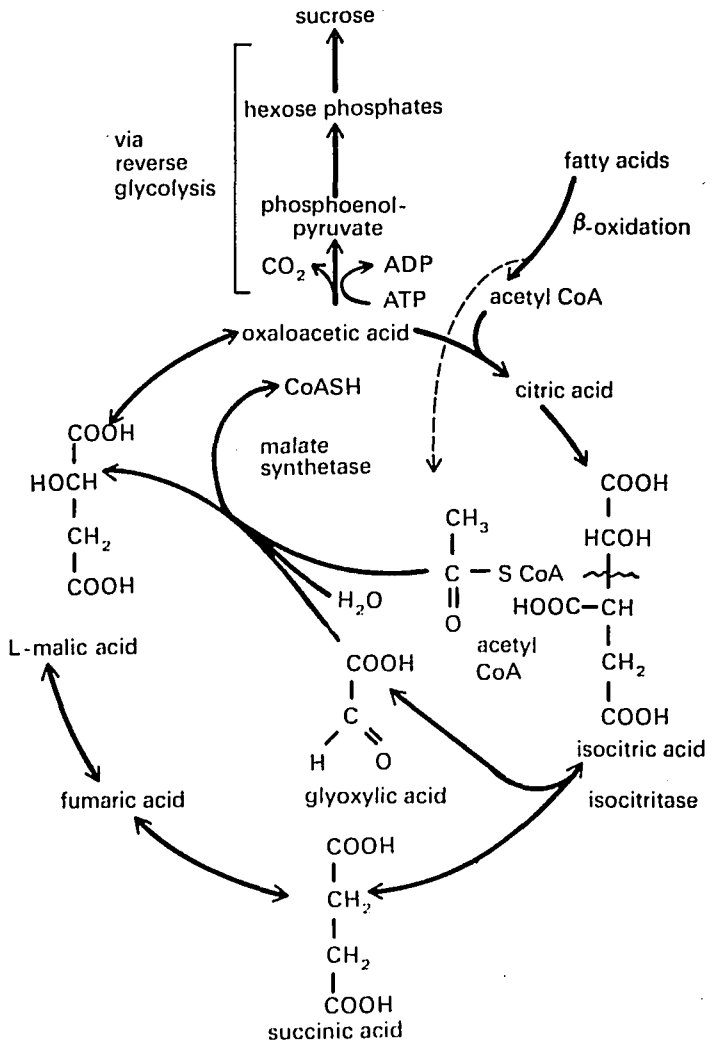

Figure 4. The glyoxylate cycle.
(according to Salisbury and Ross, 1969)

The functions of microbodies in cellular metabolism are not completely understood. No one single function is adequate to account for all microbodies (deDuve and Baudhuin, 1966; Tolbert, 1971) , and a single microbody may perform several functions. The following functions have been ascribed to microbodies:

1) Precursors of Other Organelles

Microbodies have been proposed to be precursors of mitochondria (Rouiller and Bernhard, 1956), secretion granules related to Golgi bodies (Rouiller and Jezequel, 1963; Wood, 1964; Bruni and Porter, 1965) or a type of lysosome (Novikoff and Essner, 1960; Essner and Novikoff, 1961) . All of these functions are now considered to be unlikely (deDuve and Baudhuin, 1966) .

The speculation that microbodies are mitochondrial precursors has received no support from biochemical studies which indicate that there are completely different enzymatic compositions for the two organelles (deDuve, 1960; deDuve et al., 1963; Baudhuin and Beaufy, 1963; Baudhuin et al., 1964, 1965) . Additional studies indicate differences in membrane permeability between microbodies and mitochondria (Adams, 1959; Baudhuin, 1964; deDuve, 1965; Baudhuin et al., 1965) - as a matter of fact, it is just these differences which allow microbodies to be separated from mitochondria in sucrose density gradients.

The speculation that microbodies were types of lysosomes was due mainly to earlier difficulties with differential centrifugation experiments in which both lysosomes and microbodies sedimented together, and work which reported acid phosphatase activity in microbodies of liver cells of rats injected with bilirubin

Novikoff and Essner, 1960; Essner and Novikoff, 1961) . However, present separations, achieved by sucrose density centrifugations, demonstrate that microbodies are enzymatically different than lysosomes (Tolbert, 1971) and microbodies do not stain cytochemically for acid phosphatase (Holt and Hicks, 1961; Novikoff and Shin, 1964; Trump and Ericsson, 1964) . Moreover, it is very unlikely that the enzymes of microbodies (which have an alkaline pH optimum) could function in concert with acid hydrolase found in lysosomes (deDuve and Baudhuin, 1966) .

2) Disposal of Hydrogen Peroxide

Microbodies may function to protect the cell by isolating harmful hydrogen peroxide-producing oxidases and catalase (to destroy H_2O_2) within a single organelle (deDuve and Baudhuin, 1966) . However, this protective function is not complete - liver xanthine oxidase has be localized in cellular supernatant fractions (Villela et al., 1955; Stein and Mehl, 1955) , monamine oxidase occurs mainly in mitochondrial fractions (Cotzias and Dole, 1951; Hawkins, 1952) , and a mitochondrial cytochrome c peroxidase has been identified in yeast (Altschul et al., 1940; Abrams et al., 1942) . It is conceivable that extra-microbody oxidases utilize electron acceptors other than oxygen, but no evidence has been presented for this (deDuve and Baudhuin, 1966) . Mills (1960) has postulated that catalase could diffuse from the microbodies and decompose cytoplasmic hydrogen peroxide, or else cytoplasmic hydrogen peroxide could be decomposed by cytoplasmic enzymes, such as glutathione reductase or glucose-6-phosphate dehydrogenase. Therefore, it is very unlikely that microbodies uniquely isolate and destroy hydrogen peroxide.

3) Gluconeogenesis

Glyoxysomes contain the enzymes of the glyoxylate cycle, by which carbohydrates may be synthesized from fats. Glyoxsomes and their glyoxylic cycle enzymes have been documented in liver cells (Baudhuin et al., 1965; Leighton et al., 1968) and kidney cells (Allen and Beard, 1965; Baudhuin et al., 1965) , a variety of plant cells (Breidenbach and Beevers, 1967; Ching, 1970; Gruber et al., 1970) , and microorganisms (Graves et al., 1972; White and Brody, 1973 a,b) .

4) Photorespiration

Photorespiration , or light-induced respiration , is a special type of cellular respiration which is very different from dark aerobic respiration. These differences may be summarized as :

i. Photorespiration occurs only during photosynthesis and is, therefore, light-dependent. Dark aerobic respiration is not light-dependent.

ii. Proteins, fats and carbohydrates are the major substrates oxidized in dark aerobic respiration. In photorespiration , glycolic acid (CH_2OH-COOH), a product of photosynthetic carbon dioxide assimilation, is the substrate oxidized.

iii. In eucaryotic cells, photorespiration occurs in peroxisomes; dark respiration occurs in mitochondria.

iv. Biologically-useful energy results from dark respiration; energy released in glycolic acid oxidation is not retained in a useful form.

Photorespiration is found in Calvin cycle plants, which seem to be at a disadvantage because net photosynthesis is reduced by the formation of glycolic acid which is oxidized to CO_2. Gibbs (1970) , however, believes that it is unlikely that photorespiration is an inborn error of metabolism, but is simply the result of the interaction of a glycolic acid precursor (such as fructose 6-phosphate, ribulose 1,5-diphosphate, or sedoheptulose 7-phosphate) with an oxidizing environment; and is, therefore, a mechanism for removing a harmful narcotic substance from the chloroplast. Also glycolic acid, as shown in Figure 5, may be used by plant cells for the formation of glycine and serine, which are precursors for C_1 biosynthesis and porphyrin synthesis (Tolbert, 1971) .

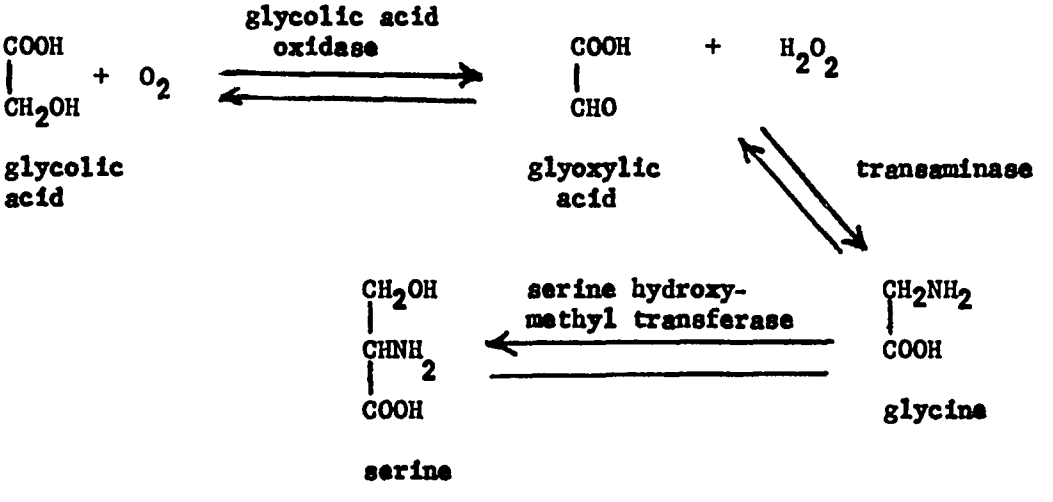

Figure 5. Conversion of glycolic acid into glycine (according to Tolbert and Yamazaki, 1969)

5) Degradation of Purines and Pyrimidines

Urate oxidase and allantoinase are involved in the degradation of purines by the following reaction:

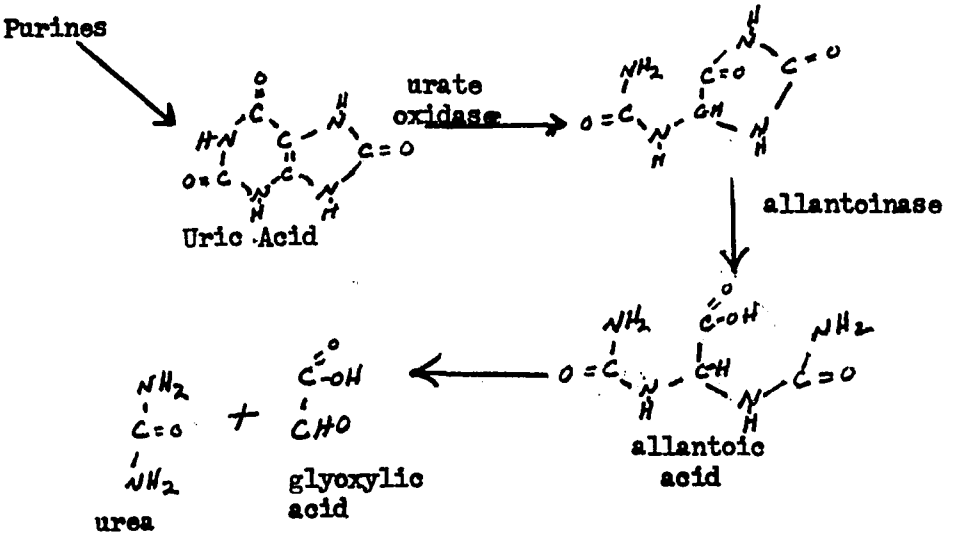

Figure 6. Enzymatic degradation of uric acid to urea and glyoxylic acid. (Davies et al., 1964)

Urate oxidase and allantoinase have been localized in peroxisomal fractions of rat, mouse, avian, amphibian, and protozoan cells (Tolbert, 1971), and crystalline cores of some mammalian microbodies are thought to be composed of urate oxidase (Hruban and Swift, 1964). These two enzymes

have also been localized in glyoxysomes of castor bean endosperm (St. Angelo and Ory, 1970; Theimer and Beevers, 1971) , but could not be found in Ricinus leaf peroxisomes (Yamazaki and Tolbert, 1970; Theimer and Beevers, 1971). Theimer and Beevers (1971) have also reported the presence of urate oxidase and allantoinase in the endosperm of sunflower, safflower, corn and Pinus, as well as in leaf cells of corn and tobacco. However, the complete metabolic sequences of purine and pyrimidine catabolism have not been found in microbodies (Tolbert, 1971).

6) Formation of Ionic Gradients and Ionic Pumps

There is no evidence to substantiate the hypothesis that microbodies function for the creation of ionic gradients and ionic pumps for the movement of cellular components (Tolbert, 1971). Tolbert and Zill (1956) and Kearney and Tolbert (1962) have suggested that glycolate excretion is a mechanism for cellular bicarbonate exchange. Furthermore, Tolbert (1971) has pointed out that since the cellular movement of glycolate is maximal during photosynthesis, it could conceivably function as an ionic pump.

7) Oxidation of Reduced NAD

Many of the substrates of microbody oxidations (e.g., ethanol and α-hydroxy acids; see Fig. 2) can be regenerated by cytoplasmic NADH-dependent dehydrogenases (deDuve and Baudhuin 1966). Thus, microbodies could participate in the oxidation of cytoplasmic $NADH^+$ and thereby function in the aerobic support of cytoplasmic metabolism as indicated in Figure 7.

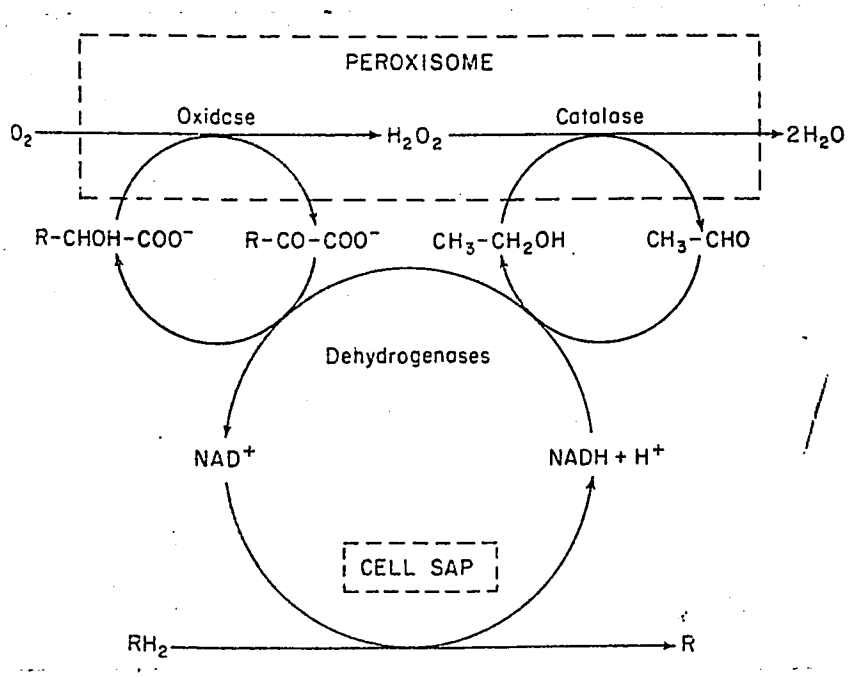

Figure 7. Possible function of microbodies in the oxidation of reduced NAD.
(from deDuve and Baudhuin, 1966)

8) Growth Regulation

It has been hypothesized (Tolbert, 1971) that liver and leaf microbodies could function as growth regulators by disposing of excess cellular energy through flavin oxidases rather than conserving energy by mitochondrial ATP synthesis.

9) Fossil Organelle

Nicholls and Schonbaum (1963) have termed catalase as a "fossil enzyme" and view microbodies as being fossil organelles which are the late descendents of a primitive respiratory system. Neither catalase nor microbodies appear to be essential for the survival of all cellular types; microbodies are found only in certain kinds of cells (deDuve and Baudhuin, 1966; Tolbert, 1971). A genetic defect in humans, characterized by lack of cellular catalase, indicates that humans can live without this enzyme (Takahara and Mijamoto, 1948)[8] . Furthermore, urate oxidase is a dispensable enzyme (it is not found in human cells) and the presence of D-amino acid oxidases in animal microbodies has long been an enigma to biochemists (deDuve and Baudhuin, 1966).

It is generally believed that life arose in an anaerobic environment and respiratory mechanisms developed with the appearance of atmospheric oxygen (mainly from photosynthesis). With the advent of oxygen, cellular hydrogen peroxide appeared; and catalase and microbodies perhaps arose to dispose of this hydrogen peroxide

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[8] This is not to imply that such genetically-deficient people do not posses some other enzyme which may be capable of performing the function of catalase.

(Nicholls and Schonbaum, 1963) . However, as deDuve and Baudhuin (1966) have pointed out, if the microbody were a fossil organelle it is difficult to visualize, a) how selective pressure would have favored its retention , and b) why microbodies are found in a variety of cellular types, if they did not fulfill a useful function.

MATERIALS AND METHODS

I. Sources and Growth of Organisms

A. Sources of Organisms

Ricinus communis, L., (Castor-oil plant) variety *Baker 296* seeds were acquired from Dr. R. Ory, Southern Regional Research Laboratory, New Orleans, Louisiana. *Spinacea oleracea* (spinach) was obtained from a local market. Leaves of greenhouse-grown *Zea mays* (corn) variety *golden midget*, *Rhipsalis cassytha* (a cactus) and *Euphorbia pseudocactus* (spurge) were obtained from Mr. Robert Dague and Mr. David Madison, Department of Biological Sciences, Hunter College, CUNY. Leaves of *Bryophyllum daigremontianum* (*Kalanchoë daigremontianum*) and *Kalanchoë blossfeldiana* were graciously supplied by Mr. George Kalmbacker and Mr. Frank Bowman, Brooklyn Botanical Gardens, Brooklyn, New York.

Anabaena cylindrica (a blue-green filamentous alga) was obtained from Dr. William Siegelman, Department of Biology, Brookhaven National Laborotories. Upton, New York. The green alga, *Chlorella pyrenoidosa*, Emerson's strain 3 (originally from the Culture Collection of Algae, Indiana University, Bloominton, Indiana) was kindly supplied by Dr. S. S. Brody, Department of Biology, New York University, New York City, New York. *Euglena gracilis Klebs* strain Z Pringsheim was obtained from Dr. S. H. Hutner of the Haskins Laboratory, New York City, New York.

B. Growth of Organisms

1. Media

Ricinus communis and Zea mays were grown from seed, and plants of Rhipsalis cassytha and Euphorbia pseudocactus were maintained in the greenhouse at Hunter College, CUNY.

Anabaena cylindrica was cultured for four days at 38 ^{0}C in 250-ml Erlenmeyer flasks containing 150 ml medium (Medium C) prepared according to Kratz and Myers (1955) and bubbled with 0.5 % CO_2 in air.

Chlorella pyrenoidosa was cultured for three days at 25 ^{0}C in 500-ml Erlenmeyer flasks containing 250 ml of media prepared according to Pirson and Ruppel (1962) and bubbled with 0.5 % CO_2 in air.

Euglena gracilis strain Z was grown at 25 ^{0}C in either 250-ml Erlenmeyer flasks (containing 100-ml aliquots) or 2000-ml Erlenmeyer flasks (containing 1500-ml aliquots) of the basal salt medium described by Cramer and Myers (1952) supplemented with either 0.015 M sodium acetate, 0.056 M glucose, 0.2 M ethanol or various concentrations of unsaturated long chain fatty acids (see below) as sole carbon and energy sources. Linoleic acid, α-linolenic acid (the latter hereafter referred to as "linolenic acid"), and arachi-

donic acid (Table 3) were prepared and added to the media, as described below. Euglena cells were either bubbled with 0.5 % CO_2 in air or aerated by vigorous agitation in air with a Burrell wrist-action shaker (Burrell Corp., Philadelphia, Pa.) . For reference and accessibility, the acetate-supplemented medium of Cramer and Myers (1952) is given in Table 2.

In all cases, Erlenmeyer flasks were cotton-stoppered. Air containing 0.5 % CO_2 was bubbled first through a flask containing water (to saturate it with water vapor) , and then through a glass "cow" and into the growth flasks.

2. Light Intensities

Anabaena cylindrica and Chlorella pyrenoidosa were grown over a bank of eight, 20-Watt Sylvania cool-white fluorescent lamps, yielding an intensity of 5.0×10^3 erg cm^{-2} sec^{-1} (incident on the bottom of the flasks), as measured by a Yellow Springs Radiometer (Precision Thermistor, Model # 65 ; Yellow Springs instrument Company, Yellow Springs, Ohio) . This radiometer was kindly provided by Dr. Shirley Raps, Department of Biological Sciences, Hunter College, CUNY.

In the case of Euglena, three types of light regimes were used for growth. " Light-grown " cells were those

TABLE 2. Acetate Medium for Cultivation of *Euglena**
(according to Cramer and Myers, 1952)

	mg/liter
$(NH_4)_2HPO_4$	1000.00
KH_2PO_4	1000.00
$MgSO_4.7H_2O$	200.00
$CaCl_2$	20.00
$Fe_2(SO_4)_3.H_2O$	3.00
$MnCl_2.4H_2O$	1.80
$CoSO_4.7H_2O$	1.50
$ZnSO_4.7H_2O$	0.40
$Na_2MoO_4.2H_2O$	0.20
$CuSO_4.5H_2O$	0.02
Sodium citrate	800.00
Sodium acetate	2000.00
Vitamin B 1	0.10
Vitamin B 12	0.0005

pH adjusted to 6.8 (with 0.1 N HCl)

* In the above medium, sodium acetate is the sole carbon and energy source. Sodium citrate is a chelating agent and is not utilized by *Euglena* as a carbon source (Cramer and Myers, 1952).

continuously cultured under conditions in which the average incident light (incandescent) falling on the culture flasks was approximately 1.9×10^3 erg cm^{-2} sec^{-1} (about 110 ft-c) as determined with the Yellow Springs Radiometer. (Average light intensities were calculated from measurement of light intensities on 4 sides of the flasks). "Dark-grown" Euglena were subcultured from cells previously depleted of their chlorophyll and chloroplasts by having been grown in aluminum foil-wrapped flasks, in a darkroom, for more than 30 generations. During the depletion process, fresh cultures were maintained by periodic transfers into fresh media under a green safe light (Lyman et al., 1961), while the cells were in the log phase of growth, i.e. about every 4 days. "Greening cells" refer to dark-grown cells placed in incandescent light (as above) for indicated periods of time.

3. Growth in CO_2-Free Air.

For these experiments, cultures of Euglena were aerated with air that had been passed first through a series of U-tubes filled with excess Ascarite[9] and then through a flask containing water. Since preliminary experiments had demonstrated that cell growth in CO_2-free air was severely retarded, large inocula ($\approx 10^8$ cells, rather than the usual $\approx 10^4$ cells) in the exponential phase of growth were used.

4. Growth in the Presence of DCMU

Photosynthesis was inhibited by adding DCMU

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[9] Ascarite is an 8-20 mesh, sodium-hydrated asbestos, used for the rapid and quantitative absorption of CO_2 (Arthur H. Thomas Catalogue, 1972).

[3(3,4-dichlorophenyl)-1,1-dimethyl urea] in ethanol (stock solution, 1×10^{-2} M) to give a final concentration of 1×10^{-5} M DCMU in the culture flasks.

The following DCMU regimes were applied to dark-grown acetate-supplemented Euglena aerated by being shaken in air:

i) Six flasks of dark-grown cells were removed from the dark and DCMU added immediately to five flasks (one served as control). The six flasks were then placed in the light. Cells in each flask were allowed to green for increasing intervals of time, i.e., 17, 24, 40, 48, 70 hours. Aliquots from each flask were fractionated for evaluation of enzyme content, or processed for electron microscopy.

ii) Five flasks of dark-grown cells were removed from the dark and allowed to green for 18 hours, and then DCMU was added to 4 of the flasks (one served as control) . The cells were then permitted to green for an additional 6, 22, 30 or 54 hours (to yield total times in the light identical to cells in section i, above). Aliquots from each flask were observed photometrically for enzyme concentrations, or processed for electron microscopy.

iii) Of four flasks of dark-grown Euglena which had undergone greening for 24 hours, DCMU was added to three. The cells were then allowed to green for an additional 16, 24 or 48 hours - to yield total times in the light of 40, 48 and 72 hours, respectively. The remaining procedures were as described above in sections i and ii.

iv) Of three flasks of dark-grown Euglena which had undergone greening for 40 hours, DCMU was added to two, and the cells allowed to green for an additional 8 or 32 hours, to yield a total of 48 or 72 hours in the light, respectively. The remaining procedures were as described above, in sections i and ii).

5. Growth in the Presence of Exogenously-Added Fatty Acids.

Linoleic acid, linolenic acid and arachidonic acid were dissolved in absolute ethanol to give stock solutions 6.5×10^{-2} M. To determine the effect of exogenous fatty acid on the growth and development of various microorganisms, stock solutions of linolenic acid were added to the culture media to yield final concentrations of: 5.0 and 1.0 [$\times 10^{-3}$ M, $\times 10^{-4}$ M, $\times 10^{-5}$ M, $\times 10^{-6}$ M, $\times 10^{-7}$ M, $\times 10^{-8}$ M, $\times 10^{-9}$ M, $\times 10^{-10}$ M, $\times 10^{-11}$ M and $\times 10^{-12}$ M, (i.e. 20 different concentrations)] . All solutions of fatty acids were prepared immediately before use.

In some experiments, Euglena were grown in Cramer and Myers basal salt media (1952) supplemented with final concentrations of 1.0×10^{-4} or 1.0×10^{-6} M linolenic acid as sole carbon and energy source.

For reference, the structural formulae of linoleic acid, α-linolenic acid and arachidonic acid are given in Table 3.

Table 3. Structural Formulae of Linolenic Acid, ∝-Linolenic Acid and Arachidonic Acid (according to West and Todd, 1971).

$CH_3(CH_2)_4CH{=}CH_2CH{=}CH(CH_2)_7COOH$	Linoleic Acid ($9,12\text{-}C_{18}$)
$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	∝-Linolenic Acid ($9,12,15\text{-}C_{18}$)
$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$	Arachidonic Acid ($5,8,11,14\text{-}C_{20}$)

6. Growth Determinations

Cell count determinations were made using a Bright Line Hemocytometer (American Optical Co., Buffalo, New York). For cell count determinations of Euglena, the cells were immobilized with 1 % KI (w/v). From such cell counts, generation times were calculated using the following relationships: growth constant, $k = (\ln N - \ln N_0)/t$; generation time, $T = \ln 2/k$. In these equations,

k = growth constant

ln = logarithm to the base e

N = number of cells inoculated into medium at zero time

N_0 = number of cells at the end of a given time period

t = the measured time period

T = generation time

II. Preparative Procedures for Isolating Chloroplasts

A. Preparation of Chloroplasts With Non-Intact Outer Membranes

Chloroplasts were prepared with non-intact outer envelopes according to the method of Jagendorf and Avron (1958). Fifty grams of pre-washed and de-petiolated lamina of Ricinus communis or Spinacea oleracea had their major veins removed, were coarsely chopped, and homogenized in a pre-chilled Waring blender with 150 ml cold 0.35 M NaCl, 0.04 M Tris-HCl at pH 7.8 for three, 5-second blendings (total 15 seconds) in a 15° C coldroom. The homogenate was filtered through 4 layers of cheesecloth and the brei centrifuged at 4° C for 90 seconds at 500 g in a Sorvall RC-2 refrigerated centrifuge (Ivan Sorvall Corp., Newtown, Conn.). The supernatant so obtained was centrifuged at 4° C for seven minutes at 1,000 g, and the resulting pellets were fixed for electron microscopy.

B. Preparation of Chloroplasts With Intact Outer Membranes

Ricinus communis and Spinacea oleracea chloroplasts with intact outer membranes ($\gtrsim$ 80 % intact, as judged by electron microscopy) were prepared according to the method of Spencer (1967) . Five grams of pre-washed, de-petiolated and de-veined lamina were finely chopped with several changes of razor blades in a glass petri dish in a 15° C coldroom for ten minutes with 10 ml of pH 7.8 medium containing 0.4 M sucrose, 0.01 M KCl and 0.05 M Tris-HCl buffer. The resultant brei was filtered through 4 layers of cheesecloth and the filtrate spun at 500 g[10] for 90

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[10] Unless otherwise specified, subcellular fractions were centrifuged out of suspension at 4° C in a Sorvall RC-2 refrigerated centrifuge (Ivan Sorvall Corp., Newtown, Conn.) at indicated speeds and times.

seconds. The supernatant so obtained was centrifuged at 1,000 g for three minutes, and the resulting pellets processed for electron microscopy.

III. Cell-Free Fractions: Preparation and Subsequent Differential Centrifugation to Yield Suspensions Rich in Specific Organelles.

A. Preparation of Cell-Free Fractions

Cells of Euglena were harvested by centrifugation at 500 g for 5 minutes and then washed once with, and resuspended in, 0.4 M sucrose in 50 mM potassium phosphate buffer, pH 7.0, to give a 20 % (v/v) cell suspension (Lord and Merrett, 1971) . The cells, in suspension (in a test tube nestled in chopped ice), were broken by sonication in a Biosonik Sonerator (Bronwill Scientific Co., Rochester, New York) for 15 seconds at a frequency of 20 kilocycles/sec. The sonerator was made available through the generosity of Dr. Shirley Raps, Department of Biological Sciences, Hunter College, CUNY. The suspension of completely broken cells (as determined by light microscopy) was centrifuged at 250 g for 5 minutes at 4^{o} C to remove large pieces of debris - the resulting supernatant constituting the "cell-free fraction" .

B. Differential Centrifugation of Cell-Free Fractions to Yield Suspensions Rich in Specific Organelles.

Cell-free fractions of Euglena were centrifuged at 300 g for 2 minutes to spin down intact chloroplasts (Ellyard and San Pietro, 1969), then at 10,000 g for 10 minutes to spin down mitochondria and chloroplast fragments (Buetow and Buchanan, 1964), and finally at 1000,000 g for 1 hour to sediment the remaining particulate matter

(Lord and Merrett, 1971)[11] . Aliquots of the pellets from these 3 spins were a) resuspended in 0.4 M sucrose in 50 mM potassium phosphate buffer (pH 7.0) for determination of enzyme content, and b) fixed for electron microscopic observation of organelle complement.

IV Sucrose Density Gradients: Their Preparation and Utilization in the Isolation of Specific Organelles.

Euglena grown under the various light regimes were harvested during the early logarithmic phase of growth by centrifugation at 500 g for 5 minutes, washed once with, and resuspended in, 0.4 M sucrose in 20 mM glycylglycine buffer, pH 7.5. The cells in suspension (1×10^6 cells/ml)[12] were broken (according to a slight modification of the method of Tolbert, 1971) by careful manual grinding with Ottawa sand (in a 1:3 volume of suspended cells:sand) for $\lesssim$ 30 seconds at 4° C. To preserve microbodies intact, the grinding was halted when $\lesssim$ 25 % of the cells were broken (as judged by light microscopy) ; Tolbert (1971) did not make such visual observations as a guide to degree of breakage. The suspension was centrifuged at 250 g for 10 minutes to remove whole cells.

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[11] Considerable controversy exists concerning a) the speeds and times required for differential centrifugation of cell-free fractions to yield suspensions rich in specific organelles, and b) the purity of these fractions. For example, Tolbert (1971) states that 100 g for 20-30 minutes is needed to isolate particles rich in whole chloroplasts; shorter periods of centrifugation not packing the cells sufficiently. He then centrifuges the supernatant at 6,000 g for 20 minutes to obtain a pellet containing broken chloroplasts, peroxisomes and mitochondria. In the present work, the 300 g, 10,000 g and 100,000 g pellets, as revealed by electron microscopy, were extremely heterogeneous in organelle composition (page 133).

[12] In general, 10 ml of cell suspension was utilized.

A. Discontinuous Sucrose Density Gradient Fractions.

8 ml of broken-cell suspension obtained as above, was carefully layered on top of a discontinuous sucrose gradient column prepared according to the procedures given by Tolbert (1971). Essentially, these gradients were made by dissolving 855 grams of sucrose in 20 mM glycylglycine buffer , pH 7.5 , to yield a total volume of 1,000 ml of 2.5 M (85.5 % w/v) sucrose solution. Successive dilutions were made with this same buffer , and all sucrose solutions were cooled to 4° C before utilization. A total of eight , 6-ml layers of the following cooled sucrose solutions were carefully pipetted , in succession , into 60-ml polycarbonate centrifuge tubes (Beckman Instrument Co., Mountainside , New Jersey) : 2.5 M sucrose (85.5 % w/v) , 2.0 M sucrose (68.4 % w/v) , 1.75 M sucrose (60 % w/v) , 1.50 M sucrose (51.0 % w/v) , 1.25 M sucrose (43 % s/v) , 1.0 M sucrose (34 % w/v) , 0.75 M sucrose (25.5 % w/v) and 0.5 M sucrose (17 % w/v) . After 4 hours of centrifugation (4° C) at 39,000 $g_{av.}$ (22,500 rev/min.) in a swinging bucket rotor SW 25.2 of a Beckman Model L-2 Ultracentrifuge (Spinco Division of Beckman Instruments , Palo Alto , California) , the tubes were removed , their lower ends pierced , and fractions collected dropwise at 4° C .

B. Continuous Sucrose Density Gradient Fractions.

Continuous sucrose density gradients were prepared in a gradient mixing apparatus (constructed by Mr. James Woodley for Dr. C. Cecciarini , both of the Department of Biological Sciences,

Hunter College , CUNY) . To prepare these gradients , 20 ml of a 34 % w/v (1.0 M) sucrose solution (in 20-mM glycylglycine buffer , pH 7.5) was placed in the inner chamber of the apparatus and 20 ml of a 68.4 % w/v (2.0 M) sucrose solution was placed in the outer chamber. The former chamber was connected to a pump which effected the transfer of dilute sucrose solution to the outer chamber . The outer chamber also contained a magnetic stirrer and an outlet for filling centrifuge tubes . To prepare the continuous gradients , the stirrer was started , the pump was activated , the connections between the two chambers made , and the outlet opened . The resulting continuous sucrose gradient was collected in a 60 ml polycarbonate centrifuge tube , over a cushion of 5 ml of 68.4 % sucrose (in 20 mM glycylglycine buffer , pH 7.5) . The broken-cell suspension (8 ml) was carefully layered on top of the 45 ml continuous sucrose gradient , and centrifuged at 4^{o} C overnight (12-16 hours) at 39,000 $g_{av.}$ in a swinging bucket rotor SW 25.2 (22,500 rev./min) of a Beckman Model L-2 Ultracentrifuge . The (3) tubes were removed from the rotor , pierced at their lower ends , and 1 ml fractions collected , at 0 - 4^{o} C.

The sucrose concentration of these 1-ml portions was measured with an Abbé Refractometer (Bausch and Lomb Optical Co., Rochester , New York) , using a standard curve I had previously plotted from solutions containing known concentrations of sucrose .

V. Methods of Determining Concentrations of Enzymes, Proteins , Chlorophyll and Nucleic Acids .

A. Enzyme Assays

Enzyme assays were performed a) on cell-free fractions , b) on suspensions rich in specific organelles , derived from differential centrifugation of the foregoing cell-free fractions , and c) on both discontinuous and continuous sucrose density gradient fractions . Unless otherwise stated , spectroscopic assays of enzymes were made with Pyrocell silica cuvettes (1.5 ml volume , 0.5 cm light-path) in a Beckman DB-G Grating Spectrophotometer (Beckman Instruments , Scientific and Process Division , Fullerton , California) which were made available through the generosity of Dr. Edward Balboni , Department of Biological Sciences , Hunter College , CUNY .

Unless specified otherwise , values reported for enzyme acitivity are based on two sets of data in which spectrophotometric assays were made in triplicate on 3 separate samples of the same cell-free extract . The means (3 samples) of each experiment were averaged ; data are given in this averaged form .

1. Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6)[13] was assayed either by the method of Lück (1963) (the one most generally used in the present work) or by the method of Baudhuin et al.(1965). By Lück's(1963) method, catalase is assayed by the initial disappearance of 12.5 mM H_2O_2, as measured(at 25°C) by a decrease in absorbancy at 240 nm. The assay mixture contained: 1.0 ml H_2O_2-phosphate buffer solution (66.6 mM phosphate buffer ; 12.5 mM H_2O_2) and 0.01-0.04 ml of the sample.Readings of optical density at 240 nm were made against a blank at 25°C containing all the components except H_2O_2. Catalase concentration, when assayed by this method, is cited in the present work as "units per

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[13] The International Commission on Enzymes was established in 1956 to study the unsatisfactory and confusing nomenclature of enzymes. In 1961, this commission published a numerical scheme for enzyme classification, by which each enzyme is numbered by four digits, separated by points, and arranged on these principles:

(i) The first digit shows to which of the six main classes the particular enzyme belongs,as follows: 1. Oxidoreductases; 2. Transferases; 3. Hydrolases; 4. Lyases; 5. Isomerases; 6. Ligases. For example, catalase belongs to the first of these main classes,i.e., it is an oxidoreductase.

(ii) The second digit indicates the sub-class(e.g., for the oxidoreductases it shows the type of group in the donors which undergoes oxidation).

(iii) The third digit indicates the sub-sub class(e.g., for the oxidoreductases it shows,for each type of donor, the type of acceptor involved).

(iv) The fourth digit is the serial number of the enzyme in its sub-sub class.

For a complete list of the numerical classification see "Report of the Commission on Enzymes of the International Union of Biochemistry"(Oxford, Pergamon Press, 1961).

mg protein" - a "unit" being that amount of catalase which decomposes half of the hydrogen peroxide present in 100 seconds at 25°C (Lück, 1963).

By the method of Baudhuin et al. (1965) catalase(in the presence of 1.5 mM H_2O_2) is determined by the formation of a peroxytitanium sulfate complex as measured at 410 nm and 0°C. In this method, preparations are pretreated with 1.0 % (v/v) Triton X-100 to release the enzyme, and then are incubated at 0 C for 10 minutes in a total volume of 5.2 ml containing: 10 mM imidazole-HCl buffer, pH 7.2; 0.1 % bovine serum albumin; and 1.5 mM hydrogen peroxide. The reaction was stopped by the addition of 3 ml of a three-fold dilution of a saturated solution of titanium sulphate [titanium (IV) oxysulphate] in 2 N H_2SO_4 , and the remaining peroxide was determined colorimetrically in this mixture as the yellow,peroxytitanium sulphate. The initial hydrogen peroxide concentration was measured similarly on a non-incubated mixture of the same composition. One "Baudhuin" unit of activity determined by this method is defined as the amount of enzyme causing the destruction of 90 % of the substrate in 1 minute in a volume of 50 ml under the above assay conditions(Baudhuin et al., 1965).

Catalase Units (Lück, 1963) , "Katalasefahigkeit" (Kat. f. , Euler and Blix, 1919), International units (Bergmeyer, 1955),and Baudhuin Units (Baudhuin et al., 1965) have been used to express catalase concentration. Table 4 facilitates the interconversion of these units.

In the present work, the smallest amount of detectable catalase was ≈ 0.1 units (Lück units) by the method of Lück (1963) and ≈ 10 units (Baudhuin units) by the method of Baud-

Table 4. Catalase Unit Conversion Table (according to Lück, 1963)

To convert from	to	multiply by
Kat. f.	Units/mg protein	0.093
	International Units/mg protein	1.2
Units/mg protein	Kat. f.	10.8
	International Units/mg protein	13.0
International Units / mg protein	Kat. f.	0.83
	Units/mg protein	0.077

Kat. F. and Baudhuin Units may be interconverted by using the following equation:

$$\text{Kat. f. (specific activity)} = \frac{\text{Baudhuin Units}}{\text{grams protein}}$$

(conversions according to Lück, 1963)

huin et al., 1965).[14]

2. Isocitrate lyase (L-isocitrate-lyase, EC 4.1.3.1) was assayed by measuring the rate of increase of O.D. at 324 nm consequent upon the formation of glyoxylate phenylhydrazone (Dixon and Kornberg, 1959) . The assay mixture contained in a volume of 1.2 ml: 8.7 x 10^{-2} M phosphate buffer (pH 6.9); 4.6 mM dithiothreitol; 8.7 mM $MgCl_2$; 1.3 x 10^{-2} M potassium L-isocitrate; 1.0 x 10^{-2} M phenyl hydrazine (freshly prepared); and the sample (containing 2 - 15 µg protein). The assay was initiated by the addition of isocitrate and absorption changes were read at 324 nm and 25 ^{0}C. After a lag of approximately 1 minute, E_{324} is linear for 4 - 5 minutes; this rate is proportional to the enzyme concentration (Dixon and Kornberg, 1959). Calculations were made assuming a molar extinction coefficient of 1.7 x 10^{4} cm^{-1} $mole^{-1}$ for the glyoxylate phenylhydrazone complex at 324 nm (Dixon and Kornberg, 1959).

3. Malate synthase (L-malate glyoxylate-lyase, EC 4.1.3.2) was assayed by following the oxidation of NADH - observed as a decrease in absorbancy at 232 nm. The rate of decrease is a consequence of the breakage of the thio-ester bond of acetyl coenzyme A in the presence of glyoxylate (Dixon and Kornberg, 1959). The enzyme was assayed in a total volume of 1.0 ml that contained: 50 mM Tris-HCl buffer (adjusted to pH 7.4); 10 mM $MgCl_2$; 0.025 mM acetyl CoA; and 10 µl of the sample. The reaction was monitored for 3 minutes to detect the possible presence of an acetyl coenzyme A deacylase (found to be absent in the present work).

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[14] According to Dr. Miklos Müller, The Rockerfeller University, New York, the method of Baudhuin should be at least an order of magnitude more sensitive in detecting catalase than the method of Lück (personal communication).

Sodium glyoxylate (5.0 mM) was then added;during the subsequent 3 minutes the rate of rapid decrease in O.D. is proportional to the amount of malate synthase. I measured O.D. for the first minute of this 3 minute interval. The blank consisted of all the above chemicals except acetyl Co A. An E_{232} for the cleavage of the thio ester bond of acetyl coenzyme A of 4.5×10^3 cm^{-1} $mole^{-1}$ (Stadtman, 1959) was used in the calculations.

4. Glycolate dehydrogenase (glycolate:DCPIP oxidoreductase; EC number has not yet been assigned [15]) was assayed anaerobically at 25 ^0C by following the reduction of 2,6-dichlorophenolindophenol (DCPIP)at 600 nm (Nelson and Tolbert, 1970). The enzyme was assayed by adding to a 3-ml Thunberg cuvette (10 mm in diameter) in the following order: 200 µmoles of pyrophosphate buffer (dihydrogen sodium pyrophosphate and normal sodium pyrophosphate, pH 8.7), 0.3 µmoles of DCPIP, the sample (containing 2-15 µg protein), and water - so that the final volume was 2.5 ml. In the sidearm of the Thunberg Beckman cuvette was placed 0.1 ml of 20 µmoles sodium glycolate. The cuvette was then flushed ten times with N_2 (which had been passed through Fieser's[16] solution to remove traces of oxygen). The reaction was initiated by tilting the cuvette

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[15] The oxidation of glycolate to glyoxylate after photosynthetic carbon dioxide fixation occurs in all green plants, both higher plants and algae (Nelson and Tolbert, 1970). Glycolate oxidase (glycolate:O_2 oxidoreductase, E.C. 1.1.3.1) has been found in all higher plants examined (Tolbert et al., 1969; Nelson and Tolbert, 1970). In green algae (including Euglena) the enzyme which oxidizes glycolate to glyoxylate is glycolate dehydrogenase - oxygen is not the terminal electron acceptor (Nelson and Tolbert, 1970).

[16] Fieser's solution is prepared by dissolving 20 grams of KOH in 100 ml of water, and then adding 2.0 grams of sodium anthraquinone-sulfonate and 15.0 grams of sodium hydrosulfite to the warm solution. The mixture is stirred until a clear blood-red solution is obtained, and is then cooled to room temperature (Reagents for Organic Synthesis, 1964, Fieser and Fieser eds., John Wiley and Sons, New York City, New York.)

so that the sodium glycolate (in the side arm) was mixed with the other reagents. Changes in O.D. were converted to moles of reduced DCPIP by using an extinction coefficient of 21.9 x 10^3 cm^{-1} $moles^{-1}$ (Armstrong,1964).

5. NADH-Hydroxypyruvate reductase, NADH-Glyoxylate reductase or D-Glycerate dehydrogenase [17] (D-glycerate: NAD oxidoreductase, EC 1.1.1.29) was assayed by following the decrease in O.D. by the oxidation of NADH after addition of hydroxypyruvate (1 mM final concentration), according to the method of Tolbert et al. (1970) . The reaction mixture contained in a total volume of 1.0 ml: 200 µl of 0.02 M phosphate buffer (pH 6.2); 50 µl of 4.0 x 10^{-3} M NADH; 30 µl of 0.5 % Triton X-100; and 620 µl of water and enzyme preparation. After a five minute period for measurement of endogenous rate, the reaction was initiated by the addition of 100 µl of 10 mM hydroxypyruvate[18] . An absorbancy change of 1.000 is equal to 32 nmoles (Tolbert et al., 1970).

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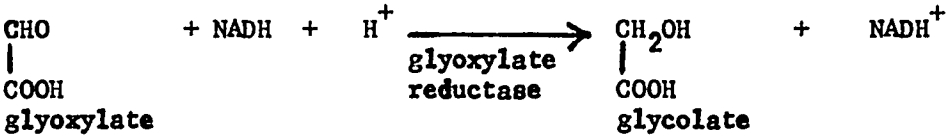
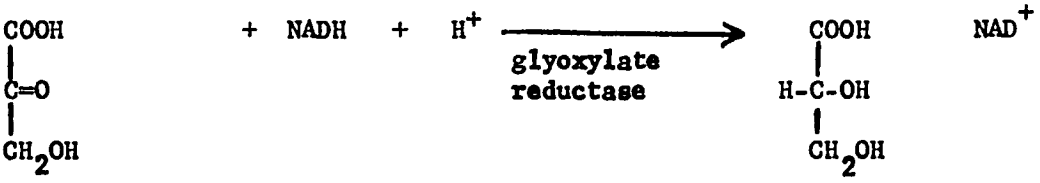
[17] Glyoxylate reductase catalyzes the reduction of glyoxylate to glycolate in the presence of NADH according to the following reaction (Zelitch, 1953) :



glyoxylate glycolate

Hydroxypyruvate is also an excellent substrate for glyoxylate reductase (Stafford and Magaldi, 1954) and the following reaction is catalyzed:



hydroxypyruvate D-glycerate

[18] Since there is a NADH-glyoxylate reductase in chloroplasts, best results are achieved with sucrose graded fractions (Tolbert, 1971). However, in the present work with Euglena, only trace amounts of this enzyme were detected in chloroplast fractions.

6. NAD- Malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) was assayed by following the decrease in O.D. by the oxidation of NADH at 340 nm with oxaloacetate according to the method of Yamazaki and Tolbert (1969). The assay mixture contained in a total volume od 1.0 ml: 0.67 ml of 0.1 M HEPES buffer, pH 7.4; 0.2 ml of 2.81 mM NADH; 1-5 µl of the enzyme preparation; and water to give a total volume of 1.0 ml. The reaction was initiated by the addition of oxaloacetate and readings of optical density at 340 nm at 25° C were made against a blank containing all the components except NADH. A unit of activity is defined as the oxidation of 1 µmole of NADH per minute at 25° C (Yamakazi and Tolbert, 1969).

7. Fumarase or fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2) was determined by the method of Cooper and Beevers (1969) which measures the rate of conversion of malate to fumarate. The reaction mixture contained in a total volume of 1.3 ml : 8.0×10^{-2} M phosphate buffer (adjusted to pH 7.5); 4.0 mM dithiothreitol; 8.0 mM sodium malate; 2 to 10 µg of the enzyme preparation; and water to give a total volume of 1.3 ml. The reaction was initiated with malate and changes in optical density at 240 nm were followed at 25° C. The molar extinction coefficient of fumarate was found to be 2.6×10^{3} cm^{-1} $mole^{-1}$, and this value was used in all subsequent calculations.

8. Aconitase or aconitate hydratase [citrate (isocitrate) hydrolyase, EC 4.2.1.3] was assayed by the method of Racker (1950) which is based on the disappearance of the double bond of cis-aconitate, resulting in a decrease of absorbancy at 240 nm. The enzyme was assayed in a total volume of 1.0 ml that contained: 100 mM Tris-HCl buffer (pH 7.4); 50 mM

trisodium isocitrate; and 100 µl of the enzyme preparation. The reaction was recorded at 240 nm and 25° C for 2 minutes, against a blank containing everything but the substrate (isocitrate). The molar extinction coefficient was found to be 4×10^4 cm^{-1} $mole^{-1}$, and this value was used in all subsequent calculations.

9. Cytochrome c oxidase (Cytochrome c: H_2O_2 oxidoreductase, EC 1.11.1.5) was assayed by the oxidation of cytochrome c at 550 nm, according to the procedure of Tolbert et al. (1968). The enzyme was assayed in a total volume of 1.0 ml that contained: 5-10 µl of the enzyme preparation; 5 µl of 4.0 % digitonin; 200 µl of a 0.1 M phosphate buffer solution (pH 7.0); and 50 µl of 1.5 mM cytochrome c (prereduced with dithionite. Cytochrome c was reduced by dithionite such that the absorbance ratio 550 nm/565 nm was ≈ 9 to 10 (Tolbert et al., 1968). Activity(nmole min^{-1}) was calculated (as the initial rate of the first order reaction) from the following equation (Smith, 1955) :

$$\frac{d\,A550}{dt} = \frac{\epsilon\,red - \epsilon ox}{\epsilon\,red} \times \frac{dA_{red}550}{dt}$$

In this equation, dA550 is the absorption change caused by both the decrease of reduced form and the increase of oxidized form of cytochrome c, $dA_{red}550$ is the absorption change due to the decrease of reduced cytochrome c, and dt is the time period measured (1 minute). Molar extinction coefficients of cytochrome c at 550 nm are ϵ red = 28.4×10^3 cm^{-1} $mole^{-1}$ and ϵ ox = 8.1×10^3 cm^{-1} $mole^{-1}$ (Van Gelder and Slater, 1962) .

B. Protein Concentration

Protein was measured by the method of Lowry et al. (1951) using a calibration curve I obtained with crystalline bovine serum albumin. Protein concentration in sucrose density gradients was corrected for absorbancy by the glycyl-glycine buffer in which the sucrose is dissolved, by using the buffer as a blank.

Unless specified otherwise, values reported for protein concentrations are based on two sets of data in which spectrophotometric assays were made in triplicate on 3 separate aliquots of the sample. The means (3 samples) of each experiment were averaged; data are given in this averaged form.

C. Chlorophyll Concentration

Concentrations of chlorophyll (a + b) as well as a and b in spinach chloroplasts were determined by the method of Arnon (1949). Concentrations of chlorophyll (a + b) as well as a and b extracted from algal cells were determined by the methanol method of MacKinney (1941). In all cases, values reported for chlorophyll are the average of three (3) determinations.

D. Concentration of Nucleic Acids

1. DNA

Microbody-containing fractions from discontinuous sucrose density gradients (2 ml) were homogenized in 8 ml of cold 0.25 M sucrose (in 0.006 N NaOH) with a Potter-Elvehjem homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) according to the method of

Shibko et al. (1967). Concentrations of DNA were estimated on these homogenates by the diphenylamine method of Burton (1956). By this method, 2.0 ml of the homogenized microbody fraction and 4.0 ml of the diphenylamine reagent (containing acetaldehyde)[19] were placed in a 10-ml test tube and mixed. Tubes containing the above reagent plus known amounts of standard DNA[20] were also prepared. A blue color is developed by incubating at 30° C for 16-20 hours, and the optical density at 600 nm is measured against the blank and compared with standard DNA values. In some preparations, DNase (40 μg/ml) was added to the samples which were then incubated at 37° C for 30 minutes, prior to treatment with diphenylamine, to verify the authenticity of DNA (Gibor and Izawa, 1963). In all cases, values reported for DNA are the average of three (3) determinations.

2. RNA

Microbodies in sucrose density gradients were fractionated by the procedure of Shibko et al. (1967), given above, and concentrations of RNA were estimated by the spectrophotometric method of Lin and Schjeide (1969). In this method, 2.0 ml of the homogenized microbody fraction and 2.0 ml of orcinol reagent[21] are pipetted into a 5-ml tear-bulb amouple (Arthur H. Thomas Co., Philadelphia, Pa.). The ampoule

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[19] The diphenylamine reagent is prepared by dissolving 1.5 grams of diphenylamine in 100 ml acetic acid and then adding 1.5 ml concentrated H_2SO_4. The reagent is stored in the dark. On the day of use, 0.10 ml of aqueous acetaldehyde (16 mg/ml) is added per each 20 ml of reagent used.

[20] Standard DNA solutions are prepared by dissolving 0.4 mg/ml calf-thymus DNA in 5 mM NaOH. From this, working standards are prepared by dilutions with $HClO_4$ and heating at 70° C for 15 minutes; these standards may be stored at 4° C for 6 months (Burton, 1956).

[21] The orcinol reagent is prepared by dissolving 1.0 grams of recrystallized orcinol in 100 ml cupric ion reagent (0.15 gm $CuCl_2.2H_2O$ in 100 ml concentrated HCL, sp. gr. 1.19) .

is then sealed with an oxygen torch, the contents rapidly homogenized with the aid of a Vortex mixer (Fisher Scientific Co., Springfield, New Jersey), and the ampoules placed in boiling water. After 35 minutes of boiling, the ampoules were removed and chilled in an ice bath and the absorbancy of each sample was measured at 666 nm. A reference consisted of 2.0 ml of distilled water and 2.0 ml orcinol reagent treated in the same manner as above. Tubes containing standard amounts of RNA were also prepared[22]. In some preparations, RNase (40 µg/ml) was added to the samples which were then incubated at 37 ^{0}C for 30 minutes, prior to treatment with the orcinol reagent, to verify the authenticity of RNA. In all cases, values reported for RNA are the averages of three (3) determinations.

VI Spectroscopy

A. Absorption Spectroscopy

Absorption spectra were determined with a Cary 14 R absorption spectrophotometer (Cary Instrument Co., Monrovia, California) at room temperature (25 ^{0}C).

B. Fluorescence Spectroscopy

Fluorescence emission spectra were determined with the apparatus shown in block diagram, Figure 8 (modified from Brody et al. , 1965) . Light, provided by a high pressure, 1800-watt Xenon arc lamp (Hanovia, Newark, New Jersey) was chopped at 450 c.p.s. (by a segmented disc) and fell upon a Bausch and Lomb grating monochromator (blazed at 400 nm and having a dispersion of 16 A^{o}/ nm). Exciting light

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[22] Standard RNA solutions were prepared by dissolving yeast RNA in 0.001 N NaOH. From this, working solutions were prepared by diluting with 0.001 N NaOH.

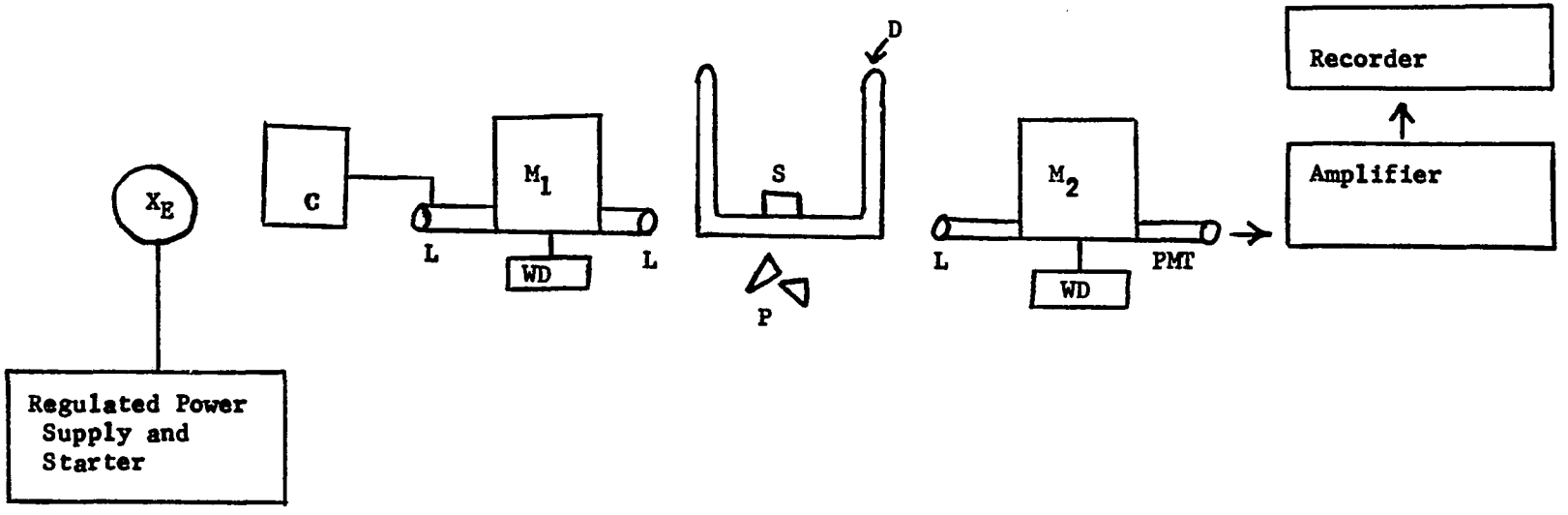

Figure 8. Block diagram of the apparatus used to measure and record fluorescence emission spectra. C, chopper; D, flat bottom dewar; L, lens; M_1 and M_2, exciting and analyzing monochromators, respectively; PMT, photomultiplier tube; P, prisms; S, sample; WD, wavelength drive; X_E, Xeon lamp. Redrawn and modified from M. Brody et al. (1965).

from the monochromator, having a maximum of 436 nm, was passed through a blue glass filter (# C.S. 5-60; Corning Glass Works, Corning, New York) to exclude second order light. At a slit width of 3 mm the incident light intensity used for exciting fluorescence was 504 erg cm^{-2} sec^{-1} (Nathanson, 1973) detemined with an Eppley thermopile (Eppley Corporation, Eppley Labs Inc., Newport Rhode Island),calibrated by the National Bureau of Standards, Washington, D.C. . This light, directed upwards by a mirrored prism towards the optically-clear bottom surface of a mirrored Dewar, fell upon the 2-ml sample, contained in a 5-ml beaker within the Dewar. Samples were cooled to 77° K (-196° C) by adding liquid nitrogen to the Dewar. Fluorescence emitted from the sample was deflected through an optical train, by means of a second mirrored prism, to an analyzing Bausch and Lomb monochromator (blazed at 800 nm and having a dispersion of 33 A°). A red cut-off filter (# C.S. 2-63; Corning Glass Works) was placed on the second mirrored prism to block all light but fluorescence. Emission was detected with a Dumont 6911 photomultiplier tube (S-1 response) - the output of which was fed into an amplifier and phase-sensitive detector , and recorded on a Varian G-11 recorder. The slit widths of the exciting and analyzing monochromators were set at 3 mm and 1.5 mm, resepctively, to yield a halfband widths of 4.8 nm.

VII. Microscopy

A. Light Microscopy

Light microscopic and phase microscopic observations were performed with a Leitz-SM-LUX microscope (E. Leitz, Inc., Rockleigh, New Jersey). Cell counts were made utilizing the light micro-

scope and a Bright Line Hemocytometer to ascertain growth rates. Thick sections (about 1 μm) of epon-embedded leaf tissue were cut for orientation prior to thin sectioning (500 A^{o}) for electron microscopy. For some statistical analyses of microbody profiles, thick sections of DAB-stained, epon-embedded cells were utilized. These thick sections were either viewed, unstained, by phase microscopy, or stained, with toluidine blue and pyronine by the method of Ito and Winchester (1963) .

B. Electron Microscopy

1. Specimens for Electron Microscopy

Algal pellets(Anabaena cylindrica, Chlorella pyrenoidosa, and Euglena gracilis) were obtained for electron microscopy by dilution with 20 mM glycylglycine buffer (pH 7.5) to give a final concentration of 0.8 M sucrose, followed by centrifugation at 39,000 g for 20 minutes in a Sorvall RC-2 refrigerated centrifuge at 4 ^{0}C, according to the procedure of Tolbert (1971). Leaf tissue (Ricinus communis, Zea mays, Rhipsalis cassytha, Euphorbia pseudocactus, Bryophyllum daigremontianum and Kalanchoë blossfeldiana) was washed several times with distilled water and cut with razor blades into small pieces, approximately 1.0 mm^{2}. Where specified in the Results, leaf epidermis (lower or upper) was stripped from fresh, turgid leaves and fixed directly for electron microscopy.

Non-intact chloroplasts and intact chloroplasts were centrifuged at 1,000 g for 10 minutes in a Sorvall RC-2 refrigerated centrifuge at 4 ^{0}C in their respective isolation media, and the pellets fixed for electron microscopy.

2. Routine Preparative Procedures

i) Fixation

The above-cited specimens were fixed for electron microscopy by either of the following procedures:

a) Modification of a procedure given by Vigil (1970) the procedure most frequently used in the present work .

The pellets or tissues were fixed for 2 hours at 4° C with 0.1 M cacodylate buffer (pH 7.4) and either used for cytochemistry (see below) or immediately placed in 2.0 % osmium tetroxide (in 0.05 M cacodylate buffer, pH 7.4) for 2 hours at 4° C. This procedure is useful for studying enzyme cytochemistry, since under these conditions, glutaraldehyde does not interfere with the enzymes (catalase and cytochrome oxidase) being investigated (Frederick and Newcomb, 1969 b; Vigil, 1970) .

b) Modification of a procedure given by Lang and Fisher (1969).

The specimens were fixed for 1 hour at 4° C in 1.0 % aqueous $KMnO_4$ (w/v), washed three times in distilled water, and post-fixed in 1.0 % aqueous osmium tetroxide (w/v). This procedure was utilized only in the case of Anabaena because, as was demonstrated by Lang and Fisher (1969), both the photosynthetic apparatus and polyglucoside granule are more clearly defined than with other methods of fixation.

ii) Post-Fixation

After fixation, the specimens were dehydrated in an ethanol series, passed through propylene oxide, and embedded in Epon

812[23]. Thin "silver-grey" sections (approximately 500 A°) were cut on a Sorvall Porter-Blum MT-2 microtome with a glass or diamond knife, stained with uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963), and viewed with an RCA EMU 3-H or Hitachi HS-8 electron microscope.

3. Cytochemistry for Detection of Catalase and Cytochrome Oxidase.

For such studies, glutaraldehyde-fixed specimens which had been washed several times in 0.1 M cacodylate buffer (page 58) were equilibrated in two ten-minute washings of 0.1 M AMP buffer (2-amino-2-methyl-1,3-propoandiol) adjusted either with 0.1 N NaOH to pH 9.0 or with 0.1 N HCl to pH 7.0, prior to incubation in the cytochemical mixture (Vigil, 1970) . The cytochemical mixture utilized in the present work for the detection of catalase and cytochrome oxidase is that of Novikoff and Goldfischer's (1968) modification of Graham and Karnovsky's (1966) original DAB (3,3'-diaminobenzidine tetrachloride) procedure for the detection of peroxidase. The DAB reaction mixture was freshly prepared before use, and contained: 5.0 ml of 0.05 M AMP buffer; 0.1 ml of 3.0 % hydrogen peroxide (diluted from 30 % superoxol with 0.05 M AMP buffer); and 10 mg DAB. The final pH and temperature of the reaction mixture was adjusted to either pH 7.0 and 25° C or pH 9.0 and 37° C. Specimens were introduced and incubated for 60 minutes. After incubation, the specimens were rinsed three times in 0.1 M cacodylate buffer, pH 7.4. Osmication, dehydration, and embedding in Epon 812 followed the procedures

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23 I found that the usual mixture of 1 part Epon A : 1 part Epon B resulted in capsules too soft for sectioning in the case of material treated or grown in fatty acids of concentrations $> 1 \times 10^{-8}$ M. A ratio of 3 parts Epon A : 7 parts Epon B was found to be satisfactory.

described above. Conditions of incubation (pH and temperature) were controlled to favor the reactivity of specific enzymes with the DAB reaction mixture. When the pH of the reaction mixture is adjusted (with dilute NaOH) to 9.0 and incubation procedes at 37° C, the DAB product of catalase activity can best be observed (Vigil, 1970); when the final pH is adjusted (with dilute HCL) to pH 7.0 and incubation procedes at 25° C, the reactivity of cytochrome oxidase is favored (Novikoff and Goldfischer, 1969; Gerhardt and Berger, 1971) .

Additional specimens were treated with inhibitors of the DAB reaction product as follows:

a) Prior to incubation in the DAB reaction mixture, aliquots of specimens which had been fixed in glutaraldehyde and washed in AMP buffer were preincubated for 20 minutes in AMP buffer containing 0.01 M KCN at pH 7.0 and 25° C or pH 9.0 and 37° C. This treatment was followed by incubation in the DAB mixture containing 0.01 M KCN at either pH 7.0 and 25° C, or pH 9.0 and 37° C. Under these conditions, KCN inhibits both the reactivity of cytochrome oxidase and catalase with DAB (Vigil, 1970).

b) Prior to incubation in the DAB reaction mixture, aliquots of specimens, which had been fixed in glutaraldehyde and washed in AMP buffer were preincubated for 20 minutes in AMP buffer containing 0.02 M aminotriazole (3-amino-1,2,4-triazole; AT) at pH 7.0 and 25° C or pH 9.0 and 37° C. The specimens were then incubated in the DAB mixture, which contained 0.02 M AT, and incubated at pH 7.0 and 25° C or pH 9.0 and 37° C for 60 minutes. Under the latter conditions, AT specifically inhibits catalase activity (Vigil, 1970).

In all experiments, the cytochemical reac-

tivity of DAB was monitored by co-processing germinating castor bean endosperm (a tissue known to exhibit catalase activity and intense DAB staining), by the above procedure.

VIII. Sources of Chemicals

All chemicals used were reagent grade and solutions were prepared using de-ionized, glass-distilled water, unless otherwise specified.

The following chemicals were obtained from Sigma Chemical Company, St. Louis, Mo. : acetyl Co-A (monosodium salt), AMP buffer (2-amino-2-methyl-1,3-propandiol), arachidonic acid, bovine serum albumin, catalase, cytochrome c (type VI), DAB (3,3'-diaminobenzidine tetrachloride), DCMU [3(3,4-dichlorophenyl)-1,1-dimethyl urea], diphenylamine, dithiothreitol, DNA (calf thymus DNA), DNase, FMN (flavin mononucleotide, sodium salt), HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid), hydroxypyruvate, imidazole-HCl buffer, linoleic aicd, α-linolenic acid, L-malic acid (monosodium salt), NADH (β-diphosphopyridine nucleotide, reduced form, disodium salt), RNA (yeast), succinic acid (disodium salt), Tris-HCl buffer (Trizma HCl), Vitamin B-1 (thiamine), Vitamin B-12 (cyanocobalamine).

The following chemicals were purchased from Baker Chemical Company, Phillipsburgh, New Jersey : DCPIP (2,6-dichlorophenol-indophenol), glycolic acid (sodium salt), glyoxylate (sodium salt), phenyl hydrazine HCl, propylene oxide, pyrophosphate buffer (dihydrogen sodium pyrophosphate and normal sodium pyrophosphate), sucrose, Triton X-100 (alkylaryl-polyether alcohol).

The following chemicals were obtained from

Fisher Scientific Company, Fair Lawn,,New Jersey: Ottawa sand (20-30 mesh), sodium acetate, sodium citrate, toluidine blue.

Arthur H. Thomas Co., Philadelphia, Penn., was the source of Ascarite. Cacodylate buffer (sodium cacodylate) and sodium antraquinone-β-sulfonate were obtained from Matheson, Coleman and Bell, East Rutherford,New Jersey. Trisodium isocitrate (d,l form) and oxaloacetate were purchased from Calbiochemical Company, La Jolla, California.

The epoxy resins used in fine structure studies were obtained from R. P. Cargille Labs., Cedar Grove, New Jersey. Osmium tetroxide (osmic acid) was acquired from United Mineral and Chemical Corporation, New York City, New York. Digitonin was purchased from Amend Drug and Chamical Company, Inc. New York City, New York. Orcinol was acquired from Aldrich Chemical Company, Cedar Knolls, New Jersey. The source of pyronine was Eastman Organic Chemicals, Rochester, New York. RNase was obtained from Schwartz-Mann, Orangeberg, New York. The source of titanium sulfate (titanium IV oxysulfate) was Merck Co., Rahway, New Jersey. CO_2 was obtained from Matheson Gas Products Incorporated,East Rutherford,New Jersey. Yeast extract was purchased from DIFCO, Detroit, Michigan.

RESULTS

I. Initial Observations on the Effects of Exogenous Fatty Acids on *Euglena* and Other Organisms.

Treatment for 60 minutes with concentrations $> 10^{-4}$ M linolenic acid produces minimal distortions of thylakoid membranes of both *Euglena gracilis* strain Z (light and dark-grown) and *Chlorella* (light-grown). With increasing concentrations of linolenic acid in the growth media there occur : 1) progressive decreases in chlorophyll content [from 12.6 µg chlorophyll (*a* + *b*)/cell in control *Chlorella* to 4.8 µg chlorophyll (*a* + *b*)/cell in *Chlorella* cultivated in the presence of 10^{-4} M linolenic acid and from 4.7 µg chlorophyll (*a*+*b*)/control 24-hour greening *Euglena* cell to 1.9 µg chlorophyll (*a* + *b*)/cell in 24-hour greening *Euglena* cultivated in the presence of 10^{-4} M linolenic acid] , 2) progressive lengthening of generation times [from 8.1 hours in control *Chlorella* to 20.3 hours in *Chlorella* cultivated in the presence of 10^{-4} M linolenic acid; from 39.8 hours in control dark-grown *Euglena* to 97.5 hours in dark-grown *Euglena* cultivated in the presence of 10^{-3} M linolenic acid; from 23.4 hours in control continuously light-grown *Euglena* to 70.1 hours in continuously light-grown *Euglena* cultivated in the presence of 10^{-3} M linolenic acid], and 3) progressive lamellar distortions.

Another effect of exogenously-added linolenic acid on greening *Euglena* is an initial retardation in lamellae formation (e.g., a plastid from a 15-hour cell cultivated in media supplemented with 10^{-4} M linolenic acid appears morphologically similar to a plastid from

a 9-hour greening control cell). The intensity of emission of the monomeric species of chlorophyll (F 685) and the aggregated species (F 720)[24] was measured at the temperature of liquid nitrogen (77^0 K) as a function of greening. In Fig. 9 may be seen ratios of these emissions (F 720/F 685) plotted as a function of greening for both control and fatty acid-supplemented cells. Cells grown in the presence of 10^{-4} M linolenic acid display an initial retardation, followed by a "catching up" in F 720/F 685 at about 44 hours of greening.

Growth experiments demonstrated that 10^{-4} M linolenic acid may serve as sole carbon and energy sources for Euglena gracilis strain Z cells grown in its presence for over 30 generations.

Since it is known (Matsuka and Hase, 1970) that Chlorella grown in nitrogen-deficient media are largely unable to convert their products of photosynthesis into protein,a means of increasing levels of endogenous cellular fatty acids is available - and may be a basis for comparison with exogenously-added fatty acids.

Cells of Chlorella pyrenoidosa grown in the light and in media containing optimal concentrations of nitrogenous salts are green in color and contain a single cup-shaped chloroplast. This chloroplast contains thylakoids (regularly arranged in bundles of 3 or 4 lamellae), and ribosome-like particles. Chlorella grown in the light for over 30 generations in media containing either 1/5 or 1/10 the optimal amount of

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[24] 720 nm is taken as the average wavelength of the maximum of aggregated chlorophyll; this wavelength actually shifts from 717 nm in dark-grown Euglena cells to 732 nm in cells allowed to green for 80 hours Brody et al., 1965).

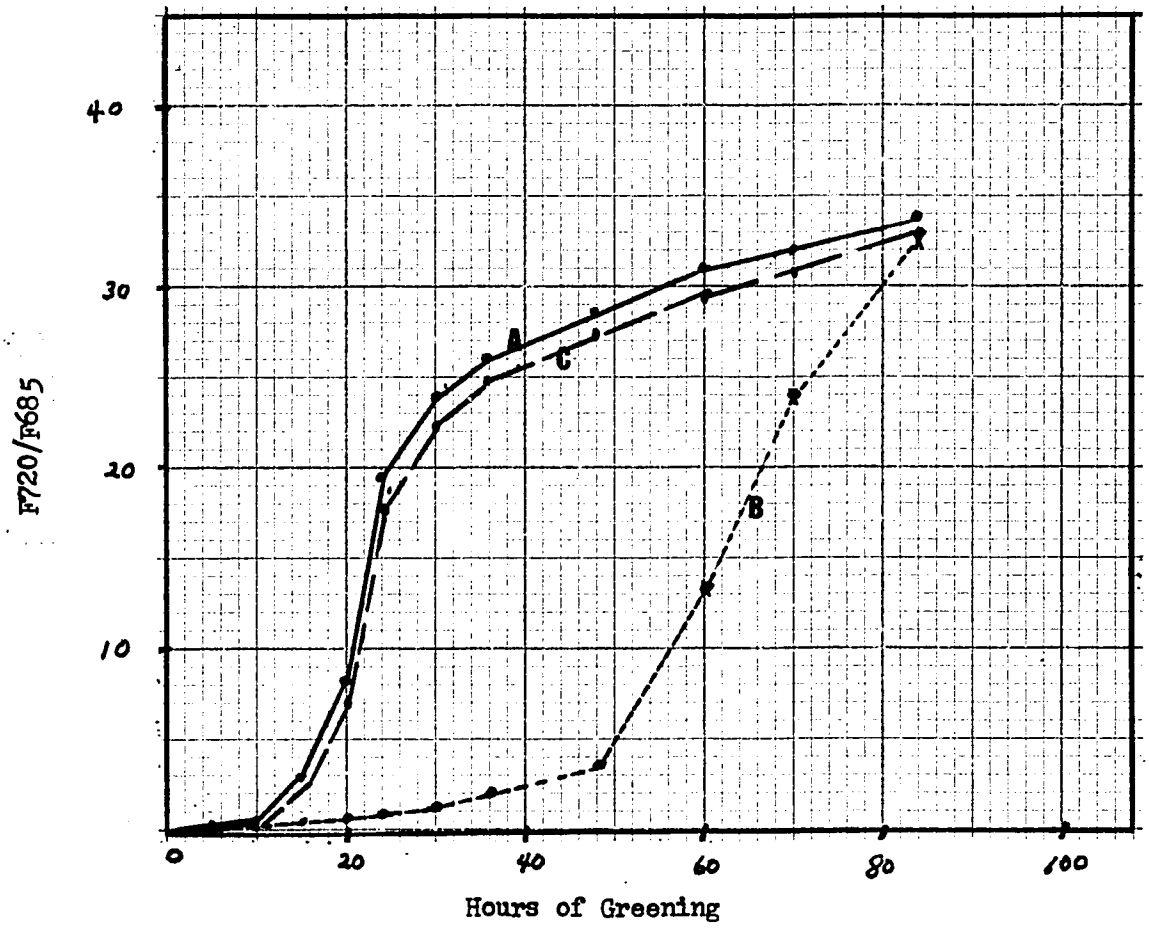

Figure 9. F720/F685 of Euglena gracilis strain Z as function of hours greening. A, control cells; B, cells grown in the presence of 10^{-4} M linolenic acid dissolved in 10^{-6}M ethanol; C, cells grown in the presence of 10^{-6} M ethanol.

nitrogen are yellowish-green in color, with chloroplasts having slightly distorted lamellae, less well-defined than those of the control. These nitrogen-deficient cells appear morphologically very similar to Chlorella grown in the presence of low concentrations (i.e., 10^{-6} to 10^{-10} M) linolenic acid.

Chlorella grown for over 30 generations in media containing 1/20 or 1/50 the optimal amount of nitrogen are yellow in color - their chloroplasts are ill-defined and distorted, appearing somewhat myelin-like. Morphologically, these nitrogen-deficient cells resemble those grown at concentrations of 10^{-4} to 10^{-6} M linolenic acid. Chlorella cultured in media containing 1/100, 1/200, or 1/500 the optimal amount of nitrogen are whitish-yellow in color and seem to possess neither chloroplasts nor lamellae. No internal structure can be observed in these cells, which appear "spore-like" and possess thick walls.

Chlorella cultured in the light in media containing decreasing amounts of nitrogen possess decreasing amounts of chlorophyll a + b. For example, cultures which were grown in optimal concentrations of nitrogen contain 12.6 μg chlorophyll per cell, while cultures grown in 1/50 the optimal amount of nitrogen have 0.7 μg chlorophyll per cell. Chlorophyll is not detectable by the method of MacKinney (1941) in cells cultivated in media containing less than 1/50 the optimal amount of nitrogen in the growth medium.

Chlorella grown in media containing the optimal amount of nitrogen have a generation time of 7.7 hours; nitrogen deprivation progressively retards growth rates. Although generation times could not be

ascertained for cells cultivated in media containing less than 1/200 the optimal amount of nitrogen, Chlorella grown in 1/50 the optimal amount of nitrogen had generation times of 25.3 hours.

Alterations in fine structure, chlorophyll content and generation time which result from growth in nitrogen-deficient media may be reversed upon 48 hours re-exposure to optimal nitrogen media (the foregoing even applied to the cells which appeared to have lost internal structure) .

In the case of Anabaena cylindrica, the major fine structure effect of either treatment with (10^{-3} to 10^{-6} M), or growth in exogenous linolenic acid (10^{-4} to 10^{-12} M ; concentrations $>$ 10^{-4} M inhibited cell division) , was on the polyglucoside granules . These granules were no longer electron-dense , as in controls, but were instead electron-lucent - appearing as "ghosts of granules" . Anabaena cells cultured for 4 days in media containing linolenic acid in the 10^{-4} to 10^{-10} M range possess a chlorophyll a concentration similar to controls , e.g. , 3.8 μg chlorophyll a/cell. Growth at final concentrations $>$ 10^{-8} M linolenic acid slightly lenghthens generation times of Anabaena cylindrica (from 23.3 hours in control cells to 29.1 hours in cells cultivated in 10^{-4} M fatty acid) . It was found that 10^{-2} M ethanol (that concentration used to dissolve 10^{-4} M linolenic acid) had negligible effects upon the generation time or chlorophyll a content of Anabaena . Therefore, most of the observed changes may be attributed to the presence of linolenic acid.

Approximately 80 % of spinach chloroplasts isolated by the

method of Spencer (1967), are whole and intact, as judged by electron microscopy. Treatment of such chloroplasts with exogenous linolenic acid in a FA/Chl[25] range of ≃ 0.001 to 0.5 results in progressive disintegration of chloroplast envelopes to produce naked lamellae, until at FA/Chl ≳ of 1.0 , intact chloroplasts are no longer observed. Concomitant with these changes in chloroplast outer membrane, changes occur in internal or lamellar membranes. At low concentrations of linolenic acid (FA/Chl ≃ 0.001 to 0.01), as in Fig. 10 A, the intergranal(stromal) membranes swell and begin to separate; at higher concentrations (FA/Chl ≳ 0.1) the granal discs become very swollen and the thylakoid fusion layers begin to separate (Fig. 10 C). Increase in FA/Chl $>$ 1.0 produces pronounced lamellar disruption and the appearance of myelin-like figures ; these myelin-like figures are sometimes accompanied by peculiar and aberrant "organelle-like" membrane structure. Similar changes in chloroplast fine structure are observed with exogenous ricinoleic and arachidonic acids , but at FA/Chl an order of magnitude higher.

II. Euglena Microbodies

Although microbodies are difficult to visualize by standard techniques of electron microscopy, the presence of catalase in these organelles makes it possible to utilize the cytochemical identifier DAB. In preliminary experiments, application of DAB incubation to dark-grown, greening and continuously light-grown Euglena gracilis (grown on media supplemented

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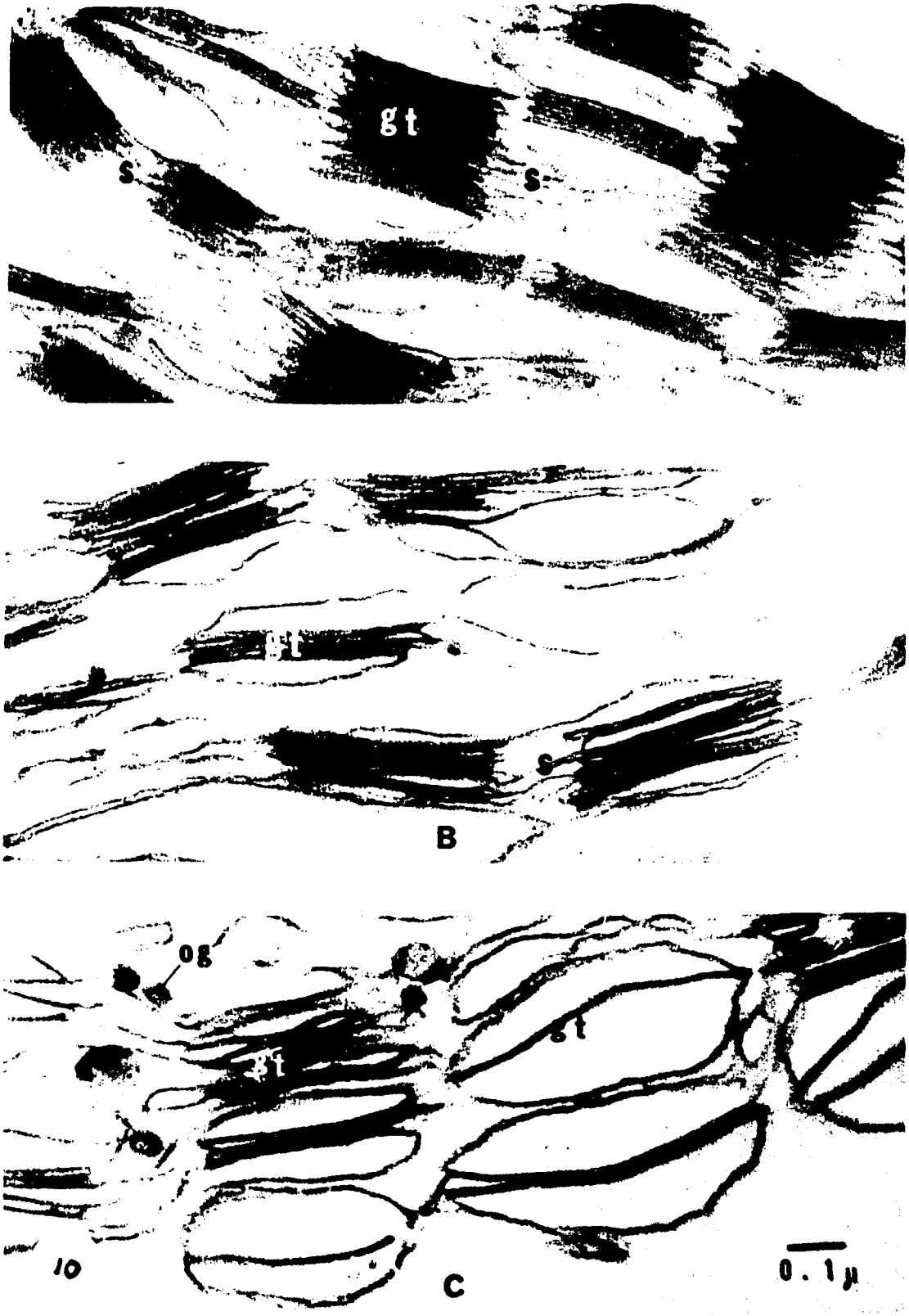
[25] Molar ratios of fatty acid/chlorophyll will be abbreviated FA/Chl.

Figure 10. Results of incubating intact Spinacea chloroplasts (prepared according to the method of Spencer, 1967) in linolenic acid for 30 minutes at 25 °C. E.M.: A, separation of (s) stromal thylakoids (FA/CHL=0.01); B, swelling of (gt)granal thylakoids (FA/CHL = 0.1); C, separation of (gt) granal thylakoids (FA/CHL=1.0). X 72,000.

from Cohen et al. (1969)


gt
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with glucose plus linolenic acid or just linolenic acid as sole carbon and energy source) gave positive cytochemical results. Just after obtaining these results, I read with surprise the papers of Lord and Merrett (1971) and Graves et al. (1971 a) stating that microbodies of Euglena do not contain catalase.

Earlier work (Reeves et al., 1962; Haigh and Beevers, 1964) had indicated that acetate (C_2) supplementation of Euglena induces enzymes of the glyoxylate cycle. Since β-oxidation has been reported to occur in microbodies (Cooper and Beevers, 1969) and the end products of such β -oxidation produce 2-carbon fragments similar to acetate, I performed further experiments to determine whether DAB-mediated polymerization could be observed in acetate-supplemented cells. Cytochemical results with this medium were also positive. I then continued my investigations on microbodies of Euglena cultivated on acetate-supplemented media.because there is an extensive literature on the physiology of such acetate-supplemented cells (Smillie, 1968; Danforth, 1968),compared to the absolute lack of literature on fatty acid supplementation. These investigations led to a major part of my dissertation research effort.

One of the first questions I attempted to answer was why my results were positive in regard to catalase in Euglena , while other workers were unable to detect this enzyme. Perusal of the literature indicated that one variable - the gas phase - was frequently unspecified in publications. Therefore, it was deemed worthwhile to determine the influence of CO_2 in the gas phase on the disposition of catalase.

A. Fine Structure Studies

1. Dark-Grown Cells

Microbodies are present in dark-grown Euglena gracilis strain Z whether carbon and energy sources in the media are glucose, glucose supplemented with linolenic acid, linolenic acid, or acetate. Since, as will be detailed below, it was observed that acetate-supplemented cells contain many more microbodies than glucose-supplemented cells, all data on microbodies (and mitochondria) , unless otherwise specified , were obtained with acetate supplemented cells.[26] Although one may describe the shape of microbodies as being generally spherical to elliptical in nature, the single limiting membrane often seems to undulate (Figs. 11, 12) . From observations of 200 sectioned dark-grown cells, the measured microbody long dimension range was 0.50 - 1.40 μm, with a median of 0.80 μm, a mode of 0.80 μm and a mean of 0.85 μm (Table 5) . The measured microbody short dimension range of dark-grown cells was 0.40 - 0.90 μm , with a median of 0.60 μm, a mode of 0.65 μm , and a mean of 0.65 μm. In some cases where sections were spherical, long and short dimensions were identical.

The fine grains of the microbody matrix tend to flocculate within the 60 - 70 A^{o} limiting membrane (Figs. 11, 12). This membrane stains only faintly - thereby, making microbodies difficult to

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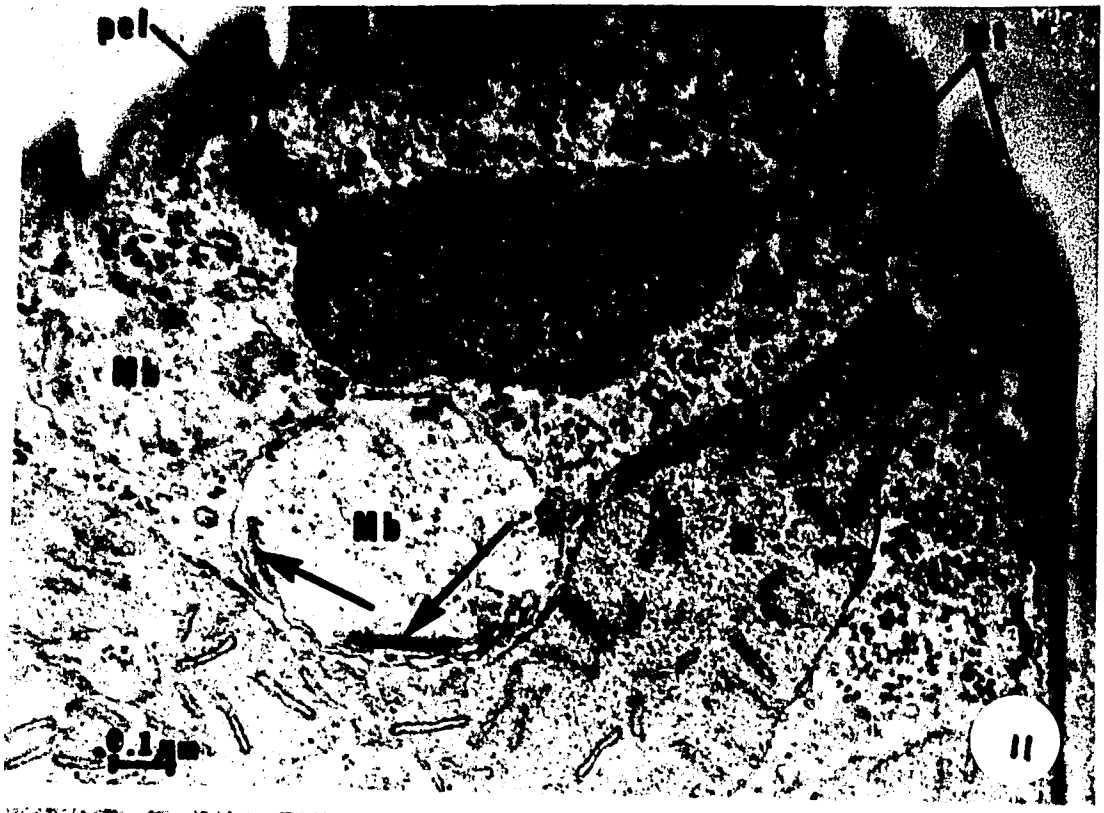
[26] Too few experiments were done to detemine accurately the number of microbodies in cells grown in media containing glucose plus linolenic acid or just linolenic acid as sole carbon and energy sources. However, the number of microbodies per cell in Euglena grown in either of these media is intermediate between cells cultured in glucose-supplemented media and cells grown in linolenic acid-supplemented media.

Figure 11. E.M. of two (non-DAB treated) microbodies (Mb) surrounded by mitochondria (M) in log-phase, dark-grown, aerated, acetate-supplemented Euglena gracilis strain Z. Arrows indicate multi-laminate extensions of limiting microbody membranes. Note the flocculent material in microbody matrices. Also seen are microtubules (Mt) and the pellicle (pel). x 70,000.

Figure 12. E.M. of two (non-DAB incubated) microbodies (Mb) surrounded by mitochondria (M) in log-phase, dark-grown, acetate-supplemented Euglena gracilis strain Z. x 70,000.

Figure 14. E.M. of multilaminate core structure (lysosome) in (non-DAB incubated) log-phase, dark-grown, aerated, acetate-supplemented Euglena gracilis strain Z. x 70,000.


pel
Mb
Mb
0.1μm
11


Mb
Mb
0.1μm
12


L
14

Table 5. Microbody* Diameters of Dark-Grown, Greening and Continuously Light-Grown Euglena Gracilis strain Z.

Data based on measurements of electronmicrographs of 200 randomly selected DAB-treated microbodies in each category. The ± values given after the mean are standard deviations.

Light Regime	Measured Long Dimension			
	Range (in μm)	Median (in μm)	Mode (in μm)	Mean (in μm)
Dark-Grown	0.50-1.40	0.80	0.80	0.80 ± 0.20
12-hr Greening	0.55-1.35	0.85	0.80	0.87 ± 0.19
24-hr Greening	0.40-1.45	0.80	0.85	0.79 ± 0.23
36-hr Greening	0.35-1.55	0.90	0.90	0.75 ± 0.25
48-hr Greening	0.55-1.40	0.85	0.80	0.85 ± 0.15
72-hr Greening	0.45-1.50	0.80	0.85	0.81 ± 0.25
Continuously Light-Grown	0.60-1.35	0.80	0.90	0.80 ± 0.13
	Measured Short Dimension			
Dark-Grown	0.40-0.90	0.60	0.65	0.65 ± 0.16
12-hr Greening	0.45-1.00	0.65	0.65	0.63 ± 0.23
24-hr Greening	0.45-1.00	0.60	0.60	0.61 ± 0.15
36-hr Greening	0.45-0.95	0.55	0.50	0.51 ± 0.13
48-hr Greening	0.40-0.85	0.65	0.60	0.63 ± 0.17
72-hr Greening	0.50-1.10	0.70	0.65	0.68 ± 2.0
Continuously light-grown	0.45-0.95	0.65	0.65	0.66 ± 1.8

*Only organelles giving DAB positive reactivity were measured.

localize by standard techniques.[27] Occasionally, there are observed multilaminate invaginations of the limiting microbody membrane into the matrix (see arrows, Fig. 11). Alternately, perhaps what is observed in this figure may be an intrusion of the endoplasmic reticulum into the microbody .

I found the favored locations of microbodies are the cell periphery and regions nearest the gullet. Dark-grown acetate-supplemented cells most frequently contain 3 or 4 (3.10 average) microbodies in any particular randomly oriented thin ($\lesssim$ 500 A^{o}) section (Table 6), with as many as 10 observed in some cells of a section(particularly in regions near the gullet). If instead of cutting thin sections to yield microbody counts as above, thick sections of epon-embedded cells are cut ($\approx$ 1-2 μm), and attention is focused on longitudinal sections in regions near the gullet, an average of 5.2 microbodies per longitudinal thick section is found (Fig. 13 A and Table 7). The situation in regard to number of microbodies in glucose-supplemented dark-grown cells in quite different - many sections must be observed to detect even a single microbody.

In order to make microbody profiles (i.e., to determine the number of microbodies per cell) serial thick sections (1 - 2 μm) of longitudinally-oriented DAB-treated epon-embedded Euglena were made, and observed under a light microscope (Fig.13 A). From the literature (Johnson, 1968) and based on my measurement of 50 cells, the average dimensions of Euglena gracilis strain Z are 30.2 μm across the horizontal axis and 155.8 μm across the longitudinal axis. Approximately

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27 Positive identification of microbodies is ascertained by enzymatic composition (Frederick and Newcomb, 1969 b). However, tentative identification may be made on the bases of organelle granularity, shape and size.

Table 6. Average Number Of Microbodies* In Thin Sections Of DAB-Treated Dark-Grown, Greening, and Continuously Light-Grown Aerated, Acetate-Supplemented Euglena Gracilis strain Z.

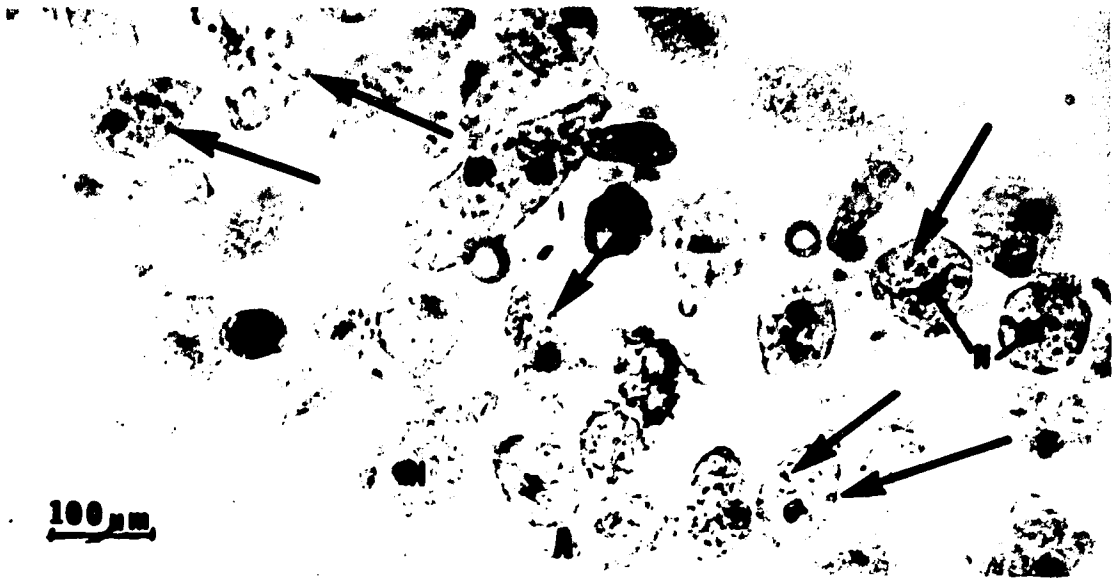
Data based on electron microscopic random counts of 200 DAB-treated, epon-embedded cells in each category. The ± values given after the mean represent standard deviations.

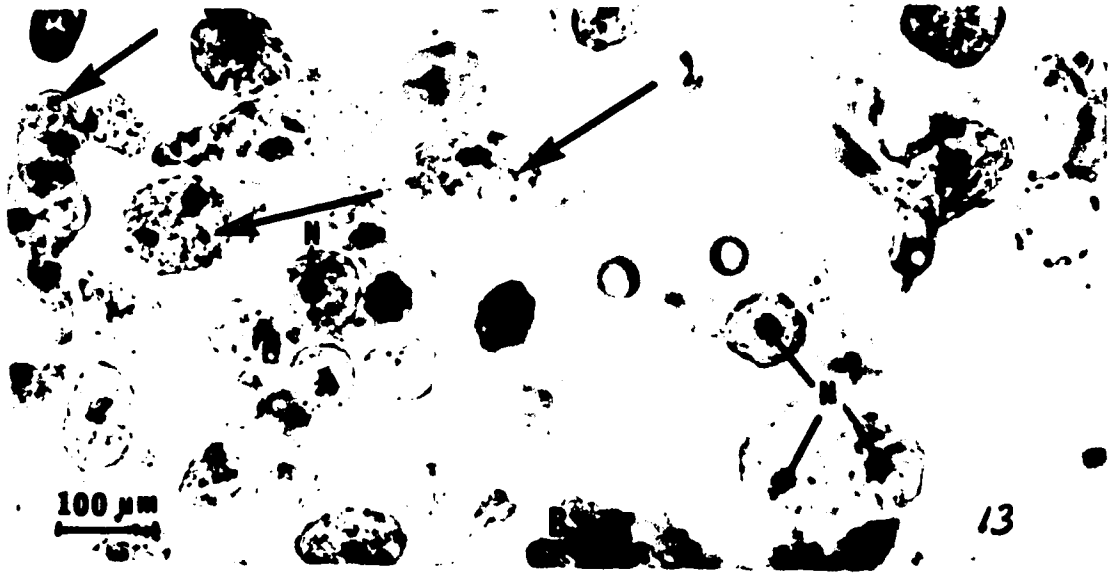
Light Regime	Mean Number of Microbodies
Dark-Grown	3.10 ± 0.53
12-hr Greening	4.63 ± 0.76
24-hr Greening	6.31 ± 0.73
36-hr Greening	6.26 ± 0.87
48-hr Greening	6.73 ± 0.83
72-hr Greening	6.53 ± 0.78
Continuous light	6.44 ± 0.81

* Only organelles giving a positive DAB reaction were counted

Figure 13. Light micrographs of thick sections (1-2 μm) of DAB-treated, epon-embedded, aerated, acetate-supplemented Euglena gracilis strain Z. A, dark-grown cells. B, 24-hour greening cells. Arrows indicate dark brown, DAB-reactive microbodies.

X 1,500.





13

Table 7. Average Number Of Microbodies* In Thick Longitudinal Sections, In Regions Near the Gullet**, In Dark-Grown, Greening and Continuously Light-Grown Aerated, Acetate-Supplemented Euglena Gracilis strain Z.

Data based on light microscope counts of 200 DAB-treated, epon-embedded cells in each category. The ± values given after the mean represent standard deviations.

Light Regime	Mean Number of Microbodies
Dark-Grown	5.2 ± 0.72
12-hr Greening	7.3 ± 0.89
24-hr Greening	9.4 ± 0.78
36-hr Greening	8.9 ± 0.85
48-hr Greening	9.3 ± 0.98
72-hr Greening	9.1 ± 0.87
Continuous light	9.4 ± 0.88

* Only organelles giving a positive DAB reaction were counted.

** Counts were made on longitudinal sections in regions near the gullet to standardize the data and also because observations had indicated that microbodies are numerous here.

30 serial sections of 10 different longitudinally-oriented cells were viewed under a light microscope, and dark brown, DAB-reactive microbodies were counted in cells in the sections (Fig. 13 A). Based on serial counts of 10 such cells, there is an average of 3.6 microbodies per section of dark-grown, acetate-supplemented Euglena; the range being 287 - 346 microbodies per cell.[28]

Thin serial sections ($\approx$ 500 A^{o}) of longitudinally-oriented DAB-stained Euglena cells were also made for viewing by electron microscopy. Since it would have required more than 600 thin sections to go through one cell, I attempted (within the limits of experimental techniques) to observe every tenth section and count the number of electron dense microbodies . This technique was utilized with 10 different cells (all in the same section) - corrections being made for sections of cells which could not be observed because of their position over the bars of the copper grid. By fine structure observations, I found an average of 276 microbodies per dark-grown Euglena cell, with a range of 243 - 316 .

Based on random counts of 200 thin sections of cells, there are $\lesssim$ 300 mitochondria to each microbody in dark-grown glucose-supplemented Euglena, while the ratio is $\lesssim$ 25:1 in acetate-supplemented cells. The number of mitochondria per cell section, however, are about equal in both of the aforementioned types of cells. There are $\lesssim$ 90,000 mitochondria/ dark grown, glucose-supplemented Euglena and $\lesssim$ 75,000 mitochondria / dark-grown, acetate-supplemented cell.

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[28] However, as pointed out by Koningsmark (1970), all total count methods of sectioned material are subject to counting errors (in which units may be missed or counted twice by the microscopist) and split cell errors (in which the unit counted is cut and is counted in two adjacent sections) .

I have found an occasional multilaminate core structure structure in Euglena (Fig. 14). Although Graves et al. (1971 a) considered these to be forms of microbodies, I believe that they are residual lysosomes[29] because of their morphological appearance, and the fact that they are electron-opaque even without cytochemical staining. In Fig. 15 other stages of lysosome activity may be observed (note the arrows to autophagic vacuoles)[30, 31].

Incubation of dark-grown aerated glucose- or acetate-supplemented Euglena in the standard DAB reaction mixture, at pH 9.0 and 37° C, results in pronounced deposition of electron-dense material in microbodies - their matrices being electron-opaque, coarsely granular, and flocculent (Figs. 15,16). The limiting microbody membrane also becomes more opaque in appearance due to accumulation of the DAB reaction product (Figs. 15,16). It is thus rewarding that the DAB reactivity used

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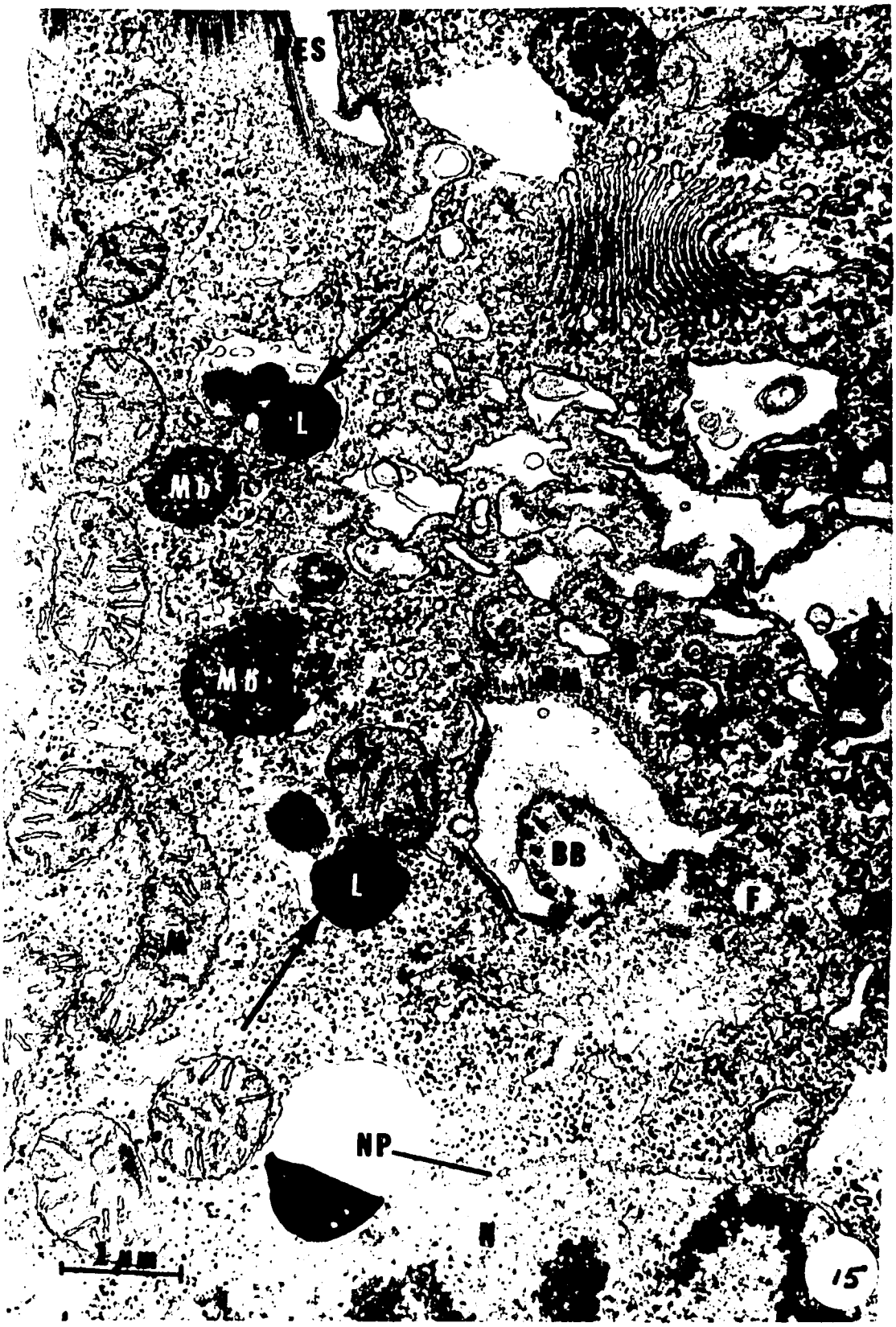
[29] The identification of lysosomes on a purely morphological basis is difficult because of their polymorphism, which is influenced by digestive activities and the array of contained substances in various tissues (de Duve, 1963). For convenience, the various stages of lysosomes have been classified (de Duve, 1963) as storage vacuoles, digestive vacuoles, residual bodies, or autophagic vacuoles (cytolysomes). The storage vacuole represents the original, or virginal, lysosomes. The digestive vacuole results partly from phagocytotic and pinocytotic processes of the cell; by these processes foreign proteins as well as large particles can be engulfed by the cell. Following enzymatic digestion within the digestive vacuole, the resulting products diffuse through the membrane into the cytoplasm. However, some of the material cannot be digested and remains within the vacuole as undigested material; this vacuole is now referred to as the "residual body" and contains multilaminate core structures. Cytolysomes (or autophagic vacuoles) are membrane-bound intracellular bodies that contain mitochondria and other cellular material in varying degrees of degeneration.

[30] Similar structures have been reported(Brandes et al., 1964) in a streptomycin-bleached strain of Euglena gracilis starved of its carbon source (acetate).

[31] It is also possible that these structures are acid phosphatase bodies (Schiff, personal communication) .

Figure 15. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C), aerated, log-phase, dark-grown, acetate-supplemented Euglena gracilis strain Z, near the gullet region. BB, basal body; F, flagellum; GB, Golgi body; L, lysosome; M, mitochondria; Mb, microbody; N, nucleus; RES, reservoir; RM, reservoir microtubules. Arrows indicate lysosomes which appear to be in the cytolysome stage. The cytolysomic action indicated by the uppermost arrow seems to be occurring within a mitochondrion. x 20,000.


ES
L
Mb
Mb
L
BB
F
NP
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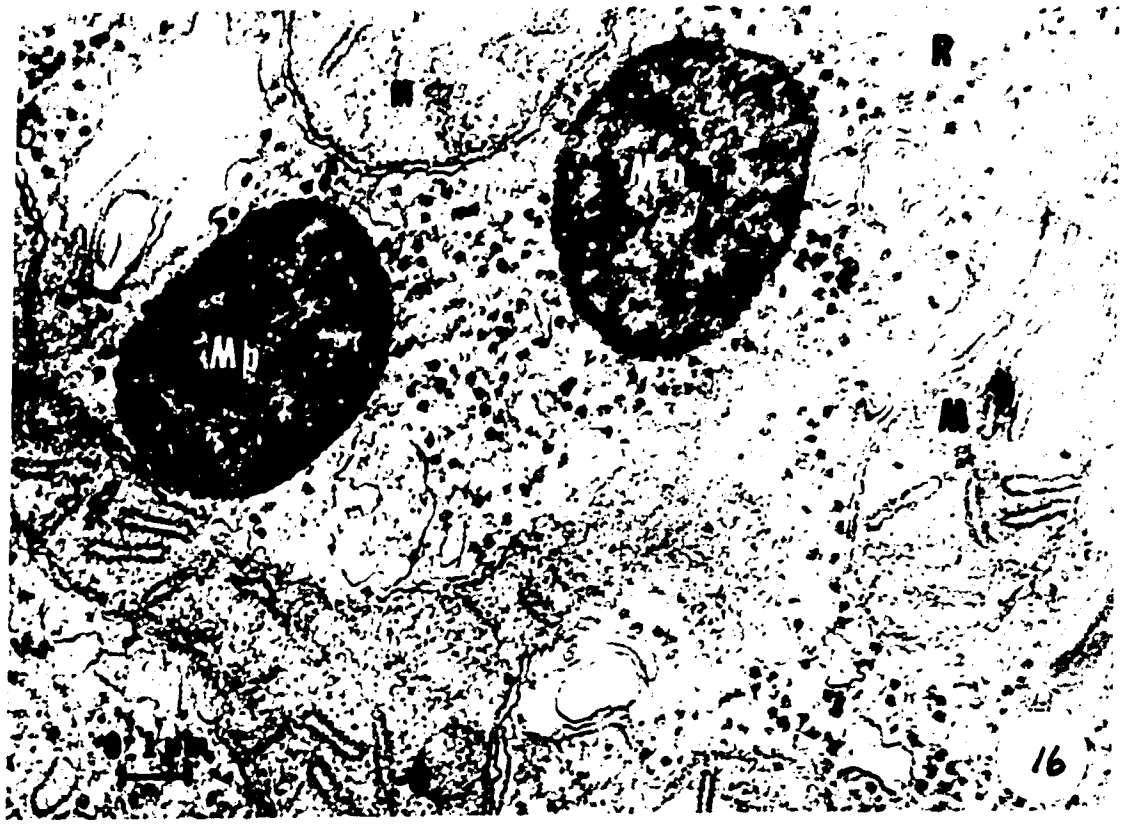
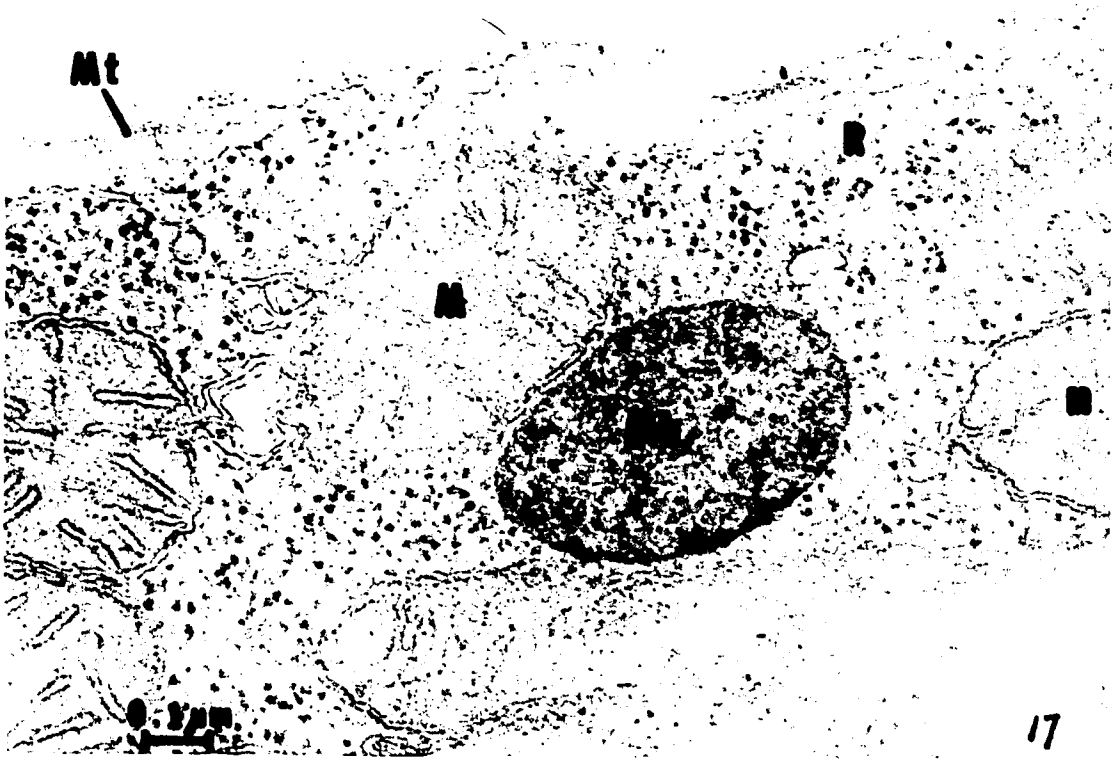
1 µm
15

Figure 16. E.M. of two DAB/H_2O_2 incubated (pH 9.0, 37 °C) microbodies (Mb) surrounded by mitochondria (M) in aerated, dark-grown, acetate-supplemented Euglena gracilis strain Z. x 70,000.

Figure 17. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) aerated, dark-grown, acetate-supplemented Euglena gracilis strain Z that was not stained with lead citrate and/or uranyl acetate. Note the electron-dense microbody (Mb), electron-lucent mitochondria (M) and microtubules (Mt). x 70,000.


R
M
Mb
M
16


Mt
R
M
M
0.5 μm
17

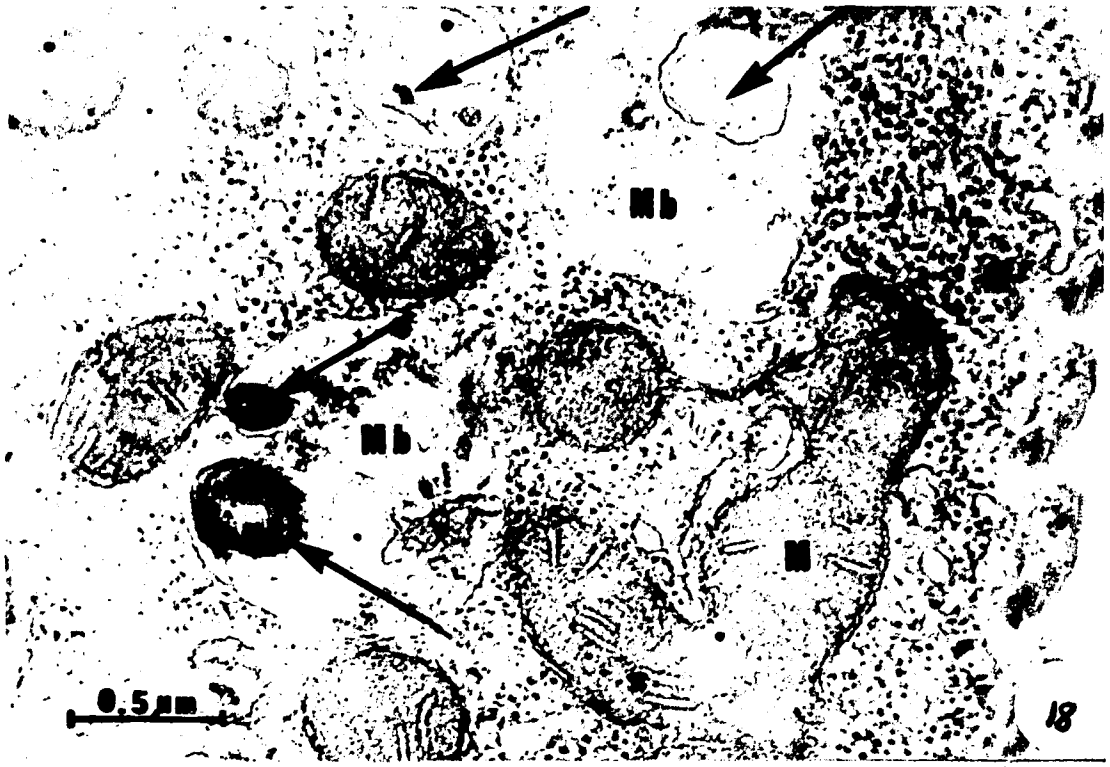
to identify catalase may be utilized for the localization of microbodies. Omission of post-staining with uranyl acetate and/or lead citrate emphasizes the difference in electron opacity between DAB-polymerized microbodies and other organelles (Fig. 17). DAB polymerization is still evident in microbodies when hydrogen peroxide is omitted from the reaction mixture. Deposition of DAB product in microbodies is completely inhibited when 0.02 M aminotriazole (Fig.18) or 0.01 M KCN are added to the reaction mixture. Catalase-mediated DAB deposition is not observed in microbodies of dark-grown acetate-supplemented Euglena grown in CO_2-free air for 24 or 48 hours; reaeration in air containing carbon dioxide for more than 2 hours restores DAB reactivity.

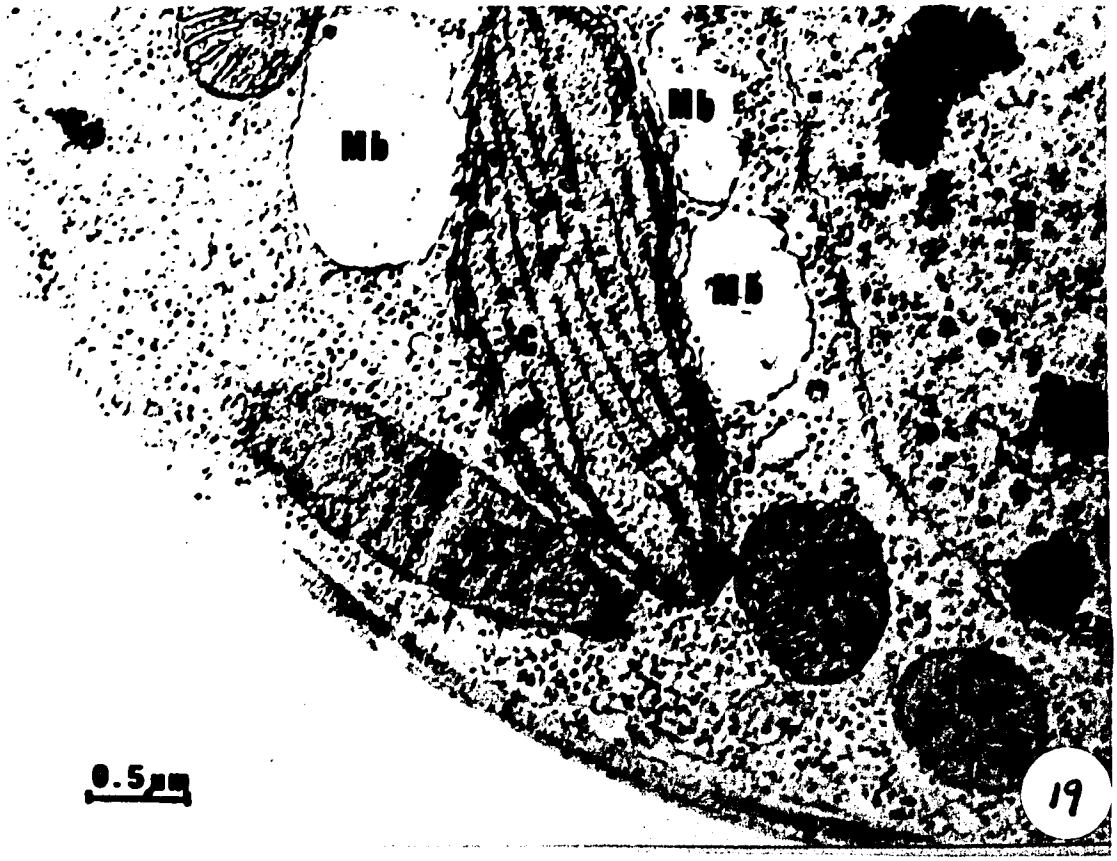
2. Greening Cells

Euglena cells grown on acetate (or glucose)-supplemented media undergo an increase in number of microbodies with greening. The increase begins after $\lesssim$ 10 hours of illumination (at an intensity of 1.9×10^3 erg/cm^2/sec) when the plastid has $\approx$ 2 straight thylakoids. Based on random electron microscopic counts of thin sections of acetate-supplemented cells, the average number of microbodies per cell section has approximately doubled by 24 hours of greening (so that the ratio of mitochondria to microbodies has decreased from $\lesssim$ 25:1 to $\lesssim$ 15:1), after this time remaining fairly constant. From Table 6 it may be seen that dark-grown, 12-, 24-, 36-, 48-, and 72-hour greening cells have an average of 3.10, 4.63, 6.31, 6.26, 6.73, and 6.53 microbodies per cell section, respectively, based on electron microscopic random counts of 200 DAB-treated acetate-supplemented cell sections of each one of these stages of greening. Additionally, light microscopic counts of thick longitudinal sections (in

Figure 18. E.M. of DAB plus aminotriazole incubated (pH 9.0, 37 °C) aerated, dark-grown, acetate-supplemented Euglena gracilis strain Z, showing several electron-lucent microbodies (Mb). Arrows indicate inclusions in these microbodies. X 40,000.

Figure 19. E.M. of DAB plus aminotriazole incubated (pH 9.0, 37 °C) aerated, acetate-supplemented Euglena gracilis strain Z after 24 hours of greening. Note several microbodies (Mb), mitochondria (M), a chloroplast (C), and a nucleus (N) containing chromatin material . X 30,000.


Mb
Mb
M
0.5 μm
18


Mb
Mb
Mb
0.5 μm
19

regions near the gullet) of DAB-treated Euglena confirm the approximate doubling in number of microbodies per section which occurs by 24 by 24 hours of greening (Fig. 13 B); such counts yield 5.2, 7.3, 9.4, 8.9, 9.3 and 9.1 microbodies per section in dark-grown, 12-, 24-, 36-, 48-, and 72-hour greening cells, respectively (Table 7).

Thick (1 - 2 μm) and thin ($\lesssim$ 500 A$^{\circ}$) longitudinal sections were made on 10 different DAB-treated epon-embedded 24-hour greening cells for purposes of obtaining microbody profiles. On the basis of thick sections (light microscopy), by 24 hours of greening, a Euglena cell contains an average of 589 microbodies - the range being between 556 and 623 microbodies per cell. Thin electron microscopic profiles revealed an average of 546 microbodies per cell, ranging from 520 - 589 microbodies per cell.

As greening procedes, microbodies are sometimes found to be located appressed to developing plastid (Fig. 19). However, this type of appression is not the usual situation; more frequently the location of these two organelles seems to be random in relation to each other. Note in Fig. 19 the flattening of two microbodies which are sandwiched between the developing 24-hour chloroplast and nucleus.

From Table 5 it may be observed that the range, median, mode and mean of both long and short microbody measured dimensions of greening cells are approximately the same as dark-grown cells. As in the case of dark-grown cells, the deposition of DAB reaction product in microbodies of greening cells is completely inhibited when either 0.02 M aminotriazole (Fig. 19) or 0.01 M KCN is present in the DAB reaction mixture.

The activity of catalase in acetate-supplemented greening cells was examined as a function of gas phase in the following manner. Catalase-mediated DAB reactivity (pH 9.0, 37° C) was investigated in the case of dark-grown cells which had been aerated in the absence of CO_2 for 48 hours and then allowed to green for 1, 2, 4, 6, 12, 18 or 24 hours, in (A) the absence of CO_2 and (B) the presence of CO_2. All cells in category (A) failed to exhibit catalase-mediated DAB reactivity. In category (B),cells which had been greening for less than 2 hours (in the presence of CO_2) failed to exhibit DAB reactivity; after 2 hours in CO_2 , microbodies were found to be electron-dense. By ≲ 12 hours of greening, microbody-like organelles (≲ 0.85 μm in long dimension, ≲ 0.65 μm in short dimension, with a granular, flocculent matrix, and bound by a single membrane) which appear to be undergoing division, are observed in acetate-supplemented Euglena . In Fig. 20 several arrows indicate regions in which divisions of microbodies seems to be occuring . At arrows "A" and "B" a type of binary fission appears to be in progress, whereas at arrow "C" a type of "budding" seems to be occurring. Note also in Fig. 20 the asterisks indicating membranous inclusion in microbodies.

A cluster of microbodies in a 24-hour greening cell appears at the top of Fig. 21 A. Upon greater magnification (Fig. 21 B) it is noted that many of these microbodies are not independent, but are part of a multilobed microbody complex, such as one might expect to result from an (as yet) incomplete series of successive divisions of a single microbody. The points of attachment are noted by arrows. Several types of inclusions are found in the lobes of this complex: 1) thin, filmy membranes, such as those observed in areas "A" , 2) electron-dense areas which are membrane-bound ("B") , 3) electron-dense whorls such as those

Figure 20. E.M. of (non-DAB treated) aerated, acetate-supplemented Euglena gracilis strain Z after 12 hours of greening, showing several microbodies (Mb) apparently undergoing division(s). Arrows "A" and "B" indicate a possible "binary fission" type of division. Arrow "C" indicates a possible "budding" type of division. Asterisks indicate membranous inclusions inside microbodies. Also seen are mitochondria (M). x 70,000.

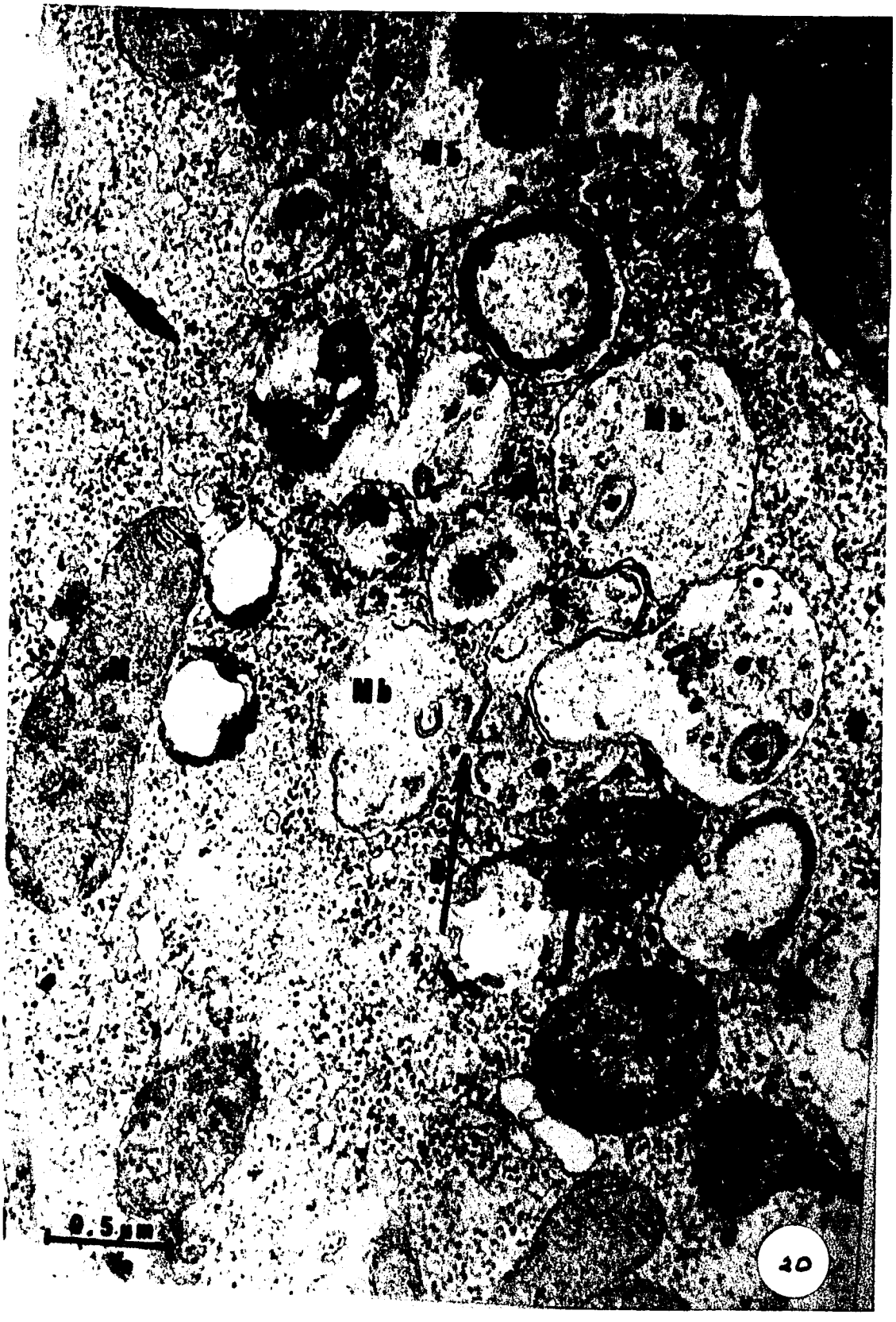

Mb
Mb
Mb
0.5 µm
20

Figure 21 A. E.M. of non-DAB treated, aerated, acetate- supplemented Euglena gracilis strain Z after 24 hours of greening, showing a "cluster" of microbodies (Mb), nucleus (N), flagellum (F), mitochondria (M), and pellicle (pel). Arrows indicate sites of possible divisions in the multilobed microbody complex.

X 30,000.

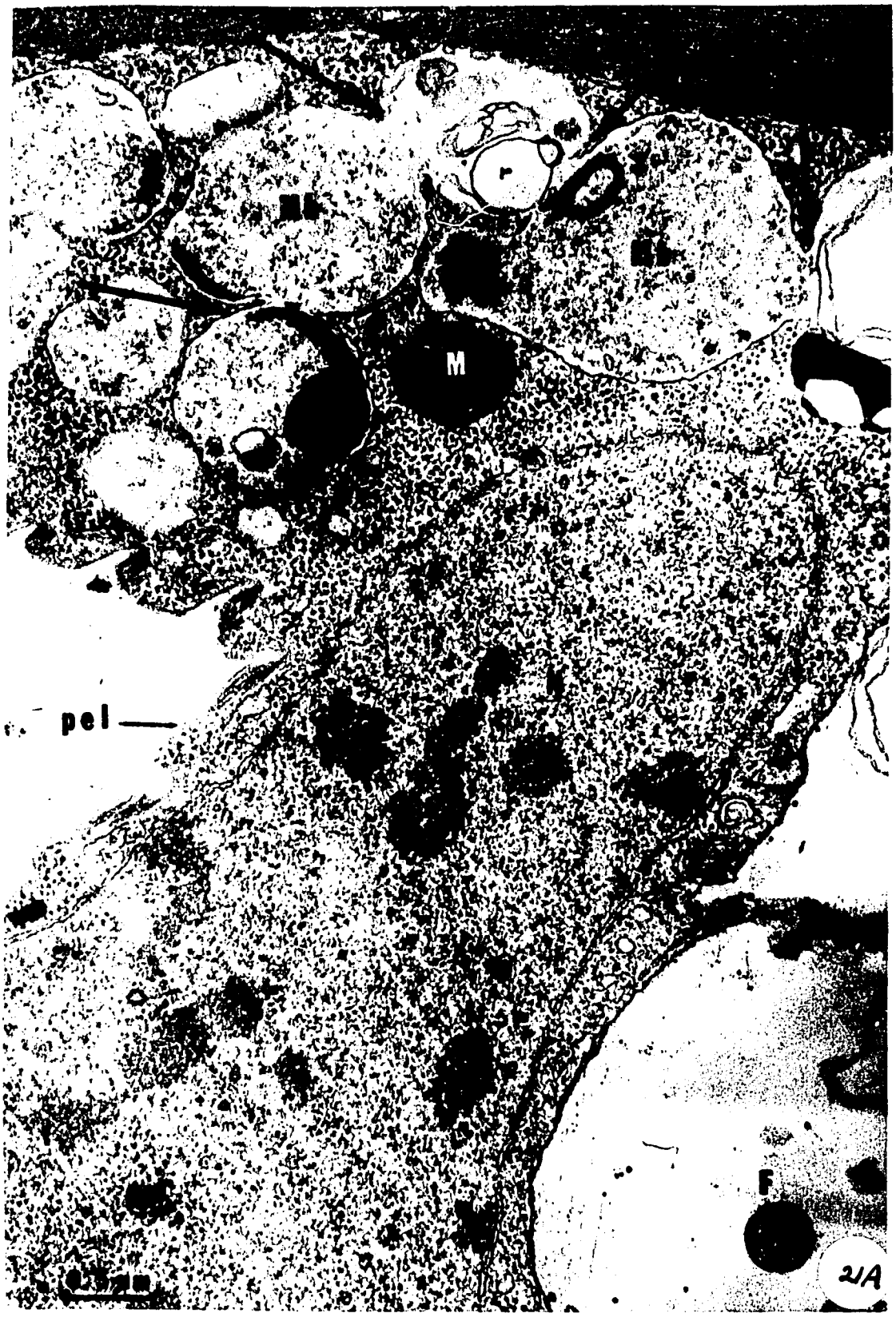
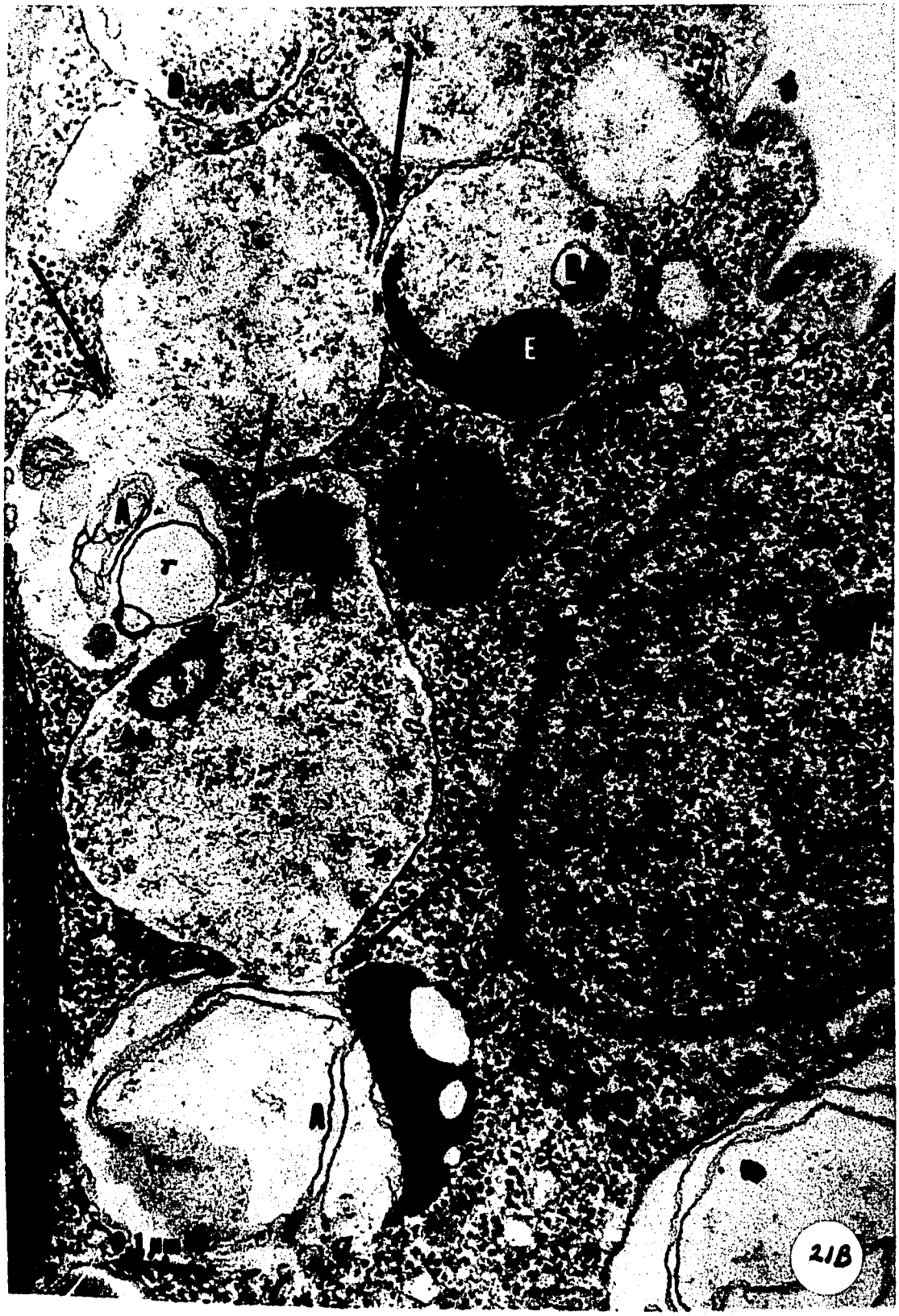

M
pel
F
2A

Figure 21 B. Enlargement of portion of Fig. 21 A, showing in greater detail the multilobed microbody undergoing divisions (arrows). Several types of inclusions are seen in the lobes of this microbody. Area "A" contains thin, filmy membranes; area "B" contains membrane-bound, electron-dense material; area "C" contains electron-dense whorls of membranes; area "D" contains compactly granular inclusions; and area "E" contains amorphous electron-dense material. x 60,000.


E
A
T
A
1 μm
21B

observed in area "C", 4) compactly granular areas such as those marked "D" , and 5) amorphous electron-dense material such as that seen in area "E" . Note that in the latter case, extending from the amorphous structure are stacks of membranes (Fig.21 C). Several arrows in Fig. 21 C indicate long (up to $\simeq$ 0.5 μm), 30-50 A^{o} wide fibrils inside the multilobed microbody which are possible strands of DNA. That this cluster of "microbody-like" organelles is indeed composed of microbodies is strongly suggested by DAB treatment (pH 9.0, 37 ^{0}C) of separate aliquots of 24-hour greening cells which yield catalase-mediated DAB reactivity in similar clusters of organelles (Fig. 22). It is observed, here too, that the "cluster" of microbodies corresponds to a multilobed complex.

Occasionally, invaginations of membrane-bound cytoplasm, containing ribosomal-like particles, may be observed in microbodies of 12 - 30-hour greening Euglena (Fig. 23). Fig. 24 demonstrates a microbody in an 18-hour greening cell in which proliferation of the limiting membrane appears to be in progress - perhaps representing an early stage of the type of cytoplasmic intrusion seen in Fig. 23. Division of microbodies (and intrusion of cytoplasm into microbodies) was noted at no time other than the 12 - 30- hour greening period.

3. Cells Cultured Continuously in the Light

Euglena cultured in continuous light possess microbodies which are similar morphologically (in appearance and size)to those of dark-grown and greening cells (Table 5). Based on random electron

Figure 21 C. Greater enlargement of a portion of Fig. 21A showing in detail the inclusions inside the multilobed microbody. Several arrows indicate long (up to 0.5 um), 30 - 50 A° wide fibrils inside the multilobed microbody which are possible strands of DNA. Area "A" contains thin, filmy membranes; area "B" contains membrane-bound electron-dense material; area "C" contains electron-dense whorls of membranes; area "D" contains compactly granular inclusions; and area "E" conatins amorphous, electron-dense material and "membrane-like" inclusions. x 120,000.

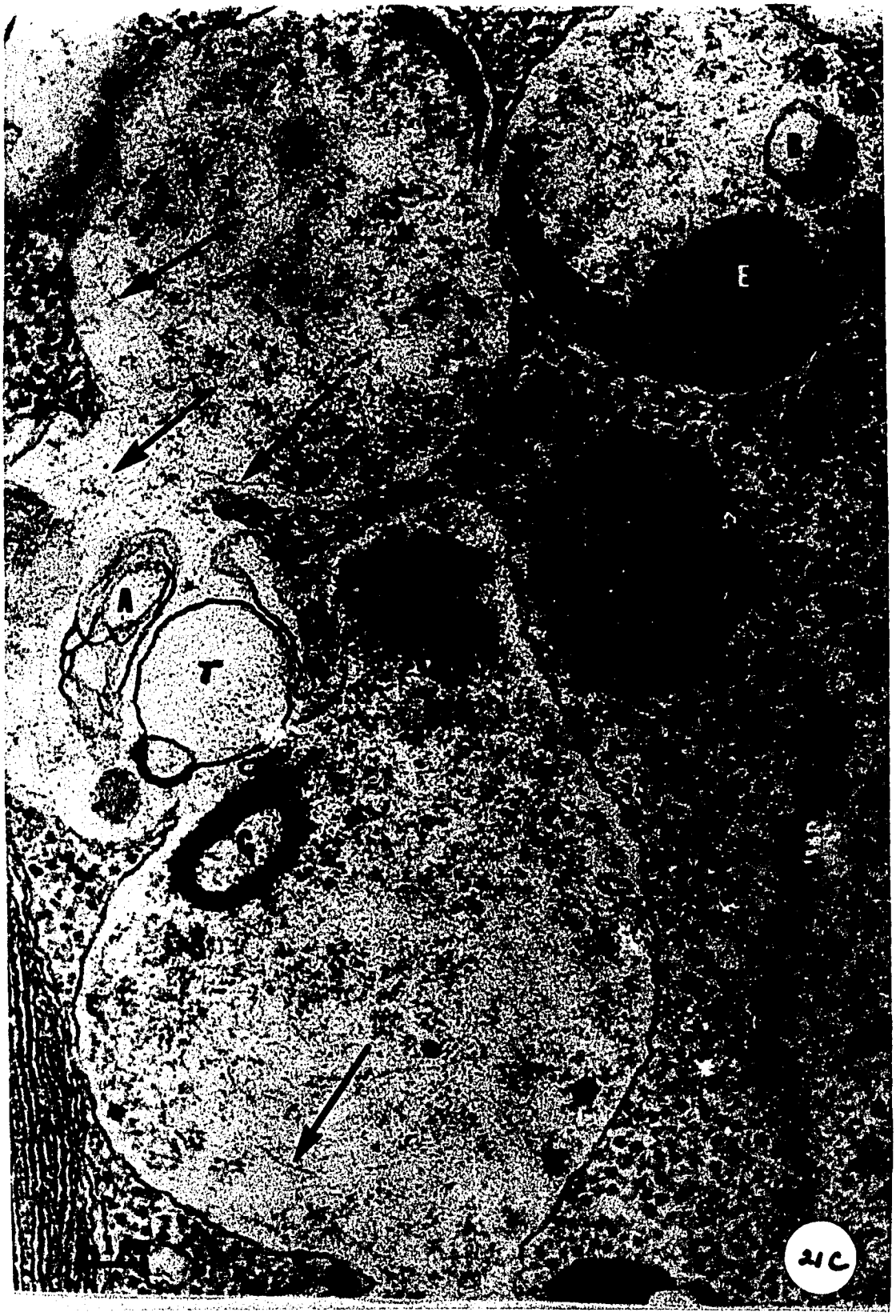
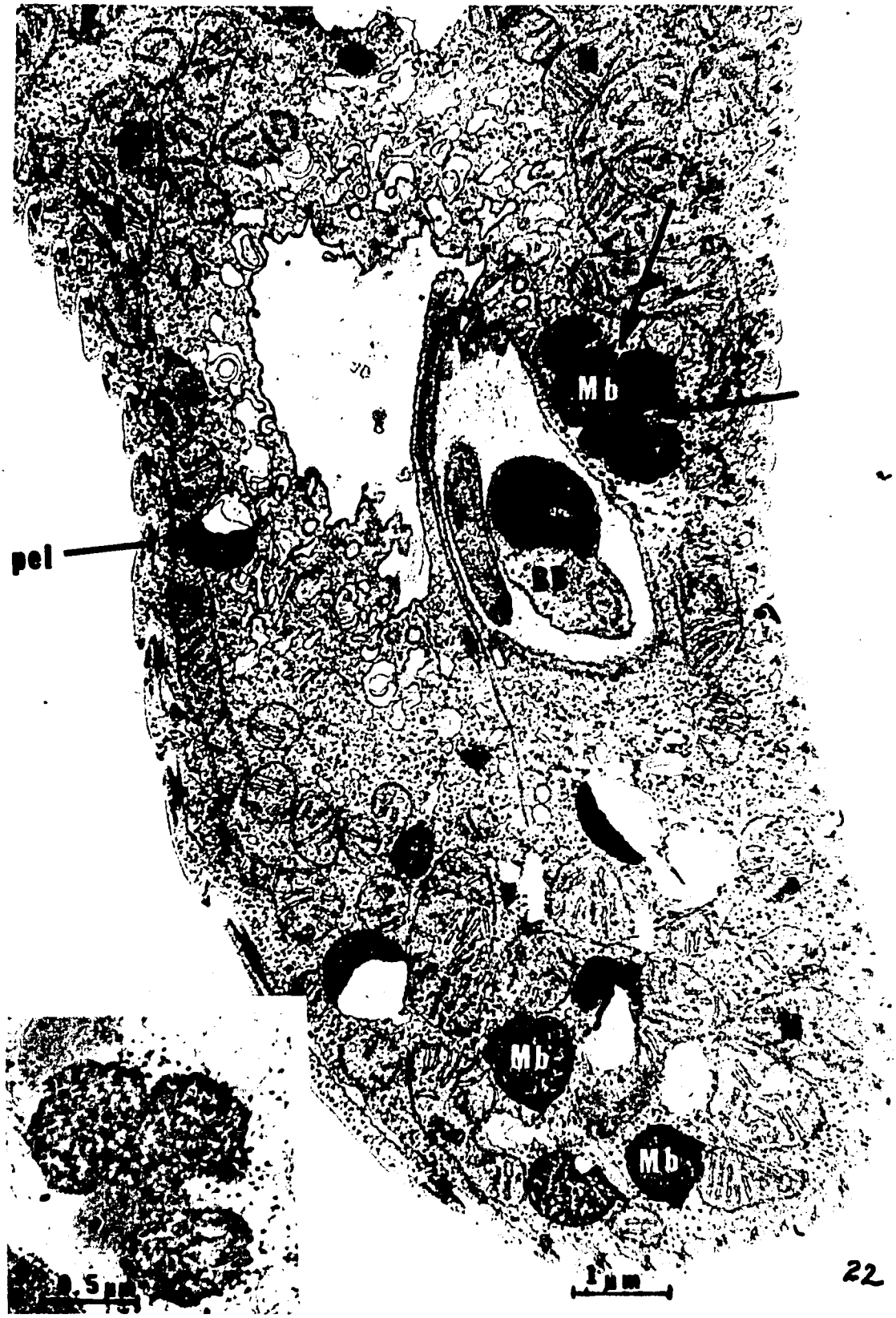

E
A
T
21C

Figure 22. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) aerated, log-phase, acetate-supplemented Euglena gracilis strain Z after 24 hours greening, showing a multilobed microbody (Mb) that is possibly in the process of division (arrows). Also seen are mitochondria (M), a flagellum (F), a basal body (BB) and the pellicle (pel). x 15,000.

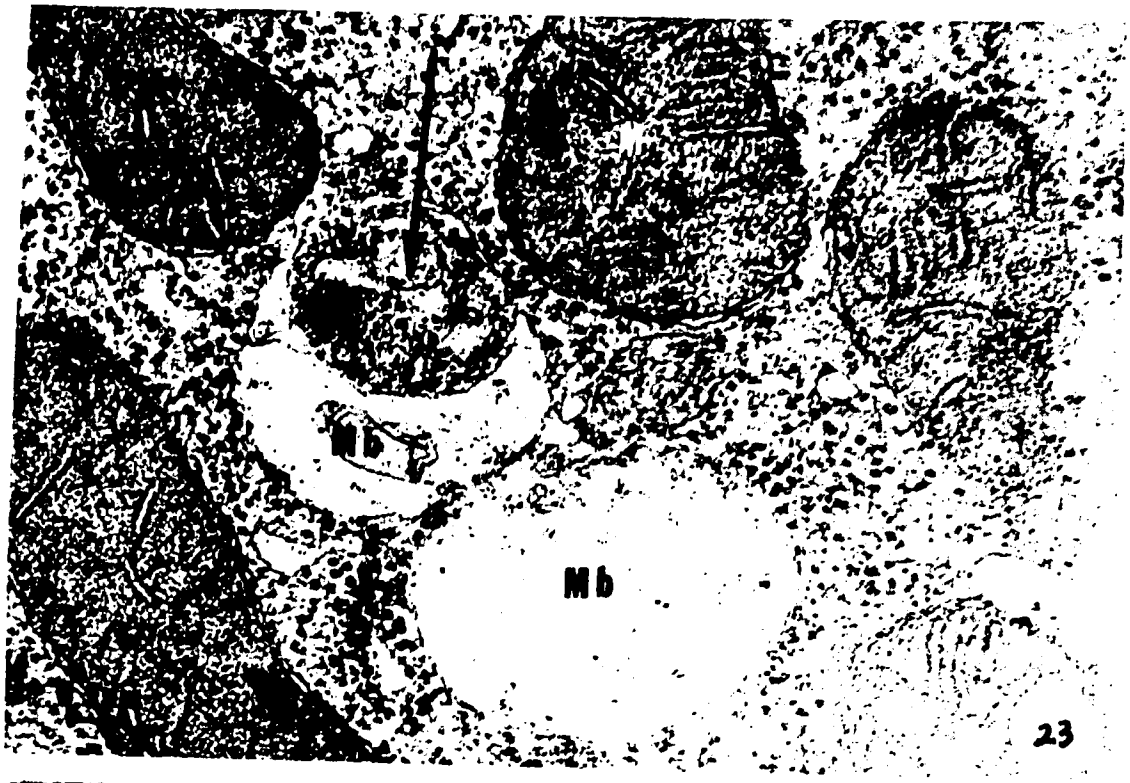
Insert: Detail of multilobed microbody
x 30,000.



22

Figure 23. E.M. of (non-DAB treated) aerated, acetate-supplemented Euglena gracilis strain Z after 24 hours of greening. The arrow denotes a possible intrusion of membrane-bound cytoplasm into a microbody (Mb). x 70,000.

Figure 24. E.M. of (non-DAB treated) aerated, acetate-supplemented Euglena gracilis strain Z after 18 hours of greening, showing possible activity of limiting membrane (arrows) of microbody (Mb). Note the close association of this microbody with mitochondria (M). x 70,000.



23

24

microscopic counts of 200 sections of continuously light-grown cells (DAB-treated), acetate-supplemented Euglena have on the average 6.44 microbodies per section of cell (Table 6). From light microscopy studies with thick longitudinal sections it was also observed that the most frequent locations of microbodies in continuously light-grown cells are also in the cell periphery and regions near the gullet (9.4 per section of cell; Table 7). Microbody profiles were performed on longitudinally oriented continuously light-grown acetate-supplemented cells. On the basis of 10 such cells in serial thick sections, Euglena contain an average of 607 microbodies per cell, the range being 567 - 634 microbodies per cell. Electron microscopic profiles of 10 continuously light-grown cells in serial thin sections reveal an average of 576 microbodies per cell, ranging from 550 - 620 microbodies per cell.

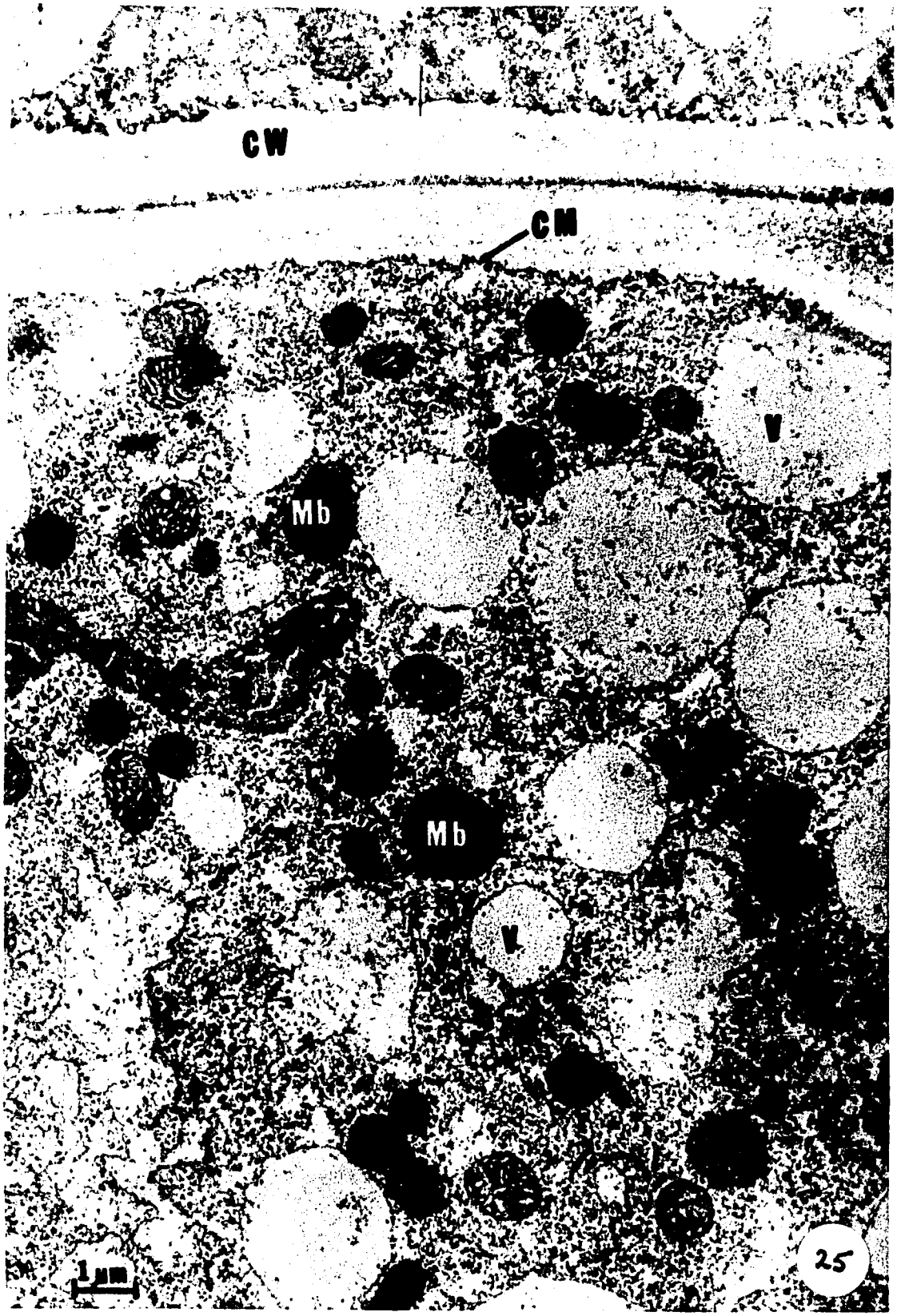
Neither multi-lobed microbodies nor cytoplasmic invaginations into microbodies were observed in continuously light-grown cells.

DAB reactivity could not be detected in microbodies of acetate-supplemented continuously light-grown Euglena which had been aerated in CO_2-free air for 24 hours; restoration of DAB reactivity was achieved within 2 hours of aeration in air containing CO_2.

4. Comparison With Microbodies of Higher Plants

In all experiments employing DAB, germinating Castor beam endosperm was co-processed to monitor the efficiency of the catalase-mediated DAB polymerization procedure. Vigil (1970) had earlier reported catalase-mediated DAB polymerization in this tissue. In Fig. 25 may be seen electron-dense microbodies in DAB-incubated, germinating (4-

Figure 25. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) Ricinus communis endosperm after 4 days' germination, showing electron-dense microbodies (Mb), mitochondria (M), fat vacuoles (V), cytoplasmic membrane (CM), and cell wall (CW) between two adjacent cells. x 10,000.


CW
CM
V
Mb
Mb
V
1 μm
25

day old) Ricinus endosperm.

In a tangential study resulting from these observations with castor bean endosperm, I also looked for microbodies in guard and leaf mesophyll cells of several higher plants; positive DAB reactions were obtained.

Although catalase-mediated (pH 9.0, 37° C) DAB reactivity has been reported in microbodies of mesophyll cells of other plants [e.g., Avena sativa, Zea mays, Chloris gayana (Frederick and Newcomb, 1971)] it has not been reported for palisade or spongy mesophyll cells of Ricinus. Neither has the fine structure of microbodies in such cells been described. Organelles, "microbody-like" in appearance (1µm in diameter, single-membrane limited, granular in nature), were observed in spongy mesophyll cells (Fig. 26) and palisade mesophyll cells (Fig. 27) of the leaf of the castor oil plant. Because of the extensive nature of the central vacuole, the microbodies of Ricinus mesophyll cells are in close juxtaposition to the mitochondria and chloroplasts - in contrast to the situation found in Euglena. The unusual structure of the chloroplast lamellae [see Figs. 26,27 ; similar to those of the bundle-sheath chloroplasts in corn as described by Frederick and Newcomb (1971)] ,poses an interesting area of potential study.

Crystalline inclusions were observed in about 20 % of non-DAB treated microbodies of castor oil plant leaf mesophyll cells (Fig. 28); the lattice of these microbodies is obscured by DAB incubation. Similar crystalline inclusions have been reported in microbodies of tobacco leaf tissue (Frederick and Newcomb, 1969 b) - but, as noted above, are not usually found in the microbodies of Euglena. Upon incubation in the DAB reaction mixture, under conditions which favor

Figure 26. E.M. of (non-DAB incubated) spongy mesophyll cells of leaf of Ricinus communis, showing a microbody (Mb), several mitochondria (M), chloroplasts (C) containing osmiophilic granules (og), a central vacuole (V) surrounded by tonoplast (Tp), cytoplasmic membrane (CM) and cell wall (CW). x 10,000.

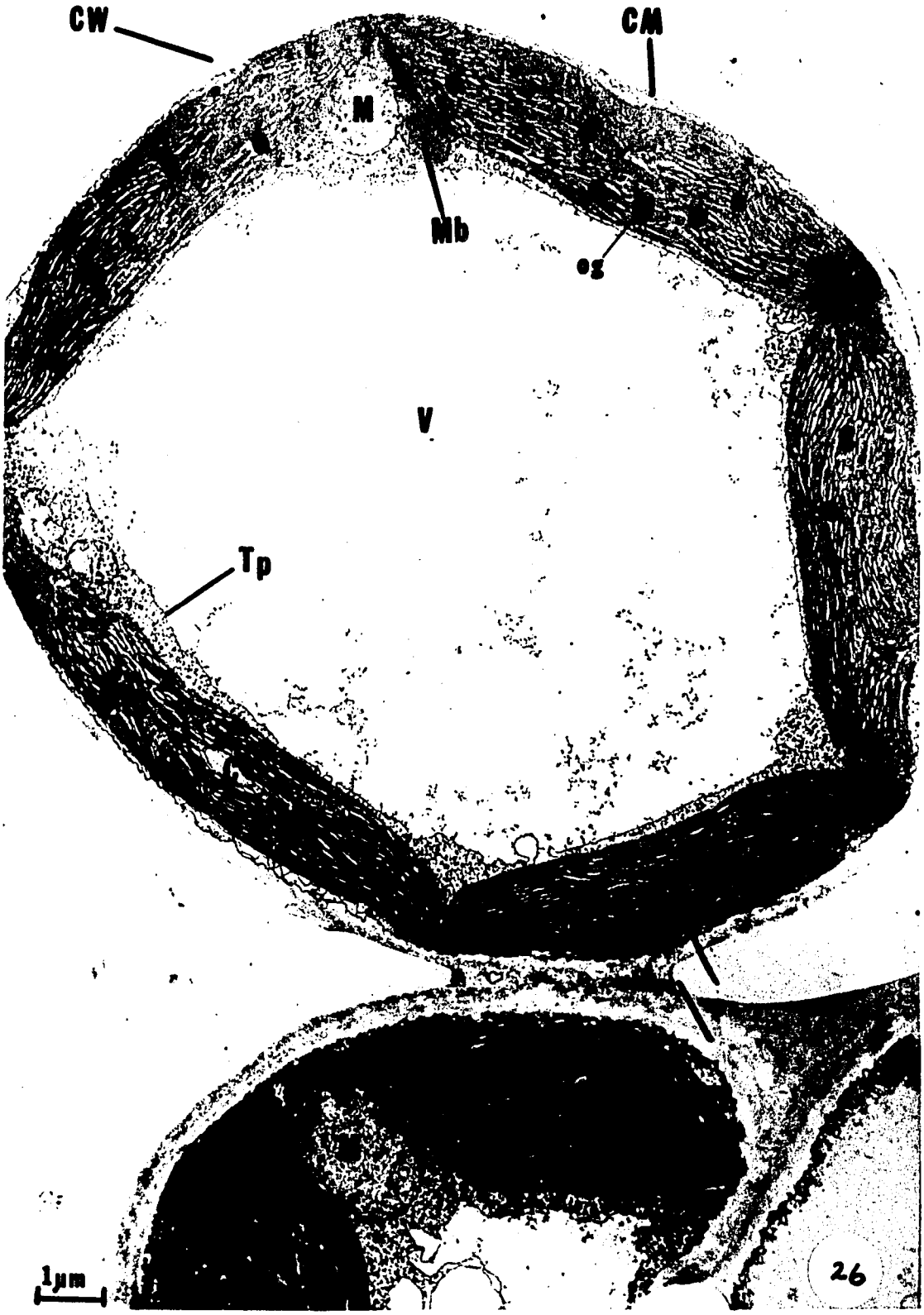
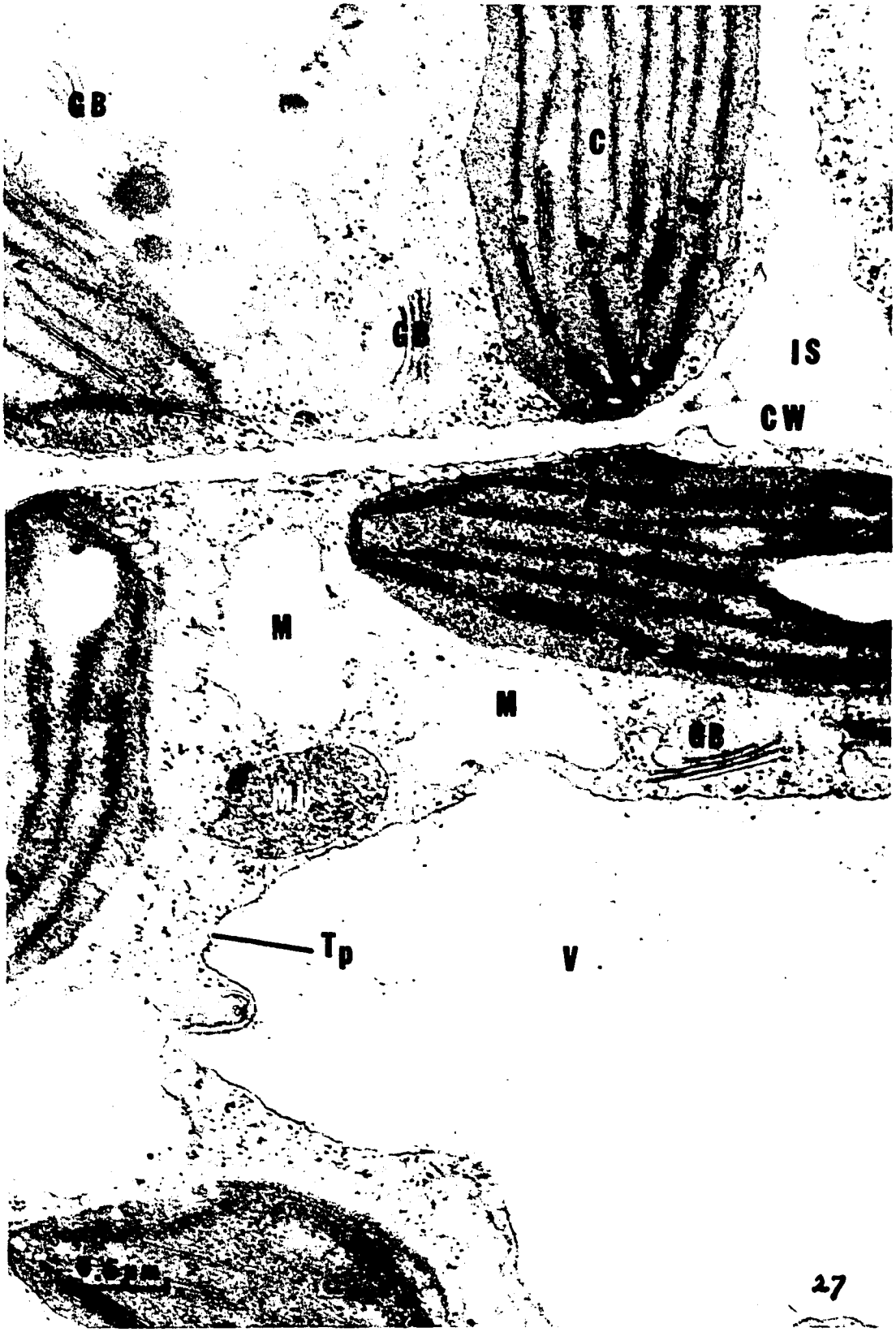

CW
CM
M
Mb
eg
V
Tp
1μm
26

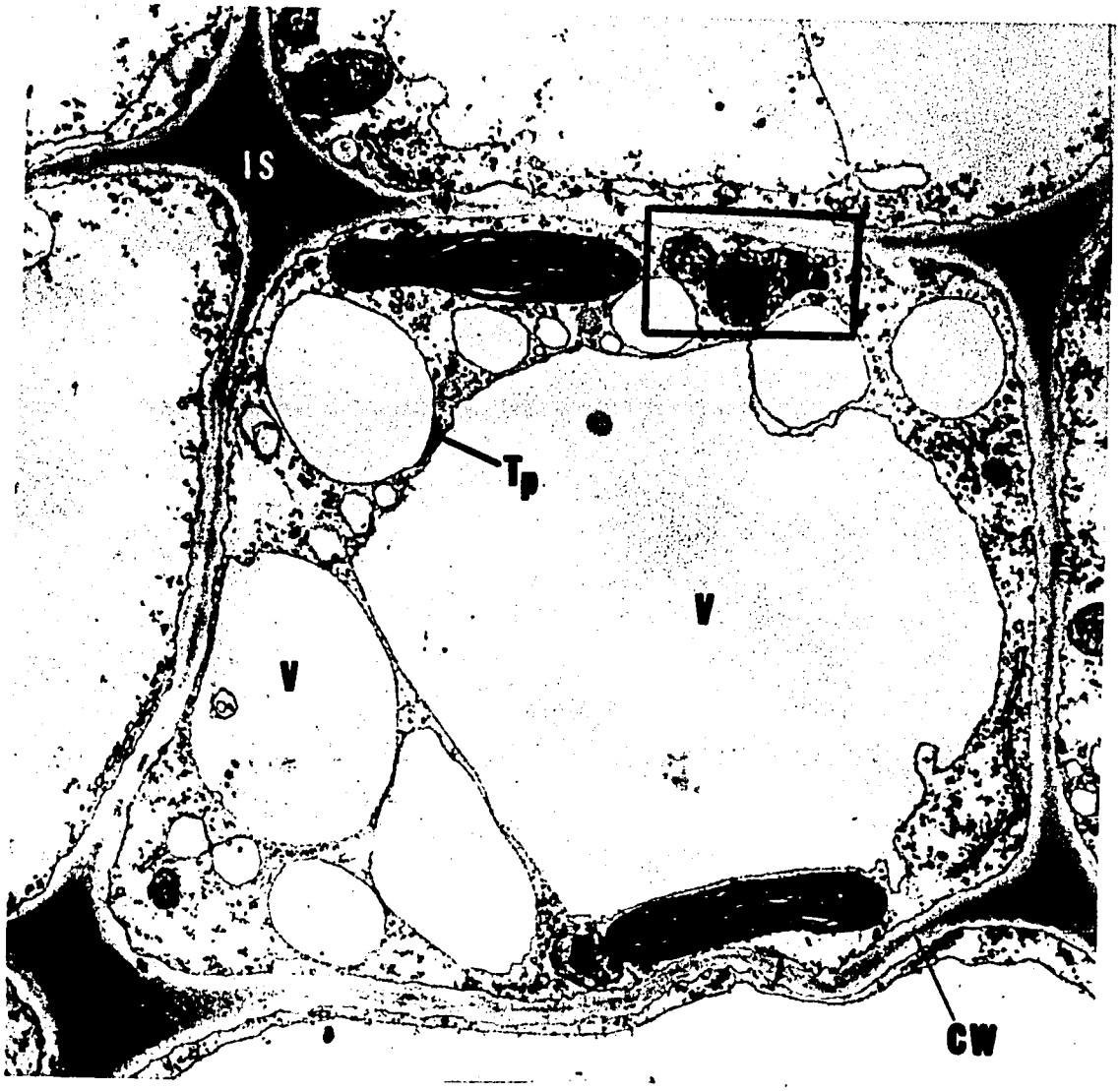
Figure 27. E.M. of (non-DAB incubated) palisade mesophyll cells of leaf of Ricinus communis, showing a microbody (Mb), mitochondria (M), chloroplasts (C), Golgi bodies (GB), central vacuole (V) surrounded by its tonoplast (Tp), intracellular space (IS), and cell wall (CW). x 30,000.



27

Figure 28. E.M. of (non-DAB incubated) spongy mesophyll cells of leaf of Ricinus communis showing crystalline inclusions inside a microbody (Mb). Also seen are chloroplasts (C), mitochondria (M), vacuoles (V), cell wall (CW) and intracellular spaces(IS). x 10,000.

Insert: Enlarged magnification of microbody crystalline inclusion. x 45,000.



1 µm

28

catalase activity, electron-dense, granular microbodies are seen in castor oil leaf palisade or spongy mesophyll cells (Fig. 29); incubation in 0.02 M aminotriazole or 0.01 M KCN completely inhibits DAB polymerization.

Although Zelitch (1963) proposed that stomatal opening is related to glycolic acid metabolism, the existence of microbodies in guard cells has not been previously demonstrated in the guard cells of any plant (on the bases of both a literature search and personal communication with Zelitch as of May, 1972). Therefore, in the present study, catalase-mediated DAB reactivity is reported for the first time in guard cells. In Fig.30 may be observed DAB-positive (catalase-mediated, aminotriazole or KCN-inhibitable) microbodies in two guard cells of Ricinus. From a comparison of microbody profile counts which I made on both Ricinus leaf mesophyll and guard cells, there is approximately an order of magnitude more microbodies in guard cells. Furthermore, the juxtaposition of microbodies with chloroplasts and mitochondria is not as intimate in guard cells as in leaf mesophyll cells - no doubt a function of the smaller vacuole in the case of guard cells.

For purposes of comparison with the castor oil plant (a dicot of the family Euphorbiaceae), fine structure and cytochemical studies were also performed on the guard cells of a Calvin cycle plant[6] (the dicot Spinacea oleracea), a Hatch-Slack plant[6] (the monocot Zea mays) , and two members of the family Crassulaceae, whose stomata are open during the night and closed during the day (the dicots Kalanchoë blossfeldiana and Kalanchoë daigremontianum). The guard cells in all the aforementioned types of plants possess microbodies which in fine structure, DAB reactivity,

Figure 29. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) spongy mesophyll cells of leaf of Ricinus communis demonstrating an electron-dense microbody (Mb). Also seen are mitochondria (M), chloroplasts (C), tonoplast (Tp), vacuole (V), cytoplasmic membrane (CM), cell wall (CW), and intercellular space (IS). x 30,000.

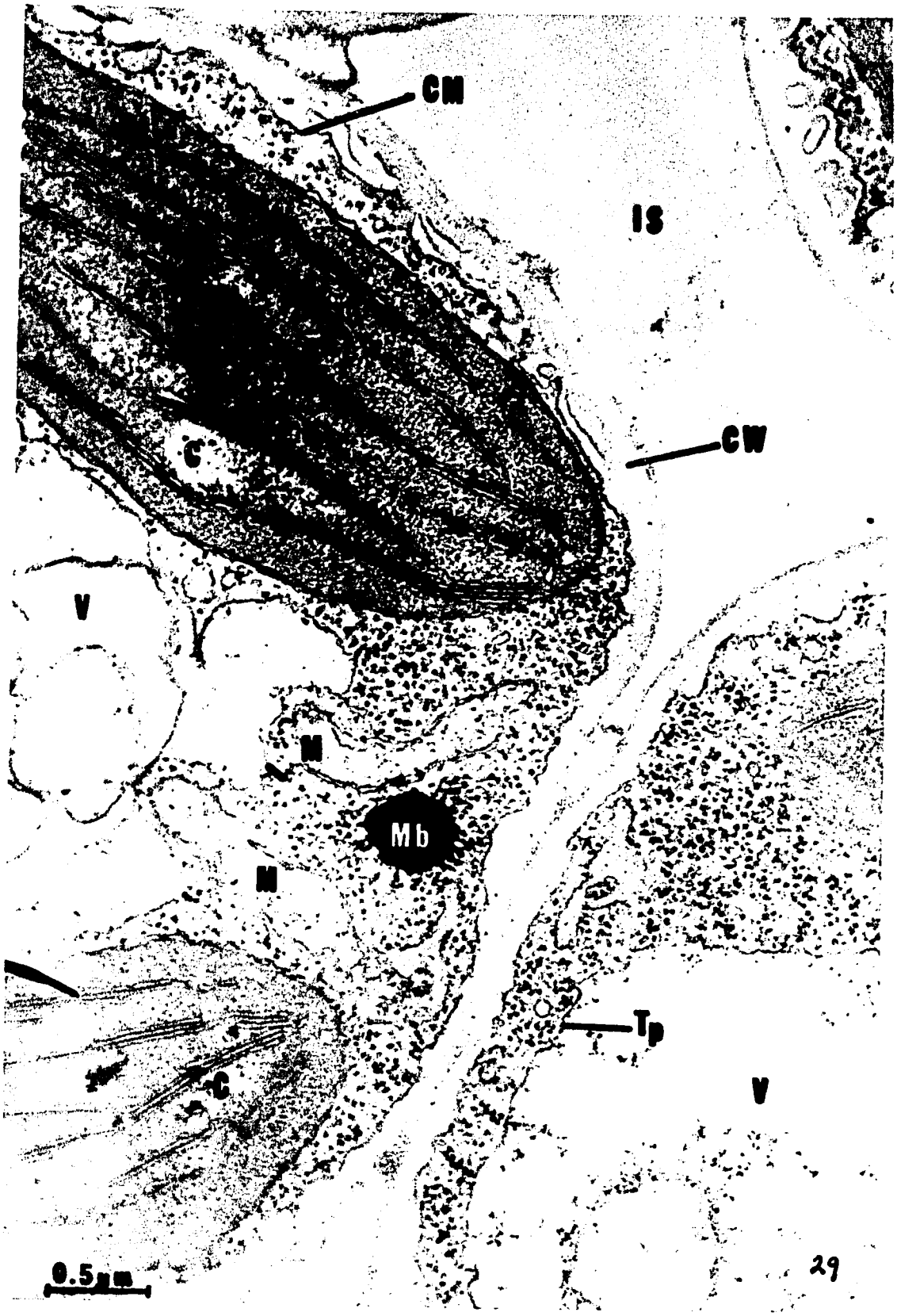
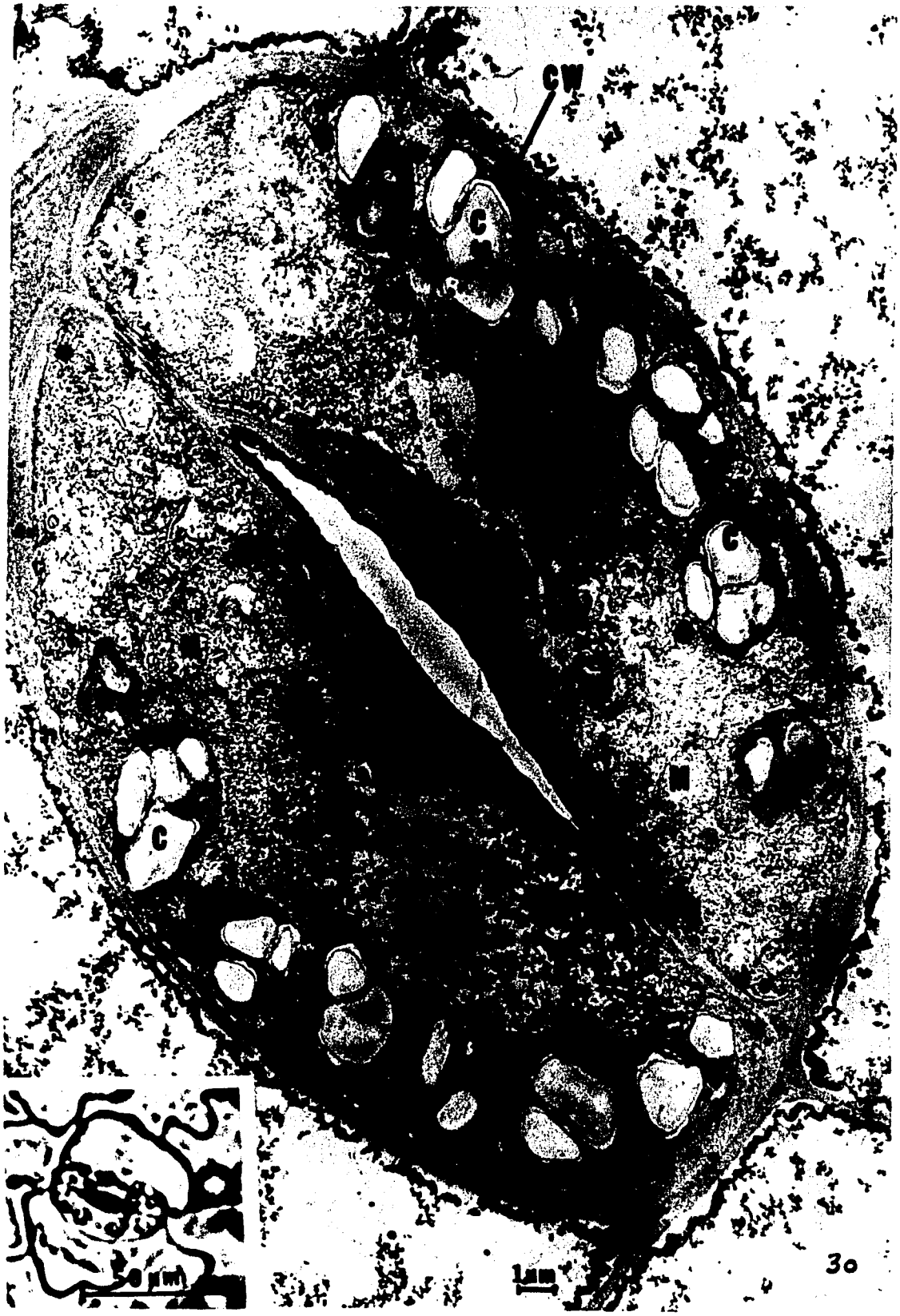

CM
IS
CW
C
V
M
Mb
M
Tp
C
V
0.5 µm
29

Figure 30. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) lower epidermis leaf tissue of Ricinus communis showing electron-dense microbodies (Mb) in two guard cells. Note the thickened inner cell walls (CW) in these guard cells surrounding the stomata and thinner outer cell walls (CW). Also seen in the guard cells are mitochondria (M), chloroplasts (C), and nuclei (N). x 5,000.

Insert: Light micrograph of two guard cells from lower epidermis leaf tissue of Ricinus communis. Tissue was prepared by stripping fresh leaf tissue. x 900.



30

number and juxtaposition in relation to other organelles were similar to those found in Ricinus.

Fine structure and cytochemical studies were additionally performed on green photosynthetic tissue of the (dicot) cactus, Rhipsalis cassytha, to determine the presence of microbodies. In this case, of course, the green tissue examined is modified stem, rather than leaf (the former being referred to as "spine"). Located between the lamellae within the chloroplasts of Rhipsalis cassytha are large (usually 1.0 - 2.8 μm in long dimension) osmiophilic structures. DAB reactivity is observed in these structures if incubation proceeds at pH 9.0 and 37° C (Fig. 31), but is not observed at pH 7.0 and 25° C. This DAB polymerization is inhibited by aminotriazole or KCN and, therefore, may be catalase-mediated. A mosaic pattern of electron opacity and lucidity is apparent on the surface of these chloroplast structures in non-DAB treated cells which have not been stained with uranyl acetate and lead citrate (Fig. 32). Extra-chloroplast microbodies of the size and type observed in Euglena and all other plants in this study were not observed in Rhipsalis. The nature of these DAB-reactive osmiophilic structures in Rhipsalis will have to wait for further study.

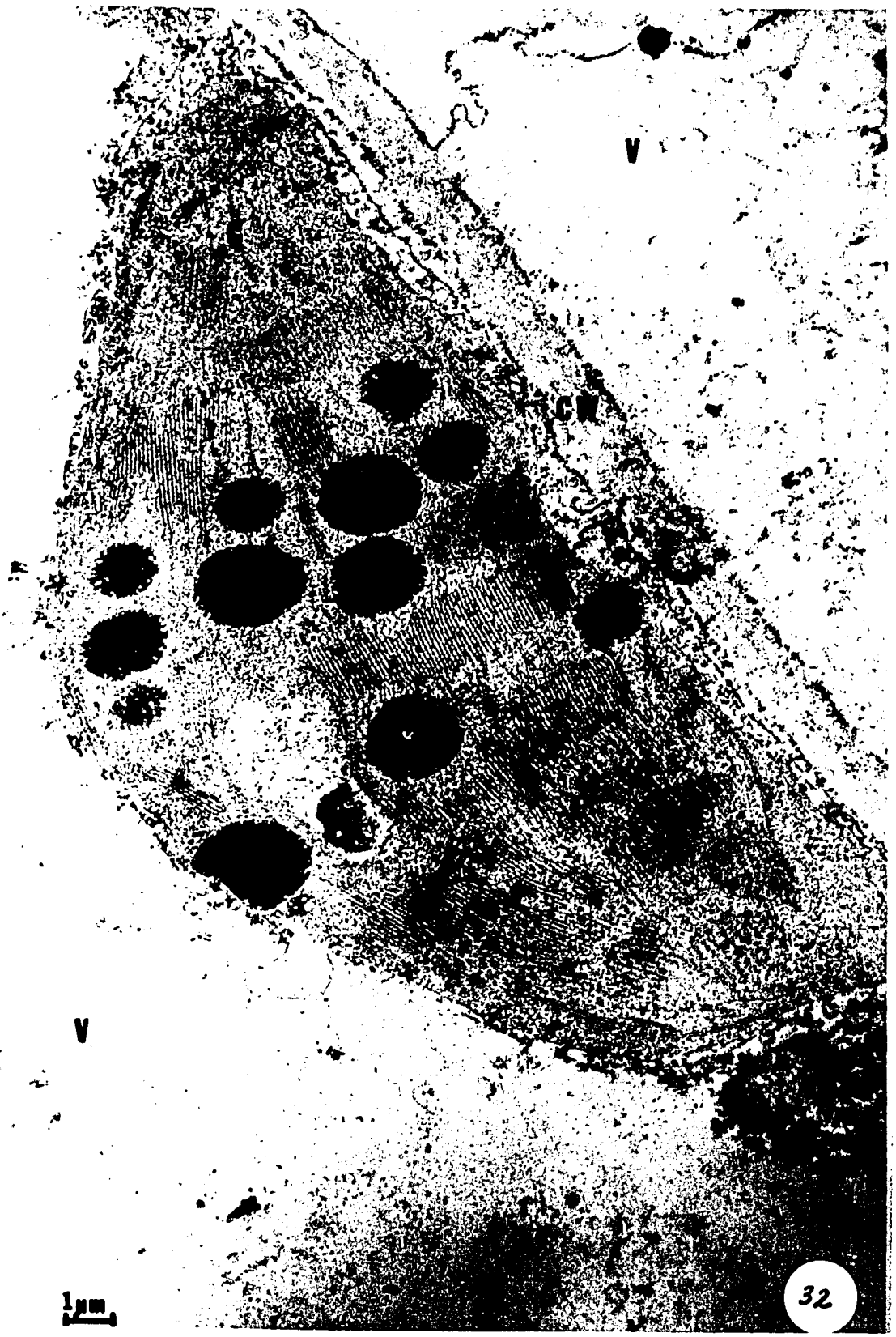
B. Isolation of Microbodies

The greatest yield of intact microbodies in Euglena was achieved by mechanical grinding in sand until ≈ 25 % of the cells were broken open, as judged by light microscopy. If grinding was continued so 100 % of the cells were disrupted, bands on discontinuous or continuous sucrose density gradients merged and were not discrete. Experimental procedures utilizing a Waring blender for cell breakage also gave

Figure 31. E.M. of section of DAB/H_2O_2 incubated (pH 9.0, 37 °C) mesophyll cell of the photosynthetic organ (flattened stem) of the cactus Rhipsalis cassytha. Sections were stained with uranyl acetate and/or lead citrate. Note the large granal stacks (B) and vacuole (V). x 30,000.


V
31
0.5 µm

Figure 32. E.M. of section of (non-DAB treated) mesophyll cell of the photosynthetic organ (flattened stem) of the cactus Rhipsalis cassytha. Sections were not stained with uranyl acetate and/or lead citrate. Note the large granal stacks (G), vacuoles (V), and cell wall (CW). x 8,000.


V
CW
V
1μm
32

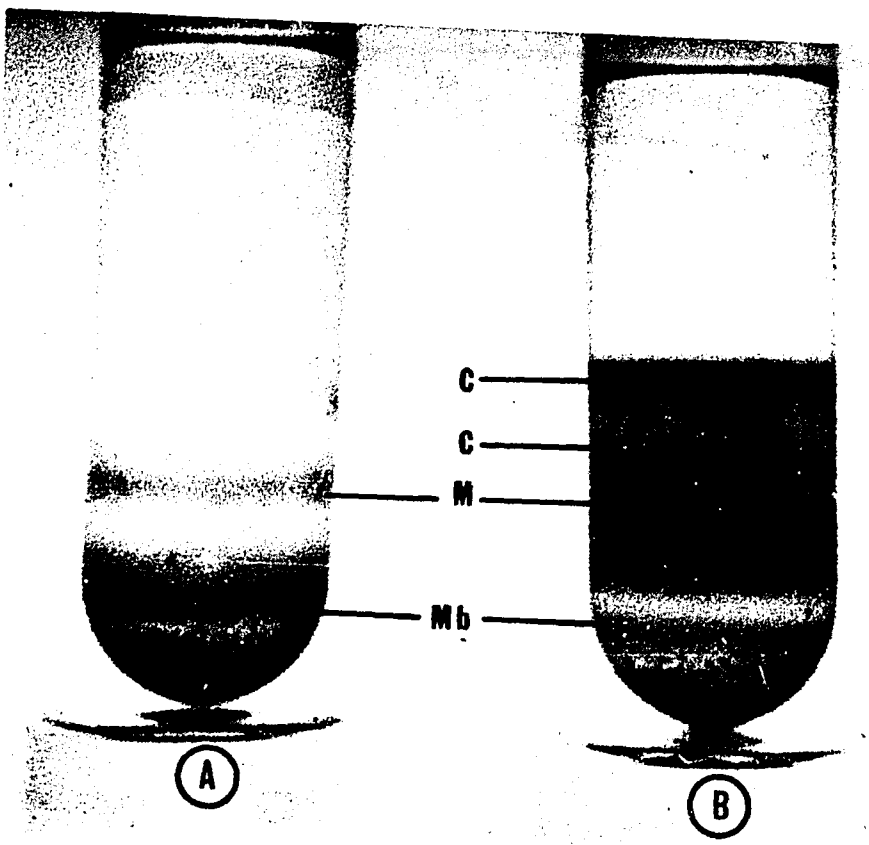
unsatisfactory microbody isolation. A photograph of two of the discontinuous sucrose gradients utilized in the present work is presented in Fig. 33.

Fine structure observations reveal that microbody-containing fractions, located at the interface between the 2.0 and 1.75 M sucrose bands on discontinuous gradients, contain fewer than 1 % intact mitochondria (Fig. 34 A). Catalase activity (pH 9.0, 37° C) is shown cytochemically in these microbodies by the oxidative polymerization of DAB (Fig. 34 B); aminotriazole (Fig. 34 C); KCN, or growth in CO_2-free air inhibit this DAB reactivity.

Electron microscopic observations of other discontinuous sucrose bands disclose that (mainly intact) mitochondria are located at the interface between the 1.75 and 1.50 M sucrose bands (Fig. 35 A), while chloroplast (naked) -containing bands appear at a) the interface between the 1.50 and 1.25 M sucrose zones [Fig. 35 B; heavy or class I plastids- see Karlstam and Albertsson (1969) for description of such plastids] and b) 1.25 and 1.00 M sucrose zones [Fig. 35 C; light or class II plastids - again see Karlstam and Albertsson (1969)] . Smaller fragments (possibly derived from chloroplasts, as judged by their pale green color) are found throughout the 1.25 M sucrose layer (Fig. 35 D).

In the present work, principal use was made of microbodies isolated on discontinuous sucrose density gradients, in contrast to the continuous sucrose gradients used by Trelease et al. (1971) and Graves et al. (1972) . Whereas, I worked with greening and continuously light-grown , as well as dark-grown Euglena , the aforementioned workers dealt only with a permanently streptomycin-bleached strain - which, of course, had no chloroplasts. Occasionally, I made

Figure 33. Photograph of two discontinuous sucrose density gradients of aerated, acetate-supplemented Euglena gracilis strain Z. A, gradient from dark-grown cells showing microbody (Mb) and mitochondrial (M) bands. B, gradient from continuously light-grown cells showing microbody (Mb), mitochondrial (M), and chloroplast (C) bands.



33

Figure 34. E.M. of sucrose fractions located at the interface between the 2.0 and 1.75 M bands of discontinuous sucrose density gradients of log-phase, dark-grown, aerated, acetate-supplemented Euglena gracilis strain Z. A, non-DAB incubated; B , DAB incubated (pH 9.0, 37 °C); C, DAB incubated (pH 9.0, 37 °C) in the presence of aminotriazole. x 8,000.

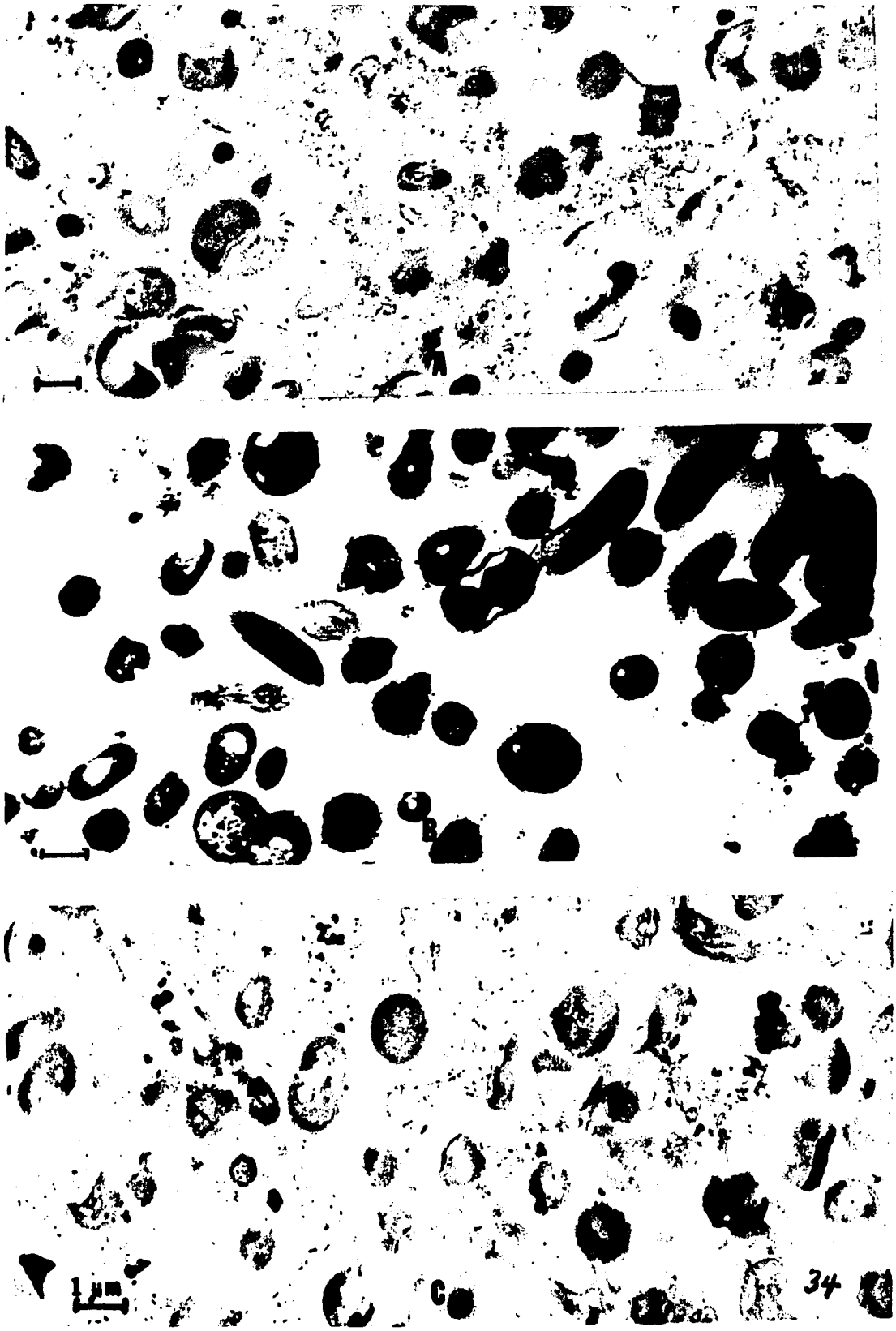
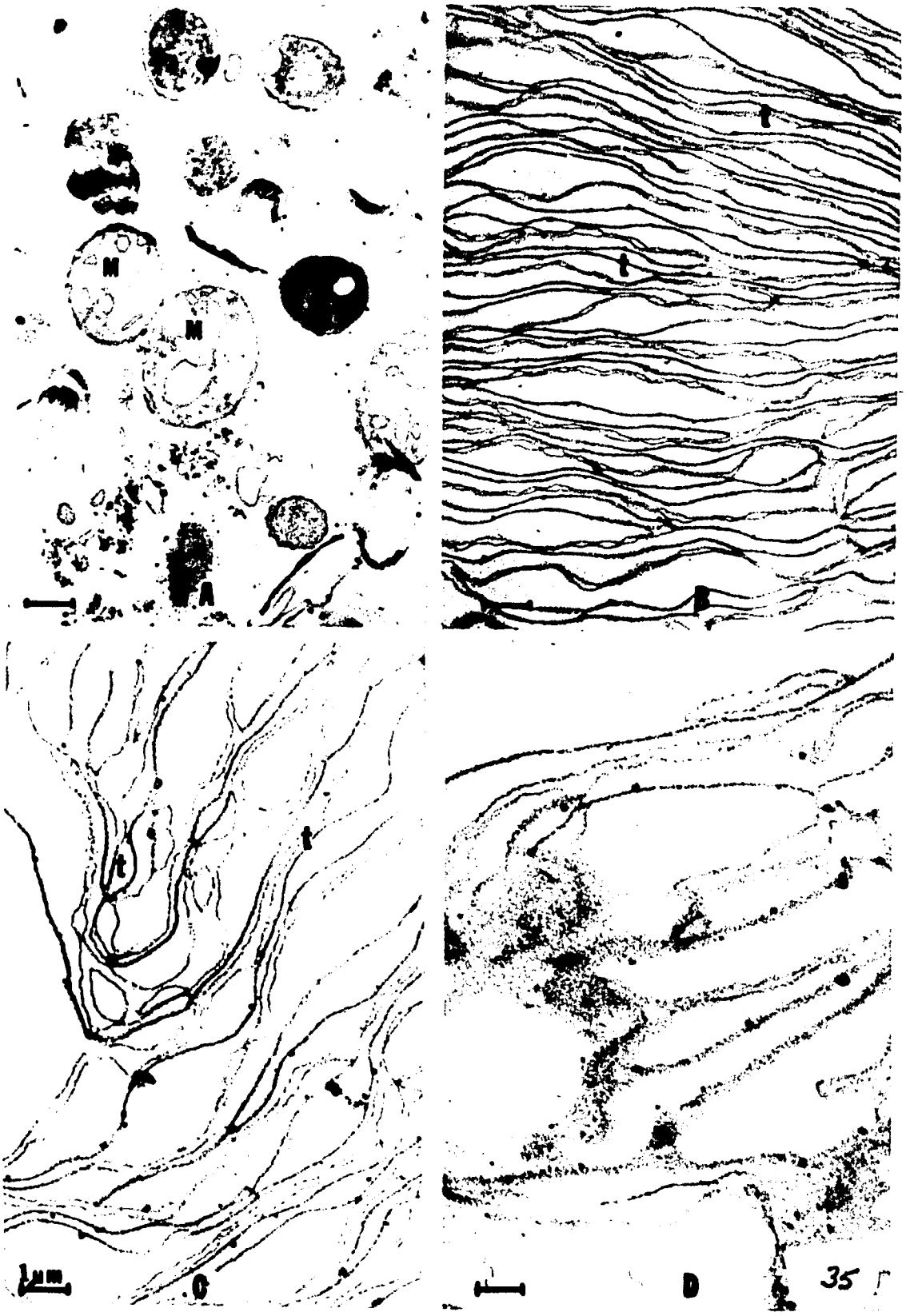

1 µm
34

Figure 35. E.M. of (non-DAB incubated) fractions from discontinuous sucrose density gradients of (4-day old) continuously light-grown, aerated, acetate-supplemented Euglena gracilis strain Z. A, band at interface between 1.75 and 1.50 M sucrose showing mainly intact mitochondria (M) and possibly lysosomes; B, band at interface between 1.50 and 1.25 M sucrose showing thylakoids (t); C, band at interface between 1.25 and 1.00 M sucrose showing thylakoids (t); D, pale green material (possibly chloroplast fragments) observed throughout the 1.25 M sucrose layer. x 8,000.


M
M
A
t
t
t
t
1 µm
C
D
35

continuous sucrose gradients of dark-grown cells a) for purposes of comparison with my data on discontinuous sucrose gradients, b) for purposes of comparison with the data of other workers who used continuous sucrose gradients (of streptomycin-bleached Euglena), and c) for purposes of concentrating the microbody fractions which I have isolated from discontinuous sucrose gradients. Since I found a great deal of chloroplast contamination in microbody and mitochondria-containing bands when continuous sucrose density gradients were used with greening or continuously light-grown cells, this technique was set aside in favor of the discontinuous sucrose gradients. Similar chloroplast contamination was noted by Tolbert (1970) in his use of continuous sucrose density gradients of leaf tissue.

Electron microscopic and enzymatic stidies in the present work reveal that on continuous sucrose gradients, microbody-containing fractions occur at densities between 1.18 - 1.22 g/cm^3, mitochondrial fractions occur at densities between 1.16 - 1.18 g/cm^3, and chloroplast fragments occur over the wide density range of $\lesssim$ 1.0 g/cm^3. Discontinuous sucrose gradient densities were measured by an Abbè refractometer; it was found that microbody, mitochondrial and chloroplast fractions occur on such gradients at densities similar to those where they reached equilibrium on continuous gradients.

Enzyme activity showed good stability if the isolated microbody bands were stored at 4° C, even for periods as long as 4 weeks. Because of this stability, storage at -18° C (utilized by Tolbert, 1970) was not deemed necessary. In all cases, however, in the present work, bands were processed for electron microscopy immediately after isolation, and enzyme assays were performed within 5 days of

isolation.

An attempt was also made to isolate Euglena cellular components by differential centrifugation. Incompleteness of isolation by this technique was recognized in fine structure studies which revealed that pellets of dark-grown, greening, and continuously light-grown acetate-supplemented cells, obtained by centrifugation: a) at 300 g for 2 minutes, contain (in the case of greening and continuously light-grown cells) both naked chloroplasts and mainly large chloroplast fragments (Fig. 36A), b) at 10,000 g for 10 minutes, contain mainly mitochondria and some chloroplast fragments (Fig. 36B), c) at 100,000 g for 1 hour contain microbodies, lysosomal-like organelles, and mitochondria (Fig. 36C). Also, enzyme studies with these three pellets revealed a heterogeneity of content (Table 8).

C. Enzyme Assays On Cell-Free Fractions

1. Cells Aerated With Air

Aliquots of log phase, aerated, acetate-supplemented Euglena (dark-grown, greening, and continuously light-grown) were completely broken by sonication. It may be noted in Fig. 37 that by 48 hours of greening a 2-fold increase occurs in amount of protein in cell-free fractions compared to cell-free fractions of dark-grown cells.

Photometric assays for catalase by the method of Lück (1963) with cell-free fractions reveal 0.68, 0.71, 0.85, 0.90, 0.98, 1.15, 1.23 and 1.25 units of catalase per mg protein for dark-grown, 12 hour-, 24 hour-, 36 hour-, 48 hour-, 60 hour-, and 72-hour greening, and

Figure 36. E.M. of fractions, isolated by grinding followed by differential centrifugation, of continuously light-grown, aerated, acetate-supplemented Euglena gracilis strain Z - not treated with DAB. A, pellet isolated by centrifugation at 300 g for 2 minutes showing a mitochondrion (M), thylakoids (t), and osmiophilic granules (og); B, pellet obtained by centrifugation at 10,000 g for 10 minutes showing mitochondria (M) and numerous other organelles (possibly, lysosomes and/or microbodies); C, pellets obtained by centrifugation at 100,000 g for 1 hour showing mainly microbody-like organelles. x 8,000.

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Table 8. Catalase Activity in 300g, 10,000g, and 100,000g Pellets and Supernatants From Differential Centrifugates Of Dark-Grown, 24-Hour Greening, and Continuously Light-Grown Aerated, Acetate-Supplemented Euglena Gracilis strain Z.

Catalase values are based on 3 determinations and reported in units per mg protein. The numbers in brackets represent the ranges of 3 determinations. "+" indicates faint traces of catalase.

Light Regime	Fraction	Catalase Activity in Linck Units/mg protein
Dark Grown	300g pellet	+
	10,000g pellet	2.1 (1.9-2.7)
	100,000g pellet	5.3 (4.5-6.4)
	supernatant	+
24-hr Greening	300g pellet	+
	10,000g pellet	4.5 (3.9-5.1)
	100,000g pellet	11.3 (9.8 - 12.6)
	supernatant	+
Continuously Light Grown	300g pellet	+
	10,000g pellet	4.1 (3.8-4.7)
	100,000g pellet	12.7 (10.8 - 13.8)
	supernatant	+

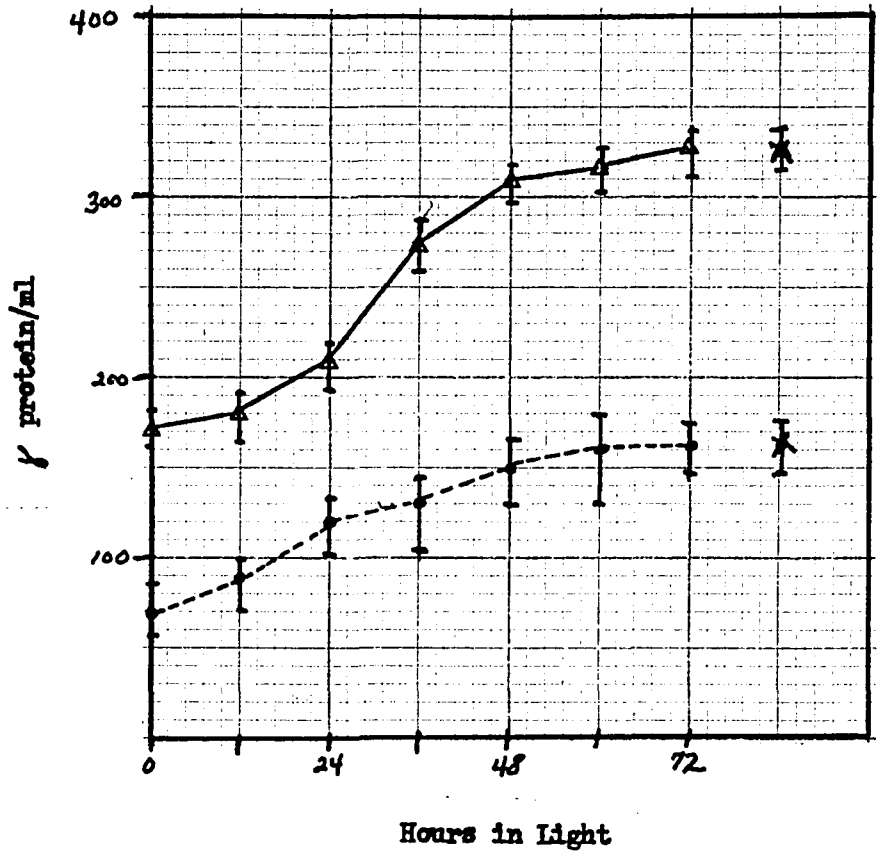


Fig. 37. Protein content of cell-free fractions (•---•) and microbody-containing fractions from discontinuous sucrose density gradients (△—△) of dark-grown, greening and continuously light-grown aerated, acetate-supplemented Euglena gracilis strain Z.

Values reported are based on two sets of data in which assays were made in triplicate on 3 separate samples of the same experiment. The means (3 samples) of each experiment were averaged; data are given in this averaged form. Ranges of data are given by vertical bars through the averaged values. "X" represents cells grown continuously in the light.

continuously light-grown cells, respectively (Table 9 and Fig. 38 B). Therefore, cell-free fractions of 72 hour greening cells display a specific activity for catalase that is comparable to continuously light-grown cells, and a 2-fold increase in specific activity of this enzyme compared to dark-grown cells. Similar specific activities for catalase were obtained by the method of Baudhuin et al. (1965).

An approximate doubling in catalase activity by 72 hours of greening is also noted in cell-free fractions when calculations are made on a per cell basis. Dark-grown, 12-hour, 24 hour-, 36 hour-, 48 hour-, 60 hour-, 72 hour-greening, and continuously light-grown Euglena contain 3.1×10^{-7}, 4.0×10^{-7}, 5.7×10^{-7}, 6.1×10^{-7}, 6.0×10^{-7}, 6.1×10^{-7}, 6.1×10^{-7} and 6.0×10^{-7} units of catalase per cell, respectively.

Specific activities of the glyoxysomal marker enzymes, isocitrate lyase and malate synthase are relatively constant in cell-free fractions of dark-grown, greening and continuously light-grown aerated, acetate-supplemented Euglena. Photometric assays for isocitrate lyase reveal specific activities of 1.65, 1.73, 1.92, 1.92, 1.78, 1.80, 1.90 and 1.80 in cell-free fractions of dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown cells, respectively (Table 9 and Fig. 38 A). Specific activities of 3.21, 3.23, 3.23, 3.30, 3.60, 3.40, 3.70 and 3.47 for malate synthase were found in cell-free fractions of dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown cells, respectively (Table 9 and Fig. 38A).

The activities of isocitrate lyase and malate synthase approximately double by 24 hours of greening in cell-free fractions of aerated, acetate-supplemented Euglena gracilis strain Z when comparisons are made on a per cell basis. Dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-,

Table 9. Enzyme Activities in Cell-Free Fractions of Aerated, Acetate-Supplemented, Dark-Grown, Greening, and Continuously Light-Grown Euglena Gracilis strain Z.

All activities are given in terms of mμ/min/mg protein (specific activity), except catalase which is reported in Lück units. Numbers given in parentheses are the measured ranges.

Enzyme / Time	Activity as a Function of Hours of Greening							
	Dark-Grown	12	24	36	48	60	72	Continuous Light
Catalase	0.68 (0.50-0.98)	0.71 (0.55-0.96)	0.85 (0.62-1.10)	0.90 (0.71-1.30)	0.98 (0.75-1.27)	1.15 (0.95-1.30)	1.23 (1.00-1.43)	1.25 (1.00-1.50)
Isocitrate Lyase	1.65 (1.30-1.90)	1.73 (1.45-1.86)	1.92 (1.56-2.20)	1.92 (1.74-2.40)	1.78 (1.52-2.22)	1.80 (1.54-2.23)	1.90 (1.67-2.23)	1.80 (1.54-2.22)
Malate Synthase	3.21 (2.78-3.85)	3.23 (2.65-3.78)	3.23 (2.67-3.86)	3.30 (3.00-4.13)	3.60 (2.96-4.00)	3.40 (3.12-3.98)	3.70 (3.56-4.00)	3.47 (3.20-3.89)
Glycolate Dehydrogenase	0.14 (0.10-0.20)	0.18 (0.13-0.27)	0.47 (0.33-0.61)	0.70 (0.55-0.86)	0.76 (0.68-0.98)	0.82 (0.65-0.98)	0.87 (0.82-0.96)	0.86 (0.70-0.98)
Hydroxypyruvate Reductase	0.16 (0.10-0.20)	0.43 (0.26-0.57)	0.75 (0.63-0.90)	0.80 (0.68-1.05)	0.87 (0.60-1.12)	0.93 (0.76-1.23)	1.00 (0.87-1.25)	0.98 (0.77-1.32)
Malate Dehydrogenase	2.3 (1.60-3.01)	2.7 (1.21-4.11)	3.0 (2.51-3.57)	2.7 (2.10-3.43)	2.8 (1.63-3.59)	2.6 (2.00-3.23)	2.9 (1.81-3.71)	2.9 (1.67-3.33)
Fumarase	0.18 (0.12-0.25)	0.23 (0.10-0.30)	0.17 (0.10-0.26)	0.15 (0.10-0.24)	0.20 (0.14-0.34)	0.28 (0.23-0.35)	0.29 (0.16-0.45)	0.29 (0.20-0.37)
Aconitase	0.32 (0.25-0.41)	0.34 (0.21-0.38)	0.32 (0.26-0.40)	0.38 (0.30-0.53)	0.39 (0.35-0.46)	0.37 (0.32-0.45)	0.32 (0.26-0.45)	0.30 (0.18-0.41)
Cytochrome Oxidase	-	-	-	-	-	-	-	-

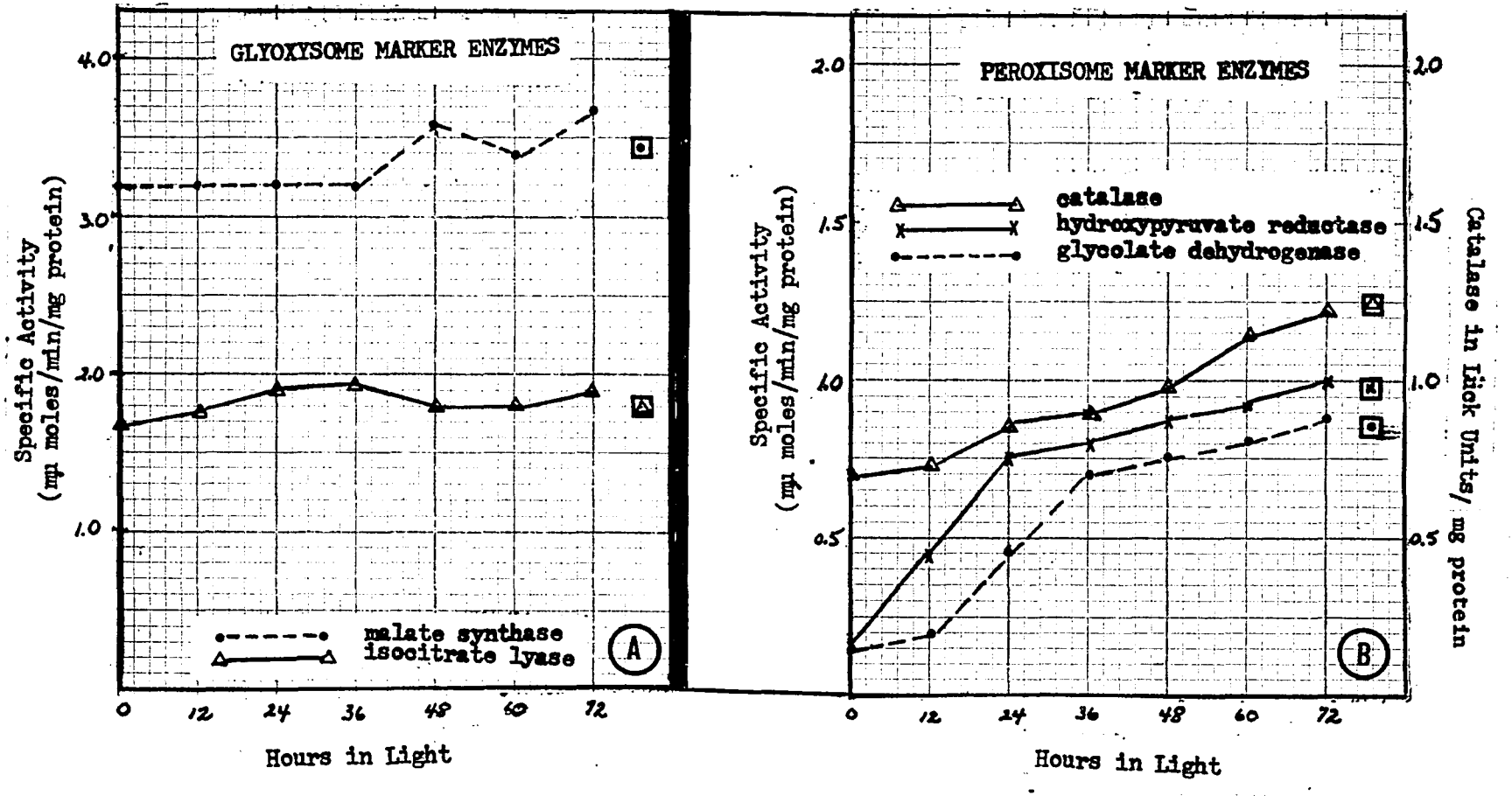


Figure 38. Glyoxysomal and peroxisomal marker enzyme activity in cell-free fractions of dark-grown, greening, and continuously light-grown aerated, acetate-supplemented Euglena gracilis strain Z. Data given by ☐ correspond to values measured with continuously light-grown cells. All values represent the mean of 6 determinations. Catalase is, of course, present in both types of microbodies.

72 hr-greening, and continuously light-grown Euglena contain 4.8 X 10^{-6}, 4.7 X 10^{-6}, 9.4 X 10^{-6}, 9.6 X 10^{-6}, 9.4 X 10^{-6}, 9.5 X 10^{-6}, 9.8 X 10^{-6}, 9.7 X 10^{-6} nmoles of isocitrate lyase per cell and 1.46 X 10^{-5}, 1.53 X 10^{-5}, 2.13 X 10^{-5}, 2.21 X 10^{-5}, 2.20 X 10^{-5}, 2.15 X 10^{-5}, 2.22 X 10^{-5}, 2.19 X 10^{-5} nmoles of malate synthase per cell, respectively.

By 72 hours of greening specific activities in cell-free fractions of the two peroxisomal marker enzymes, glycolate dehydrogenase and hydroxypyruvate reductase, increase ≈ 6-fold; an ≈12-fold increase is noted when data are expressed on a per cell basis. Photometric assays for glycolate dehydrogenase disclose specific activities of 0.14, 0.18, 0.47, 0.70, 0.76, 0.82, 0.87 and 0.86 in cell-free fractions of dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown cells, respectively (Table 9 and Fig. 38B). Specific activities of 0.16, 0.43, 0.75, 0.80, 0.87, 0.93, 1.00 and 0.98 for hydroxypyruvate reductase were found in cell-free fractions of dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown cells, respectively (Table 9 and Fig. 38B).

When data are expressed on a per cell basis, the activities of the two peroxisomal marker enzymes (glycolate dehydrogenase and hydroxypyruvate reductase) in cell-free fractions increase approximately 12-fold by 72 hours of greening. Dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown Euglena contain 0.76 X 10^{-7}, 4.4 X 10^{-7}, 8.3 X 10^{-7}, 9.1 X 10^{-7}, 9.2 X 10^{-7}, 9.1 X 10^{-7}, 9.3 X 10^{-7}, and 9.1 X 10^{-7} nmoles of glycolate dehydrogenase per cell and 0.98 X 10^{-6}, 4.2 X 10^{-6}, 9.3 X 10^{-6}, 11.8 X 10^{-6}, 11.9 X 10^{-6}, 11.8 X 10^{-6}, 11.9 X 10^{-6}, and 11.8 X 10^{-6} nmoles of hydroxypyruvate reductase per cell,

respectively.

2. Cells Aerated With Air Depleted of CO_2

Enzyme assays were also performed for catalase, isocitrate lyase, malate synthase, glycolate dehydrogenase and hydroxypyruvate reductase in cell-free fractions of dark-grown, greening and continuously light-grown acetate-supplemented Euglena which had been grown in the presence of air depleted of carbon dioxide - the air having been passed through a train of U tubes containing excess Ascarite.

Catalase could neither be detected in such cell-free fractions by the method of Lück (1963) nor by the method of Baudhuin et al. (1965), in agreement with the negative results obtained cytochemically with DAB. When carbon dioxide-deprived cells were reaerated for 24 hours with air containing CO_2, catalase levels were restored back to 0.50, 0.46, 0.79, 0.88, 0.95, 1.00, 1.00, and 1.05 units per mg protein for dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown cells, respectively. These values are very similar to those found in cell-free extracts of Euglena continuously grown in the presence of CO_2 (compare with Table 9 and Fig. 38 B).

The restoration of catalase activity to detectable levels is rapid; traces of the enzyme were found, by the method of Lück (1963) and by the method of Baudhuin et al. (1965), in cell-free fractions of carbon dioxide-deprived dark-grown, greening and continuously light-grown cells, if such cells were reaerated in air containing CO_2 for a period of time as short as 2 hours. At less than 2 hours of reaeration

in air containing CO_2, neither photometric techniques (nor the cytochemical method) reveal catalase activity.

Carbon dioxide deprivation was found to have no effect upon the levels of isocitrate lyase, malate synthase, glycolate dehydrogenase or hydroxypyruvate reductase in cell-free fractions of dark-grown, greening, or continuously light-grown cells (Table 10). In all cases, specific activities of these enzymes in cell-free fractions were similar to cells grown in the presence of air containing CO_2 (Table 9 and Fig. 38).

D. Enzyme Assays on Fractions From Discontinuous Sucrose Density Gradients.

1. Cells Aerated With Air

Aliquots of log-phase, aerated, acetate-supplemented dark-grown, greening and continuously light-grown Euglena were mechanically disrupted in sand, and layered on discontinuous sucrose density gradients. Enzyme assays were performed on the band located at the interface between the 2.0 and 1.75 M sucrose zones (Fig. 33). As mentioned earlier, this fraction contains mainly intact microbodies (Fig. 38 A). Assays for catalase, isocitrate lyase, malate synthase, glycolate dehydrogenase and hydroxypyruvate reductase, were made on this sucrose fraction.

Table 10. Enzyme Activity In Cell-Free Fractions of CO_2-Deprived, Acetate-Supplemented Dark-Grown, Greening, and Continuously Light-Grown Euglena Gracilis strain Z.

All enzyme activities are given in terms of specific activity, except catalase which is reported in Lück Units/mg protein. All values are the mean of three separate determinations.

	ENZYME ACTIVITY AS A FUNCTION OF GREENING			
Enzyme	Dark	24 hr	72 hr	Contin. Light
Catalase	-	-	-	-
Isocitrate Lyase	1.70	1.83	1.86	1.98
Malate Synthase	3.35	3.46	3.29	3.33
Glycolate Dehydrogenase	0.18	0.35	0.90	0.88
Hydroxypyruvate Reductase	0.17	0.68	0.95	0.87
Malate Dehydrogenase	0.27	0.20	0.34	0.21
Fumarase	0.14	0.16	0.21	0.22
Aconitase	0.38	0.32	0.30	0.28
Cytochrome Oxidase	-	-	-	-

Photometric assays for catalase by the method of Lück (1963) reveal 10.0, 10.6, 11.6, 12.8, 13.8, 14.2, 14.2 and 14.3 units of catalase per mg protein in microbody-containing fractions from discontinuous sucrose gradients isolated from dark grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light grown cells, respectively (Table 11 and Fig. 39 B). Therefore, microbody-containing sucrose bands from 72-hr greening cells show a 1.4-fold increase in specific activity of catalase, relative to microbody-containing fractions from dark grown cells. This increase should be compared to the 2-fold increase obtained with cell free fractions.

An increase in catalase activity by 72 hours of greening is also noted in microbody-containing fractions from discontinuous sucrose density gradients when calculations are made on a per cell basis. Dark grown, 12 hr-, 24 hr-, 36 hr-,48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown Euglena contain 3.1×10^{-7}, 3.3×10^{-7}, 3.9×10^{-7}, 4.3×10^{-7}, 4.2×10^{-7}, 4.3×10^{-7}, 4.6×10^{-7}, and 4.5×10^{-7} units of catalase per cell, respectively.

When the above mentioned microbody containing bands were assayed for catalase by the method of Baudhuin et al.(1965), similar results were obtained on the bases of both specific activity and per cell.

Specific activities of the glyoxysomal marker enzymes-isocitrate lyase and malate synthase-remain relatively constant in microbody-containing sucrose bands of dark grown, greening, and continuously light grown cells. (These findings are in agreement with the constancy of specific activity in cell free fractions.)

Photometric assays for isocitrate lyase reveal specific activities of 29.1, 30.3, 31.4, 32.0, 34.1, 32.9, 34.1, 34.8

Table 11. Enzyme Activities in Fractions Isolated From Discontinuous Sucrose Density Gradients of Dark-Grown, Greening and Continuously Light-Grown, Aerated, Acetate-Supplemented Euglena Gracilis strain Z.

All activities are given in terms of mμ/min/mg protein (specific activity), except catalase which is reported in Lück units. Numbers given in parentheses are the measured ranges.

Enzyme / Time	Activity as a Function of Hours of Greening							
	Dark Grown	12	24	36	48	60	72	Continuous Light
Catalase	10.0 (9.0-11.1)	10.6 (8.7-12.0)	11.6 (10.1-12.7)	12.8 (12.0-14.0)	13.8 (12.5-14.9)	14.2 (13.6-14.9	14.2 (13.0-16.0)	14.3 (13.1-14.9)
Isocitrate Lyase	29.1 (27.9-30.2)	31.4 (29.1-33.0)	34.1 (31.2-36.1)	32.9 (31.4-35.0)	34.1 (32.0-35.2)	34.0 (33.7-35.6)	34.8 (33.8-35.3)	33.6 (32.7-35.1)
Malate Synthase	93.3 (90.1-95.7)	95.0 (91.7-97.3)	98.0 (92.3-100.1)	99.2 (97.2-101.1)	98.7 (97.1-101.2)	98.2 (96.7-102.3)	99.0 (96.1-102.7)	99.3 (97.8-102.7)
Glycolate Dehydrogenase	5.3 (4.2-5.9)	21.4 (20.1-22.7)	27.3 (25.3-29.1)	31.8 (30.1-32.7)	32.7 (30.1-34.7)	33.0 (31.8-35.7)	33.5 (31.9-35.7)	33.4 (31.9-35.7)
Hydroxypyruvate Reductase	7.1 (6.7-7.9)	16.8 (15.2-18.1)	34.9 (32.1-36.1)	35.5 (33.2-36.8)	37.0 (35.8-38.1)	40.2 (39.1-42.7)	41.0 (39.8-42.7)	41.7 (40.7-43.5)
Malate Dehydrogenase	9.7 (9.0-10.2)	10.3 (9.1-11.0)	9.5 (9.0-9.9)	9.4 (8.7-10.1)	9.8 (8.3-11.7)	10.0 (9.1-10.9)	9.3 (8.9-10.3)	9.9 (9.0-10.5)
Fumarase	1.3 (1.0-2.0)	1.7 (1.0-2.7)	1.6 (1.1-2.3)	1.5 (1.1-2.3)	1.8 (1.2-2.7)	1.9 (1.3-2.7)	1.8 (1.2-2.9)	1.8 (1.2-2.6)
Aconitase	2.5 (1.9-3.1)	2.9 (1.8-3.7)	2.2 (1.6-3.5)	2.8 (1.7-3.5)	2.7 (1.9-3.4)	2.5 (1.8-3.3)	2.9 (1.7-3.9)	2.8 (1.9-3.5)
Cytochrome Oxidase	–	–	–	–	–	–	–	–

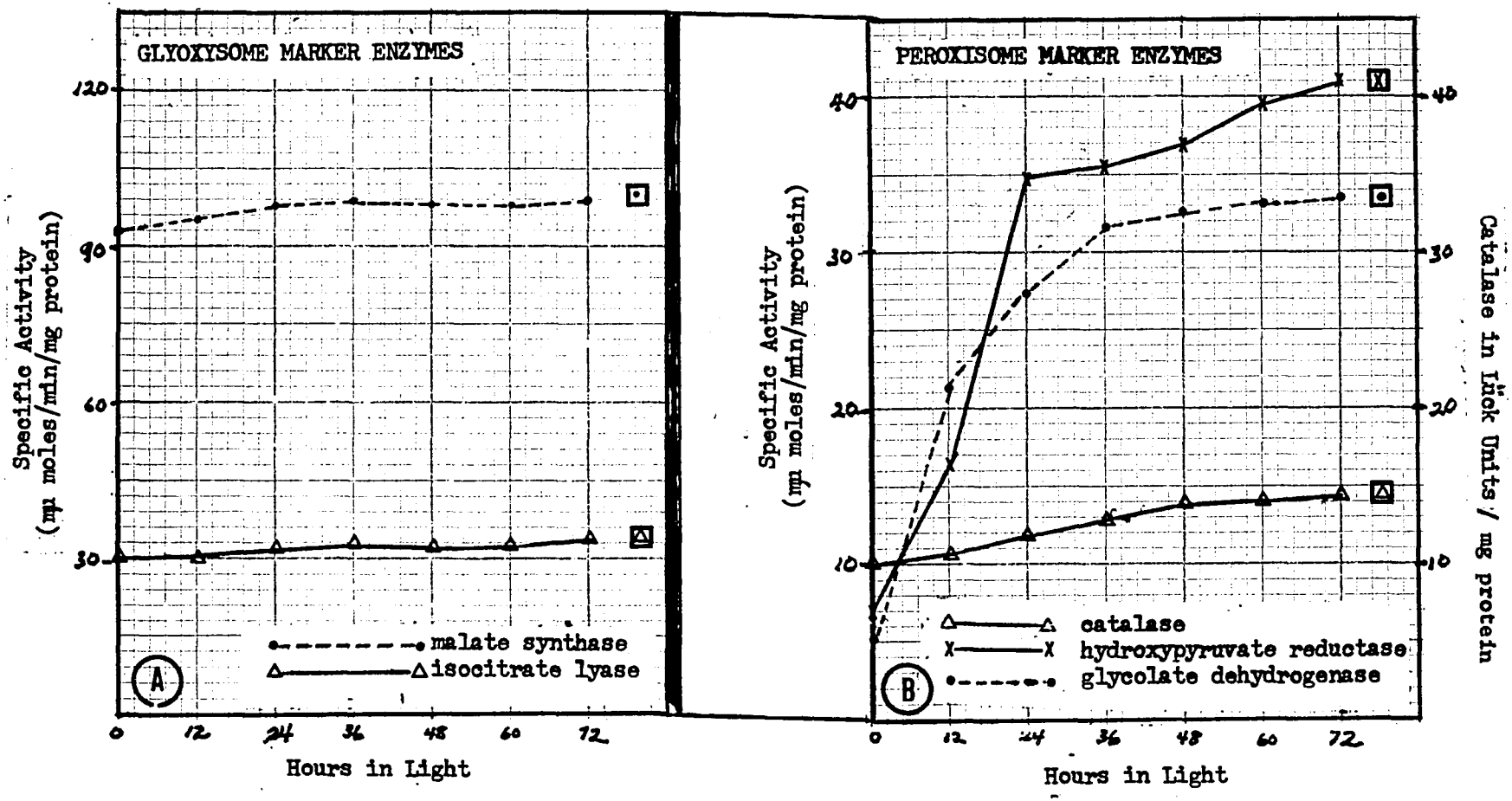


Figure 39. Glyoxysomal and peroxisomal marker enzyme activity in microbody-containing fractions isolated from discontinuous sucrose gradients of dark-grown, greening and continuously light-grown aerated, acetate-supplemented Euglena gracilis strain Z. Data given by ⊡ correspond to values measured with continuously light-grown cells. All values represent the mean of 6 determinations. Catalase is, of course, present in both types of microbodies.

and 33.6 in microbody-containing sucrose fractions of dark-grown, 6 hr-, 12 hr-, 15 hr-, 24 hr-, 36 hr-, 48 hr-, 72 hr-greening, and continuously light-grown cells, respectively (Table 11 and Fig. 39A). Specific activities of 93.3, 94.5 95.0, 97.3, 98.0, 99.2, 98.7, 99.0 and 99.3 for malate synthase were found in microbody-containing sucrose fractions isolated from discontinuous sucrose density gradients of dark-grown, 6 hr-, 12 hr-, 15 hr-, 24 hr-, 36 hr-, 48 hr-, 72 hr-greening, and continuously light-grown cells, respectively (Table 11 and Fig. 39A).

The activities of isocitrate lyase and malate synthase on a per cell basis approximately double by 24 hours of greening in microbody-containing sucrose bands of aerated, acetate-supplemented Euglena gracilis strain Z. Dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown Euglena contain 3.0×10^{-6}, 2.9×10^{-6}, 5.7×10^{-6}, 5.9×10^{-6}, 5.7×10^{-6}, 6.0×10^{-6}, 5.8×10^{-6}, 5.9×10^{-6} nmoles of isocitrate lyase per cell and 2.0×10^{-5}, 1.9×10^{-5}, 3.7×10^{-5}, 3.9×10^{-5}, 3.9×10^{-5}, 4.1×10^{-5}, 3.9×10^{-5}, and 3.8×10^{-5} nmoles of malate synthase per cell, respectively.

When enzyme assays were performed on microbody-containing fractions from discontinuous sucrose density gradients of aerated, acetate-supplemented Euglena , it was found - in agreement with data on cell-free fractions - that even dark-grown cells had low levels of peroxisomal marker enzymes. Although specific activities of catalase remain fairly constant for microbody-containing discontinuous sucrose gradient fractions derived from aerated, acetate-supplemented cells grown under the three light regimes, by 72 hours of illumination, levels of glycolate dehydrogenase and hydroxypyruvate reductase were found to increase approximately 6-fold.

Photometric assays for glycolate dehydrogenase reveal specific activities of 5.3, 9.5, 21.4, 23.6, 27.3, 31.8, 32.7, 33.5, and 33.4 in microbody-containing bands from discontinuous sucrose gradients of dark-grown, 6 hr-, 12 hr-, 15 hr-, 24 hr-, 36 hr-, 48 hr-, 72 hr-greening, and continuously light-grown, aerated acetate-supplemented cells, respectively (Table 11 and Fig. 39B). Specific activities of 7.1, 9.6, 16.8, 26.8, 34.9, 35.5, 37.0, 41.0, and 41.7 were obtained for hydroxypyruvate reductase in microbody bands isolated from discontinuous sucrose gradients of dark-grown, 6 hr-, 12 hr-, 15 hr-, 24 hr-, 36 hr-, 48 hr-, 72 hr-greening, and continuously light grown , aerated, acetate-supplemented cells, respectively (Table 11 and Fig. 39 B).

When data are expressed on a per cell basis, the activities of these two peroxisomal marker enzymes in microbody-containing bands from discontinuous sucrose gradients increase approximately 12-fold by 72 hours of greening. Dark-grown, 12 hr-, 24 hr-, 36 hr-, 48 hr-, 60 hr-, 72 hr-greening, and continuously light-grown Euglena contain 0.5×10^{-7}, 1.0×10^{-7}, 4.4×10^{-7}, 5.3×10^{-7}, 6.0×10^{-7}, 6.9×10^{-7}, 7.2×10^{-7}, 7.3×10^{-7} moles of glycolate dehydrogenase per cell and 0.7×10^{-6}, 1.4×10^{-6}, 4.9×10^{-6}, 6.0×10^{-6}, 6.7×10^{-6}, 7.8×10^{-6}, 8.0×10^{-6}, 8.1×10^{-6} moles of hydroxypyruvate reductase per cell, respectively.

The degree of contamination of the microbody-containing band by aconitase and fumarase were below limits of experimental detection. Specific activities of 0.87, 0.75, 0.88, 0.94, 0.73, 0.76, 0.77, 0.93, and 0.93 were obtained for malate dehydrogenase in the microbody-containing bands from discontinuous sucrose gradients of dark-grown, 6 hr-, 12 hr-, 15 hr-, 24 hr-, 36 hr-, 48 hr-, and 72 hr-greening, and continuously light-grown, aerated, acetate-supplemented cells, respec-

tively. Cytochrome oxidase could not be detected photometrically in any of the aforementioned microbody-containing bands. Chloroplast contamination in the microbody-containing bands from discontinuous sucrose density gradients was not apparent by visual inspection or by the photometric method of MacKinney (1941).

2. Cells Aerated With Air Depleted of CO_2

Additional aliquots of dark-grown, greening and continuously light-grown acetate-supplemented Euglena were concurrently grown in the presence of air from which carbon dioxide had been removed. Discontinuous sucrose density gradients were prepared and enzyme assays performed for catalase, isocitrate lyase, malate synthase, glycolate dehydrogenase and hydroxypyruvate reductase on microbody-containing fractions found at the interface between the 2.0 and 1.75 M sucrose bands.

In agreement with the negative results obtained upon DAB incubation, catalase could not be detected in these fractions by either the method of Lück (1965) or the method of Baudhuin et al. (1965). When carbon dioxide-deprived cells were reaerated for 24 hours, in air containing CO_2, catalase levels were partially restored, i.e. to 9.3, 9.7, 10.9, and 13.8 units of catalase/mg protein in microbody-containing sucrose bands from dark-grown, 12 hr-, 24 hr-, and 72 hr-greening cells, respectively. These values, it should be noted, are only slightly lower than those found in microbody fractions from Euglena continuously grown in the presence of carbon dioxide (Table 11 and Fig. 40B). As found with cell-free fractions, this restoration of catalase activity to detectable levels is rather rapid; traces of this enzyme were revealed by

the method of Lück (1963) and by the method of Baudhuin et al. (1965) by 2 hours of reaeration in air containing carbon dioxide, in the cases of cells grown under all three light regimes. Catalase could not be detected either by the method of Lück (1963) nor by the method of Baudhuin et al. (1965) if carbon dioxide-deprived cells were reaerated with air containing CO_2 for less than 2 hours.

Carbon dioxide deprivation had no effect upon levels of isocitrate lyase, malate synthase, glycolate dehydrogenase or hydroxypyruvate reductase in microbody-containing fractions from discontinuous sucrose density gradients for dark-grown, greening, or continuously light-grown cells (Table 12). Values for the activities of these enzymes are similar (within 10 %) to those reported in Table 11 for microbody-containing fractions from discontinuous sucrose gradients of cells continuously aerated in air containing CO_2.

E. Nucleic Acid Determinations on Microbody-Containing Fractions Derived From Discontinuous Sucrose Density Gradients.

Microbody-containing fractions from discontinuous sucrose density gradients (interface between 2.0 and 1.75 M sucrose bands) of dark-grown, greening, and continuously light-grown aerated, acetate-supplemented Euglena gracilis strain Z were assayed for the presencer of DNA and RNA.

In the case of DNA, microbody homogenates of dark-grown, 12 hr-, 24 hr-, 48 hr-, 72 hr-greening, and continuously light-grown aerated, acetate-supplemented Euglena were found to contain 1.0 ± 0.2, 1.2 ± 0.2, 1.3 ± 0.3, 1.3 ± 0.4, 1.3 ± 0.3, 1.3 ± 0.4 µg of DNA/mg protein. DNA could not be detected in aliquots of the aforementioned

Table 12. Enzyme Activity in Fractions Isolated From Discontinuous Sucrose Density Gradients of Dark-Grown, Greening and Continuously Light-Grown CO_2-Deprived, Acetate-Supplemented Euglena Gracilis Strain Z.

All enzyme activities are given in terms of specific activity, except catalase which is reported in Lück Units/mg protein. All values are the mean of three separate determinations.

ENZYME ACTIVITY AS A FUNCTION OF GREENING					
Band	Enzyme	Dark	24 hr	72 hr	Continuous Light
Microbody	Catalase	-	-	-	-
	Isocitrate Lyase	29.4	33.8	33.9	34.8
	Malate Synthase	92.9	97.8	98.9	99.0
	Glycolate Dehydrogenase	5.4	27.8	34.0	33.9
	Hydroxypyruvate Reductase	7.4	35.2	40.6	40.9
Mitochondria	Malate Dehydrogenase	10.1	9.9	9.6	10.3
	Fumarase	1.1	1.7	1.5	1.8
	Aconitase	2.3	2.4	2.3	2.5
	Cytochrome Oxidase	-	-	-	-

samples when they were pre-treated for 30 minutes at 37° C with DNase.

In the case of RNA, microbody homogenates of dark-grown, 12 hr-, 24 hr-, 48 hr-, 72 hr-greening, and continuously light-grown aerated, acetate-supplemented Euglena gracilis strain Z were found to contain 14.8 ± 0.9, 15.7 ± 0.9, 14.9 ± 1.0, 14.7 ± 1.0, 15.7 ± 1.0, 15.3 ± 1.0 μg RNA/mg protein. RNA could not be detected in aliquots of the above-mentioned samples when they were pre-treated for 30 minutes at 37° C with RNase.

The significance of these results are considered in the Discussion, page 217 .

F. Enzyme Assays on Fractions From Continous Sucrose Density Gradients

Aliquots of log-phase, aerated, acetate-supplemented dark-grown Euglena were mechanically disrupted in sand and layered on continuous sucrose density gradients. Enzyme assays on continuous sucrose density gradients were of necessity, limited to dark-grown cells since with greening, contamination by chloroplast material (which leads to errors in enzymatic determinations[18]) was noted in the mitochondrial and microbody bands. The greatest activity of the microbody marker enzymes (catalase, isocitrate lyase, malate synthase, glycolate dehydrogenase and hydroxypyruvate reductase) was found in the bands at buoyant densities of 1.18 - 1.22 gm /cm^3; minor peaks of each enzyme were also noted in the bands at 1.16 - 1.18 gm/cm^3 buoyant density ranges (Fig. 40 and Table 13);microbodies and mitochondria, respectively, are the main organelles found at these density ranges.

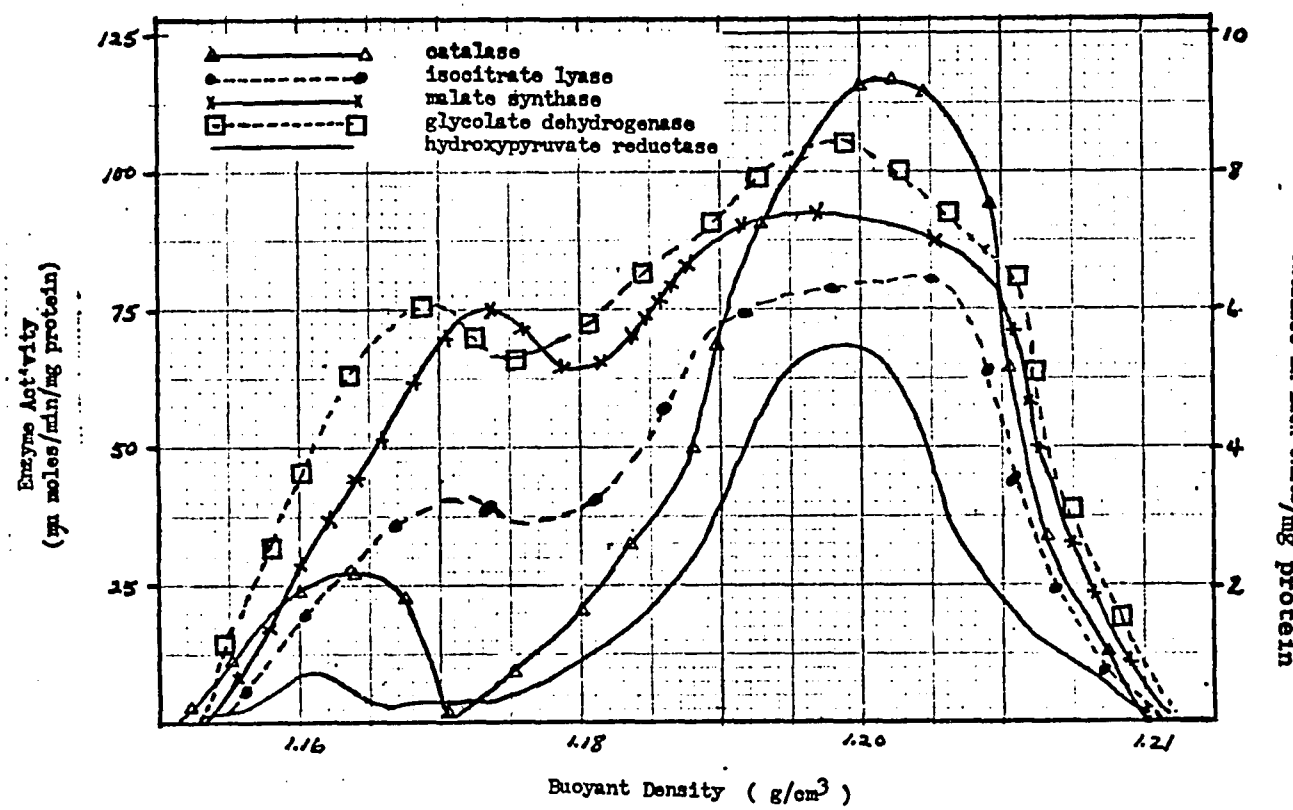


Figure 40. Microbody enzyme distribution of dark-grown, aerated, acetate-supplemented Euglena on a continuous sucrose gradient. All enzyme activities are expressed in specific activity (mμ moles/min/mg protein), except catalase which is reported in Lück units. Enzyme activities: catalase X1; isocitrate lyase X 4; malate synthase X 1; glycolate dehydrogenase X 20; hydroxypyruvate reductase X 15.

Table 13. Enzyme Distribution of Dark-Grown, Aerated, Acetate-Supplemented *Euglena Gracilis* strain Z Isolated On A Continuous Sucrose Density Gradient.

All enzyme activities are expressed in specific activity (mμ moles/min/mg protein), except catalase which is reported in Lück units.

ENZYME	MAXIMUM ACTIVITY Buoyant Density (g/cm^3)	
	1.16-1.19	1.19-1.21
Catalase	2.1	5.5
Isocitrate Lyase	8.2	20.1
Malate Synthase	75.1	88.1
Glycolate Dehydrogenase	3.2	4.2
Hydroxypyruvate Reductase	0.7	4.5

III. *Euglena* Mitochondria

During experiments utilizing catalase-mediated DAB polymerization to localize microbodies in *Euglena gracilis* strain Z, another DAB-reactive enzyme was noted by electron microscopy in some mitochondria of 20 - 72 hour greening cells. Experiments were then performed in an attempt to learn more about this mitochondrial enzyme from the points of view of its nature and factors controlling its development.

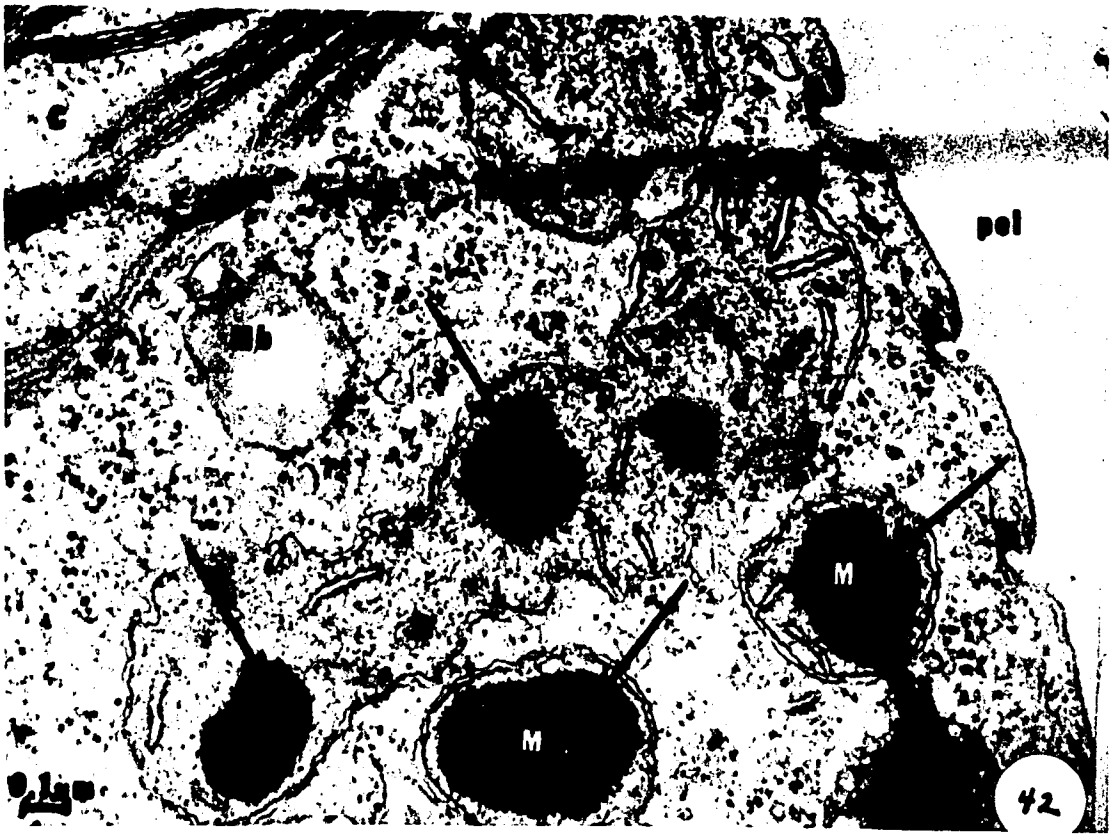
A. Fine Structure Studies

Dark-grown, aerated, acetate (or glucose)-supplemented *Euglena gracilis* strain Z exposed to light for 20 - 24 hours and cytochemically treated with DAB at pH 9.0, 37° C, not only exhibit catalase-mediated DAB polymerization in microbodies, but additionally, demonstrate faint DAB reactivity in some sections of mitochondria (Fig.41); this mitochondrial reactivity can not be observed in cells which have undergone greening for a shorter period of time. At this stage of greening (20 - 24 hours) the chloroplast is not fully developed (i.e. has only 7 - 8 lamellae compared to its full complement of 13-14 lamellae which appear by 72 hours of greening), but is photosynthetically competent (Schiff, 1963; Brody *et al.*, 1965). Mitochondrial DAB reactivity reaches a maximum intensity at ≈ 48 hours of greening (Fig. 42); note from this figure that the activity of the mitochondrial enzyme is unaffected by incubation in the presence of aminotriazole. In contrast, catalase-mediated DAB polymerization is inhibited by aminotriazole, as may be seen from the microbody in this same figure. Incubation in DAB reaction mixture containing KCN inhibits the polymerization of DAB in both organelles. There is a gradual diminution in the mitochondrial DAB

Figure 41. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) aerated, acetate-supplemented Euglena gracilis strain Z after 24 hours of greening, showing an electron-dense microbody (Mb) and several mitochondria (M). Arrow points to DAB reaction product in the matrix of a mitochondrion. x 70,000.

Figure 42. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) in aminotriazole, aerated, acetate-supplemented Euglena gracilis strain Z after 48 hours of greening, showing mitochondria (M), an electron-lucent microbody (Mb), a portion of a chloroplast (C), and the cell pellicle (pel). Arrows indicate DAB reaction product in matrices of several mitochondria. x 50,000.

41



42

stain during the interval from 48 - 72 hours of greening; by 96 hours of greening almost none of the mitochondria exhibit DAB polymerization.

Since the location of the activity of the unknown enzyme in the mitochondria appeared diffuse , an attempt was made to achieve more specific localization. Concentrations of hydrogen peroxide and DAB were reduced to 50 % of their standard amounts in the reaction mixture. However, the electron-opaque appearance of the mitochondrial matrices was unchanged in both 24 and 48 hour greening cells. The same result was obtained even when the DAB reaction mixture contained no added H_2O_2, and only half the standard amount of DAB.

It should be noted at this point, that the mitochondrial DAB reactivity reported here differs from cytochrome oxidase-mediated DAB reactivity. Firstly, optimal conditions for cytochrome oxidase activity are at pH 7.0, 25° C (Novikoff and Goldfischer, 1969; Gerhardt and Berger, 1971); the mitochondrial enzyme in the present work is absent at this pH and temperature - appearing instead at pH 9.0, 37° C (see Table 14). Secondarily, the polymerization of DAB by cytochrome oxidase manifests itself on the outer surface of the inner (or cristae) membranes (Gerhardt and Berger, 1971); the mitochondrial enzyme in the present work was never noted at this location, but always appeared in the matrix. That the particular experimental techniques employed were not the reasons for my failure to demonstrate ("normal") cytochrome oxidase is shown by the presence of this enzyme in co-processed Ricinus leaf and endosperm cells. In Fig. 43 may be seen a section of Ricinus leaf mesophyll cell incubated in the DAB reaction mixture at pH 7.0 and 25° C; note that DAB deposition is not seen in the microbody, but appears on the mitochondrial cristae membranes (compare with Figs. 27 and 29

TABLE 14. Relative Intensities * of DAB Stain in Microbodies (Mb) and Mitochondria (M) of Greening Euglena Cells As Functions of Conditions of Incubation and Time of Addition of DCMU.

Time of addition of DCMU	hours in light	pH 9.0, 37 °C						pH 7.0, 25 °C					
		DAB		AT		CN		DAB		AT		CN	
		M	Mb	M	Mb	M	Mb	M	Mb	M	Mb	M	Mb
Not added Control	18	-	+	-	-	-	-	-	-	-	-	-	-
	24	+	+	+	-	-	-	-	-	-	-	-	-
	40	++	+	+	-	-	-	-	-	-	-	-	-
	48	+++	+	+	-	-	-	-	-	-	-	-	-
	72	-	+	-	-	-	-	-	-	-	-	-	-
i) Zero Time	18	-	+	-	-	-	-	-	-	-	-	-	-
	24	-	+	-	-	-	-	-	-	-	-	-	-
	40	-	+	-	-	-	-	-	-	-	-	-	-
	48	-	+	-	-	-	-	-	-	-	-	-	-
	72	-	+	-	-	-	-	-	-	-	-	-	-
ii) After 18 Hours of Greening	24	-	+	-	-	-	-	-	-	-	-	-	-
	40	-	+	-	-	-	-	-	-	-	-	-	-
	48	-	+	-	-	-	-	-	-	-	-	-	-
	72	-	+	-	-	-	-	-	-	-	-	-	-
iii) After 24 Hours of Greening	40	-	+	-	-	-	-	-	-	-	-	-	-
	48	-	+	-	-	-	-	-	-	-	-	-	-
	72	-	+	-	-	-	-	-	-	-	-	-	-
iv) After 40 Hours of Greening	48	-	+	-	-	-	-	-	-	-	-	-	-
	72	-	+	-	-	-	-	-	-	-	-	-	-

* It is not intended to compare the relative intensities of DAB stain in mitochondria and microbodies. Intensities are relative only within the mitochondrial category; the + or - in the case of microbodies refers only to presence or absence.

Figure 43. E.M. of DAB incubated (pH 7.0, 25 °C) Ricinus communis leaf mesophyll cells showing mitochondrion (M) with electron-dense deposition on inner (cristae) membranes. The arrow indicates a crystalline inclusion inside the microbody (Mb). Note that the microbody matrix is rather electron-lucent under these conditions of DAB incubation. x 100,000.


C
V
M
Tp
43

which were not DAB incubated at pH 7.0, 25° C). It is of interest to note that neither with Ricinus endosperm nor leaf mesophyll cells did I ever observe mitochondrial enzyme activity at pH 9.0 and 37° C.

When dark-grown cells (grown either in the presence or absence of CO_2) are allowed to green in the absence of CO_2, DAB reactivity is not visualized in the mitochondria. If, however, cells which have grown in the dark in the absence of carbon dioxide, are placed in the light and aerated (with air containing CO_2), the DAB mitochondrial activity is observed by 22 - 24 hours of greening. Cells grown in the dark in the absence of CO_2 and allowed to green for 24 or 48 hours in the absence of CO_2, also display intense mitochondrial DAB reactivity by 2 additional hours of greening in the presence of air containing CO_2.

In the presence of CO_2, DAB deposition at pH 9.0 and 37° C is apparent in both mitochondria and microbodies of Euglena; in the absence of this gas the stain fails to manifest itself in either organelle..

Aliquots of aerated, acetate-supplemented dark-grown and continuously light-grown Euglena were cultivated in the presence of the (system II) photosynthesis inhibitor DCMU. As Schiff et al. (1967) have shown, chloroplast development is otherwise normal in the presence of DCMU. From Table 14 (part i at pH 9.0, 37° C) it may be seen that DCMU treatment at zero time (i.e., before the cells are placed in the light) prevents DAB deposition from occurring in mitochondrial matrices. Catalase-mediated DAB reactivity in microbodies is unchanged in the presence of DCMU (Table 14).

Although DAB reactivity is normally apparent in the mitochondria of control cells which have been allowed to green for

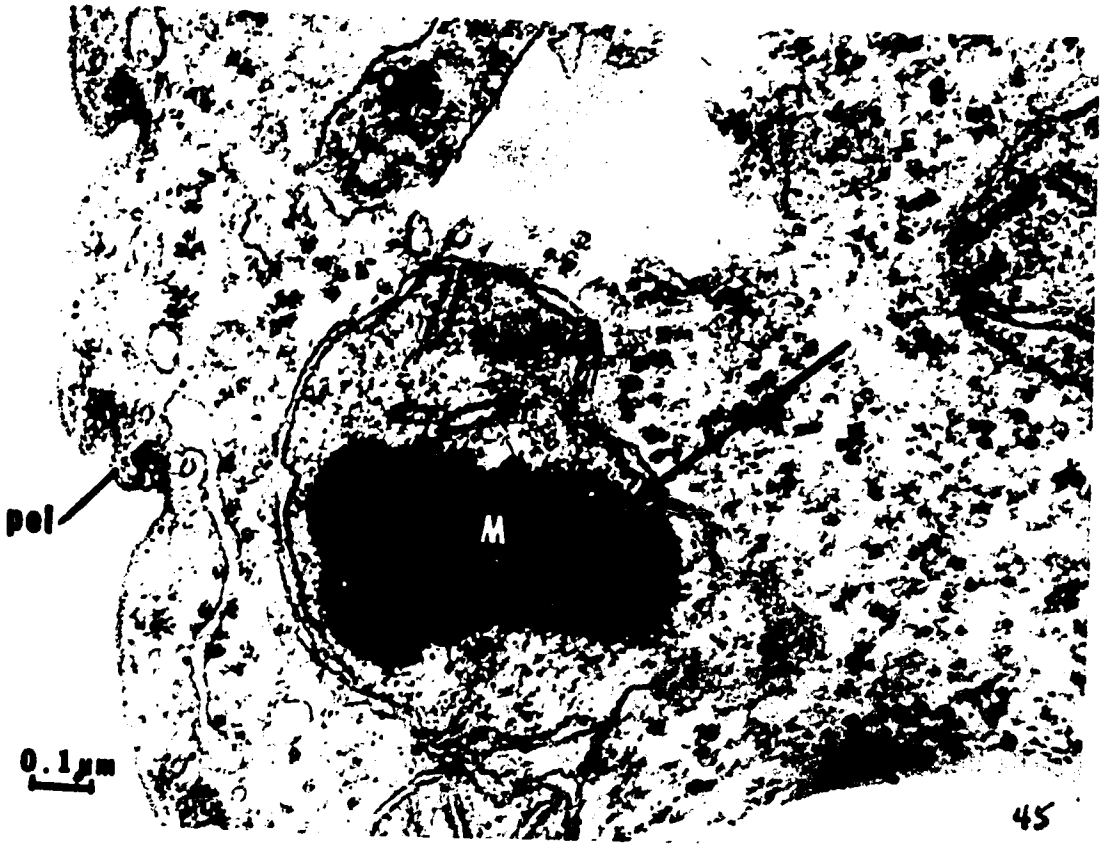
24 hours (see Control in Table 14), no DAB reactivity appears if DCMU is added at 18 hours of greening, even if cells are then allowed to green for periods up to 72 hours (see Table 14 part ii). DCMU was also added to 24 hour greening cells (Table 14 part iii) or 40 hour greening cells (Table 14, part iv) - the mitochondria of such cells exhibit intense DAB reactivity. If, after two hours of treatment with DCMU, these cells are incubated with DAB and fixed, polymerization is no longer apparent in the mitochondrial matrices.

As described in the Introduction, page 6 , changes in type and composition of fatty acids occur with greening in Euglena; dark-grown cells contain large amounts of polyenoic acids (e.g., arachidonic acid), light-grown cells contain high concentrations of - linolenic acid and smaller amounts of polyenoic acids. Therefore, it seemed interesting to investigate the possibility that this unidentified mitochondrial DAB-reactive enzyme was involved in the change-over in fatty acid metabolism. To this end, dark-grown cells which had previously been grown in acetate were allowed to green in media supplemented not with acetate, but with final concentrations of 1×10^{-4} M linolenic acid or arachidonic acid as sole carbon and energy sources. The cells were then harvested at 12, 24, 48, 60, 72, 96, 108, 120 and 132 hours of greening, and treated with the DAB incubation mixture, pH 9.0, 37^{o} C. In the case of linolenic acid-supplemented cells, the DAB reaction product appeared in some sections of mitochondria of cells allowed to green for 24, 48, 60, 72, 96, 108 and 120 hours (Fig. 44); the stain was not apparent prior to 24 hours of greening nor at 132 hours of greening. Culture grown in the presence of arachidonic acid demonstrated DAB reactivity in mitochondria of 24, 48, 60, 72, 96 and 108 hour greening cells (Fig. 45);

Figure 44. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) aerated, linolenic acid-supplemented Euglena gracilis strain Z after 120 hours of greening. Note the deposition of DAB reaction product in both the microbody (Mb) and some areas of mitochondria (arrows). x 70,000.

Figure 45. E.M. of DAB/H_2O_2 incubated (pH 9.0, 37 °C) aerated, arachidonic acid-supplemented Euglena gracilis strain Z after 108 hours of greening. Note the deposition of DAB reaction product in the matrix of a mitochondrion (arrow). x 70,000.


0.1μm
44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the stain is not apparent prior to 24 hours of greening or at 120 or 132 hours of greening. Therefore, growth in either linolenic acid or arachidonic acid-supplemented media prolonged the reactivity of the mitochondrial enzyme.

B. Enzyme Assays of Cell-Free Fractions

Cell-free fractions of dark-grown, greening, and continuously light-grown aerated, acetate-supplemented Euglena gracilis strain Z were assayed for the mitochondrial marker enzymes malate dehydrogenase, fumarase, aconitase and cytochrome oxidase. Specific acitivities of the first three enzymes were found to remain relatively constant with greening in such cell-free fractions; cytochrome oxidase, however, could not be detected (Table 9).

Specific activities of malate dehydrogenase, fumarase and aconitase in cell-free fractions of cells grown under the three light regimes, in air depleted of CO_2, were similar to cells grown in the presence of CO_2 (Table 10; compare with Table 9); again, cytochrome oxidase could not be detected photometrically.

C. Enzyme Assays on Fractions From Discontinuous Sucrose Density Gradients

Specific activities of the mitochondrial marker enzymes, malate dehydrogenase, fumarase and aconitase were found to remain relatively constant with greening in the mitochondria-containing discontinuous sucrose gradient fractions (Table 11). Note that the activities of these enzymes are approximately an order of magnitude greater in this band than they are in the microbody-containing bands. Cytochrome

oxidase could not be detected by the method of Tobbert et al. (1968) in the mitochondria-containing sucrose bands (Table 11).

Additional aliquots of dark-grown, greening, and continuously light-grown acetate-supplemented cells were concurrently grown in the presence of air from which carbon dioxide had been removed. Discontinuous sucrose density gradients were prepared and assayed for malate dehydrogenase, fumarase, aconitase and cytochrome oxidase. From Table 12 it may be seen that carbon dioxide-deprivation had no effect upon the levels of malate dehydrogenase, fumarase or aconitase, i.e., assays were similar to those presented in Table 11 for cells grown in the presence of air containing CO_2. Cytochrome oxidase could not be detected in mitochondria-containing bands of such cells grown under the three light regimes.

D. Enzyme Assays on Fractions From Continuous Sucrose Density Gradients.

The mitochondrial marker enzymes malate dehydrogenase, fumarase , and aconitase were found to band mainly at buoyant densities between 1.16 - 1.18 g/cm^3; minor peaks of each enzyme were also noted in the 1.18 - 1.20 g/cm^3 buoyant density range (microbody). Profiles of these enzymes are presented in Fig. 46. Cytochrome oxidase could not be detected at any buoyant density on these continuous sucrose density gradients.

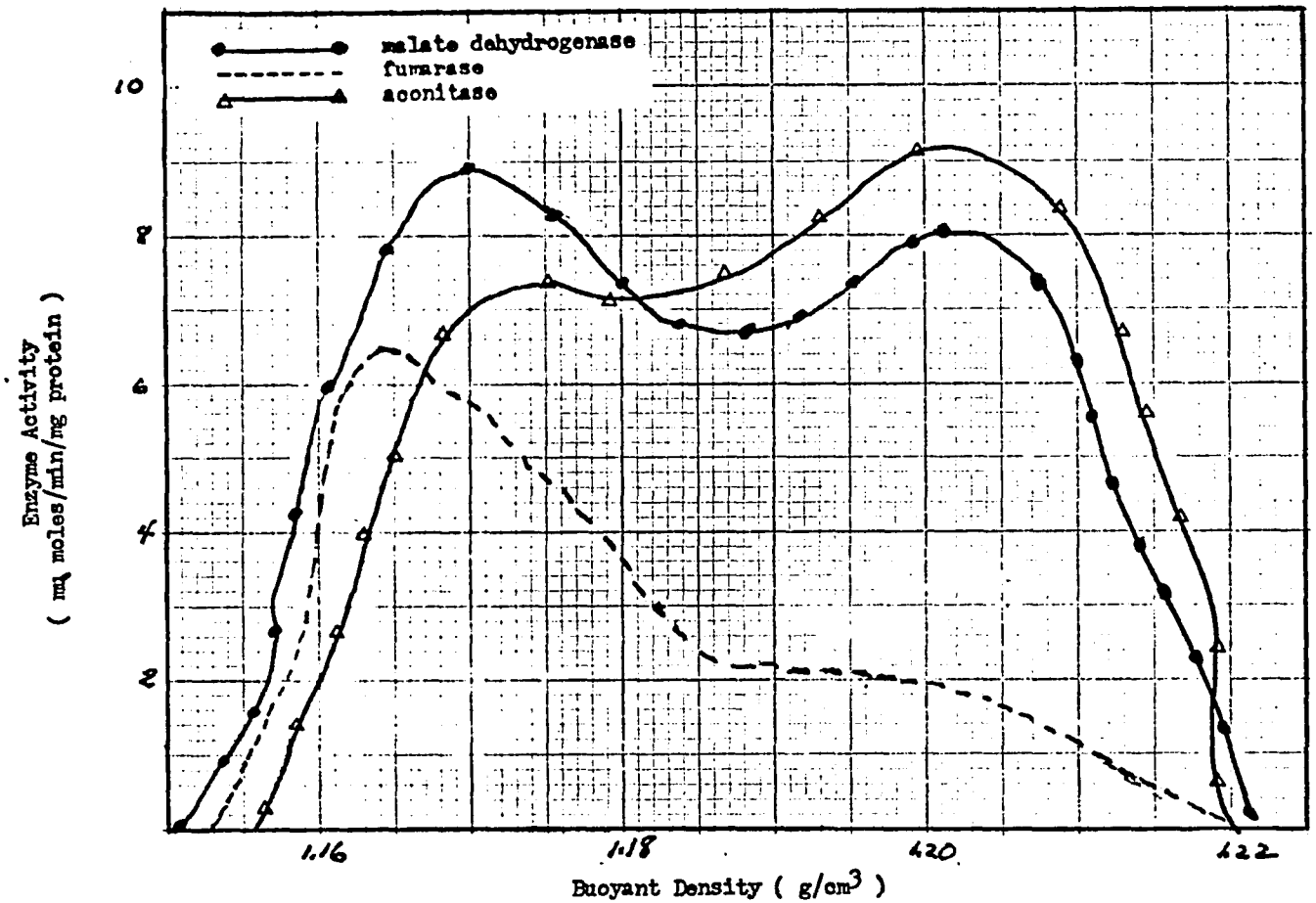

Figure 46. Mitochondrial marker enzyme profiles of dark-grown, aerated, acetate-supplemented Euglena gracilis strain Z isolated from continuous sucrose density gradients. All enzyme activities are expressed in specific activity (mμ moles/min/mg protein). Enzyme activities: malate dehydrogenase X 1; fumarase X 10; aconitase X 5.

DISCUSSION

I. Effects of Exogenous Fatty Acids on Euglena and Other Organisms

An extensive literature exists concerning environmental effects (e.g., temperature, light intensity and carbon source) on the generation time of Euglena (Cramer and Myers, 1952; Wilson et al., 1959; Cook, 1968). For example, Wilson et al. (1959) demonstrated that Euglena gracilis variety bacillaris grew twice as fast (at optimal pH) in media supplemented with acetone or succinate than media supplemented with malate or fumarate. In general, growth rates of Euglena gracilis strain Z have been reported to be proportional to the initial concentration ranges of 1.0 to 5.0 mM for succinate, ethanol, or acetate (Levedahl and Wilson, 1965).

The addition of exogenous linolenic acid (in final concentrations greater than 5.0×10^{-6} M) to glucose-supplemented, greening Euglena gracilis strain Z cells, led not only to a lengthening of generation time, but also to a retardation in chlorophyll synthesis and retardation in chloroplast development. This retardation in chloroplast development was observed directly in fine structure studies and indirectly in studies of low temperature fluorescence spectroscopy.

Since the late 1950's a great deal of evidence has accumulated for the existence of both aggregated and monomeric forms of chlorophyll in vivo. [See, for example, review by M. Brody, 1968.] Some of this evidence comes from low temperature fluorescence studies. At least three emission bands are observed from chloroplasts. The short wavelength

emitting form (F_{685} or F_M) has been interpreted as arising from Photosystem II bulk monomeric chlorophyll; an intermediate wavelength emitting form (F_{698}) has been assigned to special Photosystem II reaction center monomers; the (complex) long wavelength form (F_{720}-F_{735} or F_A) has been ascribed to emission from Photosystem I.

Brody et al. (1965) working with Euglena gracilis strain Z (cultured on the organic medium of Greenblatt and Schiff, 1959) noted a pronounced rise in the ratio of F_A/F_M at about 10 hours of greening, which heralded the beginning of photosynthetic efficiency. They postulated that sensitization of aggregated chlorophyll, as well as monomeric chlorophyll is necessary for photosynthesis, and that perhaps this steep rise in F_A/F_M signals the formation of the first effective photosynthetic lamellum. In the present work, similar results were obtained with glucose-supplemented greening cells (i.e., a steep rise in F_A/F_M occurred at about 11 hours of greening). In the case of dark-grown glucose-supplemented cells to which 10^{-4} M linolenic acid (final concentration) was added, this steep rise in F_A/F_M did not begin until after $\sim$ 42-44 hours of greening.

App and Jagendorf (1963) have demonstrated that repression of chloroplast development occurs in greening Euglena gracilis strain Z by several metabilizable carbon sources. The degree of this repression is a function of the rate of utilization of the carbon source (e.g., ethanol represses more than acetate or malate). In the aforementioned work it was also shown that pre-adaptation of Euglena to a specific carbon source (e.g., ethanol, glucose) increased the repression of chloroplast synthesis in the light by that carbon source.

In the present work, the initial retardation and then "catching up" in F_A/F_M, chlorophyll concentration and chloroplast development of Euglena cells grown in the presence of 1×10^{-4} M linolenic acid (final concentration) suggests that after an initial lag period, the fatty acid was being metabolized by the cells. While it has been shown that numerous algae [e.g., Prototheca zopfii (Barker, 1935)] may utilize fatty acids as sole carbon and energy sources, a search of the literature failed to reveal a report that Euglena gracilis strain Z could be grown on media containing long-chain unsaturated fatty acids as sole carbon and energy sources. Support for my contention that Euglena gracilis strain Z is capable of metabolizing this fatty acid, was shown in experiments in which linolenic acid (1×10^{-4} M, final concentration) served as sole carbon and energy sources for dark-grown, greening, and continuously light-grown cells. Recall that growth under such conditions was comparable to that achieved when acetate was the sole carbon and energy source (i.e., generation times and pigment synthesis kinetics were similar).

Erwin and Bloch (1963 b) demonstrated that the fatty acid composition of photosynthetically-active cell fractions from blue-green algae is very similar to that of chloroplasts of higher plants. Levin et al. (1964) have shown that in Anabaena variabilis these fractions contain large amounts of α-linolenic acid which occurs in the form of galactolipids (Kirk and Tilney-Bassett, 1967). Therefore, these chemical similarities of the photosynthetic apparatus of green plants and blue-green algae suggest that although the cytological organization of

blue-green algae and higher plants differ, the molecular structure of their photosynthetic lamellae are probably very similar.

Ris and Singh (1961) first documented polyglucoside granules in several species of blue-green algae and suggested their role as storage inclusions for the products of photosynthesis. Electron-dense granules of approximately the same size have been shown to be present in Oscillatoria amoena (Fuhs, 1963), Symploca muscorum (Pankratz and Bowen, 1963), Anacystis montana f. minor (Echlin, 1964) and Anabaena azollae (Lang, 1965). Experiments utilizing selective digestion of polyglucoside granules with diastase (Fuhs, 1963; Giesy, 1964) or α-amylase (Lang, 1968) indicate their polyglucoside nature. Pankratz and Bowen (1963) named these structures "α-granules" because they were no longer apparent after incubation in α-amylase.

However, Lang (1968) believes the term "polyglucoside granule" is more appropriate, considering their chemical composition. The number and size of these granules depends upon levels of available nitrogen, light intensities and culture age (Giesy, 1964) and are not found in developing heterocysts of Anabaena azollae (Lang, 1965) or in heterocysts and senescing cells of Gloetricha (Madsen, 1966). However, these granules were observed in germinating akinetes of Cylindrospermum (Miller and Lang, 1968), indicating an interrelationship with active photosynthesis.

As may be seen from the present work, the major fine structure effect of either treatment with, or growth in exogenous fatty acid, is on the polyglucoside granules of Anabaena - there being little

observable effect on lamellae *in situ*. After such treatment, the polyglucoside granules are no longer electron-dense (as in controls), but are electron-lucent and appear as "ghosts of granules" . One possible explanation for this electron-lucent appearance is that linolenic acid is acting as a lipid solvent. Another possibility is the masking effect of linolenic acid on the polyglucoside granules.

Barker (1935) reported that the green alga *Prototheca zopfii* grew on a) all saturated C_2 to C_{10} straight-chain fatty acids, b) even-numbered C_{10} to C_{16} saturated fatty acids, and c) isobutyric, isocaproic, α-carotonic and oleic acids. In contrast, *Chlorella pyrenoidosa* and *Chlorella ellipsoidea* were found to be limited in their utilization of fatty acids - growing on acetate,but not formate, proprionate or butyrate (Samejima and Myers, 1958). Pedersen *et al.* (1966) showed that final concentrations of 10^{-4} M lipoic acid, octanoic acid or methyl octanoate when exogenously added to *Chlorella pyrenoidosa* had inhibitory effects upon photosynthetic activities, as measured by oxygen evolution and CO_2 utilization.

Under my growth conditions, control *Chlorella pyrenoidosa* had a generation time of 8.1 hours. This compares favorably to the generation time of 10.4 hours for *Chlorella pyrenoidosa* reported by Sorokin and Krauss (1958) and Hoogenhout and Amesz (1965), considering that both groups of workers utilized light intensities of 500 ft. c. Nathanson (personal communication), working under almost the same growth conditions as I, reports a (control) generation time of 8.5 hours.

Under the conditions of growth used in the present work,

control Chlorella pyrnoidosa were found to contain 12.6 µg chlorophyll (a + b) per cell. Oh-Hama et al. (1965) and Matsuka et al. (1969) report total chlorophyll (a + b) concentrations in control Chlorella protothecoides of 10.6 and 10.0 µg/cell, respectively. Both groups of workers, however, utilized a different species of Chlorella than I and, furthermore, grew their cells at higher light intensities (500 ft. c.). Nathanson (personal communication) also reports a total chlorophyll concentration of 12.6 µg/cell working with the same strain and under the same growth conditions as I.

From my results, it is evident that with increasing concentrations of fatty acids in the growth media of Chlorella , progressive decreases in chlorophyll content and progressive lengthening in the generation times occur. Furthermore, the concentrations of ethanol used to solubilize linolenic acid have very little effect on either amount of chlorophyll or generation time.

I also undertook a study to compare Chlorella treated with or grown in exogenous fatty acid, to cells grown in nitrogen-deficient media. In the latter case, it was assumed that enhanced levels of cellular fatty acids would result. It was found with cells grown in progressively nitrogen-deficient media that a) membranes progressively became distorted, aberrant, and finally absent, b) total chlorophyll (a + b) concentrations progressively decreased until pigment was no longer detectable, and c) generation times progressively lengthened until cells no longer underwent division.

The above changes which are associated with decreased

nitrogen content - and presumably increased fatty acid content - were very similar to those observed when Chlorella was treated with or grown in fatty acid. It was not until cells were grown in nitrogen concentrations less than 1/200 the optimal amount that fine structure observations were made which were not in evidence as a result of addition of exogenous fatty acid, i.e., disappearance of all internal structures and manifestation of a "spore-like" cell. While many of the symptoms of nitrogen deficiency can be attributed to a build-up of (abnormally) high concentrations of fatty acids, a point is reached where symptoms appear that are attributable to the unavailability of nitrogen for the manufacture of certain nitrogen-requiring molecules - including structural and functional proteins. At this stage, there ceases to be a parallel with cells in which nitrogen is available but fatty acid content is increased.

Although my results on the restoration of normal fine structure, chlorophyll concentration and generation time when nitrogen-deficient cells were returned to more optimal nitrogen media might have been anticipated, the restoration of these normal parameters to cells lacking all internal structure (i.e. , the "spore-like" ones) was indeed apparently unexpected and demonstrated the astonishing "recuperative powers" of Chlorella.

Shihira-Ishikawa and Hase (1964) observed that when Chlorella protothecoides was grown in a medium rich in glucose and poor in nitrogen (in these experiments, urea was used as the nitrogen source), yellowish, chlorophyll-less cells with degenerated plastids were produced either in the light or dark. Similar results were obtained

in the present study with light-grown, nitrogen-deficient Chlorella pyrenoidosa. Moreover, green Chlorella protothecoides cells (controls) transferred into a medium void of nitrogen (both in the light as well as in the dark) rapidly become bleached, with cytological degradation of chloroplast structure occurring (Aoki et al., 1965; Matsuka and Hase, 1965; Mitsuda et al., 1970). It has been further shown (Aoki and Hase, 1964; Oh-Hama et al., 1965) that nitrogen-deficient Chlorella regain their normal complement of chlorophyll and chloroplasts when incubated in media enriched with nitrogen.

During the development of nitrogen deficiency, the cellular nitrogen content of Chlorella pyrenoidosa falls from ≈ 10 % of dry weight to ≈ 2 % (Fogg, 1959). Accompanying nitrogen deficiency, the main products of photosynthesis change first to carbohydrates, and then to lipids (Matsuka et al., 1966). Moreover, with continued nitrogen deprivation the chemical composition of Chlorella protothecoides may continuously change for several weeks (Matsuka et al., 1966). In Chlorella pyrenoidosa an accumulation of lipid commences ≈ 4 days after the onset of nitrogen deprivation, while in Chlorella vulgaris, fat accumulation does not begin until ≈ 10 days of nitrogen deprivation (Fogg, 1959). As nitrogen deficiency developes, the amount of cellular chlorophyll decreases (Fogg, 1959).

Matsuka and Hase (1965, 1970) studied the respiratory metabolism of Chlorella protothecoides and concluded that during the process of chloroplast degeneration which occurred in nitrogen-deficient media, added glucose is rapidly assimilated into carbohydrates and lipids. Otsuka and Morimura (1966) reported that: a) oleic acid is

the major fatty acid formed during nitrogen-deprived bleaching in Chlorella protothecoides, b) other fatty acids detected in the bleaching cells were linoleic (C_{18}:2), palmitic (C_{16}:0), linolenic (C_{18}:3) and myristic(C_{14}:0), c) linolenic acid concentration increased during the first 15 hours of bleaching, but decreased appreciably in terms of both mg-per-culture and mg-per-cell volume during the later period in which the bleaching of cells was most pronounced, d) the cellular increase of linolenic acid closely followed the time course of increased chlorophyll formation upon restoration of nitrogen to optimal levels. Other workers have also reported that linolenic acid disappears in parallel with chlorophyll disintegration during bleaching (Crombie, 1958; Erwin and Bloch, 1963 b; Benson, 1964).

Matsuka et al. (1969) studied the metabolism of glucose, during the process of bleaching in Chlorella protothecoides induced by nitrogen deprivation, by following $^{14}CO_2$ evolution and $^{14}CO_2$ incorporation into various cellular substances. In this study the most dramatic increases in cellular compounds (from glucose) were found to be lipids (fatty acids) and glucose polymers; when urea was added to the growth medium, the incorporation of $^{14}CO_2$ into fatty acids was greatly reduced, while the assimilation of ^{14}C into glucose polymers was increased. Although it was suggested by Matsuka et al. (1969) that the formation of large amounts of lipids (fatty acids) probably is causally related to the induction of algal cell bleaching, I believe it is logical to also consider that synthesis of chlorophyll (a nitrogen-containing compound)becomes increasingly difficult under conditions of nitrogen deprivation.

Numerous studies have shown that the fine structure of isolated chloroplasts is strongly influenced by ionic environment. Perner (1965)

and Vernon (1967) observed granal disorganization of spinach chloroplasts into single thylakoids when the cationic concentration of the isolation media was increased[32].

Itoh et al. (1963), on the basis of light microscopy, noted that dodecylbenzene sulfonate (a monovalent salt of lauric acid) induced chloroplast swelling in the dark, but inhibited it in the light. Light scattering changes (decrease), resulting from the addition of fatty acids to isolated chloroplasts, have been interpreted as arising from swelling of chloroplasts (Siegenthaler and Packer, 1965; Pedersen et al., 1966, Molotkovsky and Zheskova, 1966). Pedersen et al. (1966) showed that the reactions catalyzed by enzymes of the reductive pentose phosphate cycle, as well as photophosphorylation, are inhibited by exogenously-added fatty acids (or methyl octanoate) during carbon dioxide fixation, and concluded "the free fatty acids altered the proportions of the lamellae in such a way that photophosphorylation is blocked and a light-induced conformational change occurs." As early as 1961, Brody and Brody had suggested that photophosphorylation might produce modifications in local hydrogen ion concentration, which in turn could bring about conformation changes, resulting in alterations in the states of

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[32] Hongladarom et al. (1968) noted that aldehydes and $KMnO_4$ in concentrations used for electron microscopy induce by themselves large amplitude swelling of spinach chloroplasts. In this work, quantitative measurements by phase microscopy established that a 7-fold increase in volume and 3.6-fold increase in chloroplast face area (modal values) resulted from incubation in $KMnO_4$ within 5-10 minutes. These workers pointed out that "volumes of spinach chloroplasts isolated in NaCl as reported in the literature approach the volumes of chloroplasts swollen by HCHO and $KMnO_4$".

aggregation of attached chlorophyll chromophores.

Izawa and Good (1966) noted only a swelling effect by fatty acid (in the FA/Chl $\lesssim$ 0.001 range) on isolated spinach chloroplasts - the outer chloroplast envelopes remain intact. However, Murakami and Nobel (1967) utilizing packed weight and electron microscopy, found that 120 - 130 μM exogenously-added lauric, stearic or oleic acids (FA/Chl $\lesssim$ 0.001, my calculations) caused pronounced high-amplitude chloroplast swelling in the dark, low-amplitude swelling in the light, and removal of the chloroplast envelope.

There seems to be a close relationship between the degree of free versus bound fatty acid on configurational aspects of organelles such as chloroplasts and mitochondria. Benson (1963, 1964) postulated the likelihood that chloroplast galactolipids and fatty acids exist in dynamic equilibrium between bound and free states. Linolenic acid was found to occur in high concentrations in spinach chloroplasts by Crombie (1958); later this fatty acid was shown to be mainly incorporated into monogalactosyl dilinolenin and digalactosyl dilinolenin (Benson, 1964), with some incorporation into trigalactosyl dilinolenin (Webster and Chang, 1969). Spinach chloroplast galactolipids may possess linolenic ester contents as high as 94 - 96 percent (Benson, 1963; Sastry and Kates, 1963).

Molotkovsky and Zheskova (1965) noted that heat-induced swelling of isolated spinach chloroplasts was associated with a partial destruction of membrane lipids and that these fatty acids accumulated in the incubation medium.

Since it was my intention to study the effect of exogenous fatty acids on chloroplast ultrastructure, I wished to work under conditions in which the effects of endogenous fatty acids would be minimal. Therefore, whole spinach chloroplasts were isolated according to the method of Spencer (1967), in which the medium consists of 0.4 M sucrose, 0.05 M Tris-HCl and 0.1 M KCl, adjusted to pH 7.8. As described in the Results section, high concentration of exogenous fatty acid (or long periods of incubation in RLE) result in a sequential swelling of stromal and then granal thylakoids, accompanied by the loss of chloroplast envelopes and eventual separation of thylakoid fusion layers, to form distorted, myelin-like figures.

In an extension of the work (Brody et al., 1969; Cohen et al., 1969) in which my contribution was the observation that RLE or fatty acids bring about changes in isolated chloroplasts, two of the co-authors pursued the possibility of experimentally demonstrating that these configurational changes have their origins in conformational ones. Circular dichroism studies with chlorophyll systems of various levels of structural complexity have shown (Brody and Nathanson, 1972; Nathanson, 1973) that deaggregation of chlorophyll by exogenous fatty acid occurs by two mechanisms: a) in the case of chlorophyll in solution, the action of the fatty acid is a direct one on the chromophores, b) in the case of chlorophyll attached to protein, deaggregation is indriectly brought about through a preliminary effect on the protein (in membranous systems, the indirect mechanism seems to be the predominent one). The indirect mechanims was recognized, when it was observed that

conversion of aggregated to monomeric bands in the ultraviolet region of the C.D. spectra was preceeded by a (partially reversible) conversion of (primarily) α-helical protein to random coil - deaggregation being a secondary result of this conformational change.

The above-mentioned circular dichroism work was done with chloroplast fractions from Spinacea in which the mole:mole ratio of linolenic acid:chlorophyll was in the range of 1:1 to 10:1 (i.e., that range in which I observed disruption of chloroplast envelopes and swelling of stromal and granal thylakoids). I feel that it is very likely, therefore, that the configurational changes I observed have their origins in conformational changes.

Spinach chloroplasts treated with RLE or high concentrations of fatty acids (FA/Chl $>$ 1.0) result in the formation of myelin-like figures similar to those reported by Murakami and Nobel (1967) for chloroplasts incubated in the dark for 30 minutes in medium containing 10 - 20 % acetone. "Myelin figures" have been so named becuase of morphological similarities to nerve myelin (Stein, 1967). Surfactant lipids, which possess solubility characteristics similar to that of nerve myelin, form stable tubular extrusions when hydrated in water (Benson, 1963). Myelin forms of membranes have been made in the laboratory by mixing lecithin with water (Goedheer, 1957; Fernandez-Moran, 1962) or ammonium oleate with water (Goedheer, 1957). Such myelin-like forms (lipid micelles) have been proposed (Fernandez-Moran, 1962; Stein, 1967) to result from the imbibition of water - the structural position taken by the lipids being determined by their solubility properties (polar groups oriented towards the intercalated water and the non-polar

groups directed away). These molecular movements which result from lipid swelling, sometimes produce long, thin, myelin figures (Glickhorn, 1932) and at other times produce convoluted forms (Fernandez-Moran, 1962).

Myelin forms consisting of water, lipid and protein have been made, fixed, stained and observed by electron microscopy (Stoekenius, 1962); these artificial membranes morphologically resemble membrane strcutures of cells. The predominent lipid of the chloroplast, digalactosyl dilinolenin, forms stable myelin figures when hydrated (Benson, 1963). Therefore, it should not be surprising that mechanical or solvolytic disturbances of lamellar structures (and the "reconstitution" of solubilized protein endogenous lipid, exogenous fatty acid, etc.) would result in the formation of myelin-like figures, such as those observed in the present work.

II. Euglena Microbodies

A. Fine Structure Studies

1. Dark-Grown Cells

It is extremely difficult to locate microbodies in Euglena solely on the basis of fine structure - their limiting membranes stain very faintly and their matrices are rather electron-lucent. However,the presence of catalase in these organelles makes it possible to utilize the cytochemical identifier DAB. Despite these difficulties, Hanzely et al. (1971) and Graves et al (1971 a) very nicely demonstrated, in fine structure studies, the presence of microbodies in a streptomycin-bleached (non-photosynthetic strain) of Euglena gracilis var. bacillaris (SM-L1),grown in inorganic medium supplemented with glucose, acetate or ethanol. Both

these groups of workers, however, were unable to detect catalase, either cytochemically (under conditions of DAB incubation similar to those used in the present work) or photometrically (with cell-free fractions, using the method of Lück).

In the present study with aerated, acetate-supplemented (dark-grown, greening, and continuously light-grown) Euglena gracilis strain Z, the measured microbody long dimension range was found to be 0.50 - 1.40 μm, with a mean of ≈ 0.80 μm; measured microbody short dimension range was 0.40 - 0.90 μm, with a mean of ≈ 0.65 μm. Graves et al. (1971 a) refer to microbodies of Euglena gracilis var. bacillaris (SM-L1) as "rounded to irregularly-shaped" and reported measured dimensions in terms of "diameters". Although in the present work, the measured microbody short dimension range coincides almost perfectly with the diameters of strain SM-L1 (0.4 to 0.8 μm) given by Trelease et al. (1971) and Graves et al. (1971 a), these two groups of workers do not report data comparable to my measured microbody long dimension range. I do not know whether the differences in their data and mine represent true variances in the shapes of microbodies of the two organisms (i.e., the shape of microbodies in strain SM-L1 being rounded to irregular and that of strain Z being spherical to ellipsoidal), or whether the differences result from dissimilarities in numbers of counts made by these workers and myself - no indication made by the former workers concerning the number of measured microbodies.

In the present study, it is seen that with greening (and associated increase in peroxisomal enzyme activity) the size of the microbodies in Euglena gracilis strain Z remains relatively constant(≈ 0.85 μm long dimension, ≈ 0.65 μm short dimension). These findings con-

trast with those of Gruber et al. (1973) who noted that microbodies of primary leaf cells of etiolated Phaseolus vulgaris (bean) were smaller ($\approx$ 0.3 μm in diameter) than those of green cells ($\approx$ 1.5 μm in diameter) and suggested that this increase in size was perhaps a correlary of the increase in peroxisomal enzyme activity that accompanies greening.

My measurements of 60 - 70 A° for the thickness of the microbody-limiting membrane of Euglena gracilis strain Z compares well with the findings of 65 - 70 A° reported by Graves et al. (1971 a) for strain SM-Ll.

Graves et al. (1971 a) found that the peripheral cellular regions were favored locations for microbodies in strain SM-Ll. My findings are in agreement with this, but I have noted in addition that regions around the gullet are even more enhanced in microbodies per unit volume than in the cell periphery. Graves et al. (1971 a) observed in fine structure studies, an average of 2 or 3 microbodies per cell section, with as many as 8 in a few sections (location not specified) in SM-Ll grown in ethanol-supplemented media. I counted similar numbers in dark-grown, aerated, acetate-supplemented strain Z - an average of 3.10 microbodies per cell section, with as many as 10 in some cells sectioned near the gullet. In good agreement with Graves et al. (1971 a) I found that Euglena grown on glucose-supplemented media had far fewer (at least an order of magnitude less) microbodies than acetate-supplemented cells.

I can make no comparisons regarding the number of microbodies per dark-grown Euglena-there are no other available data. Even in regard to strain SM-Ll used by Hanzeley et al. (1971) and Graves et al. (1971 a), no estimate on total number of microbodies was given, nor can I make estimates from their data.

In the present work I have based estimates of numbers of microbodies per cell on both thick and thin sections. In general, light microscopic counts of microbodies are at least 10 % higher than electron microscopic counts. As pointed out by Koningsmark (1970), all total count methods of sectioned material are subject to both counting errors and split cell errors[36]. Also, I feel that because of the limitations of experimental techniques, more errors were introduced by attempting serial sections of every tenth thin section, than observing every thick serial section. Therefore, I feel that my data based on light microscopic counts of microbodies is more accurate.

In the present work it was found that the number of microbodies per cell section doubles by $\lesssim$ 24 hours of greening - based on random counts of (thin or thick) sections prepared for electron microscopy or light microscopy. Thick or thin serial sections examined by light or electron microscopy, respectively, also indicate an approximate doubling in the number of microbodies per cell by 24 hours of greening.

Although Trelease et al. (1971) reported that the average number of microbody profiles per cell section of dark-grown cucumber cotyledons decreased by 25 % in palisade cells and 35 % in spongy cells between days 4 and 10 of greening, perhaps this "decrease" could be attributed to an increase in cell size. Gerhardt and Beevers (1970) on the other hand, reported that in the case of castor bean endosperm, an increase in numbers of microbodies parallels the increase in specific activities of isocitrate lyase and malate synthase in glyoxysomes during the first five days of germination; after day 5 (when presumably storage lipids are dwindling) a decrease was detected in both numbers of microbodies and glyoxysomal enzymes. Vigil (1970) also worked with endo-

sperm of castor bean and noted that by day 5 of germination some microbodies(glyoxysomes) disappear *in toto*, by sequestration into autophagic vacuoles.

Very few fine structure data are available concerning relative numbers of organelles per cell as functions of development. Gruber *et al.* (1973) have shown that chloroplasts, mitochondria and microbodies occur in a ratio of 6:3:1, and occupy much of the narrow parietal band of cytoplasm surrounding the central vacuole in mature photosynthetic mesophyll leaf cells of *Phaseolus vulgaris* (bean). Frederick and Newcomb (1971) reported by fine structure studies that in *Triticum sativum* and *Avena sativa* (both of which have high CO_2 photorespiration) there were 2.65 and 2.40 microbodies per cell section, respectively, and that 89 % and 92 %, respectively, of all sections through mesophyll cells of these two species contained at least one microbody profile. On the other hand, in *Zea mays*, *Sorghum sudanense* and *Chloris gayana* (species with low CO_2 photorespiration) the average number of microbody profiles range from 0.68 - 1.09 in bundle sheath cells and from 0.08 to 0.42 in mesophyll cells; only 44 - 64 % of the sections through bundle sheath cells and 8 - 33 % of the sections through mesophyll cells contained microbody profiles in these species. The number of microbodies per cell relative to the number of mitochondria and chloroplasts is generally higher in species with high CO_2 photorespiration than in those with low CO_2 photorespiration (Frederick and Newcomb, 1971). In the aforementioned work it was also reported that bundle sheath cells and mesophyll leaf cells of *Zea mays* had profiles of 5.9 chloroplasts:7.7 mitochondria:1.09 microbodies, and 4.9 chloroplasts:2.8 mitochondria:0.29 microbodies, respectively.

In the present work it was found that there are

approximately 300 mitochondria to each microbody in dark-grown glucose-supplemented Euglena gracilis strain Z; this ratio is ≤ 25:1 in acetate-supplemented cells. However, it should once again be pointed out that the number of mitochondria per cell section are similar in either acetate or glucose-supplemented cells. There are about 90,000 mitochondria per dark-grown glucose-supplemented Euglena and about 75,000 mitochondria per dark-grown acetate-supplemented cells.

Graves et al. (1971a) noted close spatial associations among microbodies, mitochondria and endoplasmic reticulum in streptomycin-bleached Euglena. Indeed, earlier workers [e.g. Frederick et al. (1968) with root microbodies, Hruban and Rechcigl (1969) with liver peroxisomes, and Vigil (1970) with Castor bean endosperm glyoxysomes] had observed such near proximity of endoplasmic reticulum and microbodies as to suggest that microbodies may originate from endoplasmic reticulum. I have rarely found, however, such close morphological associations either between microbodies and endoplasmic reticulum or microbodies and mitochondria - their juxtaposition has most often been of a random nature. The only time I have seen anything other than a random juxtaposition is during greening.

The whorl-like configurations in Euglena gracilis strain SM-L1 which Graves et al. (1971 a) and Hanzely et al. (1971) interpreted as microbodies, have also been observed in the present work. However, I believe these organelles are not microbodies, but are residual lysosomes. I base this conclusion on the observations that these structures morphologically resemble "residual lysosomes" as decribed by de Duve (1963) and are electron-dense even without DAB cytochemical techniques. Of course, to prove with certainty that they are residual lyso-

somes, one would have to test for acid phosphatase activity (Brandes et al. 1964); I have not made such tests.

When streptomycin-bleached Euglena gracilis (SM-L1) is starved of its carbon source (acetate) many mitochondria become encapsulated in autophagic vacuoles (Brandes et al., 1964). Occasionally, I also observed such autophagic vacuoles (or cytolysomes). Their presence in non-starved organisms, therefore, perhaps demonstrates that autophagy is a part of normal catabolism.

Hanzely et al. (1971) and Graves et al. (1971 a) report that non-DAB treated microbodies of Euglena gracilis var, bacillaris possess electron-dense, granular matrices. In contrast, I have found that non-DAB treated microbodies of Euglena gracilis strain Z are electron-lucent and that the granular matrices are characteristically flocculent in texture. Electron opacity of both limiting membrane and matrix are realized only after treatment in the DAB incubation mixture at pH 9.0 and 37° C; under these conditions the flocculent nature of the granular matrix is even easier to distinguish.

Although Graves et al. (1971 a,b) and Trelease et al. (1971) are the only other workers to have elucidated the fine structure of Euglena microbodies, it is worthy to note that descriptive terms such as "electron-lucent, flocculent matrix" (Gruber et al., 1973), "finely granular matrix" (Frederick et al., 1968), and "matrices of low to moderate electron opacity" (Frederick and Newcomb, 1971) have been applied to non-DAB treated microbodies of higher plants.

Frederick and Newcomb (1969 b) found that incubation at 37° C and pH 9.0 for 50 - 60 minutes were optimal conditions for DAB deposition in microbodies of leaf mesophyll cells; little or no DAB deposition was observed in microbodies incubated at room temperature. I

also have found in the present work that pH 9.0, 37° C for 60 minutes were the most optimal conditions for DAB deposition in microbodies of Euglena.

Although Gerhardt and Berger (1971) were able to detect catalase photometrically in cell-free fractions of two acetate flagellates Polytomella caeca and Chlorogonium elongatum, they were unable to demonstrate DAB reactivity in the microbodies using various combinations of pH (6.0 - 9.5) and temperature (20° C - 37° C). Their lack of success may have been due to DAB incubation for only 30 minutes; under similar conditions I observed less electron density in the microbodies of Euglena. Alternatively, of course, they may have been working with cells partially deprived of carbon dioxide.

I observed that DAB polymerization is still evident in microbodies when hydrogen peroxide is totally omitted from the reaction mixture. Such data suggest that there is an endogenous pool of H_2O_2 within Euglena microbodies. Cooper and Beevers (1969), working with castor bean endosperm, have shown that glyoxysomes (obtained by sucrose density centrifugation) are the organelles in which β-oxidation occurs. They found that for each pair of electrons trapped as NADH, an additional pair was passed to oxygen during β-oxidation. These workers also presented evidence that a direct oxidation occurs within the glyoxysome yielding H_2O_2. Since 1969 when Cooper and Beevers wrote "the details of this reaction (β-oxidation) are not clear, but apparently H_2O_2 is produced and broken down by the very active catalase present in the glyoxysome", no new information has become available. An additional endogenous source of hydrogen peroxide would be made available in microbodies when they begin to function as peroxisomes. However, peroxisomes of higher plants contain glycolic acid oxidase and Euglena peroxisomes possess glycolate dehydrog-

enase in which oxygen does not act as the terminal electron acceptor. Other (as yet unidentified) flavin oxidase may, of course, be present in peroxisomes of Euglena.

As pointed out by deDuve and Baudhuin (1966), the activity of catalase in microbodies is peroxidative in nature. Therefore, it is important to be certain that the observed microbody DAB reaction is due to catalase, and not peroxidase. In the present work, evidence that catalase is the mediator of DAB reactivity in microbodies comes from experiments in which aminotriazole, a specific inhibitor of catalase, was utilized (Heim et al., 1956; Margolish and Novogrodsky, 1958, 1960). Rechcigl and Evans (1963) have shown in guinea pig leucocyte cells that aminotriazole inhibits catalase activity 96 % without affecting peroxidase activity. Unfortunately , no such similar experiments have been made with plant cells, but the precedent for accepting the specific inhibition of catalase by aminotriazole is well established (Frederick and Newcomb, 1969 b; Vigil, 1970).

Although 0.01 M KCN also completely inhibits DAB deposition in the present work, this poison is a general one - inhibiting catalase, peroxidase, cytochrome oxidase, enzymes of the citric acid cycle, etc. (Vigil, 1970).

Further support for the role of catalase comes from the observation that peroxidase-mediated DAB polymerization occurs at a lower pH (7.4) than catalase-mediated DAB polymerization (pH 9.0) [(Graham and Karnovsky(1966); Seligman et al. (1968)]. In the present work, DAB polymerization in microbodies optimally occurred at pH 9.0 and was absent in microbodies if DAB incubation proceeded at pH 7.0 (Table 13).

In the case of leaf microbodies, it has been well

documented that DAB reactivity is catalase-mediated rather than peroxidase-mediated. Tolbert et al. (1968) in enzyme studies with differentially-centrifuged cell-free fractions of leaf tissue, found that almost all catalase activity was located in that particulate fraction which contained single-membrane bound organelles; peroxidase, however, was detected mainly in the soluble fraction, with a small proportion in chloroplast and mitochondrial fractions.

2. Greening Cells

Counts on aerated, acetate-supplemented (or glucose-supplemented) Euglena gracilis strain Z using both light and electron microscopy reveal that the number of microbodies per cell approximately doubles by 24 hours of greening. Other than during periods of germination [Vigil (1970) with castor bean endosperm; Gruber et al. (1973) with Phaseolus vulgaris], drug induction or regeneration [Svoboda et al. (1967) with ethyl chlorophenoxyisobutyrate on rat liver microbodies] there have been no reports of increase in numbers of microbodies, such as these observed in the present work.

Microbodies of Euglena are frequently observed during the 12 - 30 hour greening period to be in a "cluster" , which upon closer scrutinization is found to be composed of a multi-lobed system, in which a single microbody appears to be simultaneously undergoing a series of divisions. Such dividing microbodies are not observed in dark-grown cells, not in cells allowed to green for more than 30 hours, and not in continuously light-grown cells; in these cases, microbodies are solitary in nature.

Although some of the microbodies of 12 - 30 hour

greening Euglena appear to be budding, this type of division is less frequently observed than the binary fission type. (It should be kept in mind that the latter may result from the knife section going through a more central region of a daughter microbody, and the former going through pronouncedly peripheral regions.)

Gruber et al. (1973) in their work with the primary leaves of bean seedlings (Phaseolus vulgaris) reported that when leaves are still within seeds and below ground (up to 3 days after germination), small microbodies $\leq$ 0.2 μm in diameter are present; microbodies in 5 day old leaves (size not specified) are the first to yield DAB-positive catalase reaction. By day 9, when primary leaves are above ground(freed from cotyledons which previously enclosed them), and have developed into deeply green photosynthetic organs, microbodies are larger than 1 μm in diameter.

In the present work, neither fine structure studies nor cytochemical tests revealed the existence of entities with dimensions less than 0.4 μm which could be positively identified as microbodies. Of course, it is possible that smaller microbodies exist in Euglena but they have a fine structure and/or catalase activity which differs from the mature form.

A phenomenon observed only infrequently during the time period in which microbodies are doubling in number (12 - 30 hours of greening), is the intrusion of membrane-bound cytoplasm. The presence of such cytoplasm in microbodies has been documented in greening cotyledons of sunflower (Gruber et al., 1970) and green beans (Gruber et al., 1973). As in the present study, Gruber et al. (1970) noted the following in greening cotyledons: a) microbody cytoplasmic invaginations were time-dependent(in higher plants usually seen at day 4 or grening - never before

day 2 or after day 7) and occurred in tandem with the development of peroxisomal enzymes, b) continuuity exists between the extra-microbody cytoplasm and the microbody-incorporated cytoplasm through a narrow inlet and c) cytoplasmic inclusions contained ribosomes, membranes and even mitochondria (I did not observe incorporated mitochondria). Trelease et al. (1971) noted that cytoplasmic invaginations into microbodies were still seen by day 10 of greening (even though their presence was optimal at day 4 of greening), and suggested that cytoplasmic intrusion is a mechanism by which information gets into microbodies.

Another fine structure observation made during the 12 - 30 hour greening period was the presence of long (0.5 μm), thin (30 - 50 A^{o}) fibrils inside the multi-lobed microbody complex of Euglena. The extreme length of these fibrils, coupled with photometric findings of the possibility of nucleic acids in microbodies, makes these strands suspect of being DNA. The implications of the findings mentioned in this section and considerations whether microbodies in Euglena duplicate by a de novo mechanism or increase in number from existing microbodies will be discussed in Section II E.

3. Light-Grown Cells

The microbodies of Euglena cultured continuously in the light morphologically resemble those of dark-grown or greening cells. As has been noted earlier, the number of microbodies per cell in a continuously light-grown Euglena is similar to cells allowed to green for more than 24 hours. Moreover, binary fission, budding and cytoplasmic invaginations were never observed in the microbodies of continuously light-grown cells.

4. Comparisons With Microbodies of Higher Plants

Neither Trelease et al. (1971) nor Graves et al. (1971a) observed dense, amorphous, crystalline regions of microbodies of Euglena gracilis var. bacillaris (SM-Li). Occasionally, such crystalline inclusions were seen, in the present work, in the microbodies of aerated, acetate-supplemented Euglena gracilis strain Z. However, they were observed only during the 12 - 30 hour greening period.

Dense crystalline inclusions have been frequently reported in microbodies of higher plant cells - e.g., in tobacco leaf mesophyll (Frederick and Newcomb, 1969 b), oat coleoptile (O'Brien and Thimann, 1967) and Ricinus endosperm (Vigil, 1970). The electron-opaque units of coleoptile crystalloids are approximately 60 by 85 A° in diameter, with lattice spacing averaging 130 and 100 A°, respectively (O'Brien and Thimann, 1967; Vigil, 1969). In Ricinus endosperm, microbody crystalloids have a uniform dimension of ≈ 60 A° and a consistant lattice spacing of ≈ 110 A° (Vigil, 1970). These structural differences in size and lattice spacing led Vigil (1970) to suggest that crystalloids in various plant microbodies may contain dissimilar proteins or be complexes of several different proteins. Even though crystalloids of various tissues differ in size and substructure, their intense DAB reactivity demonstrates that they are the principle site of catalase within the microbody (Vigil, 1970). Frederick and Newcomb (1969 b) observed with tobacco mesophyll cells, that the larger the crystal, the less intensely-stained was the amorphous matrix in the remainder of the microbody. These finding were interpreted as suggesting that the inclusions represent progressive crystallization of catalase from the microbody matrix.

Several authors have emphasized that in leaf cells,

microbodies are frequently found appressed to chloroplasts [e.g., in tobacco (Frederick and Newcomb, 1969 b) and cucumber (Trelease et al., 1971)] . Since such cells are parenchymatous in nature and have large central vacuoles, I venture to point out that it is not surprising that all the organelles are in very close spatial proximity within the small volume of peripheral cytoplasm. I, too, have noted a similar organelle relationship in leaf mesophyll cells of Ricinus communis, Spinacea oleracea, Zea mays, Kalanchoë blossfeldiana and Kalanchoë daigremontianum. In the case of Euglena, however, and to a lesser extent in the guard cells of Ricinus communis, Spinacea oleracea, Zea mays, Kalanchoë blossfeldiana and Kalanchoë daigremontianum (which have smaller vacuoles than mesophyll cells), microbodies are much less frequently seen appressed to chloroplasts.

Since microbodies in guard cells have not been previously reported, I have no basis for comparison in the present work. As far as fine structure is concerned, these microbodies are similar to those of both leaf mesophyll cells and Euglena gracilis strain Z.

B. Isolation of Microbodies

In the present work, it was found that the greatest yield of intact microbodies from dark-grown, greening, and continuously light-grown Euglena was achieved by mechanical grinding in sand at 4° C to the point where visual observation (light microscopy) revealed ≈ 25 % cell breakage. Tolbert (1971) isolated intact microbodies from sugar cane, corn and wheat leaf cells by grinding in a Waring Blender, first at several 1- to 2-second low speed bursts, followed by a 10 - 30 second burst at a higher speed. When I used this procedure with Euglena, microbody-containing bands were almost undetectable on either discontinu-

ous or continuous gradients. When Euglena were disrupted by passage through an iced French press (courtesy of Dr. Ruth Sager, Department of Biological Sciences, Hunter College, CUNY) at 600 - 800 lbs/ in pressure - a procedure described by Lord and Merrett (1971) for Euglena gracilis strain Z-I could detect, only faintly, microbody-containing bands on sucrose density gradients. Graves et al. (1971 b) first issued caution about allowing grinding past the point at which more than 33 % of Euglena cells were disrupted, since long periods of grinding resulted in loss of microbody integrity. These workers (Graves et al., 1971 b, 1972) disrupted Euglena gracilis var. bacillaris (SM-L1) by hand grinding with glass beads in a mortar and pestle for 30 seconds in the cold, and then layered the resulting (non-green) cell-free fractions on continuous sucrose gradients.

In the present work, microbody-containing fractions of dark-grown, greening and continuously light-grown Euglena gracilis were located at the interface between the 2.0 and 1.75 M sucrose bands on discontinuous sucrose density gradients; mitochondria-containing bands were located at the interface between the 1.75 and 1.50 M sucrose bands. These positions are identical to those reported by Tolbert (1971) for microbody- and mitochondria-containing fractions on discontinuous sucrose gradients of leaf cells of sugar cane, wheat and corn. It should also be recalled, that in the present work isolated microbodies gave catalase-mediated(AT inhibited) DAB reactivity.

In the present study, (predominently) microbody-containing fractions from continuous sucrose density gradients of dark-grown, acetate-supplemented Euglena gracilis strain Z were found to band at buoyant densities of 1.18 - 1.22 g/cm^3; (predominently) mitochondria-containing bands were found at densities of 1.16 - 1.18 g/cm^3. These re-

sults are in agreement with those of Graves et al. who reported, that in the case of SM-L1 Euglena, that glyoxysomal enzymes banded together on continuous sucrose gradients at a higher equilibrium density, i.e., 1.20 to 1.22 g/cm^3 (1971 b and 1972) than mitochondrial marker enzymes, i.e., 1.17 g/cm^3 (1972).

Preliminary experiments indicated a great deal of chloroplast contamination in microbody- and mitochondria-containing bands of greening and continuously light-grown Euglena gracilis strain Z when isolation was performed on continuous sucrose gradients; this contamination was minimal on discontinuous sucrose gradients. Since NADPH-glycolate reductase would interfer with enzymatic detection of hydroxypyruvate reductase (Tolbert, 1970), I mainly utilized discontinuous sucrose gradients. In general, continuous gradients were made only on dark-grown cells and for purposes of comparison to values reported on continuous sucrose density gradients of the streptomycin-bleached, non-photosynthetic strain SM-L1 of Euglena gracilis var. bacillaris (Trelease et al., 1971; Graves et al., 1971 b, 1972).

I found, in the case of organelles isolated from Euglena gracilis strain Z isolated on discontinuous sucrose density gradiwnts , values which compared well with those I measured for continuous sucrose density gradients and those reported by Graves et al. (1972) for continuous sucrose density gradients of SM-L1.

C. Enzyme Studies

Since two types of preparations for enzyme studies are utilized in the present work (i.e., cell-free fractions and isolated microbodies) and because measured enzyme activities are expressed on the

bases of "per mg protein" and "per cell" , four categories of data presentation are available for consideration. Before discussing the results of my enzyme studies, I feel that the following comments are in order concerning the relative merits of each of the four categories.

Obviously, the method of preparation utilized in obtaining cell-free fractions (i.e., total cellular disruption) has the merit of excluding errors pertaining to degree of cell breakage; the method used in isolating microbodies involves only partial disruption of cell populations, and therefore embodies these errors. I am aware of the reports that prolonged sonication destroys protein (including enzymes) - see for example Hogeboom (1955) - but fortunately the length of time required to sonicate Euglena to the point of complete cell breakage is ≲ 20 seconds. I have found that enzyme activity, when expressed on a per cell basis, is similar whether all cells are broken or whether only 25 % of the cells are broken.

The problem inherent in placing too much emphasis on cell-free fraction data (expressed on the basis of per cell or per mg protein) is that a multitude of non-microbody related proteins are present in such preparations. In addition to this situation, which exists even in regard to dark-grown Euglena, there occurs upon greening, numerous changes in structural and functional proteins related to the metabolic switch-over from heterotrophic to photoautotrophic growth. For one example, in the case of chloroplast development, there is involved the synthesis of : a) functional protein of photosynthesis [e.g., ribulose-1,5-diphosphate carboxylase and sedoheptulose-1,7-diphosphatase (Smillie, 1968)] , b) diverse electron-transfer carriers [e.g., ferredoxin (San Pietro and Lang, 1958) and cytochrome c_{552} (Perini et al., 1964 a,b)], c) structural components

of chloroplasts[e.g., protein-phospholipid complexes, etc.]. Among other

of chloroplasts[e.g., protein-phospholipid complexes, etc.]. Among othern analysis (Ouchterlony), that unique chloroplast-associated antigens are formed during chloroplast development in Euglena gracilis var. bacillaris. Also, with greening, changes occur in cytoplasmic enzymes of Euglena, e.g., the specific activity of acetate thiokinase in autotrophic cells is ≤ one-fifth that of dark-grown cells (Ohmann, 1964) and levels of paramylon synthase are lower in light-grown cells than dark-grown cells (Marechal and Goldemberg, 1964).

The above changes by no means constitute an exhaustive list [for a review of enzymatic changes which accompany greening in Euglena see Smillie (1968)], and probably accounts, only in part, for the observation made by Smillie (1968) that, by 24 hours of greening, a doubling in amount of cellular protein occurs in Euglena gracilis var. bacillaris grown on resting medium [33]. Moreover, it has been pointed out (Brawerman, 1968) that the chloroplasts comprise about one-half of the total protein content of green Euglena cells. A similar doubling in cellular protein was observed in the present work to occur by 24 hours of greening in strain Z.

Since cell-free fractions contain such a wealth of (varying) non-microbody protein, I feel that data obtained with isolated microbodies are more reliable indicators of microbody enzyme activity (much of the extra-microbody protein having been removed in the microbody

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[33] Resting medium as originally devised by Stern et al. (1964) contains 0.054 M mannitol, 0.01 M $MgCl_2$ and 0.01 M KH_2PO_4. In this medium, mannitol is the sole carbon and energy source but is not utilized for growth by Euglena; cells while in the resting medium do not divide and retain their viability for more than a month (Stern et al., 1964).

isolation process). This is not to imply that there are no sources of error in enzyme determinations utilizing microbodies isolated from sucrose gradients. Since it is impossible to break open, by grinding in sand, exactly the same number of cells in each experiment - even when light microscopic counts are made - some error is introduced when data are reported on a per cell basis.

Of the four categories of data expression the one remaining is enzyme activity in isolated microbodies related to a per mg protein basis. In this category most extra-microbody protein has been removed and activity is not expressed as a function of cell number. Therefore, of the four categories of data presentation available for consideration, it is this fourth category in which I have greatest confidence.

1. Catalase

Quite early in my research efforts I was able to identify microbodies in dark-grown, greening and continuously light-grown Euglena gracilis strain Z, both in fine structure studies, and by virtue of positive catalase reactivity - utilizing DAB/H_2O_2 cytochemically in whole cells or utilizing the photometric method of Lück (1963) in cell-free fractions. It was therefore, with great surprise that I read the papers of Hanzely et al. (1971) and Graves et al. (1971 a). Despite the fact that these workers demonstrated the presence of microbodies, by fine structure studies, in a streptomycin-bleached strain of Euglena gracilis var. bacillaris (SM-L1) grown on media supplemented with glucose, acetate or ethanol, they were unable to detect the presence of catalase in ethanol-supplemented cells, either by DAB cytochemical procedures or by Lück's (1963) photometric assay. In fact, it was even

suggested by Graves et al. (1971 a) that "microbodies can exist normally without the presence of catalase..... and an organelle should be considered to be a microbody if it satisfies the morphological criteria established for microbodies."

Graves et al. (1971 a) and Hanzely et al. (1971) had made their fine structure observations on a non-photosynthetic, streptomycin-bleached strain of variety bacillaris (grown on media supplemented with ethanol, glucose or acetate), and their catalase-negative experiments on this same organism grown on media supplemented with ethanol. At first, I attributed their negative results and my contrasting positive results (in regard to catalase) to their having worked with a streptomycin-bleached strain. Levedahl (1968) had earlier speculated that several differences in cellular metabolism probably result from permanent bleaching of Euglena with streptomycin; following his lead, I assumed that one or more of the enzymes involved in catalase synthesis (or catalase activity,itself) were inhibited or repressed by streptomycin (Brody and White, 1972).

Lord and Merrett (1971) were unable to detect catalase photometrically in Euglena gracilis strain Z (the same strain that I used) when this organism was grown either photoautotrophically in air on inorganic medium, or heterotrophically in the dark on the same medium supplemented with glucose. Since the gas phase, under conditions of heterotrophic growth,was not specified in the work of Lord and Merrett (1971), and no mention of gas phase was made in the papers of Hanzely et al. (1971) or Graves et al. (1971 a), a hypothesis was tentatively assumed that CO_2 in the gas phase was necessary for detectable levels of catalase (in non-photoautotrophic Euglena). When dark-grown, greening, or continuously light-grown Euglena gracilis strain Z were aerated with air which had its

carbon dioxide removed by passage through excess Ascarite, catalase could not be detected either photometrically [34] or cytochemically. Furthermore, addition of CO_2 to the gas phase of carbon dioxide-deprived cells resulted in restoration of catalase activity. Therefore, it appears that carbon dioxide regulates the activity (or possibly, the presence) of catalase in Euglena gracilis strain Z.

These results (while in press, Brody and White, 1972) were communicated to Graves, who subsequently reported (personal communication) being able to detect by photometric methods "low levels" of catalase in cell-free fractions of Euglena gracilis var. bacillaris (SM-L1) when such cells were bubbled with air containing 1.0 % CO_2.

In regard to the work of Lord and Merrett (1971), it should be recalled, that I had observed (as had Graves et al., 1971a) that very few microbodies are present in Euglena grown in glucose-supplemented media. Therefore, the paucity of microbodies in such cells may be what led to Lord and Merrett's (1971) report of photometrically- undetectable levels of catalase in glucose-supplemented cells. Alternatively, since gas phase was not mentioned in their work, perhaps Lord and Merrett (1971) grew cells on CO_2-deprived air.

Gerhardt and Berger (1971) also looked for catalase in microbodies (gas phase in experiments unspecified) by cytochemical (DAB) and photometric procedures (Lück, 1963) in Polytomella caeca and Chlorogonium elongatum (two acetate flagellates). They could not detect

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[34] At the time of writing this dissertation Dr. M. Müller, The Rockefeller University, New York, was also unable to detect catalase in cell-free fractions of carbon dioxide - deprived, continuously light-grown Euglena by the method of Baudhuin et al. (1965).

catalase cytochemically; however, they were able to detect it photometrically in fractions from linear sucrose gradients which contained structures morphologically similar to microbodies. It is possible that their conditions of DAB incubation (pH and temperature) were not optimal for detection of catalase, or perhaps their 30 minute incubation period was too brief to obtain a catalase-mediated DAB reaction. Recall that, in the present work, and that of Frederick and Newcomb (1969 b), it was found that 60 minutes of incubation at pH 9.0, 37 ^{0}C was optimal for detection of catalase-mediated DAB activity.

In the present work, catalase activity in cell-free fractions increased 2-fold by 72 hours of greening on either the basis of specific activity or per cell. Catalase activity in isolated microbodies also increased with greening. In this case, the increase by 72 hours of greening was 1.4-fold, whether presented on the basis of specific activity or per cell.[35] The possibility of extra-microbody catalase has already been mentioned on pages 133 and 136.

Graves et al. (1972) were unable to detect catalase

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[35] The discrepancy between the 2-fold increase in catalase levels as determined with cell-free fractions and the 1.4-fold increase as determined with isolated microbodies may have its origin in difficulties inherent in accurately measuring low levels of catalase in dark-grown cells. While the final draft of this thesis was being written, Dr. M. Müller, The Rockerfeller University, New York, kindly agreed to determine catalase levels in cell-free fractions of Euglena. While his measurements of catalase levels in cell-free fractions of aerated, acetate-supplemented, continuously light-grown cells were similar to mine, his catalase levels in dark-grown cells were so little above background that determinations could not be made with certainty.

photometrically in sucrose fractions (of presumably microbody-containing bands) from continuous sucrose density gradients of Euglena gracilis var. bacillaris. In regard to other microorganisms, Müller et al. (1969) reported catalase levels of 33 Baudhuin Units/ mg protein or 3.06 Lück units/ mg protein (my calculations based on conversions given in Table 4) for cell-free homogenates of Tetrahymena pyriformis (a colorless ciliate) grown in acetate-supplemented media. Lui et al. (1968) reported 1.7 Baudhuin Units per mg protein or 0.16 Lück units per mg protein(my calculations based on conversions given in Table 4) for cellular homogenates of Ochromonas malhamensis (green flagellate). My results for catalase activity in cell-free fractions of dark-grown, greening or light-grown Euglena gracilis strain Z are intermediate between those reported in the two aforementioned papers.

The activity of catalase as a function of greening has not been reported for other microorganisms. Therefore, comparisons of catalase activity as a function of greening will have to be made with higher plants. Different patterns of catalase activity have been reported for greening cotyledons of cucumber(Trelease et al., 1971), wheat (Feierabend and Beevers, 1972 a), sunflower (Schnarrenberger et al., 1971) and bean (Gruber et al., 1973). Most likely, these different patterns correlate with the onset and/or decline of glyoxysomal and peroxisomal activities. For purposes of comparison, patterns of catalase activity for Euglena (as determined in the present work) are compared with those of greening higher plants (Fig. 47).

Trelease et al. (1971) reported maximum levels of catalase in both homogenates (18 units/mg protein) and particulate fractions) (76 units/mg protein; Fig. 47B) of cucumber cotyledons (in which

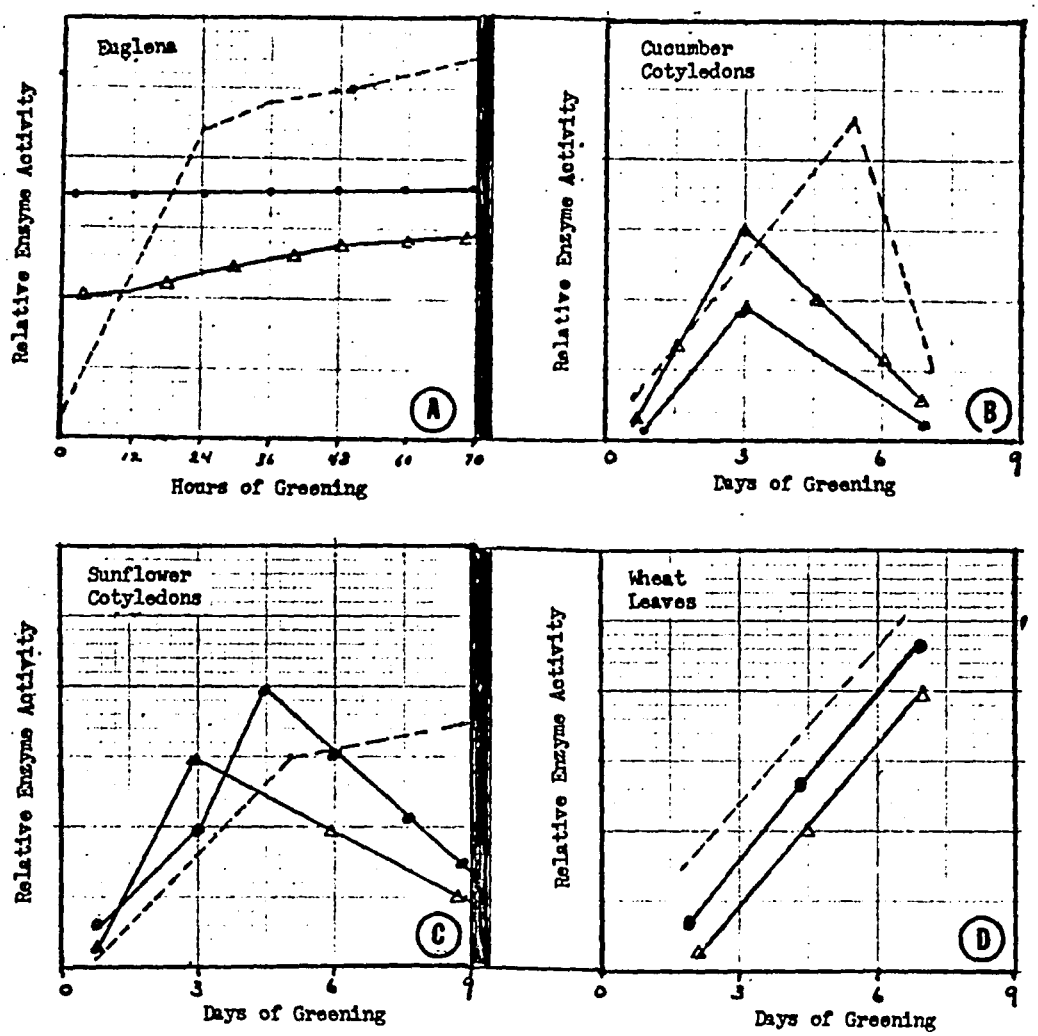

Figure 47. Comparisons of glyoxysomal and peroxisomal enzyme activity as a function of greening in aerated, acetate-supplemented Euglena gracilis strain Z and several higher plants. Specific enzyme activities are presented on a relative basis. Catalase (▲——▲); glyoxysomal enzymes (●——●); peroxisomal enzymes (-------).
A, microbody-containing sucrose gradients of Euglena gracilis strain Z (White, present thesis). B, particulate fractions of cucumber cotyledons (Trelease et al., 1971). C, microbody-containing sucrose gradients of sunflower cotyledons (Gruber et al., 1973). D, cell-free fractions of wheat leaves (Feirabend and Beevers, 1971).

lipids are the major storage form of food to be used for growth) by day 3 of greening; levels were reduced to one-sixth by day 7 of greening, probably as a result of stored lipids having undergone almost complete catabolism. Feirabend and Beevers (1971) were able to first detect trace amounts of catalase in cell-free fractions of wheat leaves (in which lipids are not the major storage form of food) by day 2 of greening; during the next 5 days of greening, a rapid and constant rate of increase occurred - to 35.6 units/mg protein (Fig. 47 D).

Schnarrenberger et al. (1971) noted that catalase had a peak activity in microbody-containing sucrose gradients of sunflower cotyledons (which store lipids) by day 3 of germination; levels declined by day 9 to only 28 % of its maximal activity. Gruber et al. (1970) detected low levels of catalase activity in cell-free fractions of sunflower cotyledons at day 1 of germination. In this study, the greatest activity of catalase per cotyledon occurred at day 4 of germination; by day 7, catalase activity declined to one-third that of day 4 (Fig. 47 C). In a later study, Gruber et al. (1973) observed that levels of catalase increased 6-fold (6.2 units/ mg protein) in homogenates of bean (Phaseolus vulgaris) leaves by day 9 of greening; activities decreased 33 % by day 11.

In summary, organs of higher plants which utilize stored lipids display initially an increase in catalase activity paralleling the increase in glyoxsomal enzyme activity; catalase activity then diminishes in concert with diminishing glyoxysomal enzyme activity even though peroxisomal enzyme activity increases (Fig. 47 B,C). Although one might anticipate an increase in catalase activity to be associated with increased peroxisomal enzyme activity, such an increase in catalase activ-

ity is not detectable in higher plants. Perhaps there is an increase in peroxisome-associated catalase activity,but to a degree insufficient to manifest itself at a time when glyoxysome-associated catalase activity is dropping at such a rapid rate. In organs of higher plants which do not store lipid, e.g., wheat leaves, catalase activity follows (probably mainly)glyoxysomal activity and peroxisomal activity and does not diminish during the greening period.(Fig. 47 D).

Perhaps it is really not appropriate to compare patterns of catalase activity in greening higher plants with catalase activity in greening Euglena - especially under culture conditions specified in the present work. Recall that Euglena were grown in acetate-supplemented media, and then allowed to green in this medium with the result that specific activities of glyoxysomal marker enzymes remained fairly constant with greening - this situation has no parallel in higher plants. To make the situation in Euglena comparable to that of higher plant organs in which lipid is the major storage form of food, Euglena would first have to be grown in the presence of acetate and then allowed to green in its absence. It is, however, just because of the fact that acetate is present during the greening of Euglena, that specific activities of glyoxysomal enzymes remain constant. Thus, in the case of Euglena, peroxisomal-associated catalase activity can be clearly noted (see Fig. 47 A); such activity is not clearly noted in higher plants.

2. Glyoxysomal Marker Enzymes

Specific activities of the glyoxysomal marker enzymes - isocitrate lyase and malate synthase - are fairly constant in aerated, acetate-supplemented, dark-grown, greening and continuously light-grown

Euglena gracilis strain Z, as judged from data obtained both from cell-free fractions and microbody-containing sucrose fractions from discontinuous sucrose density gradients.

I cannot compare my cell-free fraction data on isocitrate lyase and malate synthase expressed on a per cell basis with that of other workers, since no other workers have expressed their data in this form. However, several groups have reported specific activities for these two enzymes in cell-free fractions. Reeves et al. (1962) documented the presence of isocitrate lyase (1.72 mμ moles/min/mg protein) and malate synthase (24.0 mμ moles/min/mg protein) in cell-free fractions of light-grown acetate-supplemented Euglena gracilis strain Z. Haigh and Beevers (1964) reported specific activities of 1.7 and 2.6 for isocitrate lyase in cell-free fractions of acetate-supplemented dark-grown and continuously light-grown Euglena gracilis strain Z, respectively; they gave no data for malate synthase. In the present work, levels of isocitrate lyase in cell-free fractions (1.65 - 1.92 mμ moles/min/mg protein) agree with those reported by the two aforementioned workers, but differs considerably from that of Reeves et al. (1962) in regard to malate synthase . I found levels of malate synthase in cell-free fractions to be 3.21 - 3.70 mμ moles/min/mg protein; Reeves et al. (1962) reported levels of 24.0 mμ moles/min/mg protein. Perhaps these differences may be partially explained [as they were by Hogg (1962), working with cell-free fractions of Tetrahymena pyriformis], by the finding that cellular levels of enzymes are functions of variables such as medium, growth conditions, and growth

phase.[36] Reeves et al. (1962) used a medium enriched not only with sodium acetate, but with beef extract, tryptone and yeast extract; I used a medium in which acetate was the sole carbon and energy source. Moreoever, Reeves et al. (1962) grew Euglena gracilis strain Z at light intensities of 200 ft. c.; in the present work, cells were grown at 110 ft. c.. I believe that my specific activity data for malate synthase in cell-free fractions of Euglena gracilis strain Z are reasonable, based on my findings of specific activities of 93.3 - 98.7 for this same enzyme in microbody-containing bands of discontinuous sucrose gradients and because the latter microbody data are in good agreement with those reported by Graves et al. (1972) for microbody-containing bands from continuous sucrose density gradients of Euglena gracilis strain SM-L1.

Again, in regard to data on microbodies isolated on sucrose density gradients, I can make no comparisons for levels of malate synthase and isocitrate lyase when data are expressed on a per cell basis, because other workers have not reported their data in this fashion. Data , however, expressed in terms of "specific activity" are available in the literature.

Graves et al. (1972) noted the presence of isocitrate lyase (31 nmoles/min/mg protein) and malate synthase (95 nmoles/min/mg protein) in microbody fractions from continuous sucrose density gradients of streptomycin-bleached, ethanol-supplemented Euglena gracilis var. bacillaris (SM-L1). Although I worked with strain Z, my glyoxysomal en-

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[36] For example, Hogg (1969) reported specific activities of 0.9 and 4.7 for malate synthase in cell-free fractions of log phase and stationary phase Tetrahymena pyriformis, respectively, grown in a medium containing proteose-peptone, glucose and acetate. Cell-free fractions of log phase Tetrahymena pyriformis were also found to contain malate synthase activities of 3.5, 0, 3.0, and 5.0, in cells grown in proteose-peptone medium, proteose peptone + glucose medium, proteose peptone + sodium acetate medium and synthetic medium supplemented with sodium acetate, respectively.

zyme data for isocitrate lyase (25.5 - 32.9 mμ moles/min/mg protein) and malate synthase (93.3 - 99.2 mμ moles/min/mg protein) from discontinuous sucrose gradient microbody fractions agrees well with that reported by Graves et al. (1972).

While I would like to make comparisons of my data on the specific activities of isocitrate lyase and malate synthase as a function of greening in Euglena with that of other workers, such comparisons are not possible since the only data of isolated Euglena microbodies are from a permanently-bleached strain of this organism. Data on these enzymes is, however, available from the literature in the cases of greening cotyledons of higher plants. Gruber et al. (1970) observed optimal levels of isocitrate lyase (120 nmoles/min/mg protein) at day 4 of greening in 10,800 g particulate pellets of sunflower cotyledons; by day 7 of greening, this activity declined to 40 nmoles/min/mg protein.

Schnarrenberger et al. (1971) also noted maximal specific activities of both isocitrate lyase (820 nmoles/min/mg protein) and malate synthase (1560 nmoles/min/mg protein) by the fourth day of greening in microbody-containing bands from discontinuous sucrose gradients of sunflower cotyledons. These values decreased after the fourth day of greening; by day 11, their activities had decreased by 89 % for isocitrate lyase and 78 % for malate synthase.

Trelease et al. (1971) found maximal levels for isocitrate lyase by day 3-4 of greening in both cell-free fractions(40 nmoles/min/mg protein) and microbody-containing fractions from continuous sucrose gradients (165 nmoles/min/mg protein) of sunflower cotyledons. Similar maximal specific activities were noted by day 3-4 of greening for malate synthase in cell-free fractions (60 nmoles/min/mg protein) and

microbody-containing fractions from continuous sucrose gradients (230 nmoles/mn/mg protein) of cucumber cotyledons. Furthermore, Trelease et al. (1971) noted that after day 4 of greening, levels of isocitrate lyase and malate synthase decreased in both cell-free fractions and microbody-containing fractions from continuous sucrose density gradients; beyond day 7 these enzymes were undetectable.

Feierabend and Beevers (1972 a) have shown that in wheat leaves glyoxysomal marker enzymes increase in parallel with peroxisomal -marker enzymes (and catalase) up to day 6 of germination (when the experiment was terminated.)

Recall that both cucumber and sunflower cotyledons are organs in which the main storage form of food is lipid; in wheat leaves this is not the case - glyoxysomal enzymes continue to increase in the 6 days of greening. In regard to the pattern of glyoxysome activity as a function of greening, Euglena differs from all of these higher plants; catalase activity mainly follows glyoxysomal enzyme activity with greening.

3. Peroxisomal Marker Enzymes

A 6-fold increase was observed in the activities of the peroxisomal marker enzymes (hydroxypyruvate reductase and glycolate dehydrogenase) in cell-free fractions of aerated, acetate-supplemented Euglena gracilis strain Z by 72 hours of greening, as compared to dark-grown cells, when data are expressed on the basis of specific activity (per mg protein); a 12-fold increase was noted when data are expressed on a per cell basis. Since no other workers have expressed their peroxisomal marker enzyme data on a per cell basis, it is not possible to make

comparisons with this type of data on Euglena gracilis strain Z.

Codd and Merrett (1970) noted a 10-fold increase in the specific activity of glycollate:DCPIP oxidoreductase (glycolate dehydrogenase) in cell-free fractions of acetate-supplemented Euglena gracilis strain Z by 72 hours of greening. However, these workers grew cells under conditions of 1.0 x 10^3 erg/cm^2/sec illumination and in the present study, cells were grown under 1.9 x 10^3 erg/cm^2/sec of illumination. I wish to suggest [in accordance with Hogg (1962) [36]] that levels of glycolate dehydrogenase in Euglena are functions of growth conditions (e.g., light intensity).

Cell-free fraction data are available in the literature pertaining to activities of peroxisomal marker enzymes in higher plants as functions of greening. Schnarrenberger et al. (1971) noted in cell-free fractions of sunflower cotyledons that activities of hydroxypyruvate reductase and glycolate oxidase increased during the first four days of germination, and thereafter remained relatively constant (both enzymes had activities of ≈ 100 nmoles/min/mg protein /pair of cotyledons after day 4). Trelease et al. (1971) observed a sharp increase in specific activities of hydroxypyruvate reductase and glycolate oxidase in cell-free fractions of cucmber cotyledons between days 2 and 5 of greening; after day 5, relatively constant levels of these two enzymes were noted (i.e., 4-5 nmoles/min/mg protein).

With sucrose-isolated microbody fractions in the present work it was noted that by 72 hours of greening (by which time enzyme activities reach the levels of continuously light-grown cells), a) the specific activities of both glycolate dehydrogenase and hydroxypyruvate reductase increase ≈ 6-fold while b) the activities of both

these enzymes increase ≈ 12-fold on a per cell basis. Again, it is not possible to make comparisons of my data expressed on a per cell basis with that of other workers.

Lord and Merrett (1971) showed by discontinuous sucrose gradients of photoautotrophically-grown Euglena gracilis strain Z that glycollate:DCPIP oxidoreductase (glycolate dehydrogenase) is a particulate enzyme. Graves et al. (1972) prepared continuous sucrose gradients of a streptomycin-bleached,non-photosynthetic strain of Euglena gracilis var. bacillaris SM-L1), grown on ethanol-supplemented media and reported that, co-banding with (presumably) microbody particles that showed pronounced glyoxysomal type enzyme activity,were low levels of peroxisomal-type enzymes - glycolate dehydrogenase and hydroxypyruvate reductase (they used glycolate as substrate and therefore refer to this latter enzyme as glycolate reductase). Graves et al.(1972) report that the activities of these two peroxisomal enzymes increase upon exposure to light; in both a streptomycin-bleached and normal variety bacillaris (despite the fact that the former organism does not green upon illumination).

In higher plants, the oxidation of glycolate to glyoxylate - that follows photosynthetic carbon dioxide fixation - is mediated by glycolate oxidase (EC 1.1.3.1) which uses oxygen as the terminal acceptor (Nelson and Tolbert, 1970). Therefore, glycolate oxidase is a peroxisomal marker enzyme in higher plants. In green algae (including Euglena) the enzyme which oxidizes glycolate to glyoxylate is glycolate dehydrogenase (Enzyme Commission number not yet assigned) - oxygen is not involved as the terminal acceptor (Nelson and Tolbert, 1970).

Schnarrenberger et al. (1971) noted low specific

activities of hydroxypyruvate reductase and glycolate oxidase after one day of greening in microbody-containing fractions from discontinuous sucrose gradients of sunflower cotyledons. These activities increased rapidly by day 4 of greening (539 nmoles/min/mg protein for hydroxypyruvate reductase and 327 nmoles/min/mg protein for glycolate oxidase), and increased slightly more by day 9 of greening (when the experiment was terminated).

Trelease et al. (1971) found low levels of peroxisomal marker enzymes by day 1 of greening in microbody-containing fractions from continuous sucrose density gradients of cucmber cotyledons; maximum levels were noted by day 5 of greening ($\simeq$ 11 nmoles/min/mg protein for both glycolate oxidase and hydroxypyruvate reductase); by day 7 (when the experiment was terminated) a 60 % decrease was observed in the activities of these enzymes (Fig. 47 B).

Gruber et al. (1970) reported low levels of glycolate oxidase and glyoxylate reductase (hydroxypyruvate reductase) by the third day of greening in 10,8000 g particulate pellets of sunflower(Fig. 47 C), cucmber and tomato cotyledons; levels of both these enzymes increased $\simeq$ 9-fold by day 11 of greening (when the experiment was terminated).

Thus, peroxisomal enzyme activity is increasing while glyoxysomal activity and catalase are decreasing in organs of germinating higher plants that contain lipids as their main storage form of food (e.g., cucumber and sunflower cotyledons; Fig. 47 B,C). In contrast, in organs of plants that do not contain lipids as their main storage forms of food (e.g., wheat leaves; Fig. 47 D), peroxisomal enzymes, glyoxysomal enzymes and catalase enzymes increase in parallel with germination. In their pat-

tern of increase in activity, the peroxisomal marker enzymes in greening Euglena gracilis strain Z resemble those of wheat leaves more than cucumber or sunflower cotyledons; in the relationship between the patterns of peroxisomal and glyoxysomal activities as a function of greening, Euglena gracilis resembles none of the higher plants.

D. Nucleic Acid Determinations On Microbody-Containing Fractions From Discontinuous Sucrose Density Gradients.

Cesium chloride density gradients of DNA from continuously light-grown Euglena gracilis strain Z yield a major nuclear band at a density of 1.708 gm/cm^3 with a G + C content of 49 moles % ; and minor chloroplast DNA bands occur at densities of 1.684 gm/cm^3 with a G + C content of 25 moles % ; and minor mitochondrial DNA bands are found at a buoyant density of 1.692 gm/cm^3 with a G + C content of 33 moles % (Brawerman, 1968). Mitochondrial DNA may appear as 20 - 50 A^o thick filaments which are several mμ long, or clumped as rod-like structures with a thickness of up to 250 A^o (Brown and Bertke, 1969). Moreover, it has been observed that mitochondrial DNA from Didymium nigripes (a slime mold) cells in a rapid state of proliferation contain thicker fibers than those mitochondria of cells with lower metabolic rates (Schuster, 1965).

Euglena cells vary in their RNA content dependent upon many variables including phase of growth cycle and illumination. For example, when grown on a complex medium, dark-grown Euglena gracilis strain Z have been reported to contain 20 micrograms of RNA per 10^6 cells (Brawerman et al., 1962). Much of the extra RNA in light-grown cells has been shown to be associated with chloroplasts; the remainder being micro-

somal (Brawerman, 1968). The failure to isolate intact nuclei from Euglena gracilis has prevented studies of nuclear RNA (Brawerman, 1968).

The fact that I have detected both DNA (by the method of Burton, 1956) and RNA (by the method of Lin and Schjeide, 1969) in microbody-containing fractions from discontinuous sucrose density gradients, is by itself not conclusive evidence for the existance of nucleic acids in microbodies. Contamination of DNA and RNA from other organelles (nuclei, mitochondria amd chloroplasts), is of course, very possible. Even the fine structure observations of long ($\simeq 0.5$ µm), thin (30 - 50 A^{o}) fibrils in microbodies of Euglena in situ are only suggestive of the presence of nuclei acids. Obviously, much more work (including experiments utilizing cesium chloride density gradients) would have to be performed before the presence or absence of nucleic acids in Euglena microbodies - indeed any microbody - would be proven conclusively.

Ching (1970) reported similarly the presence of DNA (by the method of Burton, 1956) and RNA (by the method of Lin and Schjeide, 1969) in microbody-containing bands of discontinuous sucrose density gradients of germinating ponderosa pine (Pinus ponderosa Laws) seeds; washed microbody preparations were found to contain 16 ± 0.9 µg of RNA and 1.36 ± 0.23 µg DNA/mg organelle protein. In good agreement with this data, I found microbody homogenates of dark-grown, 12 hr-, 24 hr-, 48 hr-, 72 hr- greening, and continuously light-grown aerated, acetate-supplemented Euglena contain 1.0 ± 0.2, 1.2 ± 0.2, 1.3 ± 0.3, 1.3 ± 0.4, 1.3 ± 0.3, 1.3 ± 0.4 µg of DNA/mg protein, respectively. In the case of RNA in the present work, microbody homogenates of dark-grown, 12 hr-, 24 hr-, 48 hr-, 72 hr-greening, and continuously light-grown aerated, acetate-supplemented Euglena were found to contain 14.8 ± 0.9, 15.7 ± 0.9,

14.9 ± 1.0, 14.7 ± 1.0, 15.7 ± 1.1 and 15.3 ± 1.0 μg RNA/mg protein, respectively. The authenticity of DNA and RNA in the present work was verified by experiments utilizing DNase and RNase. Gerhardt and Beevers (1969) have reported similar magnitudes of RNA in microbody fractions from castor bean endosperm.

To demonstrate conclusively the presence of nucleic acids in microbodies, many experiments would have to be performed including the following: (i) electron microscopic examination of DNA in ultrathin sections of microbodies *in situ* and treatment with DNase, (ii) chemical analysis of DNA extracted from microbodies by hydrolysis procedures or isolation on the basis of specific buoyant density in cesium chloride gradients, (iii) isolation of microbody DNA in cesium chloride-ethydium bromide gradients, which separate DNA molceules on the basis of their molecular topology. I should like to continue work on fine structure observations of possible microbody DNA [i.e., (i) above].

E. Biogenesis, Development and Reproduction of Microbodies

At the present time, the nature of microbody biogenesis is uncertain. Many workers believe that microbodies originate from dilated regions of the endoplasmic reticulum [Frederick *et al.* (1968) working with cells of several higher plants, e.g., tobacco leaf mesophyll; Hruban and Rechcigl (1969) working with rat liver cells; Vigil (1970) working with castor bean endosperm]. Published electron micrographs usually fail to demonstrate a *direct* continuity between the limiting membrane of nascent microbodies and the membranes of endoplasmic cisternae (Gruber *et al.*, 1973). Such a direct continuity is a prerequisite in proving that microbodies arise by vesicle formation from the endoplasmic

reticulum. Jensen and Valdovinos (1967) , however, have demonstrated continuity of microbodies with endoplasmic reticulum in pedicel tissue of Lycopersicon esculentum. In the present study with dark-grown, greening, and continuously light-grown Euglena gracilis strain Z , at no time did I observe direct continuity between microbody membranes and the endoplasmic reticulum.

With the available data, I am unable to determine the origin of microbodies in dark-grown Euglena. However, I am able to comment on the doubling in number of microbodies which occurs between 12 - 30 hours of greening. It should be pointed out that this doubling has no exact parallel with any of the situations reported in the literature. For example, in the case of extra-cotyledonary endosperm of the castor bean there is a slow increase in number of microbodies (glyoxysomes) which reaches a maximum at day 4 of germination - when stores of lipids are being depleted. After day 4, microbodies slowly decrease in number, reportedly by autophagy (Vigil, 1970).

In the case of plants containing lipid-rich endosperm within the cotyledons, and in which the cotyledons become green and function photosynthetically, e.g., cucumber, glyoxysomally-active microbodies first appear; these are gradually replaced by peroxisomal-type microbodies. However, even this latter case has no parallel with the situation which occurs in Euglena (under growth conditions utilized in the present work) where glyoxysomal activity remains rather constant, even as peroxisomal activity increases. This may be the reason why a rapid increase (doubling) in number of microbodies is observed only in Euglena, in which there is no accompanying decrease in glyoxysomal-type microbodies. This rapid increase in number of microbodies in Euglena,

stimulated by a "normal" environmental trigger (light), may have its parallel (?) only in certain "abnormal" situations. Two such abnormal situations have been reported in the literature. The first is the rapid increase, by budding, in the number of microbodies in kidney cells of rats treated with ethyl chlorophenoxyisobutyrate (Svoboda et al., 1967). The second is the budding or fragmentation of microbodies of liver cells of clofibrate-treated rats (Legg and Wood, 1970).

The question then arises as to how Euglena microbodies double by 24 hours of greening. Does this new population arise de novo, e.g., from endoplasmic reticulum, or from division of pre-existing microbodies? Germane to this question is the work of Gruber et al.(1973) who reported that when primary bean leaves are within seeds, and below ground (up to 3 days after germination), structures morphologically resembling microbodies [but catalase (DAB/H_2O_2) negative] are found which are $\approx$ 0.2 μm in diameter; by day 9, when these leaves are above ground, and have developed into green photosynthetic organs, catalase-positive microbodies are found which are larger than 1 μm in diameter. Gruber et al. (1973) assumed that these large microbodies arose, by budding , from smooth endoplasmic reticulum - existing at the time of their release as small electron-lucent vesicles. Feierabend and Beevers (1972), on the basis of fine structure studies, believe that peroxisomes of greening leaves develop from "precursor particles of lower buoyant density".

In the present study, catalase (i.e., DAB/H_2O_2) positive microbodies of aerated, acetate-supplemented dark-grown, greening and continuously light-grown Euglena gracilis strain Z were found to have a mean measured long dimension of $\approx$ 0.80 μm and a mean measured

short dimension of ≃ 0.65 μm. I, too, observed a few "microbody-appearing" organelles having measured short dimensions less than 0.4 μm, but again these did not give DAB-positive reactivity. Whether such structures [and the catalase-negative ones observed by Gruber et al. (1973)] are indeed microbodies is open to conjecture. It may be, after all, that microbodies below a certain size do not possess active catalase. However, I have no real evidence that the doubling in number of microbodies in Euglena has a de novo origin. In spite of the fact that I have carefully scrutinized many hundreds of sections of cells from 12 - 30 -hour greening cultures, I saw neither direct continuity of endoplasmic reticulum with nascent microbodies, nor have I seen small DAB-reactive microbodies.

What I have observed to occur, exclusively during that period of time when the number of microbodies is increasing, is a kind of multilobed microbody structure which lends itself readily to the interpretation[37] of increase in number of microbodies having its basis in the division of pre-existing microbodies. Indeed, such a division could result in catalase (DAB/H_2O_2) positive organelles having minimum dimensions of approximately 0.4 μm. I will therefore, use the working hypothesis (see below) that the doubling in number of microbodies results from division of pre-existing microbodies.

A further question I would like to consider at this point is the enzymic complement of the microbodies of aerated, acetate-

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[37] It is also possible, of course, that structures similar to those observed in Figs. 21,22 are the result of a confluence or fusion of microbodies. However, the increase in numbers of microbodies which occurs at this time makes it more likely that the microbodies are dividing instead of fusing.

supplemented Euglena gracilis strain Z during the phases in which it is dark-grown, greening and continuously light-grown. In this regard, it is interesting to note that Gruber et al. (1970) and Trelease et al. (1971) investigated the relationships between functionally glyoxysomal microbodies found during early lipid-degrading stages in fat-storing cotyledons (e.g., cucumber) and functionally peroxisomal microbodies present later during greening of these cotyledons. Both groups of workers considered that the most likely explanation for the change-over in type of microbody enzyme complement, was that glyoxysomes were degraded and peroxisomes arose by de novo synthesis. Although the attempt was made, neither degradation of glyoxysomes nor de novo synthesis of peroxisomes was demonstrated by either of these groups.

Lysosomal-like activity is one possible mechanism to explain a net degradation of glyoxysomes. In greening castor bean endosperm, Vigil (1970) reported that some microbodies disappear in toto into autophagic vacuoles. However, neither Gruber et al. (1970) working with sunflower cotyledons, nor Trelease et al. (1971), working with cucumber cotyledons, found any evidence for such autophagic vacuoles. In passing, it is worthwhile to note that in the present study of Euglena (in which glyoxysomal enzyme activity remains constant and, therefore, degradation of glyoxysomes should not necessarily be expected), I also found no evidence for such autophagic vacuoles. (The only autophagic vacuoles I found were those destroying mitochondria.)

Another possible mechanism by which glyoxysomes could disappear would be by autolysis. However, there are no reports in the literature (or findings in the present work) of this phenomenon occurring.

Cytoplasmic invaginations into microbodies have been observed (Gruber et al., 1970; Trelease et al., 1971) during the period when the microbody enzyme complement is changing from glyoxysomal to peroxisomal. Since these cytoplasmic invaginations contain ribosomes, Trelease et al. (1971) have pointed out that they might function to synthesize lytic enzymes needed to degrade glyoxysomes; however, they presented no evidence for a progressive destruction of invaginated microbodies. Moreover, Trelease et al. (1971) were unable to detect acid phosphatase activity (usually associated with lysosomes) in microbody gradient fractions of cucumber cotyledons at any stage of greening.

Although several groups of workers (Gruber et al., 1970; Trelease et al., 1971) have explained the supplantation of glyoxysomal enzymes by peroxisomal enzymes on the basis of degradation of glyoxysomal-like microbodies and de novo synthesis of peroxisomal-like microbodies, another mechanism has been postulated for this replacement. The second mechanism involves a change of enzyme complement within a single continuous population of microbodies. (Trelease et al., 1971). Moreoever, Trelease et al. (1971) postulated that cytoplasmic invaginations observed in microbodies of greening cucumber cotyledons (during the microbody transition period) might be the mechanism by which this organelle changes its enzymic content. Recall, that while I, too, saw possible invaginations of ribosomes containing cytoplasm into microbodies of 12 - 30-hour greening cells, these occurrences were observed only infrequently. The rarity of these occurrences plus the fact that low levels of peroxisomal enzymes are present in dark-grown cells, makes the significance of cytoplasmic invaginations non-interpretable at the present time.

Whether in Euglena the transition from glyoxysomal to peroxisomal function involves a single population of microbodies, capable of changing their enzyme complement, or two types of organelles with different and non-overlapping metabolic roles, some sort of mechanism must exist to further synthesize or additionally activate peroxisomal enzymes. In this regard, also, the possible existence of RNA and DNA in microbodies should be further explored.

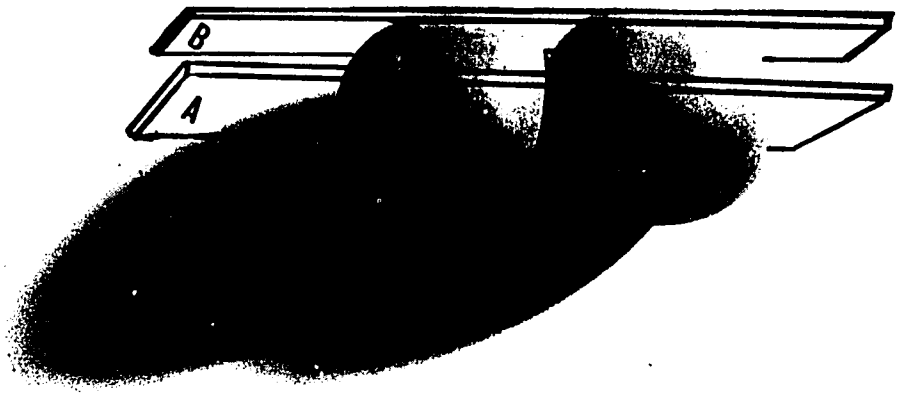
I would now like to attempt to correlate microbody data gathered in this dissertation regarding number, fine structure, protein content, and enzyme activity in aerated, acetate-supplemented Euglena gracilis strain Z. This will be done in the form of two models in which observations and assumptions are clearly distinguished.

Model I. I observed that there exists in each dark-grown, aerated, acetate-supplemented Euglena gracilis strain Z approximately 300 microbodies containing catalase, glyoxysomal enzymes, and low levels of peroxisomal enzymes. It shall be assumed in this model that each microbody contains these enzymes. By 24 hours of greening, the number of microbodies was observed (by microscopy) to double. I will assume that this doubling has its origin in a single division of each pre-existing microbody (Fig. 48) and that preparatory to (or concomitant with) this division, there occurs a doubling of all chemical constituents of each microbody. The results of such a division in each microbody would be:

a) An increase in number of microbodies from $\approx$ 300 to $\approx$ 600 per cell. This is in agreement with my observations.

b) A doubling in microbody protein content. Here, again there is no conflict with the data. A doubling in protein content was observed in the present work, by $\approx$ 24 hours of greening, on both the bases of cell-free fractions (i.e., total cell protein) and isolated microbodies (i.e.,

Figure 48. Model of microbody replication by a type of binary fission. A, plane of sectioning depicting two attached lobes. B, plane of sectioning depicting two non-attached lobes.



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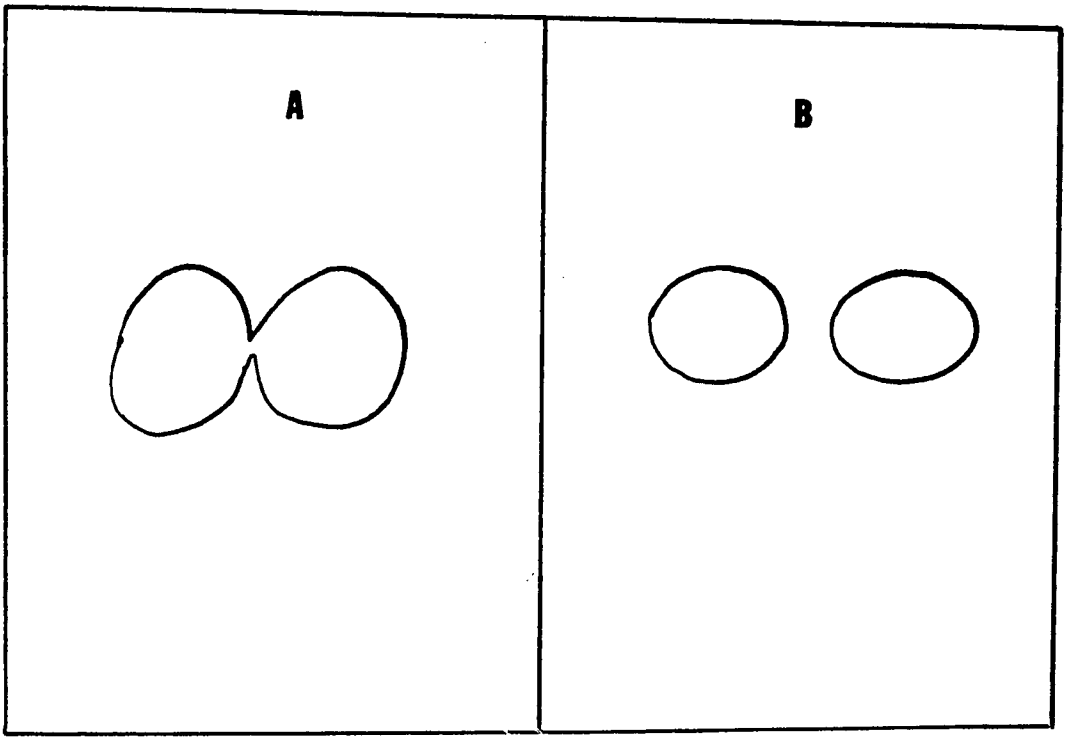


microbody protein).

c) A doubling in catalase activity on a per cell basis. In cell-free fractions such a doubling in catalase activity on a per cell basis was observed by $\lesssim$ 24 hours of greening (compared to dark-grown cells). However, catalase activity on a per cell basis in microbodies isolated on discontinuous sucrose density gradients increased $\lesssim$ 1.4-fold by 24 hours of greening. These results could be obtained if there were, for example, extra-microbody catalase or leaching of catalase from such microbodies isolated on sucrose gradients.

By this model specific activities of catalase (per mg protein) should be expected to remain relatively constant with greening. (Recall that protein content doubles by 24 hours of greening.) It was observed that the specific activity of catalase increased $\simeq$ 1.4-fold by 24 hours of greening in cell-free fractions (increasing to 2-fold by 72 hours of greening) and increased $\simeq$ 1.4-fold in isolated microbodies. Therefore, model I is in good agreement with catalase data expressed on a per cell basis, but in conflict with catalase data when expressed on the basis of specific activity.

d) A doubling in glyoxysomal enzymes. Here all the data are in good agreement with the model. Data, expressed on a per cell basis in either cell-free fractions or isolated microbodies, reveal a doubling in glyoxysomal marker enzyme activities by $\lesssim$ 24 hours of greening. Coupled with the observed doubling in total protein and microbody protein, glyoxysomal enzyme activities would be predicted to remain constant on the basis of specific activity; such observations were obtained.

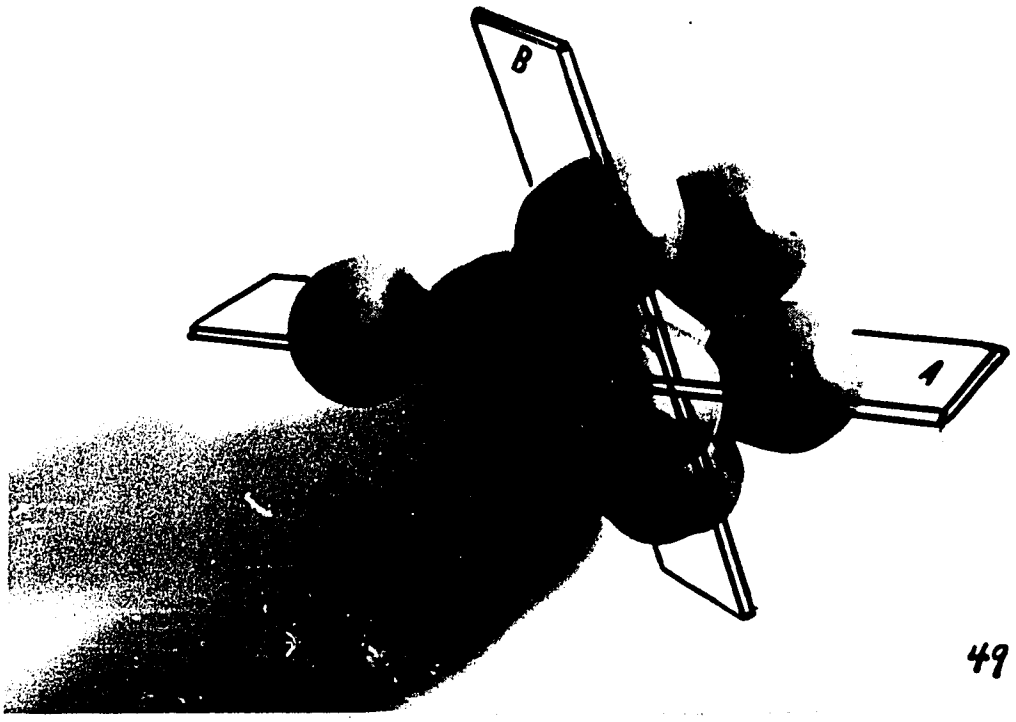
e) A doubling in peroxisomal enzyme activity. Such a doubling would be insufficient to explain the observed results. Peroxisomal enzyme

activities in cell-free fractions and in isolated microbodies, when expressed on a per cell basis, were found to increase 12-fold by ≃ 24 hours of greening (compared to dark-grown cells). Peroxisomal enzyme activities in cell-free fractions and in isolated microbodies, when expressed on a per mg protein basis, were found to increase ≃ 6-fold by 24 hours of greening (compared to dark-grown cells). In other words, superimposed upon the doubling in peroxisomal enzyme activity predicted by model I, there would have to be an additional increase in peroxisomal enzyme activity in each daughter microbody. Therefore, the model is not in good agreement with observed peroxisomal enzyme data.

Model II. There exists in each dark-grown, aerated, acetate-supplemented Euglena gracilis strain Z approximately 300 microbodies. In this second model it will be assumed that 1/10 to 1/12 of these microbodies contain low levels of peroxisomal-type enzymes accompanied by catalase. It will further be assumed that the remainder of the microbodies contain glyoxysomal-type enzymes and catalase. I have observed a doubling in the number of microbodies by ≃ 24 hours of greening. In this second model it will be assumed that the observed doubling in numbers of microbodies result because of a multilobed (12 to 10 lobes) type of division in peroxisomal type of microbodies (Figs. 49,50). I will further assume that preliminary to (or concomitant with) this multilobed type of division there is a parallel (i.e., 12- to 10-fold) increase in the levels of catalase and peroxisomal-type enzymes in these peroxisomal-type microbodies which constitute the 1/10 to 1/12 of the total microbody population in each dark-grown cell. The consequences of this type of multilobed division would be:

a) An increase in number of microbodies from ≃ 300 to ≃ 600

Figure 49. Model of microbody replication by a type of multilobed division in which all lobes are approximately the same size. A, plane of sectioning depicting a linear configuration of lobes. B, plane of sectioning depicting lobes in a spherical cluster.



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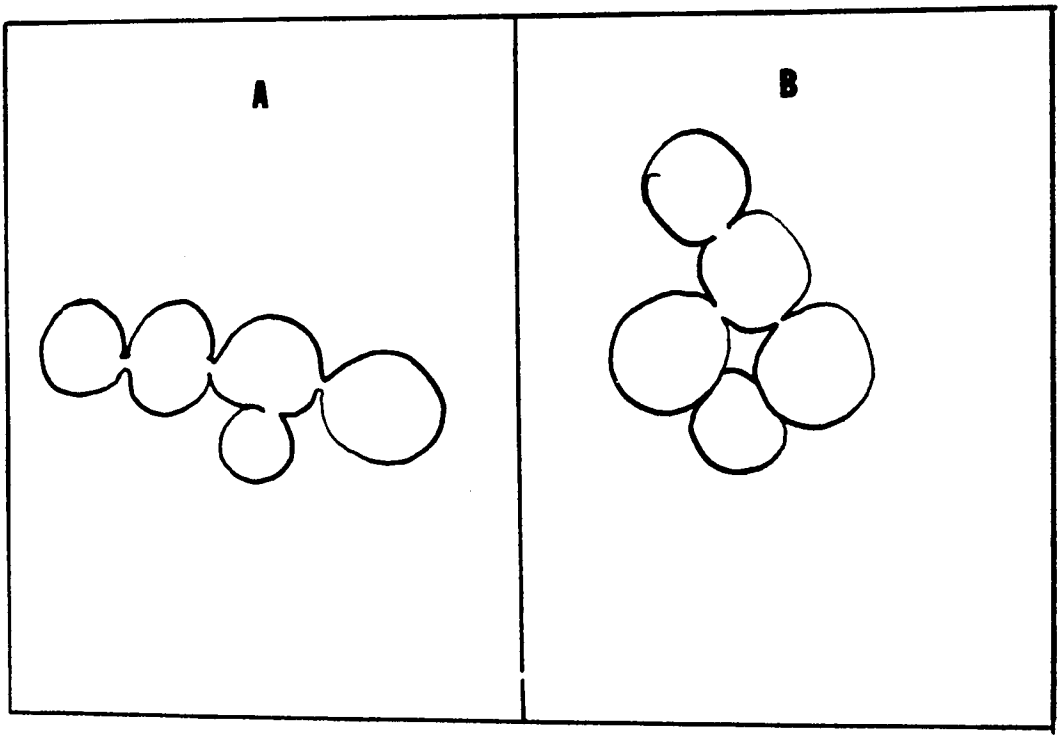
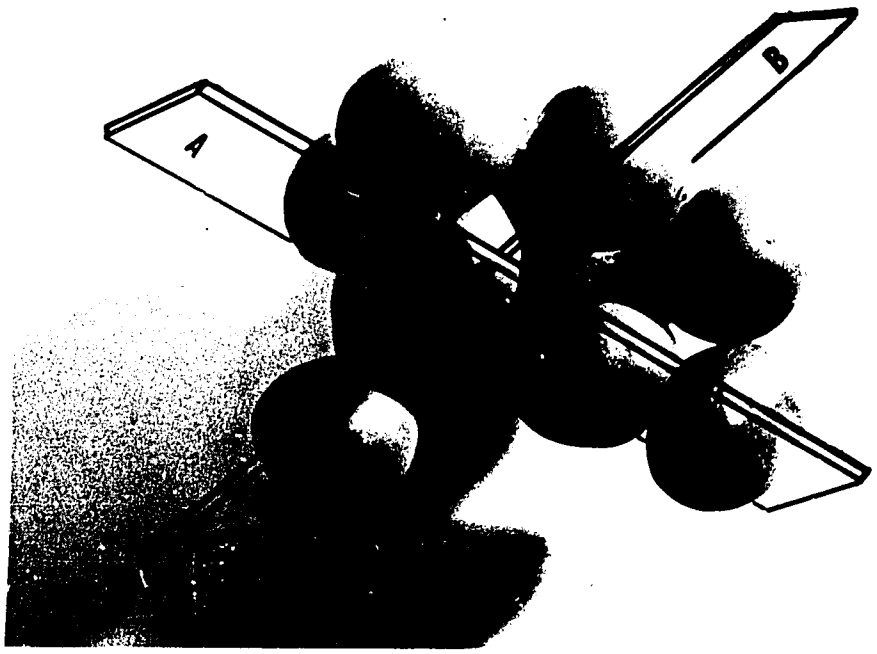
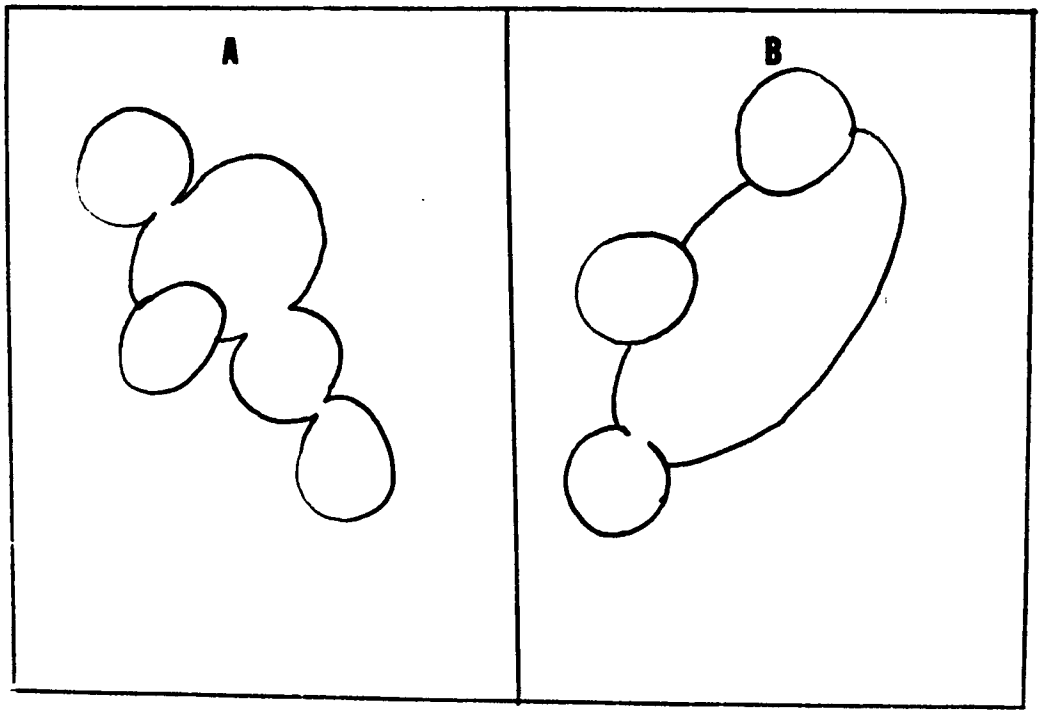


Figure 50. Model of microbody replication by a type of multilobed division in which a large "mother" microbody is present. A, plane of sectioning depicting a profile which would fail to reveal the long axis of the central "mother" microbody. B, plane of sectioning depicting a profile which demonstrates the long dimension of the "mother" microbody.



50



per cell. This is in agreement with microscopy observations.

b) A 12 to 10-fold increase in the (1/10 to 1/12 original) microbody population containing peroxisomal-type enzymes. This would lead to the observed doubling of isolated microbody content.

c) Either a 2-fold increase or less than a 2-fold increase in catalase activity by 24 hours of greening. The doubling in catalase activity would arise from the model if it is assumed that levels of catalase activity are the same in peroxisomal-type microbodies as in glyoxysomal-type microbodies. Then, for example, assume a designated value of 10 Lück units[38] of catalase in microbodies of one cell of dark-grown Euglena. Moreover, assume that 9/10 to 11/12 of these microbodies (i.e., the glyoxysomal ones) do not change their complement of catalase with greening (this corresponds to a constant 9.0 to 9.2 units of the total designated 10 Lück units per cell). Also, assume that the remaining 1/10 to 1/12 of the (peroxisomal) microbodies increase their complement of catalase by 12 to 10-fold, respectively, yielding by 24 hours of greening, 12.0 to 8.0 units of catalase, respectively. Hence, by 24 hours of greening there would be present 21.0 to 17.2 unites of catalase per cell (or a 2.1 to 1.72-fold increase) in relation to the designated number (10 units). This would be in agreement with the observed $\leq$ 2-fold increase in catalase levels by 24 hours of greening in cell-free fractions when data are expressed on a per cell basis.

If on the other hand one assumes, in application of this model, that there are lower levels of catalase in those microbodies which contain trace amounts of peroxisomal enzymes than in dark-grown cells,

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[38] For purposes of clarity, a designated value of 10 Lück units per dark-grown cell is selected. Actually, each dark-grown, aerated, acetate-supplemented Euglena gracilis strain Z cell was found to contain 2.7×10^{-7} Lück units of catalase.

less than a 2-fold increase in catalase activity would be obtained by 24 hours of greening. For example, if it is assumed that there is only half the amount of catalase in peroxisomal-type microbodies (i.e., 0.5 to 0.4 units of the total designated 10 Lücк units) compared to the amount of catalase in glyoxysomal-type microbodies (9.5 to 9.6 units of the total of 10 designated Lücк units), then by 24 hours of greening with model II, a 10 to 12-fold increase occurs in peroxisomal type microbodies (catalase activity in these microbodies would increase to 5.0 to 4.8 Lücк units). In relation to the 10 Lücк units originally designated to a dark-grown cell, there would be a 1.50 to 1.48-fold increase in levels of catalase by 24 hours of greening. This assumption is in good agreement with my data of a 1.4-fold increase in catalase activity by 24 hours of greening in isolated microbody fractions (expressed on the basis of per mg protein or per cell).

Thus, application of model II can either give the catalase activity results observed with cell-free fractions (2-fold increase) or isolated microbodies (1.4-fold increase) by assuming equal catalase activity or lower catalase activity in peroxisomal-type microbodies than glyoxysomal-type microbodies, respectively. Again, I shall assume that perhaps differences in increments of catalase as a function of greening are caused by extra-microbody catalase or leaching of catalase from microbodies isolated on sucrose gradients.

d) A constant amount of glyoxysomal enzyme activities on a per cell basis (either in the case of cell-free fractions or isolated microbodies), but a halving of specific activities of the glyoxysomal enzymes with greening. In actuality I observed that there is a doubling in glyoxysomal enzyme activities on a per cell basis and a constancy in

specific activities of glyoxysomal marker enzymes (in both cell-free fractions and isolated microbodies). These observations could result from a doubling in enzyme activity per cell, which when coupled to the observed doubling in protein content, would yield the observed constancy of specific glyoxysomal enzyme activity. Model II, per se, does not provide for this doubling.[39]

e) A 12- to 10-fold increase, with greening, in activities of peroxisomal enzymes, on a per cell basis, and a 6- to 5-fold increase on a specific activity basis. These predictions are in agreement with the findings.

In Figures 48, 49 and 50 are presented photographs of clay representations of microbodies undergoing division - the first as described in Model I, the second and third as described in Model II. In Fig. 48 a binary fission type of division is occurring. Regardless of how such a structure is sectioned, a maximum of two attached lobes can be obtained (plane A, Fig. 48A). Other planes (e.g., plane B, Fig. 48B) of sectioning result in two non-attached lobes. The most likely situation, arising from random sectioning, would be a single lobe (i.e., any section perpendicular to planes A or B). Such a microbody (Fig. 48), when sectioned, is not capable of yielding multilobes demonstrated in Figs. 21 A,B,C, 22, and 23.

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[39] Lynch and Calvin (1953) working with acetate-supplemented, continuously light-grown and dark-grown Euglena gracilis var. bacillaris and Cook (1965) working with acetate-supplemented, continuously, light-grown and dark-grown Euglena gracilis strain Z found that there was greater utilization of acetate in the light than in the dark. Both of these groups of workers also observed that under the influence of light there is increased incorporation of acetate into lipids which result in further activation of glyoxysomal enzymes needed for conversion of acetate into sugars.

Both Figs. 49 and 50 depict multilobed (i.e., 10 - 12 lobes) microbodies. Fig. 49 depicts a structure in which there is no "mother" microbody - all the lobes being approximately the same size. A section through this organelle (e.g., plane A Fig. 49 A) would result essentially in a linear configuration of lobes. Another section (e.g., plane B, Fig. 49 B) would result in a configuration in which lobes are in a spherical cluster.

Fig. 50 shows a structure in which there exists a "mother" microbody which is larger than the derived "daughter" microbodies. A section through this organelle (e.g., plane A Fig. 50 A) would result in a profile which would fail to reveal the long axis of the central "mother" microbody. Other sections (e.g., plane B, Fig. 50 B) would yield a profile in which the long dimension of the "mother" microbody would be manifested; structures showing such long axes have not been observed in the present work.

It should be recognized, however, that only those sections parallel to the long axis of the "mother" microbody would reveal its entire length - oblique cuts yielding somewhat oval and slightly larger "mother" microbodies than "daughter" microbodies.

Both Figs. 49 and 50 (microbodies undergoing multilobed division) are capable of giving types of structures observed by electron microscopy (Figs. 20, 21 A, 21 B, 21 C, and 22). More fine structure studies will have to be undertaken to determine which (if any) of the above clay representations of dividing microbodies are closer to the situation which actually obtains in aerated, acetate-supplemented, 12 - 30 hour greening Euglena gracilis strain Z.

III. *Euglena* Mitochondria

A. Fine Structure

Gerhardt and Berger (1971) noted DAB reactivity in the cristae membranes of two acetate flagellates (*Polytomella caeca* and *Chlorogonium elongatum*), especially at neutral pH and room temperature, and ascribed this reactivity to either peroxidase or cytochrome oxidase. I was unable to detect a similar pattern of deposition in *Euglena* mitochondria, although observing it on the outer surface of the cristae membranes of mitochondria of co-processed *Ricinus* endosperm and leaf mesophyll cells.

The time dependence of the mitochondrial matrix DAB reactivity in *Euglena* - its visualization at $\approx$ 20 hours of greening, its maximalization at $\approx$ 48 hours of greening, and its disappearance by 72 hours of greening - approximates the first derivative of the greening curve (Stern *et al.*, 1964; Brody *et al.*, 1965), and seems to closely relate it either to a light requirement *per se* or to the rate of synthesis of plastid constituents. Schiff *et al* (1967) noted that dark-grown *Euglena* can be light-induced to form chloroplasts in the presence of the photosynthesis inhibitor DCMU, and suggested that the developing plastid utilizes energy , not from photosynthesis, but from the cytoplasm (see also Schiff,1970).Since detectable levels of the mitochondrial enzyme(s) are inhibited by DCMU, and since the activity is not observed in CO_2-free air, its functioning may be photosynthesis-dependent. However, it should be noted that DCMU makes both Photosystems I and II inoperative in the absence of an added electron donor. Therefore, further experiments will have to be undertaken to determine whether the mitochondrial enzyme activ-

ity is a product of one or both of these photosystems.

The transitory nature of the DAB-reactive mitochondrial enzyme seems to link it with the conversion of a substrate produced in the dark into (or the synthesis of) a compound needed for new metabolism in the light. Smillie (1963) observed transient increased activity of both several enzymes of glucan metabolism and respiration, when dark-adapted Euglena gracilis were exposed to light. In this regard, it should also be recalled that Erwin and Bloch (1962) demonstrated that Euglena gracilis grown in the light on organic medium possess large quantities of α-linolenic acid(mainly in chloroplast glycolipids), while Euglena grown in the dark have large amounts of C-20, C-22 and C-24 polyeneoic fatty acids of the γ-linolenic type (mainly in the phospholipid portion of their membranes). Delo et al. (1971) have demonstrated that heterotrophic, dark-grown Euglena gracilis strain Z elaborate a single fatty acid synthetase which is a multi-enzyme complex similar to that found in yeast cells and animal tissues; fatty acid synthesis in light-grown (photoautotrophic) Euglena is performed by two independent fatty acid synthetases.

The aforementioned works suggested that the activity of the DAB-reactive mitochondrial enzyme(s) might be related to fatty acid metabolism. In order to ascertain whether this DAB reactivity was the result of an enzyme (s) involved in the conversion of "dark-associated" fatty acid metabolism into "light-associated" metabolism, experiments were performed in which Euglena were allowed to green in Cramer and Myers medium (1952) supplemented with either arachidonic or linolenic acid as sole carbon and energy sources. Assuming that the activity of the DAB-reactive mitochondrial enzyme disappeared after $\leq$ 72 hours of greening because the cellular pool of polyenoic fatty acids had been used up, I sought to

determine the effects of an exogenous pool of polyenoic fatty acid (arachidonic acid) on the kinetics of the disappearance of DAB reactivity. To this end, arachidonic acid was introduced into the growth medium, which as Rosenberg et al. (1965) have reported, account for 48 % of the total proplastid fatty acids of dark-grown and 14 % of the total chloroplast fatty acids in light-grown Euglena gracilis. With Euglena in the presence of arachidonic acid an extension of DAB reactivity in the mitochondrial matrices was observed until 108 hours of greening (i.e., 36 hours past that observed in acetate-supplemented cells). Although this extension seemed encouraging for my hypothesis, I was nevertheless puzzled as to why DAB reactivity ceased at ≤ 108 hours of greening, at a time when considerable quantities of arachidonic acid were still available in the growth medium. (A possible explanation for the loss of activity became apparent only later). As a control, to test the hypothesis that the DAB reactivity of the mitochondrial enzyme was involved in the conversion of "dark" fatty acid metabolism into "light" fatty acid metabolism, Euglena were grown in Cramer and Myers medium (1952) supplemented with linolenic acid, which Rosenberg et al. (1965) have shown accounts for 1.0 % of total proplastid fatty acids in dark-grown Euglena gracilis strain Z and 12.5 % of total chloroplast fatty acids in light-grown cells. In the presence of linolenic acid, DAB mitochondrial activity was also extended for a longer period of time than acetate-supplemented cells - to 120 hours of greening (or 48 hours past that obtained in acetate-supplemented medium).

Thus, it would seem that since DAB reactivity is extended both in the presence of a fatty acid characteristic of dark metabolism (arachidonic) and a fatty acid characteristic of light metabolism (lino-

lenic), the observed DAB reactivity is probably not a function of the conversion of "dark" to "light" metabolism. The extension of the period of time during which DAB mitochondrial reactivity is osberved in cells supplemented with either of these fatty acids may have its basis in the increased generation time which occurs when either arachidonic or linolenic acids are used as carbon and energy sources (compared to acetate).

At the present time the only conclusions which may be made concerning the DAB-reactive mitochondrial enzyme(s) are:(1) It is not catalase because aminotriazole (a specific inhibitor of catalase) does not inhibit DAB deposition in mitochondrial matrices. (2) It is not cytochrome oxidase because it is observed at pH 9.0, 37 ^{o}C and not pH 7.0, 25 ^{o}C. The latter are optimal conditions for demonstrating cytochrome oxidase-mediated DAB polymerization. Also, cytochrome oxidase could not be detected by photometric techniques. (3) It is photosynthesis-dependent since DAB deposition is not observed in mitochondria of cells grown in the presence of DCMU. (4) Its nature is transient, being first observed at $\approx$ 20 hours of greening, reaching a maximal intensity at $\approx$ 48 hours of greening and disappears by 72 hours of greening. (5) It is cyanide-sensitive. (6) Its activity depends upon the presence of carbon dioxide in the gas phase.

That the mitochondrial DAB-reactive enzyme(s) is observed neither in the presence of DCMU nor in the absence of CO_2 in the gas phase, suggests that some product of photosynthesis (either a real endproduct or "reducing power") is needed for its activity. In this regard, Palmer and Togasaki (1971) have studied ^{14}C-acetate metabolism in Pandorina and observed that light-stimulated acetate uptake is decreased by aeration with CO_2-free air. They also have demonstrated that lipid synthesis is

dependent upon both light and photosynthesis - in the dark, or in the presence of DCMU, there is severe inhibition of ^{14}C incorporation into lipid.

Among the possible mitochondrial enzymes which generate H_2O_2 and could be responsible for the observed DAB deposition in the present work are: (1) The deaminating enzyme monamine oxidase (monamine O_2-oxidoreductase; EC 1.4.3.4). (2) The decarboxylating enzyme D-lactate oxidase (D-lactate:O_2 oxidoreductase; EC 1.1.3.2.). (3) Fatty acid peroxidases (e.g., palmite:H_2O_2 oxidoreductase; EC 1.11.1.3), (4) Lipoxidases [enzymes which act on long-chain unsaturated fatty acids (e.g., linoleic, linolenic, arachidonic acids) in the presence of oxygen to form short-chain fatty acids]. (5) Transaminases involved in the conversion of glyoxylate to glycine (see Fig.5). (6) Enzymes involved in the back reaction of β-oxidation in the mitochondria.

B. Enzymes

Cytochrome oxidase, malate dehydrogenase, fumarase and aconitase were the "mitochondria marker" enzymes utilized in the present study. Two of the enzymes - malate dehydrogenase and aconitase - function both in the tricarboxylic acid and glyoxylate cycles, and were selected to ascertain their relative activities in isolated mitochondria and microbodies. Recall that Müller (1969) and Hogg (1969) had shown that in Tetrahymena pyriformis three enzymes of the glyoxylate cycle (citrate synthase, aconitase and malate dehydrogenase) are found in mitochondria and two (malate synthase and isocitrate lyase) are located in microbodies.

Attempts to photometrically detect cytochrome oxidase in cell-free fractions, discontinuous sucrose density gradients or contin-

uous sucrose density gradients of aerated, acetate-supplemented Euglena gracilis strain Z were unsuccessful. Thus, confirming previous observations made on cell-free fractions of this same organism (Krawiec and Eisenstadt, 1970; Lord and Merrett, 1971). Lord and Merrett (1971) concluded that "euglenoids lack a conventional cytochrome c oxidase". Perini et al. (1964 a and b) found an "a" type cytochrome (605) in dark-grown Euglena, but concluded that their evidence was insufficient to to characterize the enzyme as "a_3" . In contrast, Sharpless and Butow (1970), working with a permanently bleached strain of Euglena gracilis, estimated from low temperature (77° K) difference spectra that an average concentration of cytochrome oxidase of 0.25 nmoles/mg of mitochondrial protein was present. Additionally, it should be pointed out that cytochrome oxidase could not be detected cytochemically in aerated, acetate-supplemented Euglena under conditions of DAB incubation (pH 7.0, 25 °C), considered optimal for cytochrome oxidase-mediated DAB polymerization (Novikoff and Goldfischer, 1969; Gerhardt and Berger, 1971).

Specific activities of malate dehydrogenase in cell-free fractions were found in the range from 2.3 - 3.0 mμ/min/mg protein and agree well with those of 2.07 and 3.29 mμ/min/mg protein reported by Smillie (1968) for cell-free fractions of Euglena gracilis strain Z and and a streptomycin-bleached variety , respectively, grown heterotrophically at 23 °C under 700 ft-c illumination on complex organic medium. Graves et al. (1972) working with variety bacillaris (SM-L1) reported activities of malate dehydrogenase in both mitochondria-containing and microbody-containing bands isolated on continuous sucrose density gradients; the major portion being found in the microbody band. In the present work, approximately equal amounts of this enzyme were found in mitchondrial and

microbody bands isolated from continuous sucrose density gradients. This finding should not be surprising since malate dehydrogenase functions both in the tricarboxylic acid and glyoxylate cycles.

Specific activities of aconitase in cell-free fractions of aerated, acetate-supplemented dark-grown, greening, and continuously light-grown Euglena gracilis strain Z were found in the range from 0.30 - 0.39 mμ/min/mg protein. In contrast, Smillie reports a specific activity of 0.011 in cell-free fractions of this same organism grown autotrophically in inorganic medium (at 23 ^{o}C in 5 % CO_2 under 700 ft-c illumination). Perhaps these differences may be partially explained [as they were by Hogg (1969) working with cell-free fractions of Tetrahymena pyriformis], by the finding that cellular levels of enzymes are functions of variables such as medium, growth conditions and growth phase. Graves et al. (1972) working with var. bacillaris (SM-L1) reported activities of aconitase in both mitochondria-containing and microbody-containing bands isolated on continuous sucrose density gradients. In the present work with strain Z, the activities of aconitase and fumarase were localized mainly in microbody-containing bands, isolated either from continuous or discontinuous sucrose density gradients.

Carbon dioxide deprivation in the gas phase during greening of aerated, acetate-supplemented Euglena gracilis strain Z was found to have negligible effects upon the activities of malate dehydrogenase, fumarase, or aconitase in either cell-free fractions or bands isolated from discontinuous sucrose density gradients.

VI. Publications

The following publications have resulted either in part or entirety from work presented in this thesis:

Brody, M. and White, J.E. (1972). Environmental factors controlling enzymatic activity in microbodies and mitochondria of Euglena gracilis. FEBS LETTERS 23:149-152.

Brody, M. and White, J.E. (1973). Environmental regulation of enzymes in the microbodies and mitochondria of dark-grown, greening and light-grown Euglena gracilis. DEVELOPMENTAL BIOL. 31:348-361.

Cohen, W.S., Nathanson, B., White, J.E. and Brody, M. (1969). Fatty acids as model systems for the action of Ricinus leaf extract on higher plant chloroplasts and algae. ARCH. BIOCHEM. BIOPHYS. 135: 21-27.

White, J.E. and Brody, M. (1973a). Environmental regulation of microbody and mitochondrial enzymes in Euglena. BIOPHYS. SOC. ABSTR., 17th ANNUAL MEETING, Columbus, Ohio, pg. 160a.

White, J.E. and Brody, M. (1973b). Enzymatic characterization of sucrose-gradient microbodies of dark-grown, greening and continuously light-grown Euglena gracilis. FEBS LETTERS. in press.

SUMMARY

Enzymological And Fine Structure Studies On Microbodies And Mitochondria Of Euglena Gracilis Strain Z Grown Under Various Environmental Conditions.

Observations that growth in, or treatment with, several photosynthetic organisms with exogenous fatty acids brought about modifications in fine structure, led to enzymological and further fine structure studies on the microbodies and mitochondria of Euglena gracilis strain Z. In aerated, acetate-supplemented Euglena gracilis strain Z, microbodies were found to be most numerous in regions around the gullet and cell periphery. These microbodies had measured mean long dimensions of $\simeq$ 0.85 mμ and measured mean short dimensions of $\simeq$ 0.65 mμ. At least an order of magnitude more microbodies occur in cells grown on acetate-supplemented media than glucose-supplemented media. Microbodies were counted in situ using both thick and thin serial sections; $\simeq$ 300 and 600 microbodies per cell were found in dark-grown and continuously light-grown cultures, respectively. Catalase in microbodies was demonstrated by the use of the cytochemical stain 3,3'-diaminobenzidine (DAB); incubation at 37 ^{0}C and pH 9.0 for 60 minutes gives strong electron opacity, which does not manifest itself in the presence of the specific catalase inhibitor aminotriazole.

Between 12 - 30 hours of greening in Euglena (when the number of microbodies per cell doubles, compared to dark-grown cells) some microbodies (catalase-positive) appear to be undergoing "multilobed" division--a type of division not previously reported in the literature for any organelle. Also, observed during this period are long ($\simeq$ 0.5 μm), thick (30 - 50 A^{o}) fibrils inside the multilobed

microbody complex. The extreme length of these fibrils, coupled with photometric finding of nucleic acids in sucrose gradient microbodies makes these strands possible suspect of being DNA.

A comparison of microbodies of Euglena gracilis strain Z was made with those of higher plants, utilizing endosperm cells of Ricinus communis, leaf mesophyll and guard cells of Ricinus communis, Spinacea oleracea, Zea mays, Kalanchoë blossfeldiana and Kalanchoë daigremontianum. The present work contains the first report of catalase-mediated DAB reactivity in microbodies of guard cells. As far as fine structure is concerned, all the microbodies observed are similar to those encountered in Euglena.

When cells are aerated with air which had its carbon dioxide removed by passage through excess Ascarite, catalase could not be detected either photometrically or cytochemically. Since re-aeration with air containing CO_2 results in restoration of catalase (as observed both with cell-free fractions and microbody-containing fractions isolated from discontinuous sucrose gradients), it appears that carbon dioxide regulates the activity (or possibly, the presence) of catalase in Euglena gracilis strain Z.

By 72 hours of greening, in comparison to dark-grown cells in cell-free fractions, catalase activity has increased $\geq$ 2-fold on a per mg protein basis (specific activity) and $\simeq$ 2-fold on a per cell basis; a 1.4-fold increase is noted (both on the bases of specific activity and per cell) in microbody-containing fractions from discontinuous sucrose density gradients.

Specific activities of glyoxysomal marker enzymes (isocitrate lyase and malate synthase) are fairly constant in aerated, acetate-supplemented, dark-grown, greening and continuously light-grown Euglena

gracilis strain Z, as judged from data obtained both with cell-free fractions, and microbody-containing sucrose fractions from discontinuous sucrose density gradients. When such data are expressed on a per cell basis, glyoxysomal enzyme activities double by 72 hours of greening.

An ≃ 6-fold increase in the specific activities of the peroxisomal marker enzymes (glycolate dehydrogenase and hydroxypyruvate reductase) is observed in cell-free fractions and sucrose-isolated microbody fractions by 72 hours of greening, as compared to dark-grown cells; an ≃ 12-fold increase is noted when data are expressed on a per cell basis.

The observed doubling of microbodies which occurs between 12 - 30 hours of greening (associated with the conversion of "dark metabolism" to " light metabolism") has no exact parallel with any situation reported in the literature and appears to arise from the multilobed division (10 - 12 lobes) of a small fraction of pre-existing microbodies. Two models are offered to correlate microbody data in regard to number, fine structure, protein content and enzyme activity. The first model involves a binary fission type of division of each pre-existing microbody in dark-grown cells; the predicted consequences of such a division do not correlate well with some of the enzyme and fine structure data.The second model involves a multilobed type of division of peroxisomal-type microbodies (assumed to represent 1/12 to 1/10 the total number of microbodies in dark-grown cells); the predicted consequences of such a division do correlate well with the observations.

An unidentified enzyme has been demonstrated cytochemically in the mitochondrial matrices of aerated, acetate-supplemented Euglena

gracilis strain Z, under conditions of DAB incubation other than those considered optimal for cytochrome oxidase. The activity of this enzyme is transitory, light-induced, and possibly photosynthesis-dependent.

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