To the Graduate Council:

I am submitting herewith a dissertation written by Maybelle P. Gomez, entitled "Ultrastructural and Physiological Changes Associated with Aging in Euglena gracilis Klebs Strain 'Z'." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Patricia L. Walne, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

ULTRASTRUCTURAL AND PHYSIOLOGICAL CHANGES ASSOCIATED WITH AGING IN *EUGLENA GRACILIS* KLEBS STRAIN 'Z'

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

When cells become incapable of cell division as in the case of many stationary-phase cells exhibiting post-mitosis or "mitotic repression," with the passage of time, certain variables within their biological systems tend to shift their "steady state" (homeostatic capacity) from the norm. At the cellular level, with the exception of an increasing appearance of aging pigments, such age-related changes are not easily observed. The present study attempts (1) to identify such age-related cellular changes in cell cultures of Euglena gracilis Klebs strain 'Z' using cytochemical methods on both light and electron microscope levels and (2) to correlate where possible, the changes in biochemical activity, chemical composition and cellular metabolism that occur in cells as cultures age. This study reveals the appearance of different cell types in a once-homogeneous synchronized cell population. The cells become varied in size, shape and physiological condition with subsequent increase in generation time (GT) with increasing age of the cultures. Ultrastructural alterations involve apparent decrease in chromatin content of the nucleus, increase in activity in the lysosome system as shown by lytic activity of organelles, hypertrophy of dictyosomes, aging pigment and

myelin figure formation--all established features of many aging cellular systems. Cytochemical treatment of the cells shows change in latency of acid phosphatase as it increases in intracellular distribution with age and subsequent increase in lipid bodies. The chloroplasts decrease in fluorescence properties. Ruthenium Red labeling of the plasma membrane reveals decrease in the mucopolyssacharide reactions on the outer layer. Other enzymes such as catalase, cytochrome oxidase and succinate dehydrogenase manifest variable activities. The general pattern of cellular aging in Euglena resembles that of more complex multicellular biological systems. Progressive degradation followed by death occurs as cells are unable to operate efficiently in response to its environment.

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
I. MATERIALS AND METHODS	25
Growth Conditions	25
Optical Microscopy and Cytochemistry	27
Electron Microscopy and Cytochemistry	30
II. RESULTS	34
Light Microscopy	34
Electron Microscopy	52
III. DISCUSSION	101
Light Microscopy	101
Electron Microscopy	110
IV. SUMMARY AND CONCLUSIONS	135
LIST OF REFERENCES	137
APPENDIXES	164
Appendix A	165
Appendix B	170
Appendix C	174
Appendix D	187
Appendix E	191
VITA	202

LIST OF TABLES

TABLE PAGE

I. Pertinent Literature on Molecular and Biochemical Approaches to Studies of Aging 4

II. Pertinent Literature on Developmental Approach to Studies of Aging 7

III. Pertinent Literature on Genetic Approach to Studies of Aging. 10

IV. Pertinent Literature on the Phyletic Approach to the Study of Aging 12

V. Pertinent Literature on Growth of Euglena gracilis Klebs. 18

VI. Pertinent Literature on Aging Studies in Algal Flagellates and Ciliated Protozoans 20

VII. Summary of Age-Related Changes in Euglena gracilis Klebs. 94

VIII. Cytochemistry of Substances Detected in Euglena gracilis Strain 'Z' 96

LIST OF PLATES

PLATE PAGE

I. Growth Curve of Euglena gracilis Klebs Strain 'Z' 35

II. Cells in Logarithmic Phase. 37

III. Log and Stationary Phase Cells. 39

IV. Cytochemical Treatment of Log and Stationary Phase Cells 42

V. Gomori Acid-Phosphatase Cytochemistry and Sequence of Gomori Acid-Phosphatase Reaction with Increasing Age of Cultures 45

VI. Burstone's Azo-Dye Technique for Acid Phosphatase Localization. 47

VII. Fluorochrome Labelling and Fluorescence Microscopy. 50

VIII. Schematic Diagram of Life History of Euglena gracilis 'Z' in Synchronous Cultures. 53

IX. Azure Blue Staining of Thick Sections 55

X. Age-Associated Changes in Nucleus 58

XI. The Canal Region. 61

XII. Flagellum and Associated Organelles 64

XIII. Chloroplast Structure, Cytochemistry and Age-Related Changes 66

XIV. Paramylon Structure and Cytochemistry 69

PLATE PAGE

XV. Dictyosome Structure and Cytochemistry. 71

XVI. Vesicles. 74

XVII. Microbody (Peroxisome) Structure and Cytochemistry 77

XVIII. Microbodies and Related Organelles. 80

XIX. Mitochondrial Morphology. 83

XX. Age-Related Structural Alterations and Cytochemistry of Mitochondria 86

XXI. Pellicle Structure and Cytochemistry. 89

XXII. Age-Related Changes in Other Cytoplasmic Organelles. 92

XXIII. Summary of Age-Related Changes in the Ultrastructure of Some Major Organelles. 98

ABBREVIATIONS

Chemical Abbreviations

AMP	- 2 amino 2 methyl, 1,3-propandiol
AO	- acridine orange
AS-BI	- dye name designation for 6-bromo-2-hydroxy-3-naphthoyl-o-anisidine; (German: Anilid Säure)
AT	- amino triazole; 3 amino, 1,2,4-triazole
BDMA	- benzyldimethylamine
BSPT	- 2-(2'-benzothiazolyl)-styryl-3-(4'-phthalhydrazidyl)-tetrazolium chloride
DAB	- 3,3' diaminobenzidine tetrahydrochloride
DER 736	- symbol for a diglycidyl ether or polypropylene glycol (Dow Chemical Co.)
DDSA	- dodecenyl succinic anhydride
DMF	- dimethyl formamide
DMP-30	- trimethyl amino triphenol
ERL 4206	- symbol for vinylcyclohexene dioxide (Union Carbide Corp.); also known as VCD; UNOX 206; Araldite RD-4 (Ciba)
HPR	- hexazonium pararosanilin
^{3}H-uridine	- tritiated uridine
JGB	- Janus Green B
NaF	- sodium fluoride

NBT - nitroblue tetrazolium

NSA - nonenyl succinic anhydride

PO - propylene oxide

QM - quinacrine mustard

RR - Ruthenium Red; tetra amino ruthenium hydroxide chlorochloride

S-1 - symbol for dimethylamino-ethanol (Conshohocken Chemical Co.)

TPT - triphenyl tetrazolium

Tris - Tris (hydroxymethyl) amino methane; also known as THAM; Trizma Base (Sigma Chemical Co.)

Enzymes and Other Substances

AP - acid phosphatase

CyO - cytochrome oxidase

mP - mucopolysaccharide

SDH - succinate dehydrogenase; succinic acid dehydrogenase

Organelles

AV - autophagic vacuole

C - canal

CCBs - crystal containing bodies

CF - circular microtubule

Chl - chloroplast

Chr - chromosome

CO - canal opening

DB - dense body

ER - endoplasmic reticulum

F - flagellum

FCD - focal cytoplasmic degradation

FS - flagellar swelling; paraflagellar body (PFB)

GA - Golgi Apparatus

L - Lipid globule

LPG - lipofuscin pigment granule

Ly - lysosome

M - mastigoneme

MB - microbody (peroxisome)

Mito - mitochondrion

MT - microtubule

MVB - multivesicular body

MY - myelin figures; concentric bodies

N - nucleus

NE - nuclear envelope; nuclear membrane

OG - osmophilic granules (OS)

P - paramylon; paramylum grains; paramylon granules

Pel - pellicle

PM - plasma membrane; plasmalemma

Py - pyrenoid

RB - residual body

RER - rough endoplasmic reticulum

SER - smooth endoplasmic reticulum

V - vacuole; vesicle

VER - vesicular endoplasmic reticulum

Measurements and Other Symbols

ASA - American Standard Association

BL - blue light fluorescence

C - centigrade (Celsius unit)

ca. - approximately (Latin: *circa*)

cf - compare with (Latin: *confer*)

comm - communications

ft-c - foot candle; (ft-c = 10.76 lux)

FW - formula weight

GT - generation time

h - hours

K - Kelvin

KV - kilovolt

L/D - light/dark cycle

M - molar concentration

min - minutes

mg - milligram

MW - molecular weight

N - normal concentration

nm - nannometer

NMR - nuclear magnetic resonance

psi - pounds per square inch

rpm - revolutions per minute

μ - micron unit

μg - microgram

UVL - ultra violet light

v/v - volume per volume

W - Watts

x - normally preceded by an Arabic number to indicate the number of times a process is repeated; e.g., 2x to mean twice or two times.

INTRODUCTION

Aging, as a time-dependent process, is a universal biological phenomenon. Although it occurs in all living systems, its manifestations vary from one level of organization to another since not all organisms have the same genetic constitution, structural limitations and functional capacity (Strehler, 1967; 1969). There are, however, certain distinct expressions which are associated with aging--e.g., deterioration in form and function, decrement, departure from the norm and homeostatic failure (Verzar, 1969; Weg, 1969), all of which, so far, are found to be permanent and irreversible (Von Hahn, 1973).

Varied organisms have been studied, many on the complex organismal level such as mammalian systems and insects, but there have been only a few reports on ciliates and algal flagellates. The identification or detection of the onset of aging in those biological systems studied has been a major problem, particularly its origin at the molecular, ultrastructural and cellular levels. Its extent and effects, the possibility of measuring such changes, its mechanisms, the presence or absence of a common denominator are among other problems involved in the study of aging (Strehler, 1969; Weg, 1969; Woolhouse, 1969).

Strehler (1962) proposed certain criteria in order to identify the presence of a "normal ageing process" in biological systems. These criteria were enumerated by Von Hahn (1973) at the 9th International Congress of Gerontology, Kiev, Russia.

> First, it must be universal: all individuals of the species or population under investigation must undergo the changes observed, or predicted by the hypothesis. This criterion is extremely difficult to fulfill, as of necessity the experimenter must take a sample of his population. The proper choice of a sample which is really characteristic of the whole population is one of the most difficult problems in biology in general, and in gerontology even more, since individual variability in all biological parameters increases progressively with age even in the most strictly inbred populations, and makes the calculation of "mean values" and "averages" of any sample of individuals almost meaningless. Another problem is that with increasing age fewer and fewer of the population survive. The survivors may in themselves be a very biased sample of the original population.
>
> Second, the proposed mechanism must be deleterious: this is a logical criterion, since ageing is by definition that phase of the lifespan in which a general decline of the capacity of the individual to adapt to the manifold stresses of life occurs. This criterion poses relatively few problems, since our knowledge concerning the conditions of adult life, characterized by the dynamic equilibrium of metabolism called "homeostasis" has been greatly improved by the recent advances of biochemistry and molecular biology.
>
> Third, the proposed mechanism must be progressive: this criterion too, appears logical. If ageing is deleterious and damages the organism, organ or cell, the damage will eventually accumulate and lead to the decrease in adaptability which can generally be observed for all species, organs or cells with advancing chronological age. Cells and organs have "repair" mechanisms built into their metabolism, which can eliminate many errors and damages that occur constantly.

> But these repair mechanisms clearly have a limited capacity, and in spite of their activity damage will progressively accumulate.
>
> Finally, the proposed mechanism must be intrinsic: this last criterion is perhaps the most controversial among biological gerontologists, since it seems to exclude a priori all external causes of ageing. On the other hand, it is almost a semantic question to separate "intrinsic" from "extrinsic" causes of ageing. For example, let us consider the effect of ionizing or cosmic radiation. This is without doubt an extrinsic event. When such radiation passes through a cell, it can collide with atoms and cause certain quite specific chemical reactions. Since cosmic rays are not limited geographically and their effect is influenced only by altitude, these special chemical events will occur in every type of animal cell, in every type of animal or plant. The radiation-caused damage will probably occur at a constant rate throughout the lifespan of each individual. Since the event itself (the "hit" in the cell) cannot be observed, and since similar chemical events can be caused by internal chemical mechanisms, one can with good justification say that the primary event is intrinsic as far as ageing is concerned. (Von Hahn, 1973)

There are many research approaches to the study of aging. The major ones can be classified as follows: (1) molecular and biochemical, (2) developmental, consisting of morphology and physiology, (3) genetic and (4) phyletic. Each approach has made considerable contributions of its own (Tables I-IV) most of which tend to support the two proposed major mechanisms of aging : (1) aging program and (2) error accumulation (Orgel Hypothesis) (Von Hahn, 1973).

There are, however, only a few reports of aging in unicellular organisms such as the algal flagellates,

TABLE I

PERTINENT LITERATURE ON MOLECULAR AND BIOCHEMICAL APPROACHES TO STUDIES OF AGING

Year	Author	Contribution
1961	Riemschneider, R. et al.	Suggested that amino acid content in the blood is age-dependent and can be normalized by cysteine.
1963	von Hahn, H. P., and F. Verzar	Reported physical alteration in the DNA in systems of advancing age.
1964	Oota, Y.	Observed RNA in senescing plant cells exhibit "marked structural instability of ribosomes"
1964	Sinex, F. M.	Reported role of cross-linkage of collagens in maturation and senescence in man and mice.
1967	Houck, J. C. et al.	Reported increased cross-linkage of collagen with age.
1967	Wulff, V. J. et al.	Studied RNA content of tissues of rodents of different ages.
1967	Medvedev, Zh. A.	Reported alterations in DNA, RNA and proteins, with increasing age of test systems.
1967	Alexander, P.	Observed accumulation of DNA lesions in highly differentiated post-mitotic cells which may not have repair systems. Interference with proteins and RNA synthesis causes cellular aging and death.

TABLE I (continued)

Year	Author	Contribution
1969	Atkin, R. K. and B. I. S. Srivastava	Observed changes in soluble protein of excised barley leaves during senescence and after treatment with kinetin.
1969	Holliday, R.	Tested Orgel's Hypothesis by introducing non-toxic analogues that reduce amino acid incorporation, increasing senescence and "errors" in protein synthesis in Aspergillus, Neurospora and Podospora.
1970	Pelc, S. R.	Calculated loss of function or death as due to aging of metabolic DNA.
1971	Adelman, R. C.	Suggested that enzymatic induction in response to environmental changes is a good biochemical expression of aging.
1971	Miguel, J.	Observed decrease in regularity of cell formation as organisms (Drosophila) age. Varied nuclear size, intensity of staining and increase in lipofuscin pigment shown.
1972	Cristofalo, V. J.	Observed decrease in RNA synthesis, decrease in number of cells in population capable of incorporating radiolabeled DNA precursor; increase in heterogeneity of cell population shown by varied cell size, organelle alteration and change in mitotic capacity.

TABLE I (continued)

Year	Author	Contribution
1972	Cutler, R. G.	Reported the occurrence of spontaneous DNA mutation in sequences, mechanical breakage of DNA during mitosis, possibly due to oxidative effect of free radicals on membranes.
1972	Gelfant, S. and J. Graham Smith, Jr.	Described aging as a progressive conversion of cycling to non-cycling cells in cells capable of proliferation like human fibroblasts, using autoradiography.
1972	Lewis, C. M.	Reported that starvation results in decreased rate of formation of altered proteins; suggested that restrictive growth conditions may prolong life.
1973	Bermiller, J. N. et al.	Observed variable protein synthesis in Zea mays L.
1973	Buetow, D. E. and P. S. Gandhi	Observed decrease in protein synthesis in microsomes isolated from senescent rat liver.
1973	Wright, R. D. et al.	Reported loss of specific tRNA during senescence of pea and soybean leaves associated with loss of tRNA in the chloroplasts.

TABLE II

PERTINENT LITERATURE ON DEVELOPMENTAL APPROACH TO STUDIES OF AGING

Year	Author	Contribution
1908	Minot, C. S.	"Natural death"--a consequence of cell differentiation.
1926	Murneek, A. E.	Removal of flowers and fruits delays onset of senescence.
1927	Molish, H.	Sudden death in many crop plants and desert ephemerals due to formation and ripening of fruits.
1937	Ingle, L., et al.	Physical and environmental functions affect life span of Daphnia longispina (waterflea).
1939	McCay, C. M. et al.	Rats remained immature up to 900 days when kept on low-calorie diet.
1953	Hämmaerling, J.	In Acetabularia, formation of a cup or grafting of a cup to a young cell initiates nuclear division followed by death, even if the cup were removed.
1956	Wareing, P. F. and D. M. Roberts	In Robinia pseudoacacia, inhibiting influences of leaves are responsible for photoperiodically induced death and shedding of buds.
1962	Maynard Smith, J.	Drosophila lives longer when maintained at 20° C than at 26° C.

TABLE II (continued)

Year	Author	Contribution
1965	Fanestil, D. D. and C. H. Barrows	Aging in rotifers dependent on temperature and food intake.
1965	Hayflick, L.	Experimented on limited lifespan of human diploid cell strains *in vitro*.
1967	Osborne, D. J.	Hormonal regulation of leaf senescence in *Combretum*, *Phaseolus*, *Rhododendron* and *Ligustrum*.
1967	Park, D. and D. M. Robinson	Fungal hormone that controls internal water distribution in the cell associated with aging in: *Aspergillus niger*, *Fusarium oxyporum*, *F. solani*, *Mucor plumbens*, *Cunninghamella elegans*, *Abscidia glauca* and *Physarum* sp.
1967	Woolhouse, H. W.	*Perilla frutescens* (SDP), normally an annual, may survive for many years in vegetative state. Color changes in leaves accompany senescence.
1968	Hay, R. J. *et al.*	Division potential of cells in continuous culture compared to cells subcultured after maintenance in stationary phase.
1971	Butler, R. D. and E. W. Simon	Reviewed ultrastructural aspects of senescence in plants.

TABLE II (continued)

Year	Author	Contribution
1971	Curtis, H. J. and J. Tilley	Repeated cell division does not lead to chromosomal deterioration and does not support limited division hypothesis of Hayflick. (Somatic cells of man and rats were used.)
1971	McHale, J. S. et al	Human embryonic diploid fibroblast cultures may not be related to cell doubling potential but may be a function of total metabolic time in vitro.
1974 a,b	Lockshin, H. A. and J. Beaulation	Cytochemical appearance of lysosomes associated with muscle breakdown in Antheraea polyphemus and Philosamia cynthia (moths).
1974	Rose, R. J.	Dilution of cytoplasmic ribosomes reduced nucleolar activity associated with plant cell differentiation following termination of meristematic growth in Avena coleoptiles.

TABLE III

PERTINENT LITERATURE ON GENETIC APPROACH TO STUDIES OF AGING

Year	Author	Contribution
1901	De Vries, H.	High proportions of phenotypic variants derived from old Oenothera seeds compared to fresh ones. (Evidence of altered hereditary constitution with age.)
1936	Nawashin, M. S. and H. Gerassimova	High frequency of chromosomal aberration in roots of old seeds of Crepis.
1957	Kallman, F. J.	Direct relation between life span of parents and progeny.
1961	Clark, A. M. and M. A. Rubin	Radiation reduces life span in Habrobracon (wasp) causing recessive mutations.
1966	Curtis, H. J.	Frequency of chromosomal abnormality increases with aging and is accelerated by radiation.
1969	Woolhouse, H. W.	Some insects and short-lived plants have programmed genetic life span and death.
1970	Wanscher, J. H.	Genic time factor (Activator) present in the development of juvenile and adult stages of some plants and animals. Highly significant differences can be noted, particularly in systems that undergo metamorphosis.

TABLE III (continued)

Year	Author	Contribution
1971	Baird, M. B. and A. M. Clark	Life span decrease in Habrobracon males when exposed to x-rays; a single gene mutation could have mimic termination of an adult development.
1971	Goldstein, S.	Reviewed hereditary disorders in man associated with aging.

TABLE IV

PERTINENT LITERATURE ON THE PHYLETIC APPROACH TO THE STUDY OF AGING

Year	Author	Contribution
		Plants
1927	Molish, H.	Compiled data on longevity of plants including Pinus aristata (bristle cone) with 4900 growth rings.
1958	Stebbins, G. L.	Studied subfamily Cichoreae (Compositae) and illustrated tendency toward short life span in relation to environmental instability within taxonomic groups.
1960	Leopold, A. C.	Observed onset of senescence in annual plants hastened by presence of seeds in Spinacea oleracea (spinach). Male plants of dioecious species that lack fruits have slower aging process.
1965	Allsopp, A.	Observed progressive heteroblasty with increasing age in Gossypium hirsutum (cotton), and Campanula rotundifolia.
1965	Ehrendorfer, F.	Observed that 300 species in 10 genera of Dypsaceae are perennial but in unstable habitat--semi-desert annual species have evolved; Cephalaria, Kaoutia, Pterocephalus and Scabiosa.
1966	Krizek, D. I. et al.	Observed that particular photoperiods affect leaf senescence of some plants.

TABLE IV (continued)

Year	Author	Contribution
1967	Carr, D. J. and J. S. Pate	Reported that leaf senescence occurs after small plantlets arise from vegetative propagules of Bryophyllum-like gemmae from leaves of mosses.
1970	Dodge, J. D.	Observed changes in chloroplast ultrastructure during autumnal senescence in Betula leaves.
1971	Miller, M. M. and N. J. Lang	Associated presence of granular inclusions and change in thylakoid configuration with aging in Gloeotrichia sp.
1973	Harris, J. B. and H. J. Arnott	Correlated fine structure of chloroplast senescence in Nicotiana tabacum (Tobacco) with rate of oxygen evolution and protein composition of plastids.
1974	Caponetti, J. D.	Observed fluctuating levels of soluble proteins; decrease in chlorophyll content with age in Osmunda cinnamomea (cinnamon fern); treatment with kinetin retarded chlorophyll breakdown.
1974	Cran, D. G. and J. V. Possingham	Suggested that chloroplast degeneration involves loss of thylakoid membranes and appearance of osmophilic granules in spinach.
		Animals
1903	Kellog, V. L. and R. G. Bell	Reported that underfeeding extended the lifespan of silkworms.
1915	Child, C. M.	Observed that Planaria (flatworm) shrinks when starved but can be rejuvenated when re-fed or fragmented; sexual reproduction not necessary for the species.

TABLE IV (continued)

Year	Author	Contribution
1917	Loeb, J. and J. H. Northrup	Suggested that Poikilotherms (cold blooded) like Drosophila tend to have shorter lifespan when reared at high temperatures than at low temperatures.
1924	Belár, K.	Reported that cultures of Actinophrys sol lived for 1244 generations or 32 months.
1929	MacArthur, J. W. and W. H. T. Baillie	Found that lifespan and final size of Daphnids vary inversely with environment and temperature.
1932	Alpatov, V. V. and N. A. Gordeenko	Reported that mated male moths live longer due to differences related to exhaustion of food and other energy reserves. Mated females have shorter lives and lay eggs frequently.
1936	Rey, P.	Reported that male moths live twice as long as females.
1948	Lansing, A. I.	Observed that rotifer clones propagated from old mothers become extinct.
1950	Rockstein, M.	Found honeybee brain to have similar pattern of nerve cell depletion as human brain. Sudden deterioration evident as worker bee engages in complicated ritualistic activity before death.
1951	Schulze-Robbecke, G.	Reported manifestations of Daphnid senescence to be: reduction in ovarian activity, increase in fat bodies in intestinal epithelium and musculature.
1952	Lansing, A. I.	Observed that rotifers from old mothers have shorter lifespan until line is unable to reproduce and becomes extinct.

TABLE IV (continued)

Year	Author	Contribution
1953	Willensiek, U.	Reported that teleosts show reproductive senescence; each sex has definite lifespan.
1953	Wimpenny, R. S.	Observed that male plaice (flat fish) age faster and have shorter lifespan compared to females which continue to reproduce.
1959	Danielli, J. F. and A. Muggleton	Observed that starvation in Amoeba induces growth, division, formation of new clones and then the whole line becomes extinct.
1961	Comfort, A.	Reported that continued growth and death rate rise steadily with age in Lebistes (guppy).
1961	Robertson, O. H. et al.	Observed that salmonids die after spawning upstream. Non-migrating populations live longer and breed many times. Early castration of fish prevents migration and prolongs lifespan.
1962	Strehler, B. L.	Reported that Hydra has irregular senescence depending on food supply.
1964	Comfort, A.	Compiled lifespan tables of different organisms.
1966	Lloyd, M. and H. S. Dybos	Reported that life cycle of Magicicada fluctuates with changes in environment.
1970	Herold, R. C. and N. D. Meadow	Found pigment and membrane-bounded organelles with inclusions in a rotifer; acid phosphatase correlated with increasing number of inclusions.
1972	Epstein, J. et al	Correlated increase in lipofuscin pigment with appearance of acid phosphatase in intestinal epithelium of Caenorhabditis briggsae (nematode). Lipofuscin regarded as products of peroxidation of cellular constituents.

TABLE IV (continued)

Year	Author	Contribution
1972	Kritchevsky, D.	Found increase in lipids with age in man and rats.
1973	Spence, A. M. and M. M. Herman	Suggested premature aging in man (progeria) possibly due to developmental disturbance or degenerative process in mesodermal tissues rather than accelerated aging process.

possibly due to the misconception that unicellular organisms are potentially immortal (Danielli, 1956). Age-related changes, however, have been identified in a number of stationary-phase cells, non-dividing highly differentiated cells with determinate pattern of growth and development (Frolkis, 1969). Table V shows a partial survey of significant observations noted in the life span of Euglena, while Table VI presents a partial survey of pertinent literature on aging in algal flagellates and other allied organisms on which the present study is based.

It is the purpose of this investigation to (1) identify the age-related changes in populations of the unicellular alga Euglena gracilis strain 'Z' in a developmental sequence of its life span and (2) to evaluate and to correlate ultrastructural and cytochemical observations with known biochemical activity, chemical composition and cellular metabolism that might occur in such a cellular system as cultures age.

TABLE V

PERTINENT LITERATURE ON GROWTH OF *EUGLENA GRACILIS* KLEBS

Year	Author	Contribution
1902	Dangeard, P. A.	Noted nocturnal periodicity of mitosis in *Euglena gracilis*.
1926	Baker, W. B.	Observed nocturnal periodicity in *Euglena* grown in split pea infusion.
1952	Cramer, M. and J. Myers	Made quantitative measurements of growth characteristics of both light-grown and dark-grown species using turbimetric methods; noted some essential nutrients for growth of *Euglena*.
1957 a,b	Corbett, J. J.	Studied cell counts of *Euglena gracilis* (Damon strain); determined cell volumes from microscopic measurements.
1959b	Leedale, G. F.	Observed time-scale of mitosis; established periodicity of different euglenoid flagellates.
1960	Cook, J. R. and T. W. James	Studied light-induced synchrony; observed inhibitory effects of light to mitosis; generation time in different periods and light conditions.
1962	Buetow, D. E. and B. H. Levedahl	Observed decline in cellular content of RNA, protein and dry weight during logarithmic growth phase.
1962	Gross, J. A. and T. L. Jahn	Reported "monster" *Euglena* with more than one nucleus grown at high temperatures; also made cell counts of their growth behavior.
1963	Cook, J. R.	Observed occurrence of "giant cells" at 3000 ft-c; noted that growth increases linearly with light intensity.
1963	McCalla, D. R.	Observed depletion of nitrogen in growth medium as cells reach stationary phase.

TABLE V (continued)

Year	Author	Contribution
1964	Wilson, B. W. and B. H. Levedahl	Studied synthetic and division rates of Euglena gracilis grown in batch cultures; made some quantitative determination on dry weight, protein, carbohydrate, RNA and DNA content at exponential phase.
1966	Edmunds, L. N., Jr.	Demonstrated circadian, endogenous nature of rhythmic cell division under certain conditions of varied illumination and temperature and L/D cycles.
1968	Cook, J. R.	Provided detailed study on culture and growth of Euglena; suggested techniques in quantitative studies.
1970	Walther, W. G. and L. N. Edmunds	Observed periodic increase in deoxyribonuclease activity during cell cycles in synchronized Euglena.
1971 a,b	Cook, J. R.	Established periodicity of cell division in a synchronized culture; suggested possible factors in bringing about synchrony.
1971	Edmunds, L. N., Jr., et al.	Reported phasing or synchronizing cell division by L/D cycles and temperature.
1971	Votta, J. J., T. L. Jahn and B. H. Levedahl	Determined changes in intracellular pH of Euglena gracilis during growth cycle; observed role of succinate in causing reversal of stationary phase to logarithmic phase.

TABLE VI

PERTINENT LITERATURE ON AGING STUDIES IN ALGAL FLAGELLATES AND CILIATED PROTOZOANS

Year	Author	Contribution
1889	Maupas, E.	First report on intraclonal events during maturation and senescence in ciliates; occurrence of patterned sequential changes in ciliates.
1919	Calkins, G. N.	Uroleptis mobilis underwent senescence and finally became extinct if the cultures were kept isolated without conjugation.
1921	Hartmann, M.	Kept Eudorina elegans in active asexual reproduction for 8 years.
1929	Jennings, H. S.	First detailed report on the life cycle of ciliates.
1937	Sonneborn, T. M.	Interclonal and intraclonal hereditary variations in the mating types of Paramecium.
1942	Jennings, H. S.	Senescence in Paramecium accompanied by decrease in fission rate, appearance of abnormal cell types and reduction in the frequency of viable progeny.
1942	Kimball, H. F.	Interclonal conjugation followed by senescence in Euplotes.
1954	Sonneborn, T. M.	Decrease in fission rate in Paramecium; loss of ability to undergo autogamy or to survive after autogamy.
1954	Tartar, V.	Increase in anomalies in organelle structure after regeneration in starved Paramecium.

TABLE VI (continued)

Year	Author	Contribution
1955	Nanney, D. L. and P. A. Caughey	Progressive reduction in percentage of viable progeny in aging alones of Tetrahymena.
1956	Cirillo, V. P.	Reduction in rate of enzymatic induction of fatty acid oxidase in aging Polytoma.
1957	Nanney, D. L.	Presence of genetic abnormality and nuclear degeneration in aged cultures of Tetrahymena.
1959	Padilla, G. M. and D. E. Buetow	Reduction in number and size of paramylon bodies, decrease in rates of oxygen utilization and other comparable aging changes in the ultrastructure of streptomycin-bleached E. gracilis var. bacillaris.
1960	Gibbs, S. P.	Report on degenerated plastids, dense bodies, cytolysomes and vesicles of lipoidal nature in Euglena gracilis.
1960	Nanney, D. L. and M. A. Rudzinska	Age-dependent variations in Paramecium bursaria cells; loss of ability to survive conjugation until eventually all cells entering sexual reorganization die (clonal alteration).
1961	Novikoff, A. B.	Unidentified vesicles in euglenoid cells compared with lysosomes of animal tissues.
1961	Rudzinska, M. A.	Morphological changes in organelles with accumulation of age pigments in aging cells of Tokophrya infusionum.
1962	Siegesmund, K. A. et al.	Altered mitochondrial cristae, formation of concentric membrane configurations in chloroplasts in aging Euglena.
1962	Buetow, D. E. and B. H. Levedahl	Decline in RNA and proteins with age in Euglena.

TABLE VI (continued)

Year	Author	Contribution
1963	Brown, J. S.	Changes in absorption and separation of chlorophyll a in Euglena during aging.
1963	De Duve, C.	Proposed Lysosome Concept involved in aging; proposed similar activity in aging Euglena cells.
1963	Lang, N. J.	Dense bodies of unknown composition and origin in Polytoma described.
1963	Ringo, D.	Dark bodies, irregular in shape, possibly a cytolysome in non-dividing cultures of Astasia longa.
1964	Brandes, D. and F. Bertini	Role of Golgi apparatus in formation of cytolysomes after onset of starvation in Euglena.
1964	Brandes, D. et al.	Lysosomes involved in cellular lytic process observed in carbon-starved Euglena.
1964	Malkoff, D. B. and D. E. Buetow	Ultrastructural changes comparable to aging changes in carbon-starved Euglena.
1965	Blum, J. J.	Presence of acid phosphatase in Euglena gracilis using Gomori technique of cytochemical determination.
1965	Brandes, D.	Apparent mode of formation of pure lysosomes in Euglena gracilis.
1965	Brody, M. et al.	Changes in chlorophyll formation and lamellae as a function of age in Euglena gracilis.
1965	Leedale, G. P. et al.	Brownish-orange droplets in phospholipid vesicles in old cultures of Euglena spirogyra.

TABLE VI (continued)

Year	Author	Contribution
1965b	Sommer, J. R. and J. J. Blum	Cytochemical localization of acid phosphatase in Euglena.
1966	Siegel, R. W. and K. Heckmann	Intraclonal conjugation (selfing) occurring during senescence in Euplotes sp.
1967b	Leedale, G. F.	Presence of insoluble residues, brown pigment granules similar to lipofuscin pigments in human liver cells, correlated with age in euglenoids.
1968	Schuster, F. L. et al.	Increase in lipids and coalescence of vacuoles with age in Ochromonas.
1969	Grusky, G. E. and S. Aaronson	Decrease in mitochondrial activity, increase in activity in lysosomes of Ochromonas with age.
1970	Esteve, J. C.	Presence of acid phosphatase in Paramecium caudatum.
1970	Lucas, I. A. N.	Presence of multivesicular bodies, "Corps de Maupas" organelle compared to lysosomes in Cryptomonas.
1970	Moore, J. et al.	Formation of storage vesicles prior to encystment in stationary cultures of Polytomella.
1971	Aaronson, S., et al.	Abscised concentric lamellae from chloroplasts, resembling myelin figures in aging Ochromonas cultures.
1971	Messer, G. and Ben-Shaul, Y.	Increase in lipids in senescent cells of dinoflagellate, Peridinium westii.
1971	Schwender, U.	Pigments in aging cultures of Euglena gracilis.

TABLE VI (continued)

Year	Author	Contribution
1972	Bibby, B. T. and J. D. Dodge	Dense vacuoles accumulate, possibly autophagic vacuoles in encysting dinoflagellates.
1972	Palisano, J. R. and P. L. Walne	Accumulation of acid phosphatase activity in maturing phase of dictyosomes, abscission of "whorled lamellae" from chloroplasts in Euglena granulata (Klebs) Lemm.
1974	Schuster, F. L. and B. Hershenov	Increase in paramylon granules in aged cultures of Khawkinea quartana.

I. MATERIALS AND METHODS

Growth Conditions

Culture media. Cells of Euglena gracilis Klebs, strain 'Z', were obtained originally from the Culture Collection of Algae at Indiana University (No. 753) and were grown in both defined and undefined liquid, solid and biphasic culture media. The defined culture media used consisted of Euglena Broth BM (Difco), modified Cramer and Myers medium (Cramer and Myers, 1952) and Euglena Agar (Difco). The undefined media were mostly biphasic, consisting of sterilized soil-water or soil in combination with various growth media of varying proportions, and other vitamin as well as carbon and nitrogen sources (Appendix A, page 165).

Axenic culture methods. Axenic cultures of Euglena gracilis 'Z' were obtained by using the following: (1) isolation of selected cells with micropipette; (2) plating with spray technique onto agar plates of previously washed and sonicated cells (Wiedeman, Walne and Trainor, 1964), and (3) standard dilution methods.

At regular intervals, aliquots were inoculated into tubes containing different standard bacteriological media such as proteose peptone, nutrient broth and thioglycollate

in order to insure the axenic condition of the cultures (Appendix B, page 170).

Synchronization techniques. Axenic cultures of Euglena gracilis 'Z' were synchronized (1) by isolation of single cells of the same size, shape and mitotic condition; (2) by differential centrifugation at ca 2000 rpm to separate young from old, dying cells and (3) by means of light/dark shifts at 16/8 h light/dark cycles, with an initial light intensity of 800 ft-c and a constant temperature of 22° C (Cook, 1971).

Growth and maintenance. One ml of cells from a 7-day-old stationary phase axenic culture was inoculated in various media at weekly intervals to obtain samples of population of different ages. Liquid cultures were shaken periodically; populations were synchronized with a light/dark cycle in a growth chamber (Sherer Model RI-48-LTP), under conditions described by Cook (1971). Light was supplied by 40-W cool-white fluorescent bulbs. At the onset of stationary phase, cultures were transferred to lower light intensities of 150 and 40 ft-c.

Sampling for cell counts was done before and after the dark period with the use of either an Improved Neubauer Haemocytometer or an Electronic Coulter Counter (0-200h) (Hialeah Instruments, Inc.) Cells at stationary phase were treated

with Trypan Blue to test their viability while making the cell counts (Appendix C, page 174). Cell counts were recorded and processed on computer cards provided by The University of Tennessee Computer Center. Generation time was estimated according to the methods of Buchanan and Fulmer (1928) and Stanier, et al. (1971).

Optical Microscopy and Cytochemistry

Bright-field, dark-field, fluorescence and phase-contrast microscopy were used in observing living, stained and fixed materials for comparison with electron microscopy. Fluorescence microscopy permitted observation of auto-fluorescent properties of various organelles under blue light and ultraviolet light (UVL) at wavelengths of 420 nm and 365 nm, respectively.

General staining procedure for thick sections. The quality of fixation and embedding, the general condition of cells and selection of suitable areas for ultra-thin sectioning were determined with bright-field optics on thick sections prior to examination under the electron microscope. Thick sections (ca. 0.3-2.0 μ) of cells embedded in epoxy, maraglas and araldite resins were cut with glass knives and were carefully floated on a small drop of Azure II staining solution (Appendix C) on slides pre-cleaned with chromic acid. The stain was evaporated on

a hot plate (70-80° C) until the sections were flattened and dried (Jeon, 1965). The sections were either examined immediately or mounted for later examination.

Acid phosphatase. Cells were fixed in 2.5% glutaraldehyde (Eastman) with 0.3 M phosphate buffer. The pH was adjusted according to the final pH of the growth medium (Appendix A, page 165). Samples were incubated in Gomori reaction medium with sodium-β-glycerophosphate (Sigma Chemical Co.) as substrate (Appendix C, page 174) or in the presence of naphthol AS-BI substrate (Sigma Chemical Co.) with hexazonium pararosanilin (HPR) as coupling agent (Appendix C). Incubation times varied from 30 minutes to 17 hours. Preliminary results showed the samples from old cultures to be more sensitive to the substrate than those from younger cultures. Incubation periods as long as 20 hours have been used by other workers (Holcomb, Hildebrandt and Evert, 1967). Acetate buffer (0.05 M) was used at pH 5.0, and the incubation temperature was maintained at 37° C in a water bath. Controls consisted of cells kept at 90° C for 5 minutes and then incubated in standard medium either without substrate or with 10^{-4} N sodium fluoride (NaF) inhibitor (Appendix C). Fresh mounts were made for light microscopy, and other cells were processed for subsequent examination with the electron microscope.

Succinate dehydrogenase (SDH). Cells were centrifuged at 2000 rpm for 3 minutes. The supernatant was decanted and replaced with SDH reaction medium which consisted of sodium succinate substrate and nitro-blue tetrazolium (NBT) or triphenyl tetrazolium (TPT) (Dajac Lab) as coupling agents. Phosphate buffer (0.5 M) was used at pH 7.8, and a temperature of 37° C was maintained in a water bath. Incubation times varied from 30 minutes to 6 hours until a color reaction was visible. Controls were prepared by adding 0.05 M sodium malonate inhibitor to the complete reaction medium and incubated as described (Appendix C, page 174). Cells were mounted on glass slides and were examined and photographed using bright-field optics according to the methods described below (page 30).

Fluorochrome labelling. Either Acridine Orange (AO) (Sigma Chem. Co.) or Quinacrine Mustard (QM) (Dr. Archer, Sterling Winthrop) was added (0.2 mg) to the Euglena broth BM cultures just prior to the dark period (Appendix C, page 174). Flasks were covered with aluminum foil to insure darkness. Cells without fluorochrome served as controls. After 3-8 hours, the cells were washed with sterile growth medium in order to remove background fluorescence and were then fixed in 2.5% glutaraldehyde buffered with 0.2 M sodium cacodylate, pH 7.2. For the

demonstration of lipid fluorescence, cells were centrifuged at 2000 rpm for 3 minutes. The supernatant was discarded and replaced with benzpyrene reagent (Sigma Chem. Co.). The cells were allowed to stand for 1 hour in test tubes wrapped in aluminum foil and were then washed in sterile distilled water and fixed, according to the method previously described (Appendix C, page 174). The fixed cells were mounted on glass slides and were examined with blue light fluorescence and UVL at wavelengths of 420 nm and 365 nm, respectively (Appendix C).

Photomicrography. A Nikon camera (Microflex Model) was mounted on a Wild M-20 binocular research microscope equipped with an AFM adapter. Black and white photographs were taken using Panatomic X films with either a blue or a green filter. Colored photographs were taken using high speed Ektachrome B (Daylight or 3200 K Tungsten) and Kodachrome II Professional films in combination with appropriate color-balancing filters.

Electron Microscopy and Cytochemistry

General procedure. Preparation of specimens for electron microscopy involved modifications in each of the following standard procedures: (1) fixation, (2) cytochemical treatment, (3) dehydration, (4) infiltration,

(5) embedding, (6) polymerization, (7) ultramicrotomy, and (8) poststaining prior to observation under the electron microscope (Pease, 1964; Sjöstrand, 1967; Kay, 1965; Hayat, 1970; Hayat, 1972). Fixatives commonly used were glutaraldehyde and paraformaldehyde in combination with various buffers. Cytochemical treatment depended on the nature of chemical or enzyme to be tested, while post-fixation utilized buffered 2% osmium tetroxide at room temperature. Dehydration was effected in a graded ethanol series, followed by two quick changes of either anhydrous ethanol (100%), acetone or propylene oxide (PO). Either Mollenhauer's (1964) or Spurr's (1969) mixtures were used for infiltration, embedding and polymerization procedures (Appendix D, page 187). Ultrathin sections were made using either glass or diamond knives (DuPont) on either a Porter Blum MT-1 or a Sorvall MT-2 ultramicrotome. Sections were mounted on copper grids (variable mesh) which were uncoated or coated with formvar and carbon. Post-staining was done with either 2% water-based or methanolic uranyl acetate followed by lead citrate (Appendix E, page 191). Thin sections were examined with a JEM 6-C electron microscope at an accelerating voltage of 80 KV or with a Zeiss 9S-2 transmission electron microscope at 50 KV. Photomicrographs were taken using 6.5 x 9 cm contrast glass plates (Kodak, JEM 6-C) or 7 x 7 cm x 0.007 sheet film (DuPont COS-7 Graphic Arts

Film or Agfa-Gevaert Scientia film 23 D 56-46010076).

Lysosomes. Either fixed whole cells or unstained ultrathin section mounted on copper grids were incubated in freshly prepared Gomori acid-phosphatase reaction medium. Sodium-β-glycerophosphate was used as substrate at pH 5, 37° C and was later coupled in 2.0-0.5% ammonium sulfide. Controls consisted of cells heated to 90° C for 5 minutes and then incubated in the reaction medium either without substrate or with 10^{-4} M sodium fluoride (Appendix E, page 191).

Microbodies. Fixed cells previously stabilized in AMP buffer were incubated in DAB reaction medium, using H_2O_2 as substrate at pH 9, 37° C. Controls were heated to 60° C for 5 minutes in a water bath before incubation. Amino triazole (AT) inhibitor was added to the above DAB reaction medium (Appendix E).

Mitochondria. The reaction medium used to demonstrate oxidase enzyme activity of mitochondria, particularly cytochrome oxidase (CyO), consisted of lowering the pH of DAB medium described previously to pH 7.8. H_2O_2 was also omitted. The inhibitor used for controls was sodium azide or sodium cyanide, diluted 1:1 with DAB reaction medium (Appendix E).

For the visualization of SDH activity, the reaction medium consisted of BSPT dissolved in DMF, with succinate as substrate. Incubation was at 37° C, pH 7.2. Controls consisted of omission of the substrate or addition of malonate inhibitor. Incubation was quickly followed by osmication in Tris buffer (Appendix E, page 191).

Plasma membrane and cytoplasmic vacuoles. A final concentration of 0.1% Ruthenium Red (RR) (Dajac Lab.) was added to 2.5% glutaraldehyde buffered with 0.5 M sodium cacodylate, pH 7.2, room temperature. Cells were rinsed in 0.05 M sodium cacodylate and osmicated with 0.1% RR, room temperature (Appendix E).

II. RESULTS

Light Microscopy

Growth and morphology. Plate I, Fig. 1, shows a typical growth curve of Euglena gracilis 'Z' under the conditions described. Cultures underwent a lag phase of ca. 40 h before entering positive growth acceleration at log phase. The GT at the log phase was ca. 6-8 h as doubling of cell counts occurred after every dark period. Cell division was initiated ca. 2 h before each period. Plate II, Figs. 2 and 4, represent cells sampled 8 h after the dark period in the log phase (ca. 50 h), wherein most cells were at cytokinesis following mitosis. In the middle of the light period, most cells remained "at rest" or underwent mitotic inhibition by light. In highly synchronized populations, most cells were homogeneous in size, shape, motility and physiological activity (Plate III, Fig. 6).

At ca. 240 h, stationary phase began as GT reached infinity. At the transition of log-stationary phase, various cell types were observed. Some of the highly motile elongated cells from log phase became spherical and decreased in motility. A small number of cells was still capable of dividing. In the stationary phase, after

Plate I. Growth curve of Euglena gracilis Klebs strain 'Z'.

Fig. 1. The "typical" growth curve of Euglena gracilis 'Z' shows a lag period of ca. 40 h prior to onset of logarithmic phase. Doubling of populations occurred after every dark period during the log phase with a generation time (GT) of ca. 6-8 h. Stationary phase began at ca. 240 h. Circles indicate cell counts during the light period; dots indicate cell counts during the dark period.

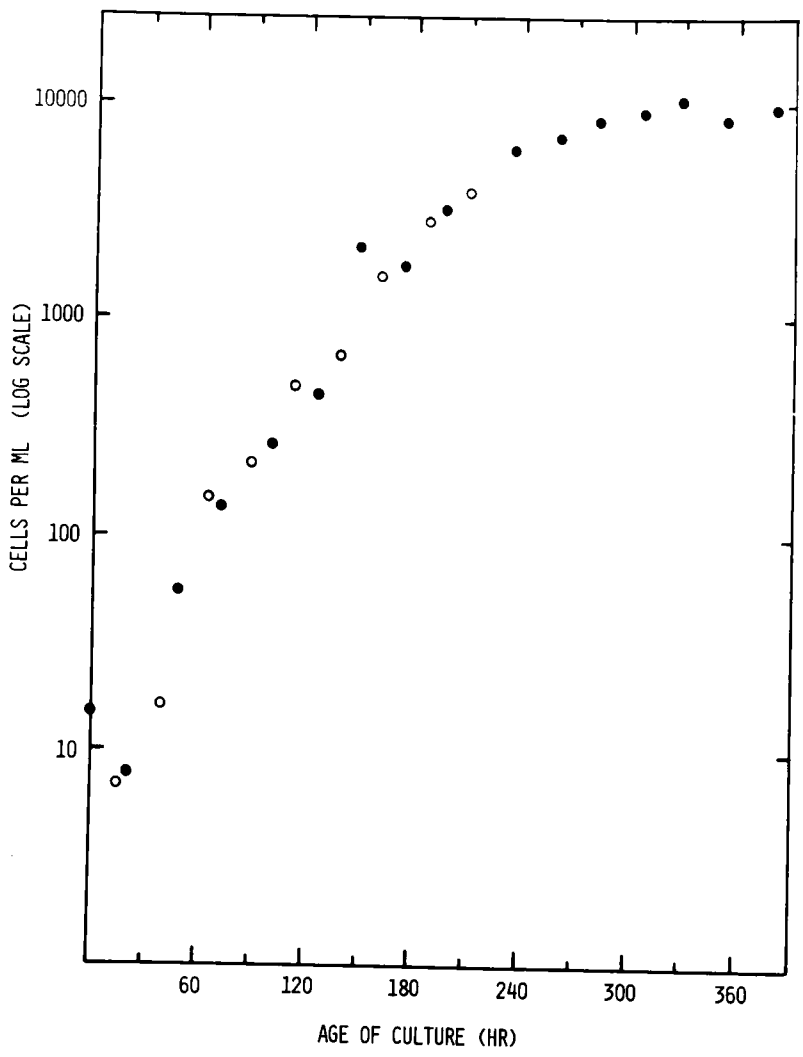

10000
1000
100
10
CELLS PER ML (LOG SCALE)
60
120
180
240
300
360
AGE OF CULTURE (HR)

Plate I

Plate II. Cells in logarithmic phase.

Fig. 2. Log-phase cells sampled 6 hours after the onset of the dark period. SDH reaction of mitochondria following treatment with NBT is localized in blue threadlike-granular reticulum. X750.

Fig. 3. Close-up of cell treated with NBT. Note blue SDH reaction in threadlike-granular mitochondria at periphery of cells and some in close association with chloroplast. X750.

Fig. 4. Log-phase cells during cytokinesis treated with TPT sampled 8 hours after onset of dark period. SDH reaction is shown by faint pink-reddish color of mitochondria. X400.

Fig. 5. Close-up of cell treated with TPT. SDH reaction of mitochondria is shown in round-threadlike, pink-red structures. X750.

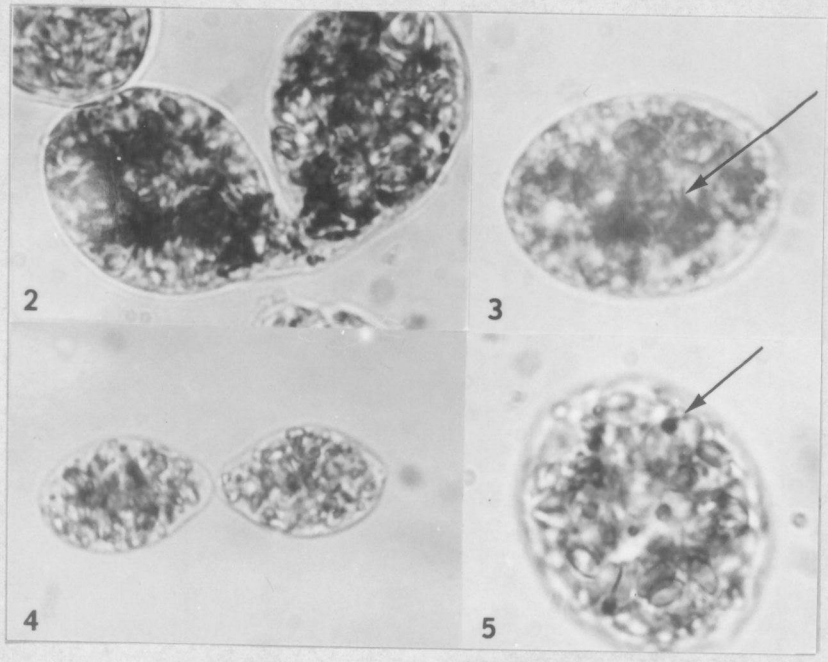


Plate II

Plate III. Log and stationary phase cells.

Fig. 6. At ca. 3-4 days, cell population is fairly synchronized. Note homogeneous population of cells similar in morphology and physiological activity. Phase contrast microscopy during light period. X75.

Fig. 7. At the mid-stationary phase (ca. 10-20 days) incipient clumping shown by aggregation of some cells. X70.

Fig. 8. Close-up of clumping phenomenon. X300.

Fig. 9. Two giant cells (from 15-day-old culture). Note paramylon granules and contractile vacuole. Phase contrast microscopy. X600.

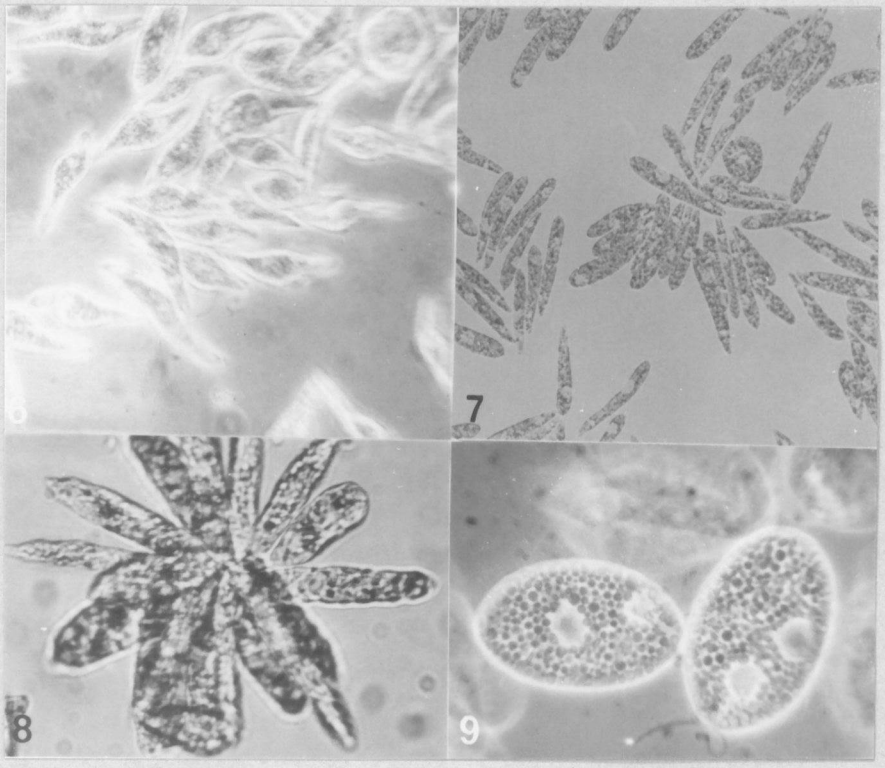


Plate III

360 h, clumping was evident (Plate III, Figs. 7-8; Plate IV, Figs. 10-12), together with the appearance of giant cells (Plate III, Fig. 9; Plate IV, Fig. 13). The giant cells were considerably larger than the "normal" cells and were less motile. A number of cells decreased in viability as revealed by their deep-staining reaction with the Trypan Blue test for viability (Plate IV, Fig. 14). Viable cells did not stain.

Concomitantly, discrete aging pigment bodies, dark orange at first, became more widely distributed with age. A corresponding change in pH of the growth medium from pH 3.6 to 7.5 was also noted in the latter part of stationary phase.

Succinate dehydrogenase. Localized sites of SDH activity in cells were either stained blue with NBT (Plate II, Figs. 2-3) or red with TPT (Plate II, Figs. 4-5). The granular-to-threadlike structures can be correlated with the various sizes, shapes and distribution of mitochondria. Positive SDH activity was also detected in some areas of the chloroplasts. Controls, containing sodium malonate inhibitor failed to show any SDH activity, depending on the physiological state of the cells. In some cases, increased, decreased or slight change in SDH activities were noted in cells from cultures of comparable ages.

Plate IV. Cytochemical treatment of log and stationary phase cells.

Fig. 10. Early clumping stage (from 10-day-old cultures). Note presence of dark-staining positive acid-phosphatase reaction at certain portion of each cell following treatment with Gomori reaction medium. X300.

Fig. 11. During late clumping stage, dark-staining reaction is denser and more widely distributed (from 15-day-old cultures). X350.

Fig. 12. No dark staining acid phosphatase reaction is evident in controls. Sodium-β-glycerophosphate substrate omitted. X350.

Fig. 13. Giant cell compared to "normal" cells. Length of a "normal" cell is ca. 50 microns; by contrast, giant cell is ca. 150 microns in length. X450.

Fig. 14. With Trypan Blue, viable cells are readily identifiable compared to the less viable and lysed cells (arrows). Dead cells take up stain more rapidly and more intensely than do viable cells. X75.

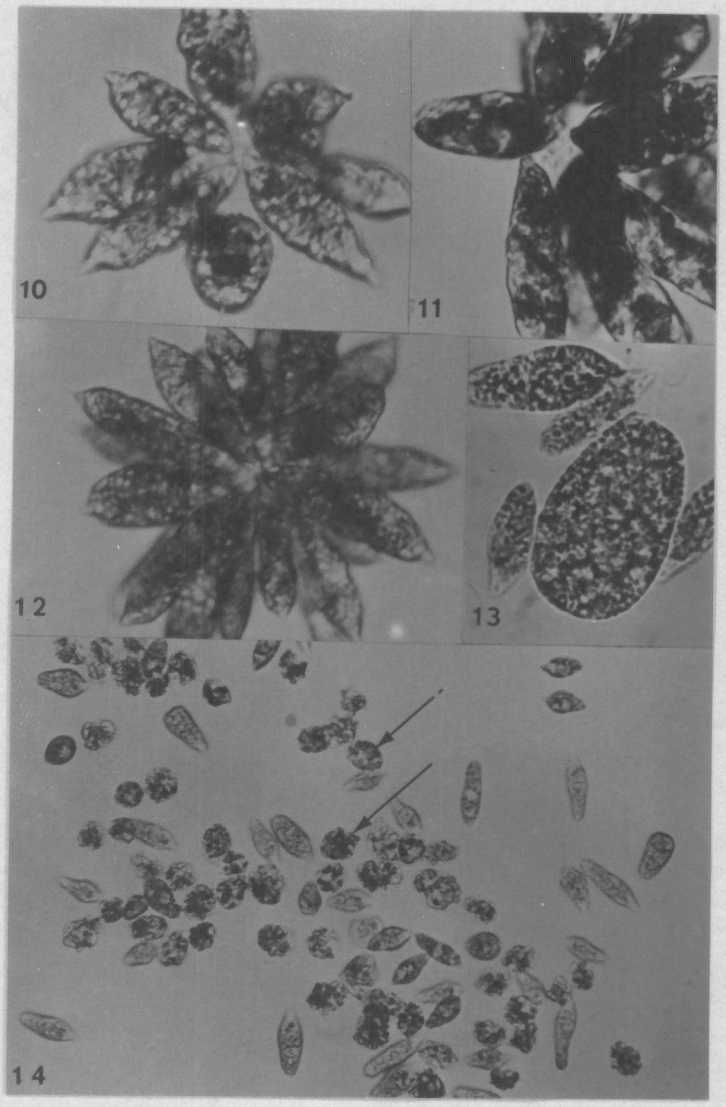


Plate IV

Acid phosphatase. Acid phosphatase reaction appeared initially as a dark precipitate near the reservoir (Plate IV, Fig. 10; Plate V, Fig. 15) and subsequently also at the perinuclear region of the cell (Plate V, Fig. 16). Discrete vesicles resembling lysosome-like bodies, appeared in later stages (Plate IV, Fig. 11; Plate V, Fig. 17) and increased in size and number, accumulating until the acid phosphatase reaction product was dispersed throughout nearly the entire cell (Plate V, Fig. 18). That this sequence of changes actually occurred and was not artifactual was further confirmed with the use of another cytochemical test which was a modification of Burstone's azo-dye technique. In this test, the acid-phosphatase-positive reaction appeared as a faint pink-to-red precipitate which localized initially near the reservoir of young cells (Plate VI, Fig. 23) and gradually became more widely distributed in the cytoplasm of cells from older cultures (Plate VI, Figs. 24-26). Some of the cells at late stationary phase (Plate VI, Fig. 27) were also deeply stained red for acid phosphatase; however, non-viable and lysed cells all gave negative reactions. The controls, treated with either acid phosphatase inhibitor (NaF) or denatured by heat with the omission of the substrate, failed to show any characteristic acid-phosphatase-positive reaction (Plate V, Figs.

Plate V. Gomori acid-phosphatase cytochemistry and sequence of Gomori acid-phosphatase reaction with increasing age of cultures.

Fig. 15. Two-day-old sample showing discrete localization of acid phosphatase precipitate (arrow) near region of reservoir. X925.

Fig. 16. Nine-day-old sample showing acid phosphatase localized at the reservoir and in vicinity of the nucleus (arrow). X750.

Fig. 17. Thirty-day-old sample showing more widespread distribution of acid phosphatase in diverse parts of cytoplasm (arrow). X600.

Fig. 18. Sixty-day-old sample showing acid phosphatase precipitate reaction throughout cell. X925.

Fig. 19. Close-up of control cell from a 2-day-old culture. Sodium fluoride inhibitor has been added to medium. X925.

Fig. 20. Close-up of control cell (from 9-day-old culture) in which sodium-β-glycerophosphate substrate has been omitted. Note absence of acid-phosphatase precipitate. X600.

Fig. 21. Several cells from which the cell in Fig. 19 was sampled show similar negative reaction with sodium fluoride. X300.

Fig. 22. Control cells without sodium-β-glycerophosphate substrate from which the cell in Fig. 20 was sampled. No acid-phosphatase precipitate is evident. X300.

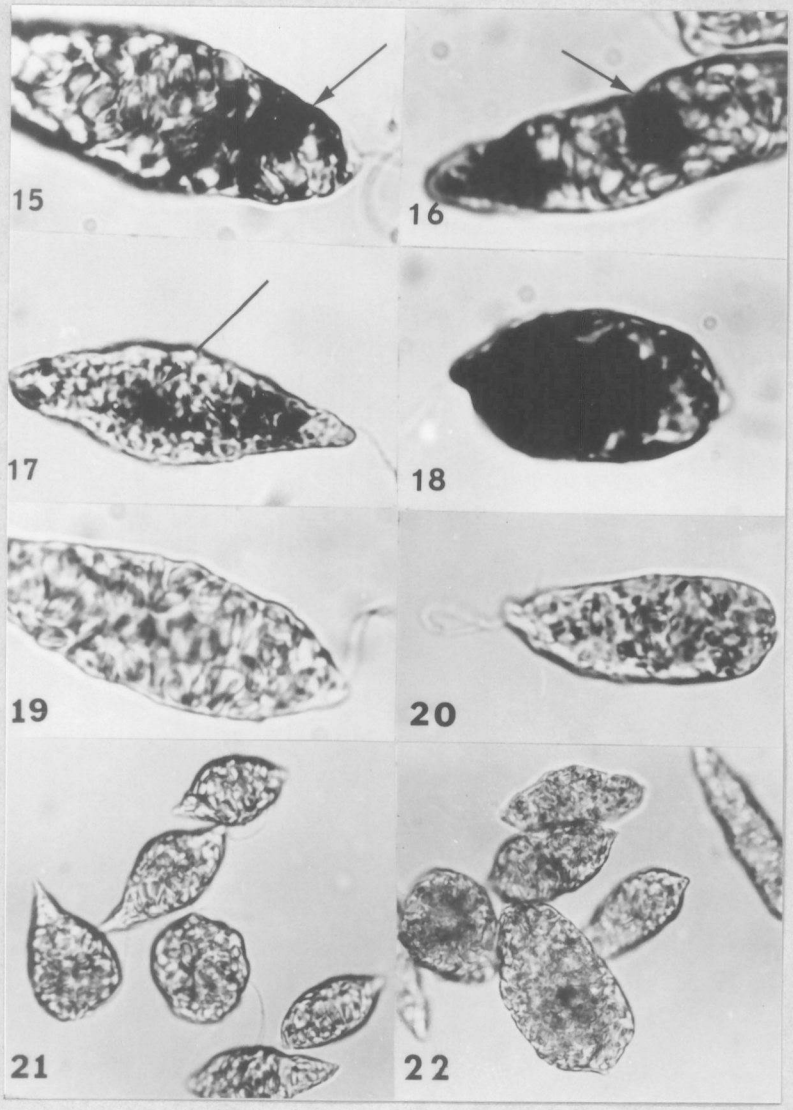


Plate V

Plate VI. Burstone's azo-dye technique for acid phosphatase localization. Sequence of reaction with increasing age of culture.

Fig. 23. Cell from 2-day-old sample treated with Burstone's azo-dye method using AS-BI substrate. Note red acid-phosphatase staining reaction (arrow), localized at reservoir region. X300.

Fig. 24. Cell from 10-day-old culture. Red staining reaction has spread around reservoir region and toward nearby nucleus. X300.

Fig. 25. Red acid-phosphatase reaction is more intense and shows more widespread distribution in cell (from 25-day-old culture). X300.

Fig. 26. Cell from 30-day-old culture showing discrete, intense, red acid-phosphatase precipitate reaction at reservoir, near nucleus and in various parts of cytoplasm. X300.

Fig. 27. Group of cells from 30-day-old sample. Some cells show red reaction for acid phosphatase; others show incipient autolysis. Viability of cells decreases at late stationary phase. X75.

Fig. 28. Control cell. AS-BI substrate has been omitted. Note absence of red staining reaction for acid phosphatase. X300.

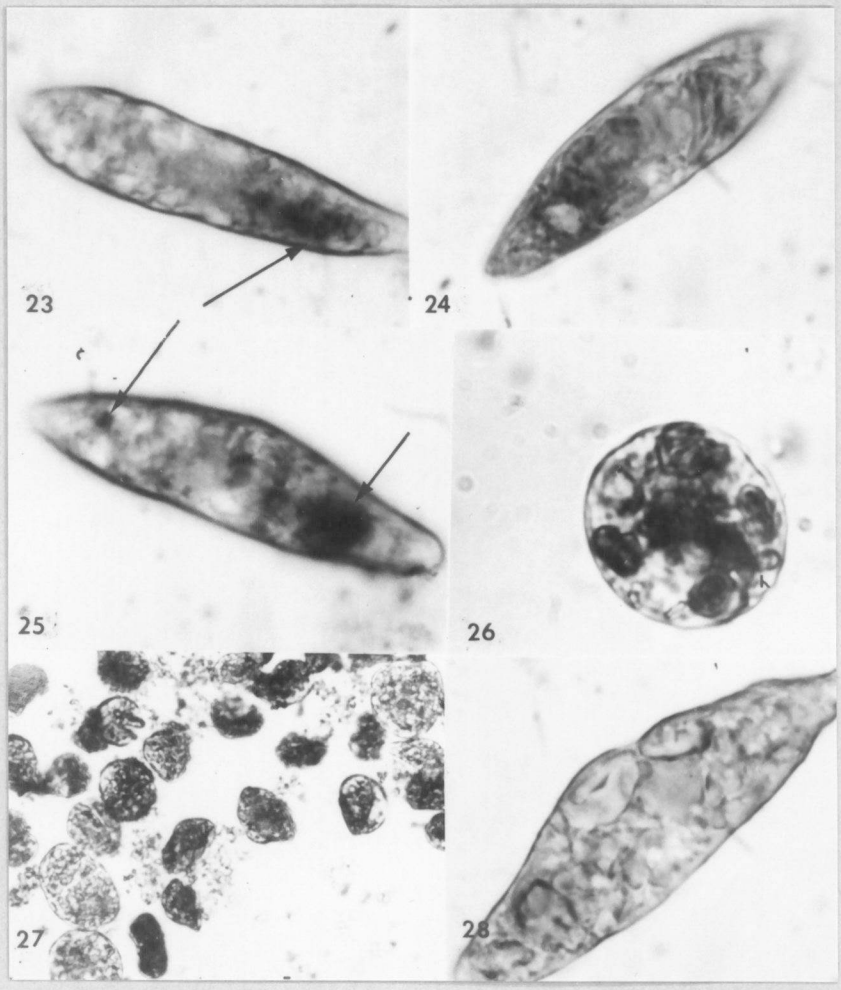


Plate VI

19-22). Results were similar in untreated cells (Plate VI, Fig. 28).

Fluorochrome labeling. Cells with chloroplasts fluoresced under blue light (BL) or ultraviolet light (UVL). Prolonged exposure, however, caused fading of fluorescence. Treatment with fluorochrome therefore became necessary in order that fluorescence properties of cell organelles could be observed for longer periods under continuous UVL. Plate VII, Fig. 29, shows a stationary-phase population treated with acridine orange (AO) and exposed to BL fluorescence. Some cells changed from motile, elongated forms to less motile round ones. Plate VII, Fig. 30, shows the same cell population under brightfield optics. With quinacrine mustard (QM) fluorescence could be observed for longer periods, compared to those treated with AO. Yellow fluorescing bodies interspersed with the bright red fluorescing chloroplasts which were faintly observed became more evident with QM treatment (Plate VII, Fig. 32). Less viable cells, however, became more yellow. Plate VII, Fig. 33, shows a dead cell exhibiting yellow fluorescence throughout.

Visualization of lipid bodies, abundant in aged cells, was enhanced with benzpyrene labeling. They occurred as bright yellow fluorescing granules (Plate VII,

Plate VII. Fluorochrome labelling and fluorescence microscopy.

Fig. 29. Fluorescence microscopy of quinacrine mustard labelled cells at transition of log-stationary phase of growth. Elongated, motile cells and spherical quiescent cells (arrow) are evident; the latter become dominant in late stationary phase cultures (ca. 20 days). X90.

Fig. 30. Same as Fig. 29 but with brightfield optics instead of fluorescence optics. X90.

Fig. 31. In cell from a 14-day-old culture, aging pigment (arrow) first appears as discrete bodies which accumulate and coalesce to form larger bodies in older cells. X300.

Fig. 32. At mid-stationary phase (ca. 10 days) viability of cells decreases. With acridine orange (AO) labelling, less viable cells (arrow) do not show the typical bright-red fluorescence of chlorophyll but tend to fluoresce yellow. X90.

Fig. 33. Note dead cell (arrow) showing bright-yellow fluorescence. Viable cells are bright red in color. X200.

Fig. 34. Lipid bodies increase in size and number as cells age (ca. 360 hours) and are visualized with fluorescence optics as bright yellow after benzpyrene labelling. X150.

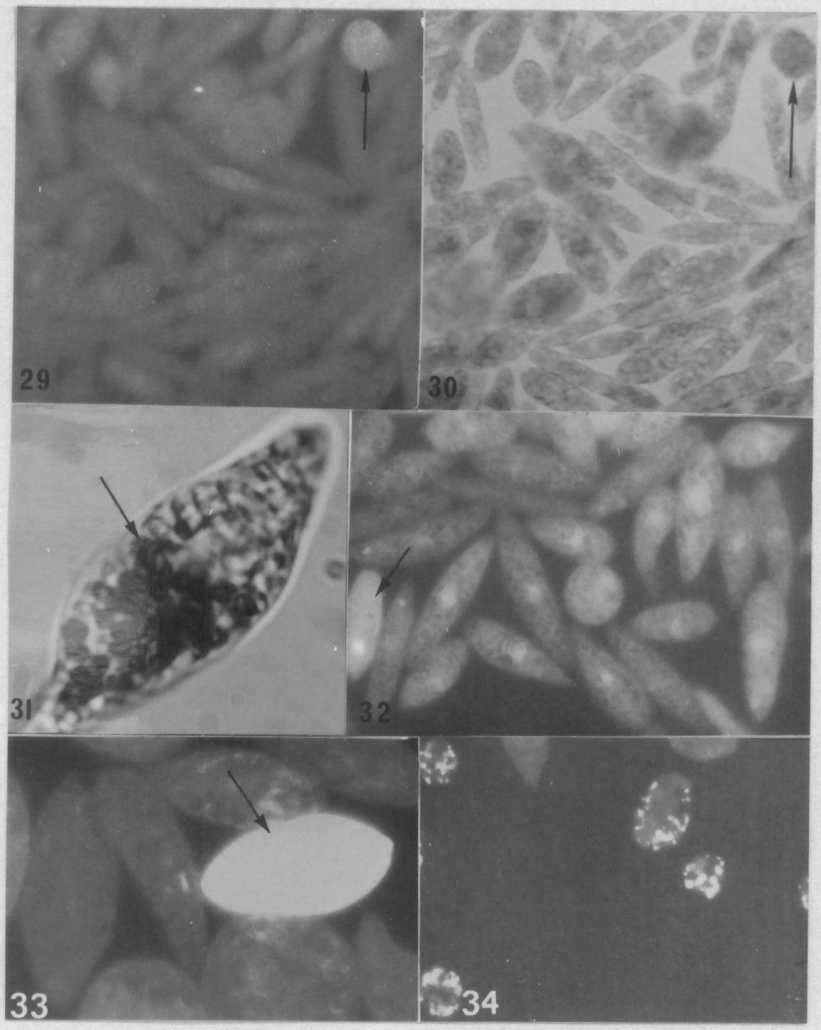


Plate VII

Fig. 34), although there were a few which fluoresced silver to blue. The controls which were not treated with fluorochrome could not be observed for long periods under UVL. They faded easily, and other naturally fluorescing bodies were not as discernible.

The general results of studies made under the light microscope are summarized on Plate VIII in which the morphological pattern of cellular aging in the life history of Euglena gracilis 'Z' is shown.

Electron Microscopy

General cell morphology. Prior to ultrastructural observation, "microthin" sections which were too thin for observation with light microscopy but too thick for electron microscopy (ca. 0.2 microns) were sectioned with glass knives in order to examine the general cell structure and the quality of fixation. Plate IX, Fig. 36, shows an elongated young cell with good membrane preservation as revealed by Azure II staining. The anteriorly located reservoir-canal region (C) and the centrally located nucleus are shown. Small, elongate paramylon granules were abundant in young cells, particularly around the nucleus (Plate IX, Fig. 36). Plate IX, Fig. 37, shows a rounded cell from late stationary phase. This cell, showing black precipitate in some portions of the cell,

Plate VIII. Schematic diagram of life history of Euglena gracilis 'Z' in synchronous cultures.

Fig. 35. Morphological pattern of cellular aging in Euglena gracilis 'Z' grown in liquid cultures.

A. The cell population is synchronized with a L/D cycle of 16/8 hours. After ca. 3 days, logarithmic phase is initiated with cell division occurring mostly in darkness. Generation time is ca. 6-8 hours.

B. At the transition of log to stationary phase, morphological differentiation occurs. Some cells become rounded while others remain elongate.

C. Giant cells appear in stationary phase; some cells become non-motile, possibly by shedding or resorbing flagella; only a few elongated motile cells persist.

D. Clumping of cells occurs, accompanied by secretion of mucilaginous substances.

E. Lysis of many cells occurs. Others remain in clumps and are embedded in a mucilaginous substance, reminiscent of palmelloid stages in other algal forms. Few cells are motile and capable of dividing, similar to the initial population shown in A. Most cells are stationary and continue to accumulate aging pigment bodies.

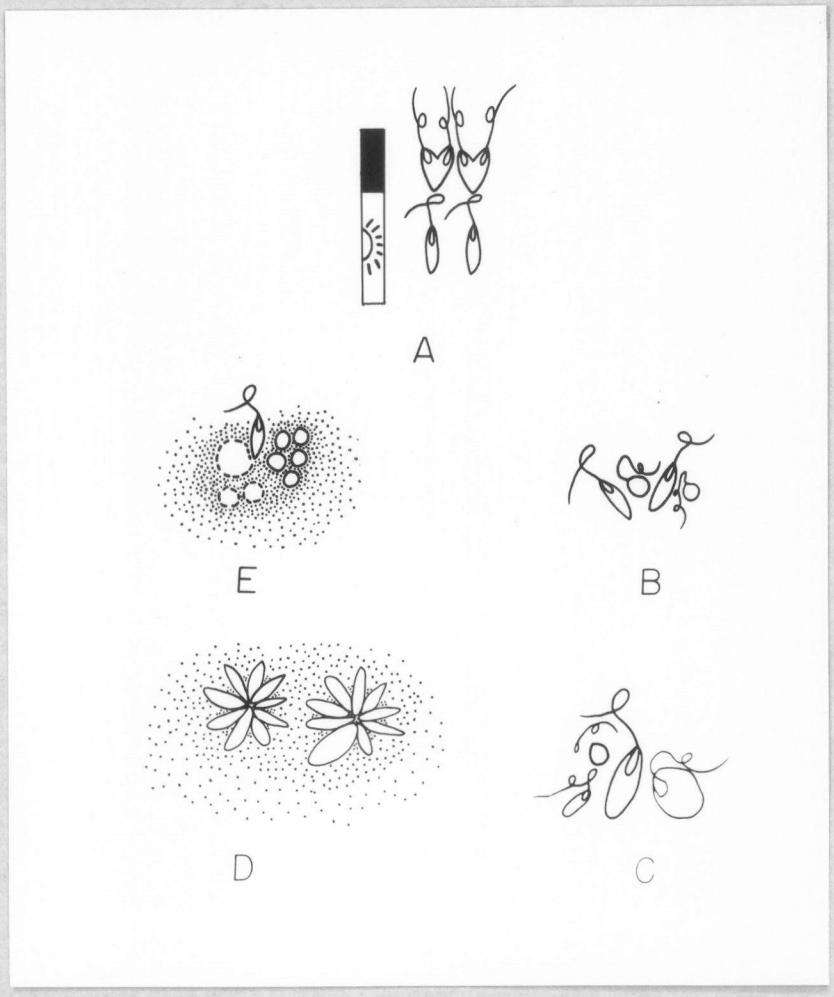
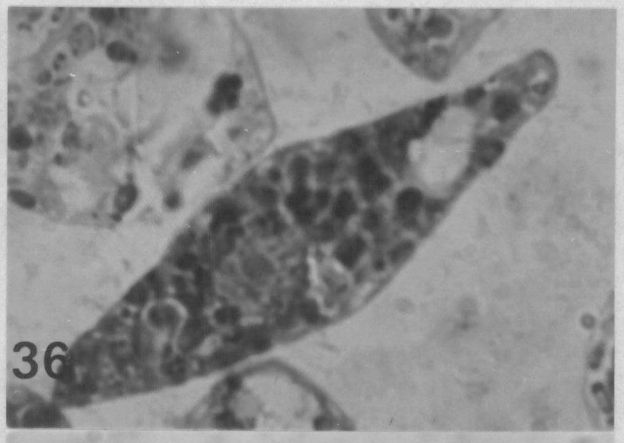


Plate VIII

Plate IX. Azure blue staining of thick sections.

Fig. 36. Longitudinal section of cell from 2-day-old culture showing nucleus with chromatin materials. X1900.

Fig. 37. Cell fixed according to Gomori--acid-phosphatase procedure and counterstained with Azure II. Note black Gomori acid-phosphatase reaction (arrow). Membranes of various organelles are stained blue. X1000.



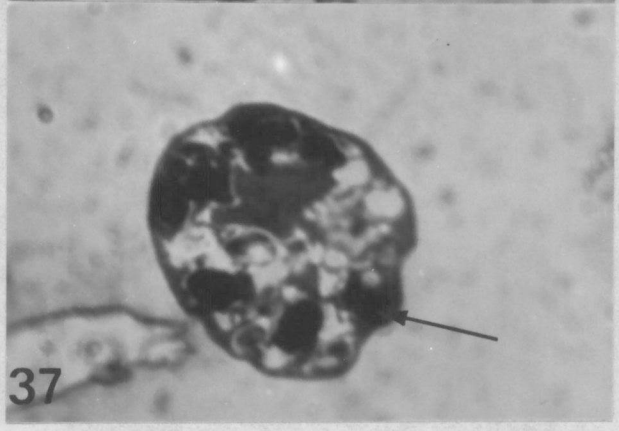


Plate IX

was treated with Gomori reaction medium for acid phosphatase. Details of this treatment will be described elsewhere in this thesis.

Nucleus. The nucleus was surrounded by a "honeycomb" nuclear envelope as shown in Plate X, Figs. 38-39. The nuclear pores measured ca. 0.1 μ in diameter. In cells sampled from stationary phase (Plate X) where nuclear division was "inactive," the chromosomes were found to be persistently condensed, and a number were attached to the nuclear envelope (Plate X, Figs. 40-41; 43; 45-46). The nucleoplasm remained fairly homogeneous until later stages when the endosome began to decrease in stainability indicated by some "fading" at the periphery (Plate X, Fig. 41). In some cases, the endosome consisted largely of a fibrous meshwork (Plate X, Fig. 42) and formed "vacuoles"--regions of low electron density (Plate X, Fig. 43). Increase in vacuolization of the endosome occurred with advancing age of the cell (Plate X, Figs. 43-45), and further degradation resulted in decrease in chromatin content of the nucleoplasm (Plate X, Fig. 46). This was also accompanied by general deterioration of cellular membranes; their fragility made fixation of aged cells difficult.

Plate X. Age-associated changes in nucleus.

Fig. 38. Tangential section through nucleus showing "honeycomb" structure of nuclear envelope with nuclear pores. X7000.

Fig. 39. High magnification of nuclear pores. X20,000.

Fig. 40. In non-dividing cells from 9-day-old cultures, persistently condensed chromosomes are shown in close association with nuclear envelope. X20,000.

Fig. 41. As cells progress towards stationary phase, some chromosomal components, notably the endosome, decrease in stainability. Note attachment of some chromatin material to nuclear envelope. X7000.

Fig. 42. Section showing large endosome composed of anastomosing fibrils and a granular core of low electron density. X8000.

Fig. 43. Central core of endosome showing marked granularity and opacity. X7000.

Fig. 44. In late stationary phase from 30-day-old culture, electron-opaque areas increase within endosome. Some vacuolate areas are indicated; a fibrillar meshwork characterizes other regions. X8000.

Fig. 45. Section of nucleus showing vacuolate endosome. Some chromatin material shown in association with nuclear envelope. X7000.

Fig. 46. In very old cultures, integrity of nuclear envelope may be lost, with concomitant loss of most nucleoplasm. Remnants of chromatin remain, and the endosome persists but is greatly reduced in electron density. X7000.

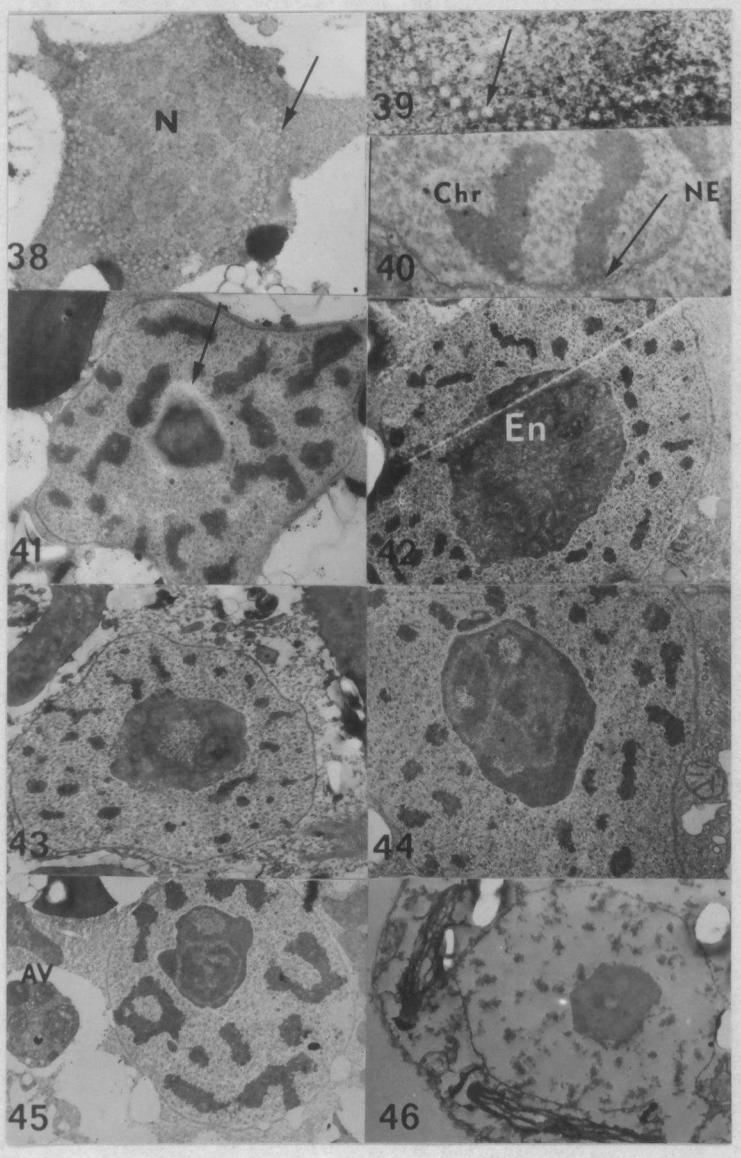


Plate X

Canal region, reservoir and associated organelles. Transverse sections through the distal region of the canal reveal a complex network of anastomosing circular microtubules (CF). Round configurations of CF in cross section are surrounded by an adjacent layer of CF in longitudinal section, forming a filamentous meshwork (Plate XI, Fig. 47) suggesting a definite angular orientation of the two sets of microtubules. The MT in Plate XI, Fig. 48, are in perpendicular array beginning from the canal opening (CO) and continuing posteriorly into the reservoir. This perpendicular orientation is further illustrated by the longitudinal MT aligned near the canal with an adjacent layer of MT in cross section a little farther away from the canal proper (Plate XI, Fig. 49). Deeper in the canal, CF are less prominent (Plate XI, Fig. 50). Plate XI (Fig. 51) reveals a longitudinal section of the flagellum in the reservoir. RR labeling shows the flagellar membrane (FM) and various projections. Plate XI (Fig. 52) shows a transverse section of two flagella, together with some mastigonemal projections. A close-up of RR-labeled mastigonemes closely associated with the reservoir is shown in Plate XI (Fig. 53).

The flagellar swelling (FS) is closely associated with the basal portion of the flagellum and appears as a deep-staining body with a crystalline core in transverse

Plate XI. The canal region.

Fig. 47. Transverse section through distal region of canal (C) showing complex network of circular microtubules (CF). Smooth endoplasmic reticulum (SER) is abundant in this region. X38,000.

Fig. 48. Surface section through the upper portion of canal showing microtubules in perpendicular array (arrows) leading to canal opening (CO). X15,000.

Fig. 49. Median longitudinal section through proximal region of canal showing perpendicular arrays of microtubules. X15,000.

Fig. 50. Microtubules at canal (C). X28,000.

Fig. 51. Canal region showing a flagellum (F) in longitudinal section; elements of smooth endoplasmic reticulum (SER) in adjacent cytoplasm. X28,000.

Fig. 52. Transverse section through proximal portion of canal showing two flagella in cross section and associated mastigonemes, labelled with ruthenium red. X28,000.

Fig. 53. Mastigonematal projections in canal labelled with ruthenium red. X40,000.

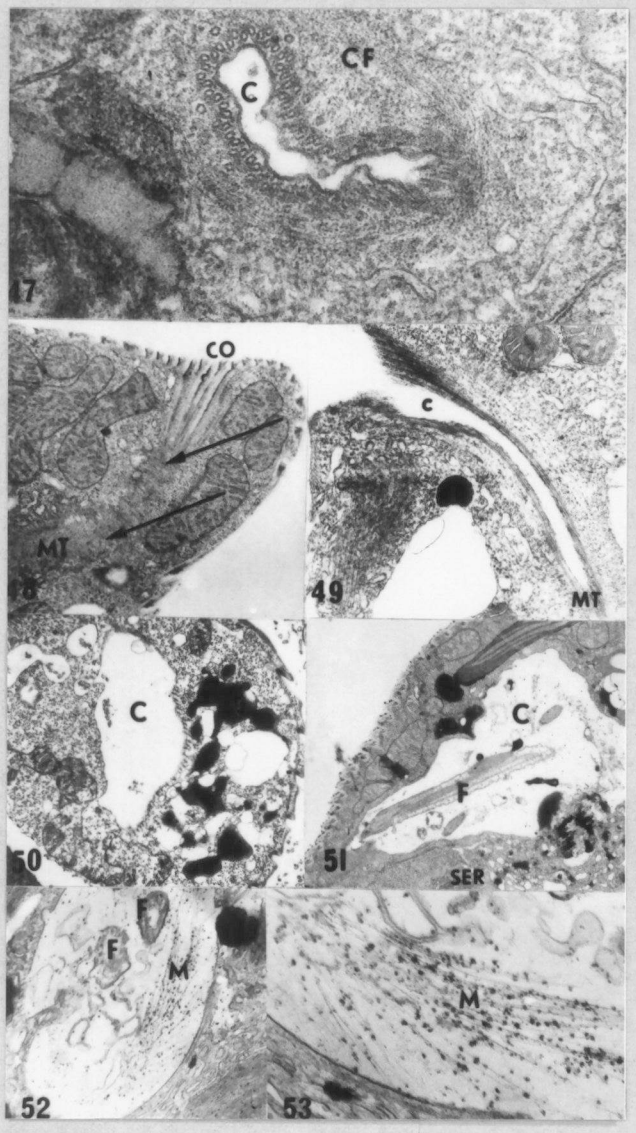


Plate XI

section (Plate XII, Figs. 54-55). A common membrane surrounds the emergent flagellum and FS as revealed by RR labeling (Plate XII, Fig. 55). The irregular membrane of the flagellum, together with projections that could be mastigonematal remnants are stained readily with RR (Plate XII, Fig. 56). Both outer and inner MT are seen in parallel orientation with the longitudinal section of the flagellum (Plate XII, Figs. 56-58). Other associated vesicles, vesiculate ER (VER) and smooth ER (SER) are abundant in the reservoir region; some portions are labeled with RR (Plate XII, Figs. 51 and 59).

Chloroplasts. *Euglena* chloroplasts exhibit long continuous internal lamellar systems and homogeneous matrices unlike those of higher plant chloroplasts which manifest alternating grana-stroma-lamellae (Plate XIII, Fig. 60). Most often, they are associated with other cytoplasmic organelles such as mitochondria and paramylon granules (Plate XIII, Fig. 61). Near the mid-portion is a differentiated region, the pyrenoid (Plate XIII, Fig. 62), in which the thylakoids are tightly-packed (Plate XIII, Fig. 63). In chloroplasts of some aged cells (20 days), electron-dense osmophilic bodies formed and increased in number, size, and distribution with increasing age (Plate XIII, Figs. 64-66). Later, they were lysed into the cytoplasm wherein the chloroplast membranes

Plate XII. Flagellum and associated organelles.

Fig. 54. Association between flagellar swelling (FS) and locomotory flagellum is shown. X41,500.

Fig. 55. Transverse section of canal (C) showing flagellum and flagellar swelling (FS). A common membrane surrounds the emergent flagellum and flagellar swelling. X41,500.

Fig. 56. General view of emergent flagellum that has been shed by cell. Note projections from flagellar membrane as revealed by ruthenium red labelling (arrow). X8000.

Fig. 57. View from base of flagellum. Both center and peripheral microtubules are seen. X38,000.

Fig. 58. Portion of flagellar tip showing mastigonematal projections. Microtubules are prominent. X38,000.

Fig. 59. Vesiculate endoplasmic reticulum (VER) and other vesicles in cytoplasm adjacent to canal. X48,000.

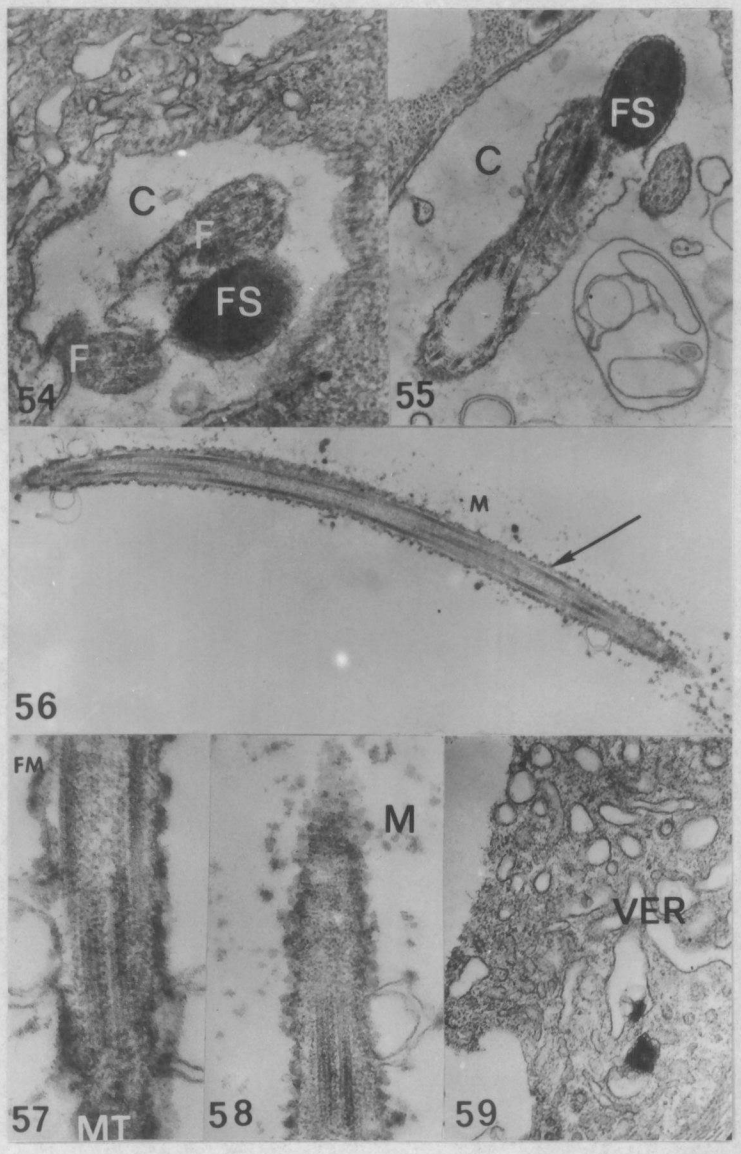


Plate XII

Plate XIII. Chloroplast structure, cytochemistry and age-related changes.

Fig. 60. Portion of a typical euglenoid chloroplast (Chl) from a 3-day-old culture showing matrix and internal lamellar (thylakoid) system. X33,000.

Fig. 61. A chloroplast is seen adjacent to a mitochondrion (from a 3-day-old culture). X16,700.

Fig. 62. Median section through chloroplast of cell from old culture (20 days) shows constriction in pyrenoid (arrow). X9000.

Fig. 63. Close-up of pyrenoid (Py) showing tightly packed arrangement of lamellae. X27,000.

Fig. 64. In older cells (from 20-day-old culture), osmophilic granules appear in pyrenoid region. X27,000.

Fig. 65. Osmophilic bodies in matrix and between thylakoid membranes (arrow). X30,000.

Fig. 66. Osmophilic bodies (OS) increase in size, number and electron density with advancing age of cell. X26,000.

Fig. 67. Chloroplast-derived osmophilic bodies (OS) released into cytoplasm after chloroplast lysis. X26,000.

Fig. 68. Chloroplast vesiculation, disorientation of thylakoids occur in cells from old cultures. X26,000.

Fig. 69. Myelin figures characterize membrane degeneration in chloroplast. X27,000.

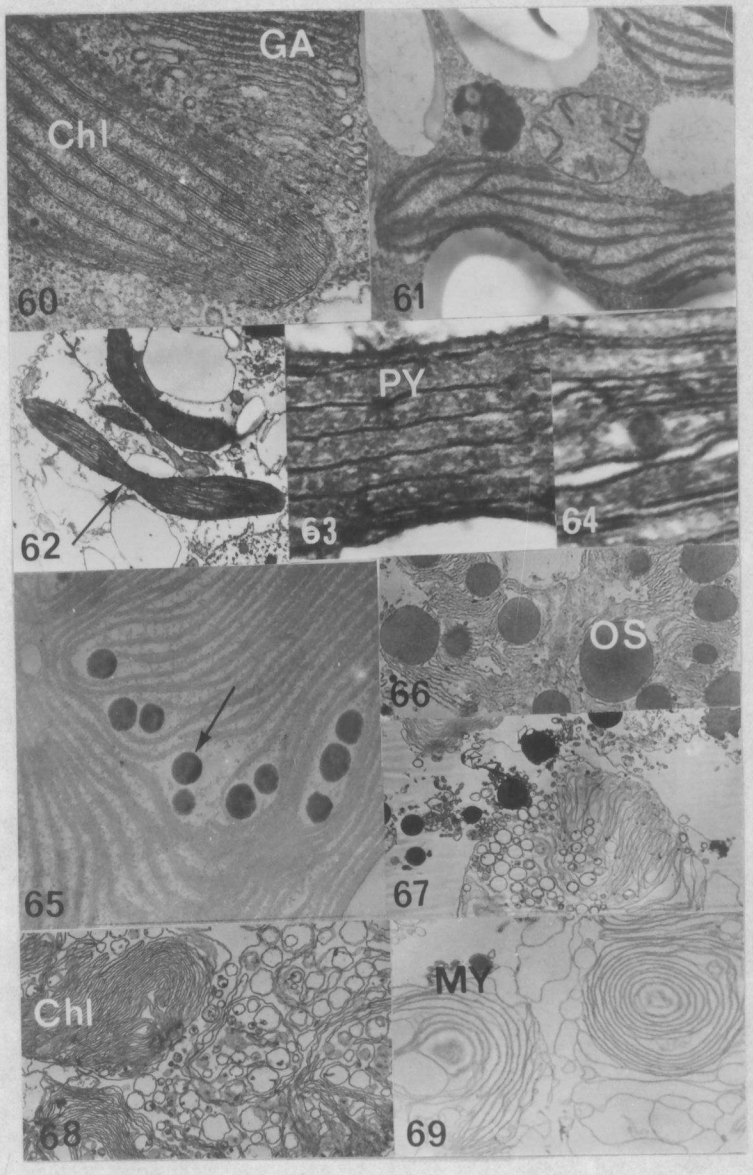


Plate XIII

subsequently degenerated (Plate XIII, Fig. 67). Further degradation of the chloroplast with age resulted in the formation of myelin figures consisting of concentric arrangement of membranes (Plate XIII, Figs. 68-69).

Paramylon. In young cells, paramylon granules (P) appear as lenticular beads dispersed throughout the cytoplasm (Plate XIV, Fig. 70). They are associated with most organelles, particularly the chloroplasts and other cytoplasmic vacuoles. Some paramylon granules group together to form large clumps (Plate XIV, Fig. 71). Treatment with RR reveals some localization of precipitate around those paramylon granules in close association with the pellicle, suggesting the presence of some kind of pectinous material. The surrounding membrane is indiscernible (Plate XIV, Fig. 72). As cells age paramylon granules undergo alteration in structure to form plate-like aggregates which may coalesce (Plate XIV, Figs. 73-74).

Dictyosomes (Golgi apparatus). Stacks of tightly packed cisternae, deeply stained at the intercisternal margins, comprise dictyosomes of healthy Euglena cells (Plate XV, Figs. 75-76). In face view, cisternae appear as concentric flattened stacks of saccules (Plate XV, Fig. 77). In actively metabolizing young cells,

Plate XIV. Paramylon structure and cytochemistry.

Fig. 70. In young cells from young cultures (ca. 4-5 days old), paramylon granules (P) are small (ca. 2-3 μ), elongated in shape and distributed throughout cytoplasm. In this cell treated with Gomori reaction medium for acid-phosphatase localization, the paramylon granules are in close association with vesicles containing acid-phosphatase (arrow). X16,000.

Fig. 71. Cluster of paramylon granules in localized portion of cell. X18,000.

Fig. 72. With RR labelling, membranes of paramylon granules near pellicle show positive reaction for acid mucopolyssacharides (arrows). X20,000.

Fig. 73. Small (ca. 2-3 μ) elongate paramylon granules characteristic of young cells are not as evident in older cells (ca. 30 days). Narrow, rectangular arrays of paramylon are formed and are in close association with vesicles containing acid-phosphatase. X12,000.

Fig. 74. Coalescence of rectangular paramylon granules. X15,000.

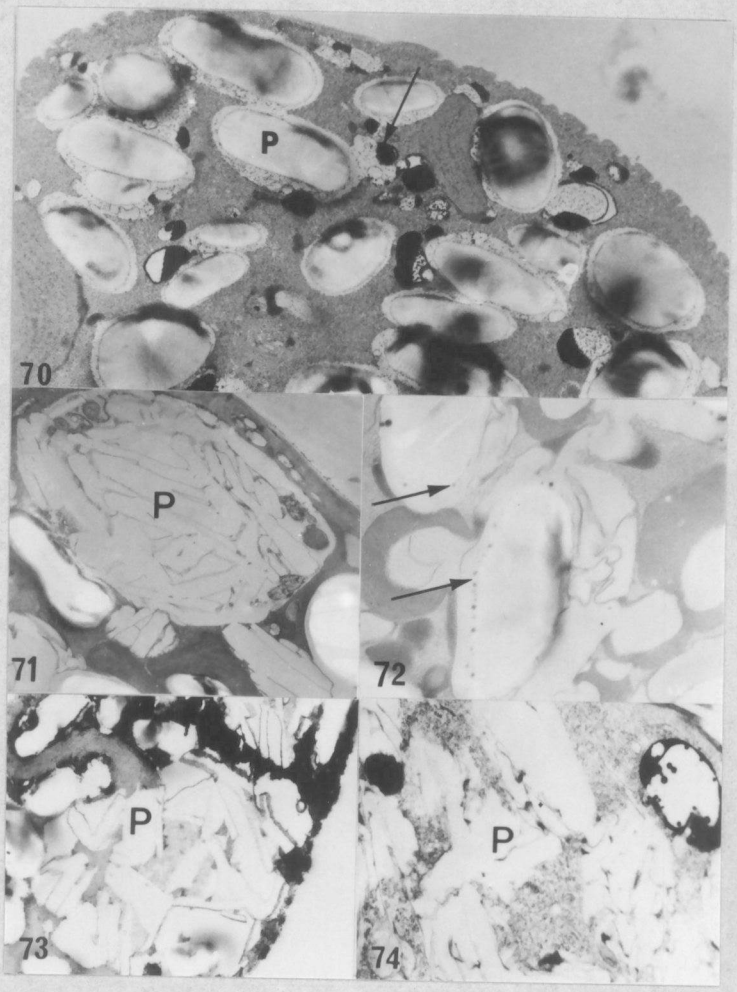


Plate XIV

Plate XV. Dictyosome structure and cytochemistry.

Fig. 75. Dictyosome structure typical of euglenoid organisms. X25,000.

Fig. 76. Dictyosomes sectioned in different plane; face view indicated by arrow. X25,000.

Fig. 77. Metabolically active cells contain several dictyosomes, frequently occurring adjacent to each other. X25,000.

Fig. 78. At the quiescent stage from 20-day-old culture, dictyosome saccules separate and undergo hypertrophy to form enlarged vesicles.. X25,000.

Fig. 79. After treatment with Gomori reaction medium, heavy labelling of acid phosphatase is visualized in dictyosomes of cells from mid-logarithmic phase (ca. 5 days). X25,000.

Fig. 80. In early stationary phase (ca. 10 days) acid-phosphatase label within the dictyosome diminishes, and the precipitate is localized largely in dictyosome-derived vesicles. X22,000.

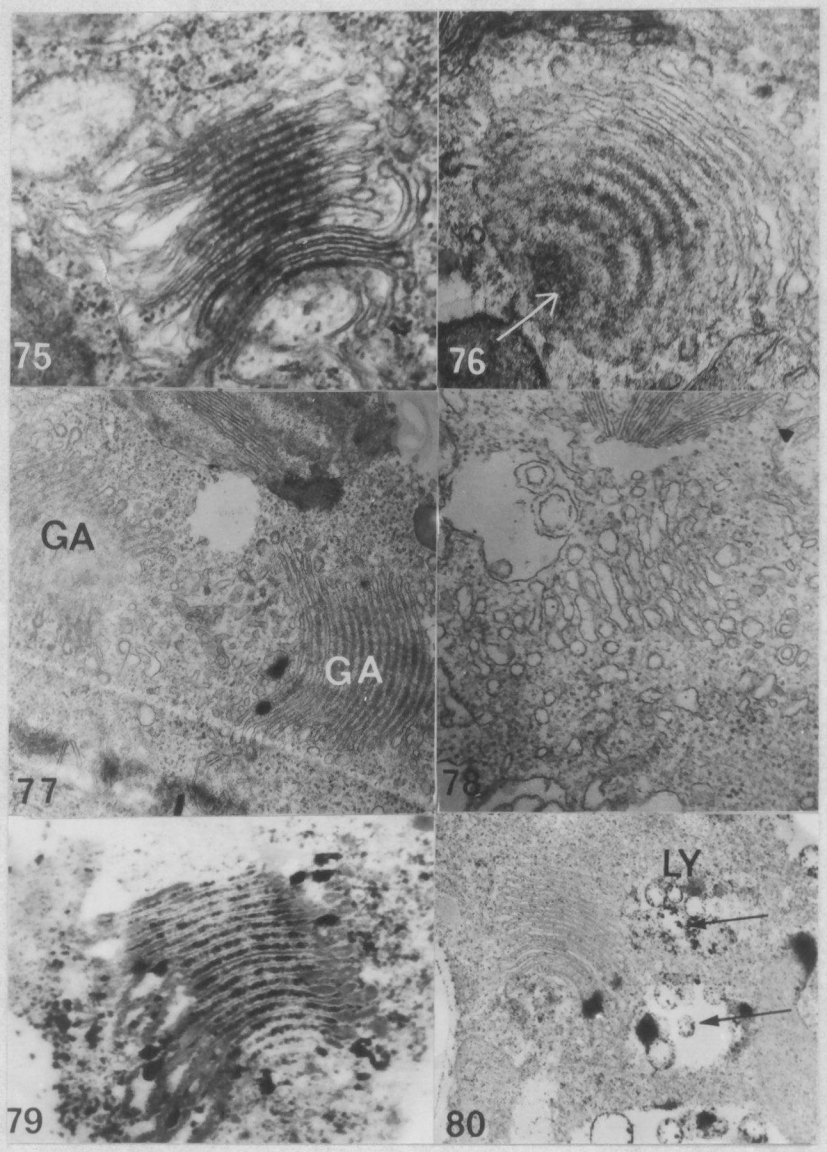


Plate XV

dictyosomes are numerous and adjacent to each other (Plate XV, Fig. 77). As cells age, they tend to undergo hypertrophy as intercisternal spaces widen and some cisternae dilate to form large vacuoles (Plate XV, Fig. 78). With the use of Gomori reaction medium for acid phosphatase, cells from mid-logarithmic phase are heavily labeled (Plate XV, Fig. 79). In contrast, older cells show little labeling with acid phosphatase precipitate (Plate XV, Fig. 80).

Lysosomes. Acid phosphatase, an enzyme marker for lysosomes, was found in Euglena. Younger cells did not stain readily for acid phosphatase, suggesting enzyme latency. As shown in Plate XV, Fig. 80, dictyosome-related vesicles were readily labeled in older cells. With advancing age of cells, such vesicles associated with other cytoplasmic vesicles of different electron densities (Plate XVI, Figs. 81-84). Some of the vacuoles enlarged, and those with heterogeneous matrices were predominant. Such heterogeneity suggested possible "coalescence" of dark-staining lysosome-like vacuoles with those with lighter matrices. In some portions of the cell, homogeneous osmophilic bodies of presumptive lipoidal nature were surrounded by acid-phosphatase-containing vesicles (Plate XVI, Fig. 83) which subsequently underwent

Plate XVI. Vesicles.

Fig. 81. Heterogeneous matrices of large vesicles is suggested by differential heavy labelling of acid-phosphatase. X10,500.

Fig. 82. Vesicle matrix shows different electron densities. Acid-phosphatase precipitate is shown by the electron-dense granules at periphery. X10,000.

Fig. 83. In this aged cell, the granular acid-phosphatase label is localized at the periphery of a vacuole, while the central portion is highly osmophilic suggesting a lipoidal composition. X10,000.

Fig. 84. This vesicle has the lipoidal portion at the periphery, while acid-phosphatase portion occupies the major central portion. X10,000.

Fig. 85. Note diminishing size of lipoidal portion of vesicle during lysis (see Fig. 83). X10,000.

Fig. 86. Granular and osmophilic constituents of vacuole are dispersed into surrounding cytoplasm upon lysis. X10,000.

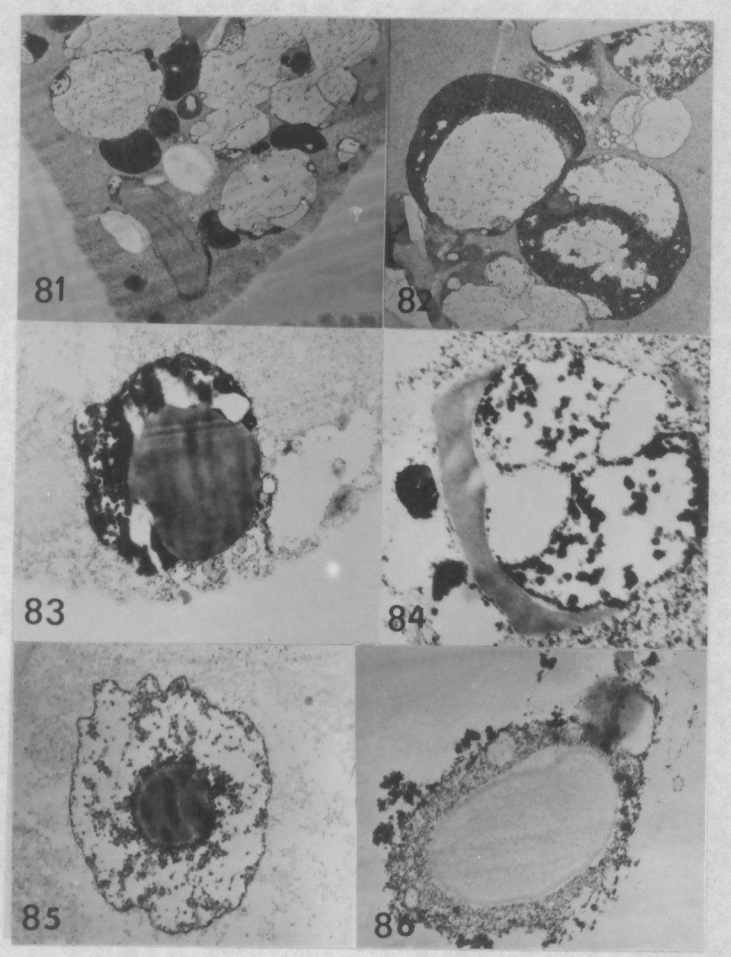


Plate XVI

alteration in matrix composition (Plate XVI, Figs. 84-86), suggesting the occurrence of some kind of biochemical, hydrolytic activity.

The absence of acid-phosphatase precipitate in the untreated cells and in controls made it difficult to discern in them the distribution and nature of vesicles containing hydrolytic enzymes such as acid phosphatase. The lipoidal and lightly stained vacuoles, however, were still recognizable; together with mitochondria that exhibited morphological variations that will be discussed later in this thesis.

Microbodies (peroxisomes). In younger cells, (two days) microbodies (MB; peroxisomes) were not numerous or especially reactive to DAB medium at pH 9 (Plate XVII, Fig. 87). They were usually round, sometimes of variable shapes and bounded by a very thin single limiting membrane. The matrices were finely granular, of uniform distribution and mostly of the anucleoid type, unlike those that occur in most animal and higher plant microbodies with crystal-containing bodies (CCBs). Occasionally, they occurred together with organelles that contained electron-dense whorl-like cores that appear "hollow" in cross section (Plate XVII, Figs. 89-90).

The staining reaction of microbodies in DAB medium

Plate XVII. Microbody (Peroxisome) structure and cytochemistry.

Fig. 87. Cell from a 2-day-old culture, incubated in DAB medium (37 C pH 9) shows slight staining reaction in microbody. X40,000.

Fig. 88. Microbody (MB) in an untreated cell from a 2-day-old culture. X40,000.

Fig. 89. Electron-dense "cores" are occasionally observed. X55,000.

Fig. 90. Electron-dense cores with whorl-like configuration appear "hollow" in cross section. X55,000.

Fig. 91. Upon treatment with aminotriazole inhibitor (0.04 M, pH 9) peroxidative activity in microbodies (MB) is inhibited as shown in this 2-week-old sample. X28,000.

Fig. 92. Cell from 30-day-old culture shows a denser reaction product in the microbodies with DAB medium, at pH 9. Also note subtle staining in mitochondrial cristate membranes. X25,000.

Fig. 93. Dense reaction product with DAB medium at pH 9 is more intense in microbodies of this cell from a 60-day-old sample compared to those of younger cells in Fig. 92 (30-day-old culture). The uniform, electron-dense staining makes the limiting membrane nearly indiscernible. No observable reaction is evident in adjacent mitochondria. X10,000.

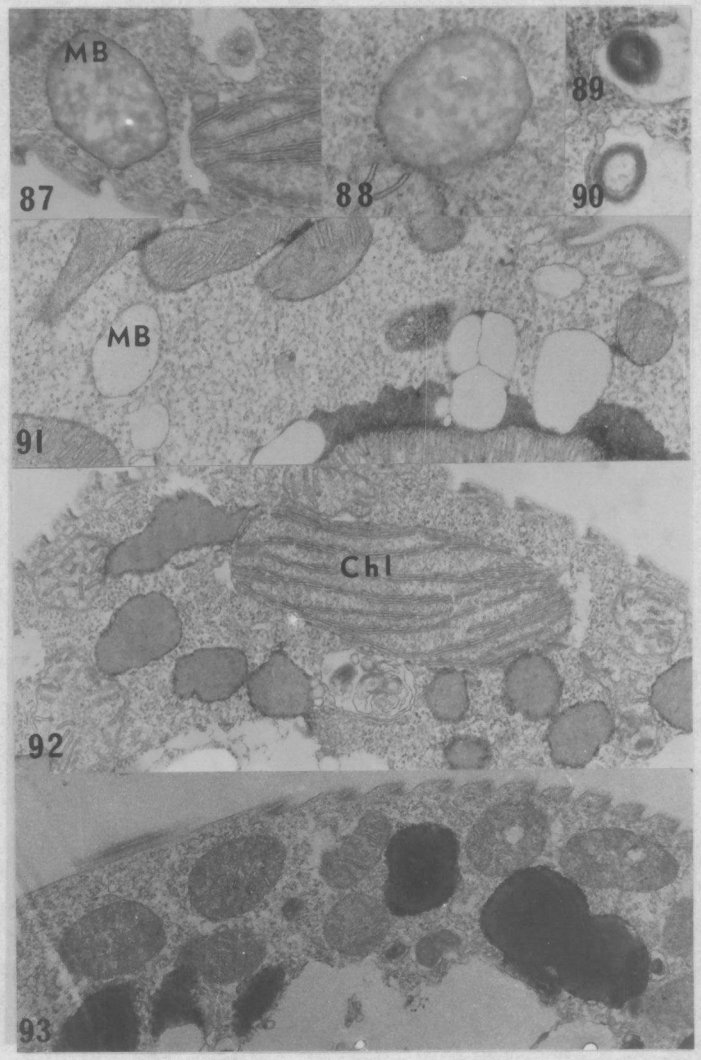


Plate XVII

was indistinguishable from the untreated cells (Plate XVII, Fig. 88). Except for a few reactive organelles, peroxidative activity in the microbodies was inhibited by catalase inhibitor, aminotriazole (AT) at pH 9. As shown in Plate XVII, Fig. 91, there was no positive-staining reaction either to DAB or to the catalase inhibitor in the microbodies. The reaction product that resulted from treatment with DAB and its inhibition by AT at pH 9, therefore, suggested that some peroxidative process was involved, indicative of the enzyme, catalase.

As cultures reached stationary phase from 15 days on, microbodies were more numerous and more electron-dense, particularly in the subpellicular regions (Plate XVII, Fig. 92). In 60-day-old samples, staining was even more intense, with some microbodies showing some evidence of coalescence (Plate XVII, Fig. 93). The intense and uniform distribution of stain in peroxisomal matrices made their thin limiting membranes indiscernible.

It was of common occurrence to see microbodies in close association with other organelles. Plate XVIII, Fig. 94, shows a microbody between a mitochondrion and a chloroplast; others were associated with dictyosomes (Plate XVIII, Fig. 95). Microbody-associated organelles likewise exhibited positive reaction to DAB at pH 9, such as multivesicular bodies (Plate XVIII, Fig. 96), minute

Plate XVIII. Microbodies and related organelles.

Fig. 94. A microbody in close association with a chloroplast and mitochondrion. X28,000.

Fig. 95. Some microbodies with positive staining reaction to DAB medium are seen in close proximity to dictyosomes and dictyosome-associated vesicles. X25,000.

Fig. 96. In this early stationary-phase cell (from 9-day-old culture) dense reaction product with DAB medium at pH 9 is visualized in a multivesicular body and a microbody in close proximity to a mitochondrion. X28,000.

Fig. 97. DAB reaction product is seen in endoplasmic-reticulum-associated vesicles and in some incipient multi-vesicular bodies. X28,000.

Fig. 98. Staining reaction of DAB medium resembles those of lipofuscin-like aging pigment granules (LPG) of this untreated aged cell grown in biphasic medium (4 years). X2500.

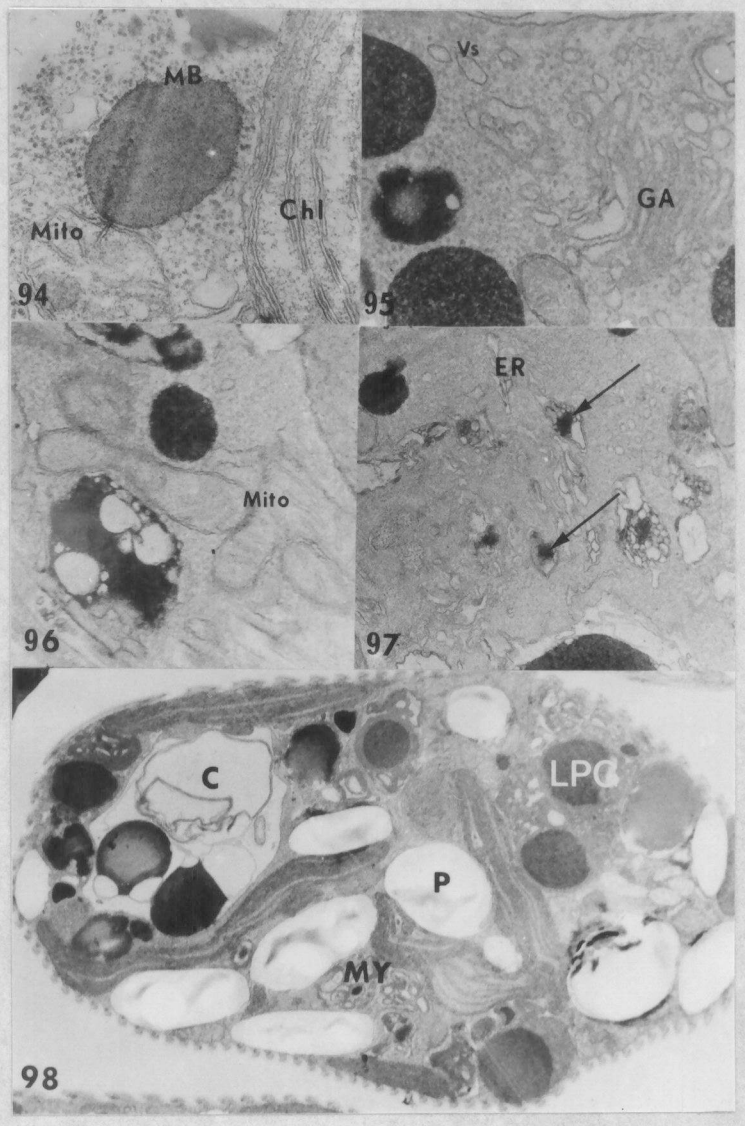


Plate XVIII

ER-related vesicles (Plate XVIII, Fig. 97), termed by Novikoff, et al. (1973) as "microperoxisomes."

Compared to lysosomes, the limiting membrane of MB may exhibit a greater degree of permeability as visualized by the staining characteristics of their matrices. Whereas lysosomes contained coarser granules with intense staining, microbodies possessed homogeneous matrices, which made discernment of limiting membranes difficult. In comparison to lipofuscin-like aging pigments, however, some MB became indistinguishable with regard to electron density. Plate XVIII, Fig. 98, shows an old cell (4 years) with lipofuscin-like aging pigments that were largely osmophilic as DAB-treated microbodies. The cell in Plate XVIII, Fig. 98, however, was not treated with DAB. The dark-staining reaction of aging pigments was attributed to some kind of lipoidal substance in the nature of unsaturated fatty acids, as shown by their positive response to osmium tetroxide.

Mitochondria. Mitochondria in Euglena were elongate (Plate XIX, Fig. 99), rod-shaped (Plate XIX, Fig. 100) or long and branching, forming a mitochondrial reticulum (Plate XIX, Figs. 101-103). Younger cells (two days) treated with modified DAB medium, pH 7.8, exhibited intense deposition of staining reaction product in the inner mitochondrial membranes, specifically at the outer margins of

Plate XIX. Mitochondrial morphology.

Fig. 99. Long mitochondrion (arrow) in close proximity to vesicles. X10,000.

Fig. 100. Round-to-elongated mitochondria are shown in close association with pellicle. X10,000.

Fig. 101. Mitochondrial reticulum near canal region in close association with microtubules. X10,000.

Fig. 102. Mitochondrial reticulum can be long and branching, extending from one end of cell to the other. X7000.

Fig. 103. Branching mitochondrial reticulum in close association with the pellicle and a multivesicular body. X28,000.

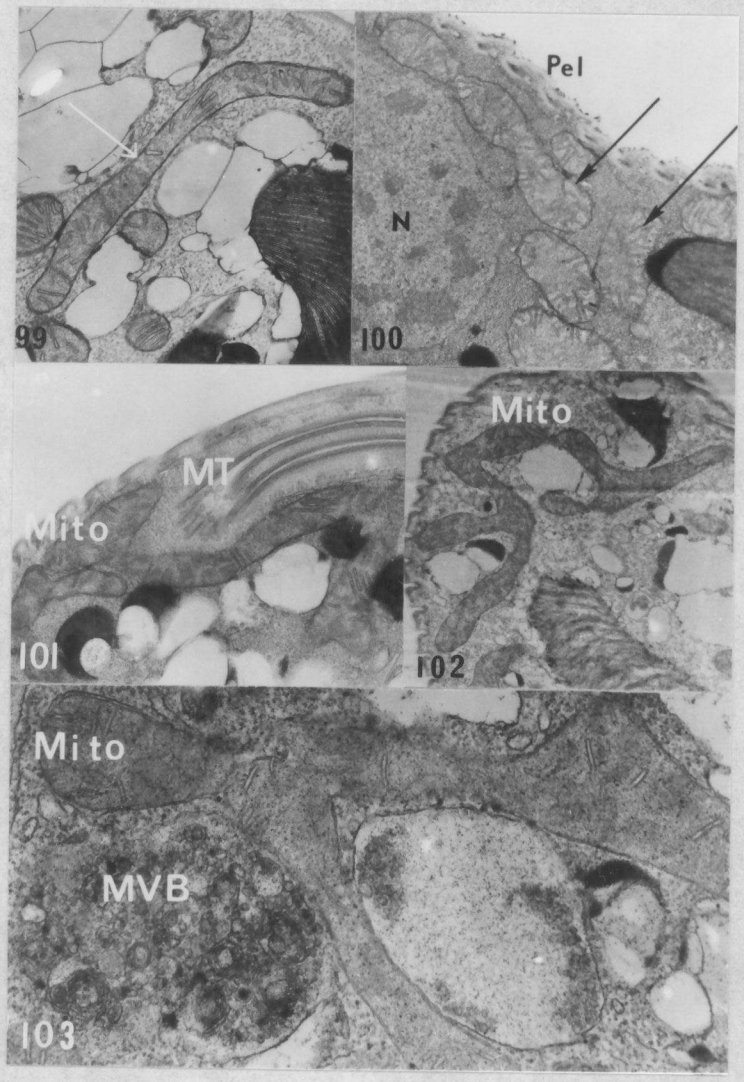


Plate XIX

cristate membranes (Plate XX, Fig. 104), suggesting cytochrome oxidase activity. The reaction product apparently localized on the surface of the cristate membranes in the form of linear deposits; occasionally, they appeared in the intracristate spaces. The use of sodium azide, however, did not completely inhibit the staining reaction of young cells (Plate XX, Fig. 105). Slight deposition of the reaction precipitate could still be observed in the cristate membranes. Cytochrome oxidase activity in mitochondria in cells from old cultures, however, was completely inhibited by sodium azide (Plate XX, Fig. 106), since no staining reaction was detectable. Cells from 30-day-old cultures treated in DAB medium at pH 7.8 responded very slightly to the treatment, and the reaction product was confined to small deposits on the surfaces of cristae (Plate XX, Fig. 107). Intracristate deposits were minimal. The majority of mitochondria in cells from 60-day-old cultures failed to react with DAB medium, and some of them could be observed in the lytic process (Plate XX, Figs. 108-109). As cultures aged, mitochondria underwent fragmentation initiated apparently by the breakdown of the outer membranes; others were "engulfed" in autophagic vacuoles. There were, however, few cristae which remained intact for quite some time and could still respond to DAB treatment.

Plate XX. Age-related structural alterations and cytochemistry of mitochondria.

Fig. 104. Mitochondrion from 2-day-old culture treated with DAB medium (pH 7.8, 37 C) shows some staining reaction along the inner cristate membranes. X42,000.

Fig. 105. Oxidative activity in mitochondria is not completely inhibited by sodium azide (0.2 M, pH 7.8) in this cell from a 2-day-old culture. Note staining reaction along the inner cristate membranes and intercristate matrix. X42,000.

Fig. 106. Cell sample from 60-day-old culture shows complete inhibition of oxidative activity with treatment of sodium azide at pH 7.8. Note absence of reaction products in microbody and cristate membranes. X20,000.

Fig. 107. Oxidative reaction with DAB medium is not as intense in inner cristate membranes of this cell from a 30-day-old culture as in younger cells (cf. Fig. 104). X55,000.

Fig. 108. Mitochondria from old cultures (60 days) fail to react with DAB medium. X25,000.

Fig. 109. Mitochondrial contents are dispersed in the cytoplasm when organelles degenerate in aged cells. X22,000.

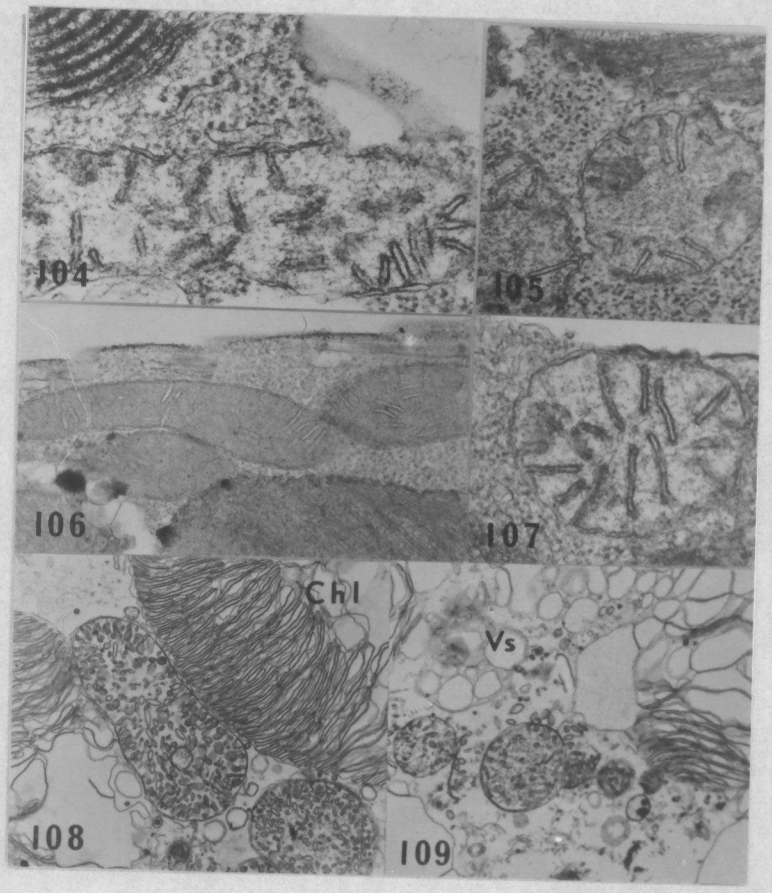


Plate XX

Plasma membrane and other cytoplasmic organelles. Younger cells (three days) were more responsive to labeling with RR than were cells from older cultures. In the younger cells, RR labeling on the surfaces of the plasma membrane was thicker, slightly fibrillar and amorphous (Plate XXI, Figs. 110-111) in comparison with the reaction of cells from 30-day-old cultures (Plate XXI, Figs. 112-113) which were somewhat obliterated due to the reaction product. They appeared as a "fuzzy" mass of interwoven fibrils. RR precipitate was also localized in the muciferous canals along the articulating surfaces of the pellicle strips near the grooves, and on the ridges of the pellicle (Plate XXI, Figs. 112-115).

No evidence of invagination of the plasma membrane or of its association with autophagic vacuole formation as described in some animal cells was observed in our preparations, except in few isolated cases where some RR reaction product was localized in some portions of paramylon membranes (Plate XIV, Fig. 72, page 69) which were in close proximity to the pellicle and the subtending muciferous bodies.

Sections through older cells that manifest increased cytoplasmic vacuolization did not reveal any plasma-membrane-derived vacuoles. Myelin figures and lipofuscin-like aging pigments were prominent in these aged cells

Plate XXI. Pellicle structure and cytochemistry.

Fig. 110. Ruthenium red labelling is evident along periphery of the plasmalemma of early log-phase cells (from 3-day-old culture). X11,000.

Fig. 111. Section through the "knob-bearing" pellicular strip shows heavy RR labelling along plasmalemma and in knob-like protrusion. X20,000.

Fig. 112. Oblique section through pellicle showing RR labelling in peripheral layer of the plasmalemma (7 days). X25,000.

Fig. 113. Median longitudinal section through pellicle showing RR precipitate on plasmalemma between pellicular strips (arrows). X25,000.

Fig. 114. RR labelling is restricted to outermost layer of the plasmalemma. X8000.

Fig. 115. RR labelling in plasmalemma of older cells (30 days) decreases with age. Note uneven distribution of RR staining in longitudinal section of pellicular strip of this stationary phase cell (arrows). X28,000.

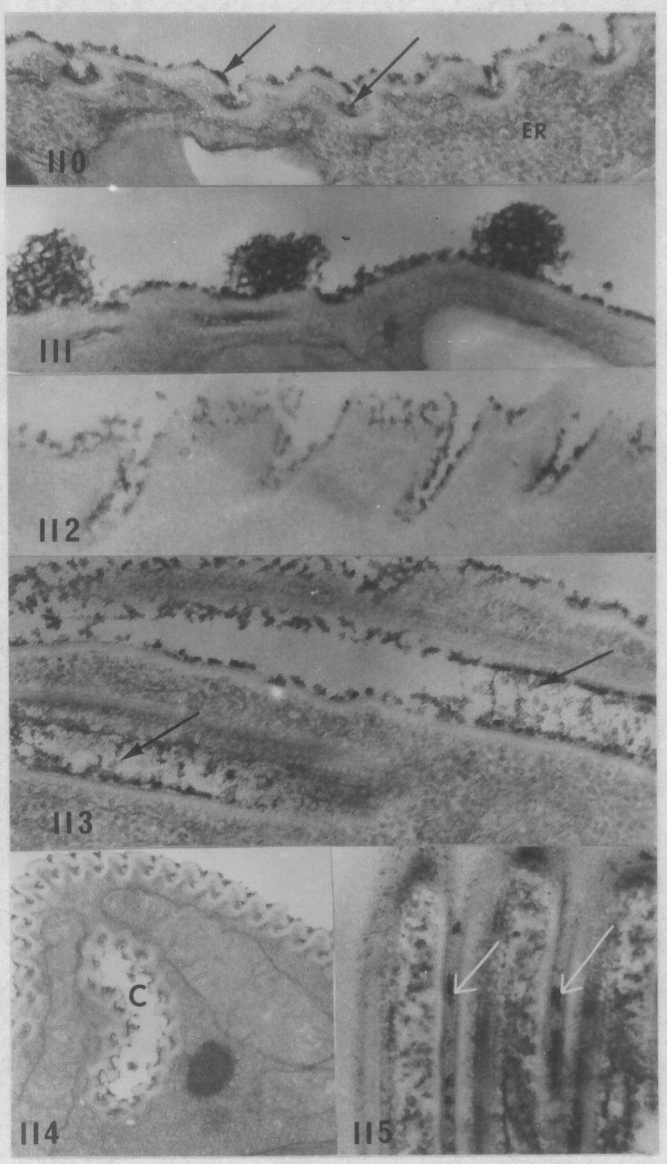


Plate XXI

(Plate XXII, Figs. 116-117). Occasionally, some autophagic vacuoles contained some recognizable organelles within them, such as mitochondria (Plate XXII, Fig. 118). Further cytoplasmic degradation with age revealed lysis of many cytoplasmic organelles such as multivesicular bodies, dense bodies, chloroplasts and lipid bodies, all of which dispersed in the cytoplasm (Plate XXII, Fig. 119).

Table VII presents a general outline of age-related alterations in Euglena cells. The results of some of the cytochemical tests are shown in Table VIII. A schematic diagram of the general pattern of age-related changes in the various organelles studied in Euglena appears on Plate XXIII, Fig. 120.

Plate XXII. Age-related changes in other cytoplasmic organelles.

Fig. 116. Cell from a 60-day-old culture showing increased vacuolization and myelin figure formation (MY). X40,000.

Fig. 117. Lipofuscin-like pigment is prevalent in cells which have remained in late stationary-phase for extended periods (4 years). X40,000.

Fig. 118. Lysis of some organelles occurs occasionally in cells from late stationary phase culture (60 days). Mitochondria, presumptive microbodies and other vesicles can be discerned. X10,000.

Fig. 119. Lysed organelles such as mitochondria and other membrane-bound vesicles interspersed with the matrices of pooled cytoplasmic constituents such as lipids (L). X12,000.

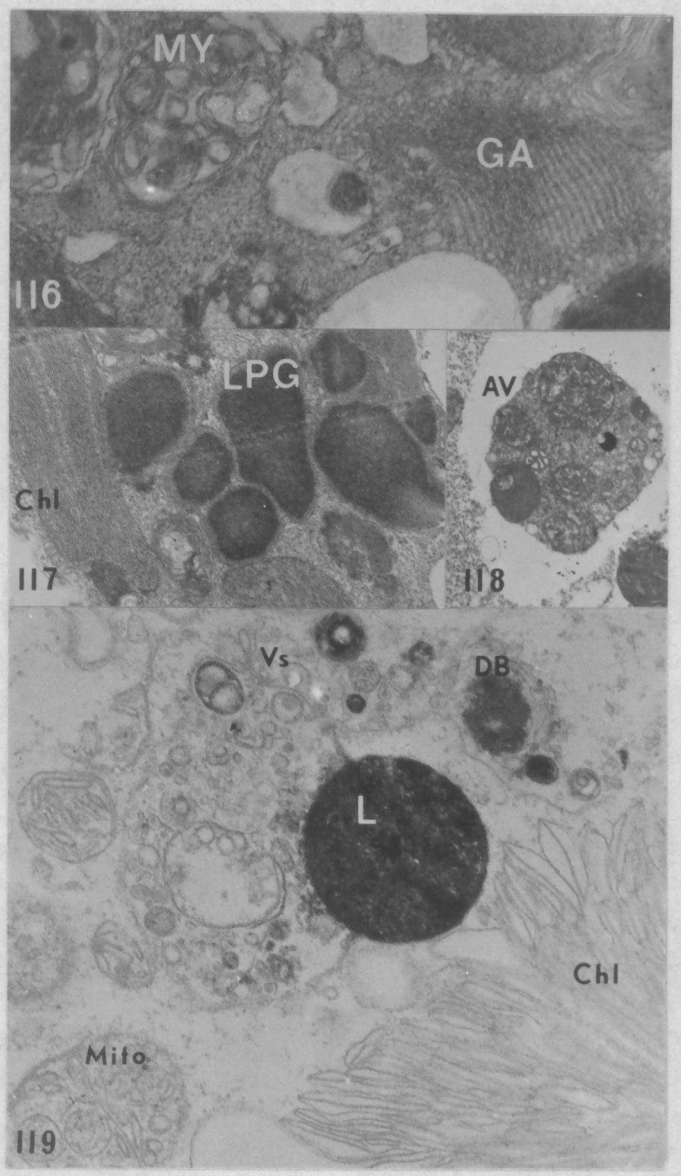


Plate XXII

TABLE VII

SUMMARY OF AGE-RELATED CHANGES IN *EUGLENA GRACILIS* KLEBS

Category	Alteration
Growth Rate	
Generation time	Increased
Cell counts	Decreased
Viability of cells	Decreased
pH of culture medium	Increased
Gross Morphology	
Cell shape	Rounding up of cells, clumping, formation of giant cells
Motility	Loss of motility, shedding of flagellum
Color	Decrease in greening of chloroplasts; accumulation of orange-to-brown aging pigments
Ultrastructure	
Nucleus	Decrease in chromatin content; "vacuolization" of the endosome; decreased stainability
Cytoplasm	
Lysosomes	Secondary lysosome formation; coalescence with other vacuoles; alteration in size and in appearance of matrix
Microbodies	Increase in size and in electron density of matrix; coalescence with other vacuoles
Mitochondria	Alteration in size, shape and distribution; fragmentation and lysis
Golgi apparatus	Fragmentation, hypertrophy; cisternal dilation; decrease in number of cisternae

TABLE VII (continued)

Category	Alteration
Endoplasmic reticulum	Decrease in incidence of associated ribosomes; increase in association with other vesicles; coalescence, autophagy or sequestration
Chloroplast	Loss of lamellar configuration; increase in size and number of osmophilic granules; lysis.
Paramylum granules	Variation in size and number; alteration in morphology; formation of plate-like aggregates
Other vacuoles	Coalescence, autophagy sequestration
Cytoplasmic membranes	Myelin figure formation
Plasma Membrane	Change in appearance of outermost surface; decrease in localization of mucilaginous secretions as revealed by RR labelling
Pellicle	Pellicular microtubules become less prominent
Cytochemistry	
Acid phosphatase	Increase in distribution; change in latency
Succinate dehydrogenase	Variable activity
Cytochrome Oxidase	Variable activity
Catalase	Variable activity
Lipids	Increase in distribution
Chlorophyll	Loss of fluorescence
Mucopolysaccharides	Decrease labelling in plasma membrane

TABLE VIII

CYTOCHEMISTRY OF SUBSTANCES DETECTED IN EUGLENA GRACILIS STRAIN 'Z'

Substance	pH	Substrate	Coupler	Visible Reaction	Major Reference
mP	7.2	-	RR/Osmium	Pink to red (bright-field) black (EM) (ionic coupling)	Luft, 1971; Chambers, 1973
AP	5.0	Sodium-β-glycero-phosphate	NH_4S	Yellow, brown to black (lead sulfide)	Gomori, 1952; Berjak, 1968, 1972
AP	5.0	AS-BI	HPR	Red (naphthol hexazo-tized HPR complex)	Barka and Anderson, 1963a,b; Pearse, 1968
Catalase	9.0	H_2O_2	DAB/Osmium	Black (osmium black)	Novikoff and Gold-fischer, 1969; Seligman, et al. 1973
CyO	7.2-8.0	-	DAB/Osmium	Black (osmium black)	Novikoff and Gold-fischer, 1969
Lipid	7.2	-	Benzpyrene	Yellow, silver to blue fluorescence (ionic tagging)	Chayen, et al. 1969
Fatty acids (unsaturated)	7.2	-	Osmium	Black (osmium black)	Pease, 1964; Hayat, 1972

TABLE VIII (continued)

Substance	pH	Substrate	Coupler	Visible Reaction	Major Reference
SDH	7.8	Succinate	TPT	Pink to red (formazan)	Chayen, et al. 1969
SDH	7.8	Succinate	NBT	Blue (formazan)	Chayen, et al. 1969
SDH	7.8	Succinate	BSPT/Osmium	Black (benzothiazolyl chelate)	Kalina, et al. 1972

Plate XXIII. Summary of age-related changes in the ultrastructure of some major organelles.

Fig. 120. Cell organelles are plotted with corresponding age of culture.

A. Nucleus (N):

1. In the initial stage, the chromatin content of the nucleus is highly electron-dense. The chromosomes and endosome are deeply stained and condensed. When in a "resting stage," the persistently condensed euglenoid chromosomes are attached to the nuclear envelope.

2. At logarithmic phase (ca. 3 days) during active mitosis, the chromosomes detach from the nuclear envelope and divide synchronously with the endosome.

3. At stationary phase, when the cell stops dividing for long periods, the chromatin content of the nucleus decreases; the endosome becomes vacuolate but condensed chromosomes remain attached to the nuclear envelope.

B. Chloroplast (Chl):

1. The euglenoid chloroplast in young cells (from ca. 5-day-old culture) exhibits a homogeneous matrix and continuous lamellar system.

2. It is postulated that chloroplasts may divide symmetrically as shown or asymmetrically and may be in synchrony with the nucleus (Cook, 1971a,b; Boasson and Gibbs, 1973).

3. With advancing age, osmophilic bodies appear in the matrix, between thylakoids, followed by lysis and myelin figure formation.

C. Dictyosome (GA):

1. The dictyosome in young cells (from 5-day-old culture) has a compact arrangement of cisternae.

2. Division may be longitudinal as proposed by Mignot (1965a,b) or transverse as suggested by Leedale (1968).

3. As the cell ages, hypertrophy occurs as the inter-intracisternal spaces increase; cisternae dilate to form large vacuoles and may coalesce with other vesicles to form multivesicular bodies, dense bodies and autophagic vacuoles.

D. Mitochondrion (Mito):

1. Mitochondria may be either rod shaped or in the form of a long and branching mitochondrial reticulum.

2. Reproduction may occur by budding or abscission.

3. In aged cells, mitochondrial fragmentation occurs, followed by lysis; other mitochondria become sequestered by autophagic vacuoles.

E. Pellicle (Pel):

1. Ruthenium Red labelling is heavy in the outermost portion of the plasmalemma of the pellicular region of young cells. Microtubules are prominent in actively motile cells.

2. At cell division, the pellicular strips divide at the incipient stages of cytokinesis.

3. Ruthenium Red labelling decreases as the cell ages, suggesting the shedding of the acid mucopolysaccharide layer possibly due to copious secretion of mucilage during the quiescent palmelloid stage. The decrease in prominence of pellicular microtubules, together with the decrease in acid mucopolysaccharide layer which may serve as a lubricant for the sliding pellicular strips during metaboly, could be factors in the decreasing motility of the cells when they age.

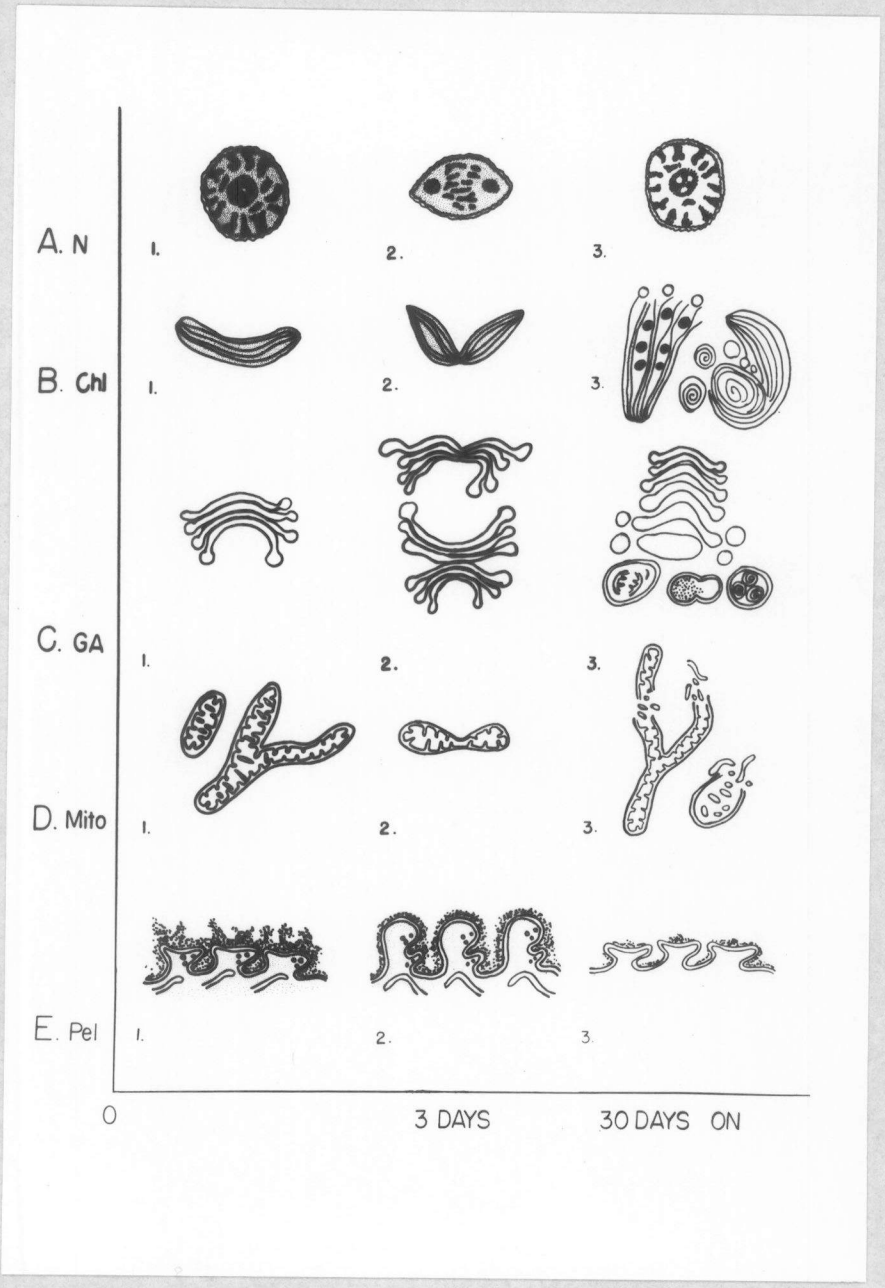


Plate XXIII

III. DISCUSSION

Light Microscopy

Growth characteristics. Doubling of cell counts occurred mostly after every dark period wherein the findings of Leedale (1959a), Cook (1963) and Cook and James (1960) become relevant in this discussion. According to Leedale (1959a) such nocturnal periodicity of mitosis occurs when nutrient supply is low, suggesting that rich food supply removes such periodicity. Cook (1971) found that high external phosphate levels, together with an optimum pH of either 3.3, or 6.8, enable light to stimulate periodicity. The cell is "flooded" with phosphates upon its entry into the light period, causing intracellular pH changes, modifying ratios of phosphorylated compounds, which result in mitotic inhibition (Cook, 1971).

Aside from intracellular pH change as reported by Cook (1971), a corresponding extracellular pH change in the culture medium from pH 3.6 to 7.0 was observed in my cultures. Kempner and Miller (1965) also noted similar pH changes in their cultures from pH 3.4 to 7.0. They attributed such change to the exhaustion of L-glutamic acid as shown by their analysis of amino acid composition. Elemental analysis of lyophilized cells revealed a subsequent decrease in exogenous phosphorus concentration which was correlated with decreasing growth rate of the cells

until a stable cell count was reached at stationary phase. Restoring the exhausted compounds, however, reversed the "retarded" growth rate to normal. The estimated GT of their cultures was ca. 16 h, and they were not synchronized. My cell cultures had a GT of ca. 6-8 h and were synchronized.

Light has been the synchronizer (Zeitgeber) of many autotrophs. According to Cook (1973) energy is stored during the light period. The build up of excess energy causes a sudden burst of cell division which occurs at the latter part of the light period or at the onset of the dark period, as was also observed in my study.

The findings of Winet and Jahn (1971) in Tetrahymena showed that such mitotic inhibition followed by a sudden burst of cell division could have been brought about by the formation and destruction of an "inhibitory layer" caused by the intermittent shaking of flasks during sampling. Cells are drawn to the more nutritious portion of the medium, and growth-inhibitory factors in the subsurface are dispersed. It is, however, doubtful if this type of growth limitation is a factor in the sudden burst of cell division in the case of our synchronized cultures of Euglena.

As shown in Plate I, Fig. 1, page 35, cell division diminished at the onset of stationary phase as GT reached infinity. Following this stage there was a progressive conversion of dividing cells into non-dividing cells, appearance of various cell types, giant cell formation, decrease in cell viability,

eventually death. Morphological variation of cell types is a common occurrence at this stage of culture among ciliated protozoans such as Paramecium (Jennings, 1942; Sonneborn, 1954; Nanney and Rudzinska, 1960); Tetrahymena (Nanney and Caughey, 1955; Nanney, 1957) and Tokophrya (Rudzinska, 1961). The appearance of giant cells, however, is of interest. Such phenomena have been reported in a number of aging cell cultures, such as in human parenchyma cells (Tauchi, 1972); in late stationary phases of Aspergillus niger (Stanier, et al. 1968); in bacteria and yeast cells (Buchanan and Fulmer, 1930); in enlarged cells of Anabaena (Bausor and Agona, 1973); Chroococcus and Gloeocapsa (Shoun, et al. 1973); and in two forms of light-grown Chroomonas salina (Antia, et al. 1973). There were earlier reports (Gross and Jahn, 1962; Cook, 1963) of "monster" Euglena cells, which seem to be similar morphologically to the giant cells found in my cultures.

In the aforementioned cases, the cells were all non-dividing but possibly still retained their "potentiality" for cell growth. In the case of human cells in culture, Tauchi (1972) interpreted such an occurrence as part of the programmed and controlled aging process. Buchanan and Fulmer (1930) and Stanier, et al. (1963) suggested that giant cell formation could be due to a chemotropic response for survival. In 1973, Bausor and Agona hypothesized that "autointoxication," as a result of CO_2 depletion and pH change, might be implicated in their appearance, whereas

Gross and Jahn (1962), in their experiment with thermal and photo stress in Euglena, postulated that "such induced teratism could be a genetic mutation or only a cytoplasmic mutation with the capacity of reverting to normal."

Antia, et al. (1973), in their aging culture of Chroomonas salina, observed abnormal liberation of starch into the culture medium. They interpreted such a phenomenon as a "suicidal tendency" of cultures to continue the reproduction and maintenance of the cell line. It is possible that depleted nutrients in the medium are replenished by cellular autolysis or decomposition of less-viable cells, thereby contributing to the maintenance of surviving cells and stabilizing the population at stationary phase. The different levels of biological organization are inherently determined both with regard to their size limitation and to various control mechanisms from molecular to ecosystem levels (Strehler, 1962). The stationary populations of the cultures are found to persist for countless generations with GT at infinity, with some cells dying and very few dividing. Populations maintained for many months without renewal of growth medium apparently function "normally" only to the extent that cellular control mechanisms permit, considering age and genetic constitution. Survival at this stage, therefore, could possibly be a matter of evolutionary selection.

Another morphological change observed among old cultures of non-dividing cells is clumping, accompanied by

loss of motility and, occasionally, the copious secretion of mucilaginous substances. Cook (1963) observed such a phenomenon in Euglena grown under high light intensities. Similar clumping and secretory activity were reported in "palmelloid" stages of Anabaena (Bausor and Agona, 1973). Very little, however, is known about the possible causal mechanism in such clumping phenomena.

Acid phosphatase. The increasing amount of acid-phosphatase precipitate, which is considered to be age-related (De Duve, 1969) has been previously reported in Euglena granulata (Walne, et al. 1970; Palisano and Walne, 1972), in two species of Ochromonas (Lui, et al. 1968; Grusky and Aaronson, 1969), in stationary-phase cultures of aging Tetrahymena populations (Elliot and Bak, 1964; Elliot, et al. 1966), in some rotifers (Herold and Meadow, 1970), in starved cultures of Euglena gracilis (Brandes, et al. 1964; Malkoff and Buetow, 1964) and in Tetrahymena (Klammer and Fennell, 1961; Nilsson, 1970).

As shown in Plate IV, Figs. 10-11; Plate V, Figs. 15-18; Plate VI, Figs. 23-26, the labeling of acid phosphatase is first localized at the reservoir region. Like Tetrahymena, this area contains a large number of smooth-membrane-bound vesicles associated with the Golgi complex (Nilsson, 1970; Gomez, et al. 1973; 1973b; 1973c) whereas in Cryptomonas, it is associated with multivesicular bodies (Lucas, 1970). Acid phosphatase, a type of hydrolytic

enzyme, is considered to be one of the biochemical markers of lysosomes. According to the "lysosome concept" (De Duve, 1969), primary lysosomes are Golgi-derived, containing hydrolytic enzymes which exhibit latency. In younger cells, acid phosphatase may show such marked latency that its presence may not be as evident in this stage of cellular development.

The increasing distribution of acid phosphatase may be attributed to a change in latency of hydrolytic enzymes--either by change in membrane configuration as postulated by De Duve (1969), or by a time-release process caused by gradual electrostatic changes taking place within the lysosomal matrix (Koenig, 1969). This time-release process could have been triggered by some catabolic process, brought about by macromolecular breakdown (Klamer and Fennell, 1961; Grusky and Aaronson, 1969), accompanied by coalescence of other lysosomes and other vacuolar systems (Nilsson, 1970; Gomez, et al. 1973a; 1973b; 1973c; 1974a; 1974b). Ultrastructural cytochemistry, which will be discussed elsewhere in this thesis, confirms such morphological observations.

Succinate dehydrogenase (SDH). Decline in SDH activity has been reported in many aging biological systems (Toth, 1968; Grusky and Aaronson, 1969), although there are other reports of constant SDH activity (Tauchi and Sato, 1968). Decrease in respiratory activity has been directly

correlated with protein degradation, liberation of amino acids and bases into the medium, increase in acid-phosphatase activity, and hence, cellular autolysis (Nilsson, 1970). On the other hand, Tauchi and Sato (1968) noted few, but greatly enlarged, mitochondria in aging human cells in culture, interpreting such morphological changes as a compensatory mechanism of the cells for decrease in number of mitochondria. Barrows, et al. (1960), in their studies dealing with age-related succinoxidase contents in mitochondria, noted a similar decrease in enzyme activity but attributed such results as due to decrease in number of mitochondria rather than to change in relative amount of the enzyme per mitochondrion.

A possible cause of variable SDH activity noted in my study lies in the nature of the mitochondria complex in euglenoid flagellates. Leedale and Buetow (1970) observed in untreated, unstained Euglena dynamic activity of the mitochondrial threads which underwent morphological changes such as branching, elongation, fragmentation, and fusion to form a mitochondrial reticulum. With the use of Janus Green, they further observed the appearance of coarse mitochondrial threads associated with reduced oxidative activity, whereas longer, thin mitochondria were associated with higher oxidative activity. In some cytochemical procedures, however, the concentration and quality of the stain used could be a factor. When a mitochondrial stain such as Janus Green B (JGB) is used

in low concentration, shortening and coarse granulation of mitochondria occur, resulting in decrease in SDH activity (Koide, et al. 1970). The cytochemical studies on SDH activity carried out showed increase, decrease or very slight change in enzyme activity. Objective measurements are therefore needed in order to assess the true SDH activity in relation to the age of the cells.

Fluorescence. Some lysosomes that are found to contain lipoproteins exhibit fluorescence due to their electrostatic binding ability with basic dyes or fluorochromes. Their dependence on acidic binding sites makes pH and concentration of fluorochrome of important consideration in fluorochrome labeling experiments (Allison and Young, 1969; Koenig, 1969). Most of my fluorochrome studies were done at pH 3.6, 5.0 and 7.8, depending on the pH of the particular growth medium. Fluorescence tended to fade at low pH. Koenig (1969) postulated that such fading was possibly caused by cationic molecules competing for acidic binding sites of lysosomes.

The aging pigment in Euglena, which is possibly similar to lipofuscin as reported earlier (Leedale, 1967a,b), accumulates in non-dividing cells. It is believed to be lipoprotein in nature and exhibits golden-yellow fluorescence (Allison and Young, 1969; Strehler and Barrows, 1970). Bastos, et al. (1966), reported a correlation between Gomori-positive granules and the distribution, form, and

size of fluorescent granules. Delayed fluorescence was likewise attributed to latency of hydrolases within lysosomes.

As shown in Plate VII, Figs. 29, 32, 33 and 34, page 50, in my study, bright-yellow-fluorescing granules were observed, particularly in late-stationary phase cells (Plate V, Fig. 17, page 45). No correlation could be made, however, with the distribution of acid phosphatase in Gomori-treated cells in Plate III, Figs. 6-9, page 39. It is then possible that diffusion of acid phosphatase in the cell could have altered some fluorescence properties due to molecular competition for the binding sites of the fluorochrome.

Benzpyrene labeling showed increase in lipids with increase in age (Plate VII, Fig. 34) in a majority of the cell population. Similar accumulation of lipids has been reported in Ochromonas (Schuster, et al. 1968), in phospholipid vesicles of Euglena spirogyra (Leedale, et al. 1965), in degenerated plastids of Euglena gracilis (Gibbs, 1960) and in Polytoma (Cirillo, 1956), exhibiting reduced fatty-acid oxidase activity. Products of lipid autoxidation, which could possibly be derivatives or precursors of the aging pigment lipofuscin (Plate VII, Fig. 31) could have caused the bright-yellow fluorescence of granules shown in Plate VII, Figs. 29, 32, 33 and 34.

Electron Microscopy

Nucleus. Although the nucleus of Euglena followed "classical" mitosis like those of higher organisms, the persistent condensation of chromatin at the "resting stage," specifically of the endosome (nucleolus), was considered a deviation (Leedale, 1967a,b). The nuclear envelope was equally persistent during active mitosis. It resembled the honeycomb nuclear envelope of Amoeba proteus (Flickinger, 1973), differing only in the outer layer of the double membrane. The inner portion of Amoeba nuclear envelope has the honeycomb layer with fine fibrils in hexagonal array of pores while those of the outer layer, towards the cytoplasm, resembled thin cisternae of rough ER. In Euglena, both inner and outer double membranes seemed to fuse to form the pores (Plate X, Figs. 39-40, page 58).

There have been no studies on isolated nuclei of Euglena although the biochemical aspect of its nucleic acid composition has been reported (Brawerman, 1968). In synchronously dividing populations, the DNA level was found constant during the first 8 h of the light regime, doubling 6 h hence (Edmunds, 1964; Walther and Edmunds, 1970). During active mitosis, in the dark, the DNA level became constant once again, suggesting that Euglena followed a normal cell cycle. In cells with low GT (high growth rate), a corresponding high DNA level was noted (Cook, 1963).

Brawerman (1968), however, attributed such high DNA level to growth conditions such as growth medium and light intensity.

As other organelles degenerated with age, the nucleus remained relatively intact until later stages. As shown in Plate X, Figs. 41-46, page 58, a corresponding change occurred as the nucleus began to age. The endosome notably became vacuolate and some regions decreased in stainability. As cells moved on from log phase to stationary phase, Pogo, et al. (1966) found a corresponding decrease in RNA content in Euglena. In wheat coleoptile, low ^{3}H-uridine incorporation was correlated with inactive rRNA synthesis, appearance of nucleolar fibrils and vacuoles as cells stopped dividing (Rose, 1974). Franke, et al. (1973) associated the appearance of nucleolar granules and fibrils with reduced rRNA synthesis in generative cells of pollen.

Acid phosphatase was detected in the nucleus of animal cells in culture which accounted for the presence of hydrolyzed end products of RNA degradation (Love, et al. 1968; 1969). Degradation of RNA yields 2',3' nucleotides. Together with the sugar phosphates, hydrolysis of these nucleotides by acid phosphatase could increase the level of orthophosphates and other phosphates in the pool. Orthophosphate activates the enzyme polynucleotide

phosphorylase responsible for degrading nuclear RNA. It is then possible that increased acid phosphatase localization with age includes nuclear acid phosphatase which could then correspond to increased vacuolization of the endosome.

In animal cell cultures, nucleolar vacuoles have been associated with microspherules. It is postulated that they are sites of DNA inactivation (Recher, et al. 1973). In stationary-phase cultures of Euglena, a variable level of DNA was noted (Brawerman, 1968) which could be interpreted as a kind of shifting in the steady state of DNA in the nucleus during stationary phase, rather than DNA inactivation.

Canal, reservoir and related organelles. Microtubules manifest certain definite orientation around the canal region and reservoir regions. Distinct types of MT were recognized: (1) circular microtubules situated immediately below the canal opening (Plate XI, Fig. 47, page 61) had angular orientation of less than 90°, possibly occurring in helical patterns, and (2) filamentous MT (Plate XI, Figs. 48-49) aligned at right angles to each other around the canal proper. Buetow (1968a) interpreted the MT arrangement to be helical throughout but probably failed to recognize some of the definite angular patterns observed in this study.

Certain variations in MT have been identified (Frey-Wyssling, 1973) based on the diameter of each MT, helical angle with respect to its tubular axis and inclination angle. There are also MT which can be isolated such as those found in the mitotic spindles, whereas these in the cytoplasm have not yet been isolated. Frey-Wyssling (1973) postulated that such structural variation could be influenced by chemical differences, ontogeny, sensitivity to fixation and temperature and effects of various enzymes. It is highly probable that age could also be a factor influencing structural variation of MT, and likewise the decreasing number in aged cells, particularly at the subpellicular region. Since increase in acid phosphatase localization has been observed in Euglena (Gomez, et al. 1973a; 1973b; 1973c; 1974a; 1974b), decreasing numbers of MT in aged cells may be attributed to the action of lytic enzymes within the lysosome system. Such turnover process, however, is irreversible (Frey-Wyssling, 1973).

At the subapical portion of the canal lies the reservoir. MT align in parallel array encircling the surface of the reservoir (Plate XI, Fig. 50, page 61). In certain food-ingesting algal flagellates such as Peranema, this type of MT is absent in the reservoir proper (Mignot, 1966; Nisbet, 1974). The absence of MT allows considerable elasticity and expansion of the membranes during

ingestion in Peranema (Nisbet, 1974); in Euglena, MT are numerous and there are no reports of food-ingestion into the reservoir. It is postulated that these MT may be functioning as a cytoskeleton providing rigidity (Leedale, 1967a,b; Nisbet, 1974).

Although MT are not found in the reservoir proper of Peranema, occasionally some MT similar to pellicular MT are found in isolated portions of the canal (Nisbet, 1974). Due to their association with muciferous bodies and dark staining bodies in the canal, it was postulated that they may have some secretory function. Actual secretory activity has not been observed in Euglena, but fibrillar mastigonemes were found in the reservoir (Plate XI, Figs. 52 and 53, page 61). Positive AP and RR labeling of the smooth membrane vesicles found in this region (Plate XI, Fig. 51; Plate XII, Fig. 59, page 64), however, suggest the possible occurrence of secretory activity. Secretion of mucilaginous substances have been observed in Euglena particularly during the clumping stage in old cultures.

Two flagella lie below the surface of the membrane lining the reservoir in which a complex system of MT (basal bodies or kinetosomes) is found at the base. Beyond this region in the long flagellum is an adjacent body with a crystalline inclusion, the flagellar swelling (Plate XII, Fig. 54). A common membrane "wraps" the flagellum with the

FS; some MT were found to be continuous with this membrane (Plate XII, Figs. 55-58, page 64). This type of association has also been observed in Astasia (Sommer and Blum, 1965) and Khawkinea (Schuster and Hershenov (1969, 1974) and has been related to active metaboly (Mignot, 1965b; 1966). Hence it is possible that the decrease in MT observed in aged cells is associated with decreased motility of the organism. Flagella were also shed at this stage, although there were reports of flagellar resorption in starved cultures (Leedale, 1972, personal comm.). The manner of shedding and regeneration has not yet been clarified. Flagella origin, however, has been commonly traced to the basal body which is possibly autonomous (Grell, 1973). The presence of DNA, however, has not yet been established in basal bodies (Younger, et al. 1972; Flavell and Jones, 1971; Grell, 1973). In Paramecium (Dippell, 1968; Grell, 1973) new basal bodies arise near mature ones and the perpendicular orientation of new and old MT is of significance to this study.

Chloroplasts. In healthy young cultures of Euglena, cells exhibited bright, grass green coloration. Under the electron microscope, chloroplasts revealed thylakoids which were aligned approximately in parallel array in the homogeneous stroma. Actively dividing chloroplasts were

not demonstrated in my study, but data from other investigators suggest the presence of information DNA units which may or may not be independent of the nucleus (Gibor and Herron, 1968; Schiff and Epstein, 1968; Buetow, 1968b).

As cultures reached stationary phase, a corresponding decrease in greening of the cells was observed. The fine structure of chloroplasts showed prominently the appearance of electron-dense osmophilic bodies in the chloroplast matrix between thylakoids, accumulating with age. Subsequently, chloroplasts ruptured and lysed, releasing contents into the cytoplasm. The concomitant appearance of osmophilic bodies with age seems to be an established feature of chloroplast aging, particularly in higher plants. There have been a number of reports in Phaseolus (Barton, 1966), Cucumis (Butler, 1967), wheat (Haber, et al. 1969) oat (De Vecchi, 1971), tobacco (Arnott and Harris, 1973; Harris and Arnott, 1973) and spinach (Cran and Possingham, 1974). Harris and Arnott (1973) called the OS, plastosomes, and suggested that they originated from primordial discs within the grana and thylakoids. Bailey and Whyborn (1963) isolated two kinds of globules from chloroplasts of Beta vulgaris. One type was a lipid plastoquinone and the other a protein component. The lipid component roughly corresponded with the number of

OS which led them to postulate that lipids may be sources of energy in the same way as starch. Harris and Arnott (1973) hypothesized that OS may be an initiating step prior to chloroplast breakdown. Cran and Possingham (1974), however, suggested that chloroplast degeneration could have been brought about by the inability of the plastids to utilize certain substances produced by dividing cells. It is possible that this particular substance is in the nature of a ribosome. Reduction in chloroplast ribosomes, nucleic acids, chlorophyll and soluble proteins have been observed in a number of studies (Buetow, 1968a; 1968b; Cocucci and Marré, 1966; De Vecchi, 1971 and Butler, 1971). Butler (1971) found free ribosomes in the ground plasm following chloroplast breakdown. It is possible that these ribosomes were released into the ground plasm, forming aggregates (polysomes) or helical coils (cytonemata) which later on attached to ER. The ER vesiculated and later on disappeared (Butler, 1971) possibly in the lysosome system.

Paramylon. Morphologically, paramylon granules resemble starch grains and are found outside chloroplasts. The negative reaction to an iodine test indicated they were not starch. Tests by other investigators have shown them to be β-1,3-glucan (different from starch which is a

β-1,4-glucan, Leedale, 1967a). Recent studies, however, failed to show evidence of glucose or free sugars which were associated with β-1,3-glucan (Kempner and Miller, 1972).

Large quantities of D-mannitol and significant traces of glycerol were detected instead using tracer studies ("chase" radioactivity and proton NMR analysis) and enzymic measurements (colorimetry and chromatography). While D-mannitol was found to be "impermeable" to Euglena cells, it was suggested that positive mannitol dehydrogenase activity similar to those found in bacterial systems could have brought about rapid conversion of fructose to mannitol.

Earlier studies of its fine structure revealed membranes surrounding paramylon granules, with the fibrils arranged lengthwise (Clark and Stone, 1960). Positive RR labeling around the granule, suggesting the presence of some kind of mucopolysaccharide (pectin), was observed in this study (Plate XIV, Fig. 72, page 69). It was, however, found only in those paramylon granules in close association with the pellicle of young cells but was not detected in older cells where they occurred in clumps. Rogers, et al. (1972) attributed the presence of plate-like aggregates to bleaching rather than to age, while Brandes, et al. (1964) and Malkoff and Buetow (1964), in their studies with acetate (carbon) starvation, found fusion and depletion of paramylon.

Schuster and Hershenov (1974) observed a similar increase in paramylon in their old cultures of Khawkinea, a colorless counterpart to Euglena. It is then possible that the increasing association of paramylon with dense bodies and acid phosphatase-positive vesicles and the prevalence of paramylon in older cultures are age-related phenomena.

Dictyosomes, lysosomes and related organelles. Dictyosomes abound in healthy young cells of Euglena and were situated around the reservoir, nucleus and at the posterior portion of the cell. Long stacks of cisternae per dictyosome were estimated to be 15-30 (Mollenhauer, et al. 1968). They exhibited polarity as shown by the presence of polyribosomes at the forming face (Mollenhauer and Morré, 1973). Polyribosomes were aligned in between the ER and the first cisterna of the dictyosome.

The size and number of dictyosome varied with the age of the cell. Older and less active cells contained fewer dictyosomes and cisternae, particularly after they underwent hypertrophy and cisternal dilation.

The electron-dense staining at the center of the dictyosome has been a common observation, but its nature remains unknown. Microtubules have been reported in the intercisternal spaces (Mollenhauer, 1964). It has been postulated that they serve to transport products into and out of the Golgi apparatus. The question on the possible

function of the Euglena dictyosome has therefore been raised once again (Mollenhauer, et al. 1968). As shown in this study, it could function as a source of processing of acid hydrolases or other enzymes.

Acid phosphatase labeling was localized initially in the Golgi regions of cells from mid-logarithmic phase of growth and subsequently was transferred to dictyosome-associated vesicles of cells from older cultures. Observations from light microscopy, however, showed initial labeling of acid phosphatase in the region of the reservoir wherein smooth membranes were predominant, suggesting perhaps some association with both smooth endoplasmic reticulum (SER) and Golgi apparatus (GA) complexes. Sommer and Blum (1965b) found acid phosphatase both in the ER and GA of peri-reservoir and sub-pellicular regions of Euglena, while Nilsson (1970) interpreted such small, smooth-membraned vesicles to be primary lysosomes functioning as an enormous Golgi complex at the oral region of Tetrahymena.

With advancing age of cultures, acid-phosphatase precipitate decreased in the Golgi region but increased in near-by vesicles in the cytoplasm. Concomitant increases in numbers and kinds of vacuoles exhibiting variable matrix consistencies have been noted. In younger cells, acid-phosphatase-positive vesicles were observed in association with paramylon granules, which later decreased

in size and distribution. Malkoff and Buetow (1964) interpreted such phenomena as a means of cellular utilization of carbon reserves in case of glucose depletion. Yamamoto, et al. (1969) distinguished two types of lysosomes in HeLa cells which could parallel acid-phosphatase activity in the logarithmic and stationary phases of growth in our study: (1) primary lysosomes which are responsible for cellular growth and activity and (2) secondary lysosomes which bring about cellular degradation, in which the concept of focal cytoplasmic degradation (FCD) (Hruban, et al. 1961; 1965; Swift and Hruban, 1964; Hruban and Rechcigl, 1969) becomes significant in the latter part of the discussion.

A number of studies suggested that primary lysosomes originate from endoplasmic reticulum. Berjak (1972) postulated that acid phosphatase is initially synthesized in the ER and is later incorporated with Golgi vesicles to form secondary lysosomes. Maggi and Carbonnell (1969) detected acid phosphatase in the rough ER (RER) of mice tissues, which are, however, different in the sense that they were not inhibited by sodium fluoride (NaF). Allen, et al. (1963) in their electrophoretic studies of acid phosphatase in Tetrahymena, found one acid phosphatase in the microsomal fraction. It differed from the other 17 hydrolases they had identified because it was incapable of

hydrolyzing sodium-β-glycerophosphate. It is possible that the acid phosphatase found by Maggi and Carbonnell (1969) in the RER is related to that found in the microsomal fraction of Allen, et al. (1963).

Studies of Euglena by Brandes, et al. (1964); Brandes and Bertini (1964); Sommer and Blum (1965b); Walne, et al. (1970); Palisano and Walne (1972); Gomez, et al. (1973a; 1973b; 1973c; 1974a; 1974b) showed labeling of acid phosphatase in the Golgi region. Similar results were obtained in Tetrahymena (Nilsson, 1970). The electron microscope radioautographic studies of Droz (1965; 1967) reported that the highest peak of protein labeling occurred in the Golgi region and occasionally in the adjacent lysosomes, suggesting that the enzymes could be processed in the Golgi.

Novikoff, et al. (1964; 1966) have placed great emphasis on the Golgi-endoplasmic-reticulum-lysosome (GERL) and/or Golgi complex in the cytogenesis of primary lysosomes, providing a possible mechanism for their origin. My findings, however, suggest the active role of the Golgi in the processing of hydrolytic enzymes such as acid phosphatase. These enzymes are incorporated into small packets or primary lysosomes which, in later stages, associate with other cytoplasmic vesicles. The manner of association of primary lysosomes with these

vesicles is not known; they are, however, of great significance in the study. As the cell ages, certain unfavorable conditions in its extra-intracellular environments occur. The cell must be able to "cope" by replacing or digesting materials in its metabolic pool for re-utilization. Increased hydrolytic activity, therefore, takes place, accompanied by coalescence, increase in vacuolization and possible sequestration and autophagy. Failure of the cell to adapt to this changing environment ultimately leads to its death.

Microbodies (peroxisomes). Microbodies (peroxisomes) are recently identified organelles reported in a few algal groups such as Euglena (Graves, et al. 1971; Brody and White, 1973), Polytomella and Chlorogonium (Gerhardt and Berger, 1971) and 13 genera of dinoflagellates (Bibby and Dodge, 1973). They are characterized as ubiquitous organelles, bounded by thin, single-limiting membranes, with fine granular matrices of uniform distribution and of varied sizes and shapes (Hruban and Rechcigl, 1969; Vigil, 1970). In a number of animal and higher plant cells, microbodies are seen with cores or nucleoids, centrally located. In higher plants the cores have been identified as crystal-containing-bodies (CCBs - Frederick, et al. 1968), and the microbodies themselves

lack the often-reported association with ER (Fahimi, 1969). Their major occurrence in less-active plant cells and in storage tissues has led to the suggestion that they are symptoms of functional inactivity, and are mere modifications of microbodies of the non-crystalline, anucleoid type (Frederick, et al. 1968). In 1969, Fahimi detected the enzyme urate oxidase to be associated with nucleoid microbodies.

In this study, only microbodies without CCBs have been observed. Graves, et al. (1971), however, reported the appearance of structures with electron-dense cores with whorl-like configurations similar to those I observed (Plate XVII, Figs. 89-90, page 77). It is suggested that such structures may represent one of the multiple forms of microbodies with a different type of core (Graves, et al. 1971).

There are other criteria used in the identification of microbodies aside from morphological visualization. With the use of DAB medium, it is now possible to characterize microbodies cytochemically by means of the enzymes present in them (Graham and Karnovsky, 1966; Seligman, et al. 1968; Novikoff and Goldfischer, 1969). Although a number of enzyme systems are associated with microbodies, catalase appears to be the only enzyme present in all microbodies. It has, therefore, been regarded as a diagnostic biochemical marker (De Duve and Baudhuin, 1966;

Hruban and Rechcigl, 1969). There are, however, some fragmentary reports of microbodies that are devoid of catalase and that are unresponsive to DAB treatment, such as those found in Polytomella and Chlorogonium (Gerhardt and Berger, 1971), in dinoflagellates (Bibby and Dodge, 1973). Low levels of catalase activity have been reported in homogenates of Tetrahymena (Muller, et al. 1968) and in Ochromonas (Lui, et al. 1968). Tumor cells containing minute and only slightly reactive microbodies were described by Novikoff, et al. (1972; 1973a; 1973b). It is possible that such microbodies with little or no catalase may contain virtually undetectable levels of enzymatic activity (Brody and White, 1973), particularly in the microperoxisomes of the above-mentioned tumor cells, and in the few microbodies of algae grown in inorganic medium.

Catalase in microbodies stains well with DAB in the presence of H_2O_2 substrate at pH 9, producing an osmium black reaction with oxidized DAB (Seligman, et al. 1968). Such reaction is inhibited by aminotriazole, and no reaction can be produced at a pH lower than 7.0, suggesting the presence of the enzyme, catalase (Childs, 1973; Hand, 1973).

In some untreated preparations without addition of H_2O_2 substrate and/or DAB medium, the staining of microbodies can still be observed. This is possibly due to the

generation of endogenous H_2O_2 during incubation or to staining by some non-enzymatic mechanisms (Fahimi, 1969). The presence of catalase is important in the cell because it is regarded as a safety device to prevent the overflow of H_2O_2 generated by microbody oxidases, thus providing protection from oxygen toxicity (De Duve and Baudhuin, 1966).

As shown in the results, there is an increasing staining reaction to DAB medium as the cultures age. Similar increase in size and number of microbodies has also been associated with aging castor bean endosperm (Vigil, 1970). When DAB undergoes polymerization, the polymers exhibit different degrees of branching, depending on the conditions of oxidation at a given time. Smaller polymers that produce slight staining reaction are indicative of a slow oxidation process in the microbodies. Due to their close association with other organelles like ER, it is also possible that the size and alkaline nature of the enzymes may allow them to diffuse short distances and to couple with acidic sites of ribosomes and ER (Fahimi, 1971; Seligman, et al. 1973). There is, however, some evidence that suggests the abscission of microbodies from ER, particularly those seen in spatial proximity to ER (Vigil, 1970). The membranes of adjacent ER and microbodies are almost indistinguishable, and small membranous connections could very well be extensions of ER cisternae (Frederick, et al. 1968).

The absence of reaction within the ER has been interpreted to indicate that catalase is assembled within the peroxisome from ER-produced precursors (showing negative DAB reaction), which are subsequently transported via smooth ER connections to the peroxisomes (Hand, 1973). On the other hand, it is possible that some peroxisome enzymes may not originate from ER but may be synthesized by ribosomes, then transported to the peroxisomes, incorporated and transferred directly across the peroxisomal membrane, some of which form minute organelles (micro-peroxisomes) that are surrounded by membranous elements in the cytoplasm where the enzymes are synthesized (Hand, 1973).

During cellular lysis, like those involved in FCD, microbodies undergo fusion, and vacuolization involving sequestration by other organelles such as lysosomes and autophagic vacuoles. Some cells (e.g., castor bean endosperm) underwent condensation of the microbody-producing-matrix, forming CCBs, altering the principal sites of catalase activity in the matrix (Vigil, 1970). Decreased or variable catalase activity, therefore, could be attributed to such biological processes as FCD in the microbodies.

The identification of various enzymes in the microbodies made possible their correlation with different biochemical processes taking place in the cell, such as those involved in cellular aging, e.g., nucleic acid degradation,

gluconeogenesis, β-oxidation of fatty acids, oxidation of reduced NAD (Hruban and Rechcigl, 1969), and photo-respiration (Nir and Sligman, 1970; Brody and White, 1973).

Mitochondria. DAB medium without H_2O_2 and at pH 6-8 acted as a coupling agent to demonstrate cytochrome oxidase activity in mitochondria. Seligman, et al. (1969), in their studies of mitochondrial activity in animal cells, demonstrated cytochrome oxidase activity using DAB medium. Most of the reaction product was observed in the intracristate spaces (in the outer compartments) between the inner and outer limiting membrane of mitochondria. Their findings suggested that as cytochrome a and a_3 are oxidized, cytochrome c - located on the inner surface of the intracristate space was able to oxidize the DAB. Novikoff and Goldfischer (1969), interpreted the staining of the inner mitochondrial membrane as due to cytochrome c activity and that of the outer mitochondrial membrane as cytochrome b_5. Childs (1973) proposed the presence of two enzymes systems in the mitochondria, both capable of oxidizing DAB. One intracristate enzyme was found to be stronger oxidant than are those in the inner and outer mitochondrial membranes.

Upon treatment of Euglena gracilis 'Z' with DAB under temperature conditions not considered optimal for

detecting the presence of "conventional" cytochrome oxidase, Brody and White (1973) reported the presence of a mitochondrial enzyme in the matrix. In my results, however, the staining was localized largely to the cristal membranes and not mainly in the matrix as reported by Brody and White (1973). The staining was partially inhibited by sodium azide in younger cells and was completely inhibited in older cells, suggesting that these enzymes might be cytochrome oxidase. Without isolation of the organelles and subsequent biochemical studies, it is doubtful if such results will allow the identification of the cytochrome oxidase triplets, as suggested by Seligman, et al. (1969) and by Novikoff and Goldfischer (1969).

Another difficulty involved in the cytochemical identification of mitochondrial enzyme in Euglena gracilis lies in the nature of its mitochondrial complex. As previously described (Leedale and Buetow, 1970; Gomez, et al. 1974a; 1974b) euglenoid mitochondria are long and branching, and often form a mitochondrial reticulum that resembles somewhat the single giant branching mitochondrion of the diploid strain iso-N of Saccharomyces cerevisiae (Hoffman and Avers, 1973).

The mitochondria contain a heterogeneous enzyme system involved in a variety of chemical and biological processes. It is possible that portions of the mitochondria may be involved in different chemical reactions, depending

on their spatial location and association with other organelles in the cell. Various cytochemical responses can therefore be observed, depending on the physiological conditions of the cell at a given time.

Plasma membrane and other cytoplasmic organelles. Ruthenium red (RR), which was used in this study, has been found to be a stereospecific stain capable of labelling pectic acid and its derivatives, sites between adjoining galacturonic monomer units, and de-esterified cell walls of higher plant cells (Sterling, 1970; Luft, 1971). In studies of cytophagocytosis, the use of RR eliminates the necessity of tedious serial sectioning, because RR can penetrate crevices and small openings at the cell surface, adhere to the extraneous layer of the plasma membrane, and bind with acid mucopolysaccharides and plasma-membrane-derived vacuoles (Chambers, 1973). Ruet and Herzborg (1973) used RR in the study of cell surface activity and enzymes associated with it in animal embryonic cells. In higher plant cells, Colvin and Leppard (1973) were able to study the structure and possible composition of cell walls with osmium-RR labelling. Their presence along the outer pellicular strips on the surface of Euglena gracilis 'Z' has been previously reported (Gomez, et al. 1973c; 1974a; 1974b).

As shown in this present study, RR labelling of the outer layer of the plasma membrane revealed a slightly

fibrillar structure interwoven to form "fuzzy" fibrils. Similar fibrillar structures, particularly those occurring at the exterior surfaces of pellicular knobs of old pellicles, have been investigated by Leedale (1964) and by Schwelitz, et al. (1970). Some investigators (Buetow, 1968; Schwelitz, et al. 1970) have postulated that the substructure of threads superimposed on the smooth layer of the plasma membrane were mucilaginous secretions (mucin), secreted as such along the pellicular striae of cells. Leedale (1964) suggested that they come from the muciferous bodies and serve as a means of lubricating the surface of the pellicle, as the ridges slide along the grooves during euglenoid movement. Muciferous bodies, together with microtubules, have been observed at the pellicular pores of Euglena (Leedale, et al. 1965; Arnott and Walne, 1967), where mucus bodies deposit their contents (Dangeard, 1928), presumably produced from the vesiculate portions of the ER (Leedale, et al. 1965).

Similar mucilaginous tuft-like materials on the old walls of many higher plant cells have been previously reported (Colvin and Leppard, 1973). They appear as thick, bushy fibrils which later are sloughed off into the growth medium in huge quantities. In this study, older pellicular surfaces are found to contain little or no RR labeling, compared to those from younger cultures. It is possible that the fibrillar tufts so prominent in cells from younger

cultures have been similarly sloughed into the medium, thus accounting for the decrease in cellular motility of many aging Euglena cells as lack of lubrication may prevent the sliding of pellicular ridges along the grooves. In the red algae, Porphyridium aerugeneum and Pseudogloiophloea, similar observations have been reported during the stationary phases of growth. Large quantities of amorphous, water-soluble, polyanionic polysaccharides are excreted. Electron microscopy revealed that the polymerized polysaccharides are transported via Golgi vesicles to and through the plasma membrane (Ramus, 1972).

Preliminary studies on the chemical composition of these fibrils reveal the presence of fibrillar, a 1-4 polymer of D-galacturonic acid as a major constituent (Colvin and Leppard, 1973). Its heterogeneity and ability to absorb proteins make it a suitable substance for surface attachment between cells, such as that seen in the clumping phenomenon of aging Euglena cells, or as promotor of symbiosis and cementing substance between cells of higher plants (Colvin and Leppard, 1973).

Unlike the cell surfaces of SaI cells (Chambers, 1973) and of Amoebae (Anderson, et al. 1968; Flickinger, 1973), the plasma membrane of Euglena has not been observed to participate in cytophagocytosis by invagination of the plasma membrane. No RR labelling can be observed in the membranes of cytoplasmic vacuoles, except in a few paramylon

membranes near the pellicular region, suggesting that some paramylon membranes may contain acid mucopolysaccharides derivatives, enabling them to fuse with other paramylon granules as reported earlier (Buetow, 1968). Furthermore, the complex structure of the pellicle surface as compared to surfaces of Amoeba and SaI cells may not allow the plasma membrane to invaginate.

Although there has not been any trace of plasma-membrane-derived cytoplasmic vacuoles, autophagic vacuoles or cytolysomes and multivesicular bodies, together with lipofuscin-like pigments and myelin figures have been observed. When a cell isolates "faulty organelles" and harmful substances presumably by "sequestration" of the smooth ER, such cytoplasmic components are formed (e.g., cytolysomes). Isolated substances are degraded by autolysis and are subsequently recycled in the metabolic pool for re-utilization (Swift and Hruban, 1964). The heterogeneity of matrices found in the various cytoplasmic vacuoles may represent possible sites of such sequestration, different stages of lysis and other physiological processes related to FCD. It is suggested that coalescence is not involved in FCD (Swift and Hruban, 1964), but it is also possible that both coalescence and sequestration could occur simultaneously.

Observed in many aging cells, the increasing occurrence of myelin figures, lipofuscin-like pigments and dense

bodies are established symptoms of protoplasmic degeneration involving the complex lipoprotein framework of the cell. The increase in lipid content of the cell (Swift and Hruban, 1964; Gomez, et al. 1973a; 1973b; 1973c) and its resistance to inhibitors including boiling (Fahimi, 1969) suggest that the protein portion of the lipoprotein complex in the cell is degraded at an early stage, increasing in rate as the cell ages. FCD, therefore, may be a survival mechanism or response of a cell, as it undergoes aging, involving isolation and recycling of harmful or useless products. Failure of a cell to recover or to adapt to the changing environment eventually would lead to its death.

IV. SUMMARY AND CONCLUSIONS

1. This investigation involved two research areas: (a) the study of cellular aging using Euglena gracilis 'Z' and (b) the use of ultrastructural cytochemistry in the identification of age-related changes in those unicellular organisms.

2. While age-related changes were not easily identifiable, stationary-phase cells with long generation times (GT) manifested "mitotic repression" or post-mitosis. When such a condition occurred, cells tended to shift in intraorganismal balance, departure from their normal "steady state" and hence became susceptible to changes related to aging.

3. Among the age-related alterations observed in the cell cultures were: changes in morphological and physiological conditions of the cells; alteration of cell organelles, as the nucleoplasm in the nucleus decreased in content, occurrence of lysis in various organelles such as mitochondria and chloroplasts; possible increase in activity in the lysosome system; hypertrophy of dictyosomes; aging pigment and myelin figure formation.

4. Cytochemical changes showed variable activities in catalase, cytochrome oxidase and succinate dehydrogenase. There were concomitant decreases in the fluorescence

property of chlorophyll and in Ruthenium Red labeling of the mucopolyssacharide layer of the plasma membrane, autolysis and increase in activity of the lysosome system were correlated with change in latency of acid phosphatase, as it increased in distribution within the cell with increasing age.

5. When cells failed to cope with these changes, the general deterioration in their form and function was consequently followed by death.

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APPENDIXES

APPENDIX A

CULTURE MEDIA

Defined Culture Media

1. Euglena Broth BM (Difco-0610-01)

Component	g/Liter Medium
L-Glutamic acid	3.0
DL-Aspartic acid	2.0
Glycine	2.5
Sucrose	15.0
Succinate	0.52
DL-Malic acid	1.0
Thiamine Hydrochloride	0.06
Ammonium Bicarbonate	0.36
Calcium Carbonate	0.08
Monopotassium Phosphate	0.3
Magnesium Sulfate	0.2

Dissolve the above components with distilled water and bring to a final volume of 1 liter. Adjust the pH to 3.6 at 25° C.

2. Cramer and Myers Medium (Cramer and Myers, 1952)

Basal Medium

Component	g/Liter Medium
$(NH_4)_2HPO_4$	1.0

(Component)	(g/Liter Medium)
KH_2PO_4	1.0
$MgSO_4$	0.2
$Na_3C_6H_5O_7 \cdot 11\ H_2O$	0.8
	ml/Liter Medium
$Fe_2(SO_4)_3 \cdot 6\ H_2O$	3.0
$MnCl_2 \cdot 4\ H_2O$	1.8
$Co(NO_3)_2 \cdot 6\ H_2O$	1.3
$ZnSO_4 \cdot 7\ H_2O$	0.4
H_2MoO_4	0.2
$CuSO_4 \cdot 5\ H_2O$	0.02
Thiamine·HCl	0.01
Vitamin B_{12} (Merck Cobione)	0.0005
Added for stocks:	g/Liter Medium
Agar	16.0

3. Euglena Agar (Difco Lab 0596-01) (Greenblatt and Schiff, 1959)

Medium A (pH 3.3)

Component	g/100 ml Distilled Water
KH_2PO_4	0.04
$CaCO_3$	0.02 (as Ca)
$(NH_4)_2HPO_4$	0.02
L-Glutamic Acid	0.5
DL-Malic Acid	0.2
$MgSO_4 \cdot 7\ H_2O$	0.05

(Component)	(g/100 ml Distilled Water)
$FeCl_3 \cdot 6\ H_2O$	0.5 (as Fe)
Thiamine·HCl	0.1 mg%
Vitamin B_{12}	0.02 µg/%

Trace metal solution

containing the following:	g/1000 ml Distilled Water
$ZnSO_4 \cdot 7\ H_2O$	4.4
$MnSO_4 \cdot 4\ H_2O$	4.08
$Na_2MoO_4 \cdot 2\ H_2O$	1.008
$CoCl_2 \cdot 6\ H_2O$	0.0807
$CuSO_4 \cdot 5\ H_2O$	0.039
H_3BO_3	0.0283
NaI	0.00118

Add 2 ml of trace metal solution per 100 ml of medium.

4. Euglena Medium (Greenblatt and Schiff, 1959)

Medium B

Component	g/100 ml Distilled Water
K_2HPO_4	0.01
$CaCl_2 \cdot 2\ H_2O$	5 mg (as Ca)
NH_4Cl	0.04
Sodium Acetate .3 H_2O	0.1
n-Butanol	0.3
EDTA	0.05
$MgSO_4 \cdot 7\ H_2O$	0.05
$FeCL_3 \cdot 6\ H_2O$	0.3 mg (as Fe)

(Component)	(g/100 ml Distilled Water)
Thiamine·HCl	0.1 mg
Vitamine B_{12}	0.04 µg
Trace metal solution	1.0 ml
(Same as in Medium A)	

Undefined Culture Medium (Biphasic Medium)

1. Regular Soil-Water Medium
   - (a) Nashville garden loam soil — 15 g
   - (b) Diluent or solvent — 150 ml
   - (c) Additives — speck or pinch
2. Variations of b and c:
   - (a) 150-ml of the diluent may include any of the following:
     - Distilled Water
     - Tap Water
     - Millipore-Filtered Pond Water
     - Bold's Basal Medium
   - (b) Any of the following additives may be submerged beneath the soil:
     - Whole green pea — 1 seed
     - Split or minced pea — 1 seed
     - Pearl barley grain — 1 grain
     - Calcium carbonate — 0.1 g

Procedure:

1. Grind Nashville soil by means of a mortar and pestle.
2. Sieve the soil to separate rocks and other large particles.
3. Distribute the soil in wide-mouth milk bottles or glass jars (14.5 x 5 cm), 15 g soil per bottle (ca. 0.5-1.0 cm from the bottom of the jar).
4. Submerge the additives beneath the soil by means of forceps.
5. Fill each bottle with 150 ml of the diluent.
6. Plug or cover the jars either with cotton plugs carefully wrapped with cheesecloth or glass covers from small Petri plates (Kimax, 6 cm diameter).
7. Sterilize 3X at 15 psi, 160° C for 10 minutes every other day or steam for 1 hour for three consecutive days.
8. Allow the soil to settle to the bottom of the bottle until the medium is clear. The final pH should be ca. 7.2 to 7.4.

APPENDIX B

AXENIC CULTURE METHODS

A. Isolation with Micropipette

1. Heat Pasteur pipette over Bunsen burner until area behind tip is soft. Withdraw from flame and with forceps pull pipette to desired length.

2. Place a drop of sample in a well of a triple depression slide.

3. Place slide under stereo binocular dissecting microscope. Isolate desired cell with micropipette and transfer to drop of sterile medium or distilled water in second well.

4. Examine drop in second well carefully. Re-isolate cell, being careful to avoid any other organisms or contaminants that may have been carried over from first well.

5. Repeat the process using several clean depression slides and micropipettes until a single cell is isolated.

6. Test axenic condition of cultures using standard bacteriological procedures described in this Appendix, pages 171-173.

B. Plating and Streaking

1. Wash cells by sonicating them in 4% Tween 80, either

in growth medium or in distilled water followed by centrifugation at 2000 rpm in an International Clinical centrifuge. Repeat process ca. 5X or until the cells are thoroughly washed.

2. In a sterile room, flame a platinum inoculating loop until red hot. Cool and take a loop of sample.

3. Carefully lift cover of Petri plate containing agarized medium, and streak the sample either back and forth in a zig-zag manner or in parallel rows.

4. Incubate plates in a growth chamber under desired conditions of light and temperature.

5. After 3-4 days, examine last row of streaked inoculum for uncontaminated cells. This portion should be the most dilute and least contaminated. Obtain a loop from this portion and repeat the streaking process on a new Petri plate containing agarized medium.

6. Test axenic condition of cultures using standard bacteriological procedures on pages 171-173.

C. Bacteriological Tests for Axenic Conditions

1. Proteose Peptone (Difco Lab 0120-02)

For detection of fastidious pathogens, particularly for toxin-producing bacteria. Medium turns from clear to cloudy, indicating presence of bacterial contaminants.

Components	g/100 ml Medium
Proteose peptone	0.5
Distilled water	75.0-100 ml

Dissolve proteose peptone in distilled water. Distribute in test tubes (6 x 1 cm) fitted with caps or cotton plugs. Sterilize in autoclave for 15 minutes at 15 psi, 121° C.

2. Nutrient Broth (Difco Lab 0002-01)

Medium turns from clear to cloudy, indicating presence of contaminants.

Components	g/Liter Medium
Bacto-Beef Extract	3.0
Bacto-Peptone	5.0

Dissolve in distilled water and bring to a final volume of 1 liter. Dispense in culture tubes (6 x 1 cm) with caps and cotton plugs. Sterilize in autoclave for 15 min. at 15 psi, 121° C.

3. Nutrient Agar (Difco Lab 0001-01)

Recommended for determining agar plate count of bacteria in bacteriological examination of water.

Component	g/Liter Medium
Bacto Beef Extract	3.0
Bacto-Peptone	5.0
Bacto-Agar	15.0

Dissolve in cold distilled water to a final volume of 1 liter. Heat to boiling. Sterilize in autoclave at 15 psi,

for 15 min, 121° C. Cool. In the sterile room, dispense the medium in previously sterilized Petri plates.

4. Thioglycollate Medium without Dextrose (Difco Lab 0363-01)

Recommended for determination of anaerobes in carbohydrate-free medium. Blue layer of methylene blue decolorizes, indicating presence of anaerobes.

Component	g/Liter Distilled Water
Bacto-Yeast Extract	5.0
Bacto-Casitone	15.0
L-Cystine Difco	0.25
Sodium Chloride	2.5
Thioglycollic Acid	0.3 ml
Bacto-Agar	0.75
Bacto-Methylene Blue	0.002

Adjust to pH 7.2 at 25° C. Dispense in test tubes (70 x 5 mm) with well-fitted caps or plugs. Sterilize in autoclave at 15 psi for 10 minutes, 21° C. Cool until a blue layer of methylene blue appears on the surface of the medium.

APPENDIX C

OPTICAL MICROSCOPE CYTOCHEMISTRY

A. General Staining Procedure for Thick Sections (Jeon, 1965)

Azure II (G. T. Gurr, Ltd.)	0.1-0.5 g
Anhydrous sodium borate (Borax)	1 g
Distilled water	100 ml

Dissolve Azure II in 1% borax solution. Filter and store in dark polyethylene bottles with nozzles (Nalgene). In this way the stain solution can be directly applied to and spread over the slide or cover glass (No. 1, 22 x 50 mm) while being kept dust-free at the same time. This stains the membranes in general.

B. Chromic Acid Slide Pre-Cleaning Solution (Purvis, et al. 1966)

Sodium or potassium dichromate	30 g
Water	100 ml
Sulfuric acid	1000 ml

Make up 1 liter with concentrated sulfuric acid, cooling the mixture while slowly adding acid to water mixture. Soak the slides in the solution for ca. 30 minutes. Rinse thoroughly with running water and wipe them dry.

Procedure:

1. Section the blocks on an ultramicrotome equipped with a

glass knife (LKB) to 0.3-2.0 microns (purple sections) sections of cells embedded in epoxy or araldite resins.

2. Pick up sections with a platinum or nickel wire loop.

3. The sections from the wire loop can either be floated directly on a drop of Azure II stain on a glass slide or can be laid flat on a glass slide pre-cleaned with chromic acid.

4. Place a drop of Azure II directly on the sections and spread or smear the stain evenly.

5. Place slide on a hot plate (ca. 70° C) until the sections are flattened and stain is evaporated.

6. After cooling, place a drop of either Canada balsam or immersion oil (Cargille 25° C-1.5150) on top of the sections. Mount with a cover slip before microscopic examination.

C. Trypan Blue Test for Viability (Paul, J., 1970)

Trypan Blue (GibCo)	0.4 g
Sodium chloride	0.85 g
Distilled water	100 ml

Dissolve Trypan Blue stain in 100 ml of physiological saline solution containing 0.85 g sodium chloride in 100 ml distilled water. Dead cells are selectively stained with Trypan Blue while viable cells tend to resist the staining reaction.

D. Gomori Method for Detection of Acid Phosphatase (Gomori, 1952; Berjak, 1968, 1972; Pearse, 1968; Chayen, et al., 1969; Charvat, 1973, personal comm.)

1. Sodium Acetate Buffer Stock Solution (0.1 N, pH 7.2 or 5.0)

Sodium acetate (FW = 136)	13.6 g
Distilled water	1000 ml

Dissolve sodium acetate in distilled water. Adjust pH using 0.1 N acetic acid or 0.1 N sodium hydroxide.

2. Sodium Acetate Buffer Working Solution (0.05 N)

Sodium acetate buffer #1	1 part
Distilled water	2 parts

Dilute 1 part of #1 with two parts of distilled water to make 0.05 N working solution. Adjust pH to 5.

3. Substrate Solution for Gomori Reaction Medium (3% or 0.1 M)

Sodium-β-glycerophosphate (Matheson, Coleman & Bell)	3 g
Distilled water	100 ml

Dissolve substrate in 100 ml of distilled water.

4. Gomori Reaction Medium (Gomori, 1952)

Acetate buffer #2	500 ml
Lead nitrate	0.6 g

To 500 ml of #2, dissolve 0.6 g lead nitrate. Adjust to pH 5. This constitutes the Gomori reaction medium which may

also be used as control without the substrate.

5. Gomori Reaction Medium Plus Substrate (Gomori, 1952)

Substrate #3	50 ml

To 500 ml of #4, add 50 ml of #3. The complete reaction medium, #5, is left for 24 h at 37° C during which time some white precipitate should form depending upon the source of substrate. The solution must be allowed to cool before filtration. Use the fresh medium immediately after filtration.

6. Gomori Reaction Medium with Sodium Fluoride Inhibitor Used as Control (Berjak, 1972)

Sodium fluoride	0.42 g
Gomori reaction medium #4	100 ml

Add sodium fluoride inhibitor to 100 ml of #4 to make control working solution.

7. Ammonium Sulfide Coupler (Charvat, 1973, personal comm.)

Ammonium sulfide	0.02-0.1 ml
Distilled water	100 ml

Under a fume hood, stir and mix thoroughly. Use fresh.

8. Glutaraldehyde Fixative Stock Solution (3%)

Glutaraldehyde (25% in water, Eastman)	3 ml
Distilled water	22 ml

Stir and mix thoroughly.

9. Sodium Cacodylate Buffer Working Solution (0.1 M)

Sodium cacodylate 0.2 M stock solution (Appendix D, page 187) 1 part

Distilled water 1 part

10. Fixative (1.5% glutaraldehyde buffered with 0.05 M cacodylate)

Glutaraldehyde #8 1 part

Cacodylate buffer #9 1 part

Dilute 1:1 v/v to make Gomori Acid Phosphatase test fixative.

Procedure:

1. Wash cells in Euglena broth BM medium by low-speed centrifugation. Suspend cells by placing tube in a Vortex mixer (Lab Line) after every rinsing in growth medium. Rinse 3X for a total of 15 min.

2. Fix cells in fixative consisting of 1.5% glutaraldehyde buffered with 0.05 M cacodylate, pH 7.2, v/v at room temperature for 1 h.

3. Wash cells in 0.03 M sodium cacodylate, pH 7.2, room temperature 3X for 15-30 min.

4. Distribute cells in carefully labelled tubes.

5. Rinse cells in acetate buffer (0.05 M), pH 5, 5X at room temperature.

6. Incubate in a water bath at 37° C in their respective Gomori reaction media (#4, 5, 6). Suspend the cells

intermittently during incubation times of 15 min, 30, 45 min, 1 h, 2 h depending on the color reaction and on physiological age of the cells.

7. Wash cells in distilled water, 3X at room temperature.

8. Saturate with ammonium sulfide #7 for 3 min.

9. Rinse cells in distilled water, 3-5X for a total of 5 min.

10. Examine under the microscope and photograph if desired. Other cells can be prepared for electron microscopy (Appendix E, p.191).

E. Azo-Dye Technique for Detection of Acid Phosphatase (Burstone, 1958; Barka and Anderson, 1963a,b; Pearse, 1968; Chayen, et al. 1969).

1. Hexazonium Pararosanilin (HPR) Stock Solution

HPR (Polysciences)	1 g
Distilled water	20 ml
Concentrated HCl	5 ml

Dissolve HPR in distilled water. Add concentrated HCl slowly with gentle warming. Filter after cooling and set aside at room temperature.

2. Sodium Nitrite Solution (4%)

Sodium Nitrite	4 g
Distilled water	100 ml

Dissolve sodium nitrite in distilled water. Store in

refrigerator. Use freshly prepared solution #2 each week.

3. AS-BI Substrate Solution

AS-BI (Sigma Chem. Co.)	100 mg
DMF (Polysciences)	10 ml

Dissolve AS-BI in 1 ml of dimethyl formamide (DMF). Store in the cold.

4. AS-BI Reaction Medium

HPR stock solution #1	0.8 ml
Sodium nitrite solution #2	0.8

Combine #1 and #2 v/v. Dilute #3 with 10 ml distilled water. Pour #1 and #2 to AS-BI solution #3. Adjust to pH 5 with 1 N of sodium hydroxide. Bring to a final volume of 20 ml with distilled water. Adjust to pH 5. For controls, omit AS-BI substrate solution.

Procedure:

1. Pour cells in carefully labelled test tubes (100 x 13 mm).

2. Centrifuge at 2000 rpm for 2 min, room temperature.

3. Discard supernatant and add 1-2 ml cold fixative consisting of 6% glutaraldehyde buffered 1:1 with 0.3 M phosphate.

4. Set aside for 20-30 min at 4° C with intermittent agitation.

5. Wash cells (ca 7X) with cold phosphate buffer (0.15 M), pH 7.2, until fixative is thoroughly removed, gradually

increasing temperature to 25° C or room temperature.

6. Incubate cells in AS-BI reaction medium, 37° C, pH 5 for 60-90 min with intermittent shaking to hasten coupling.

7. Rinse in distilled water briefly ca 3X.

8. Cells can now be mounted on clean glass slides for observation under the light microscope. Presence of acid phosphatase is visualized by a bright red localization in the cell.

F. Succinate Dehydrogenase Cytochemistry (Chayen, et al. 1969)

1. Reaction Medium

NBT (Dajac Lab.)	0.1 g
Phosphate buffer (pH 7.8)	100 ml

Heat nitroblue tetrazolium (NBT) gently to dissolve. Cool. Filter and adjust to pH 7.8 when necessary. This gives a blue color when used as a coupler. When triphenyl tetrazolium (TPT) is used instead of NBT, a red to pink color is shown. (Warning: carcinogenic chemicals).

2. Substrate Reaction Medium

Sodium succinate (6 H_2O)	0.68 g
Reaction medium #1	50 ml

Dissolve sodium succinate in 50 ml of reaction medium #1 to make a 0.05 M solution. Adjust to pH 7.8.

3. Control solution (with inhibitor)

Sodium malonate	0.37 g
Reaction medium #1	50 ml

Dissolve sodium malonate in 50 ml of reaction medium #1, to make a 0.05 M solution. Adjust to pH 7.8.

Procedure:

1. Distribute living cells in carefully labelled tubes.

2. Centrifuge cells at 2000 rpm for 2 min, room temperature.

3. Decant the supernatant and replace with respective reaction media #2 and #3.

4. Agitate tubes in a Vortex mixer for 1 min.

5. Incubate tubes containing cells in a water bath at 37° C at pH 7.8 for 30 min to 6 h with intermittent agitation to hasten coupling.

6. Fix cells by adding 0.8 ml of 25% glutaraldehyde in 10 ml of cells. Stir and let sit for 3 min at room temperature.

7. Wash cells in sterile distilled water.

8. Mount on glass slides for optical microscopic examination.

G. Fluorochrome Labelling Method for Blue Light Fluorescence Microscopy

1. Fluorochome Labelling Solution

Fluorochrome 0.25 mg

Growth medium 10.0 ml

Most fluorochromes are carcinogenic. Handle with care. Labelling of cells can be scheduled 3-6 h prior to microscopic examination. Recommended fluorochromes are: acridine orange (AO; Dajac Lab.), Quinacrine mustard (QM; Dr. S. Archer, Sterling Winthrop); Quinacrine dihydrochloride, ethidium bromide (Sigma Chem. Co.), neutral red (fades easily) (Nutritional Biochemical Corp.) and benzpyrene for lipid fluorescence (Sigma Chem. Co.).

Procedure:

1. Weigh 0.25 mg of fluorochrome and distribute each 0.25 mg in clean oven-dried tubes (15 x 1.5 cm) with well-fitted caps and appropriate labels.

2. Sterilize tubes at 15 psi, 121° C for 10-15 min and cool.

3. In case of L/D synchronized cells, treatment during dark cycle is preferred since most fluorochromes are light-sensitive. Wrap tubes in aluminum foil (shiny side out) during the treatment. Exclusion from light enables cells to maintain their synchrony as they are sampled in their dark cycle.

4. Pour 10 ml cell culture onto sterile tubes aseptically in a sterile transfer room with green filters in order to simulate dark conditions. Be careful to wrap

each tube with aluminum foil to keep from light.

5. Agitate each tube in a Vortex mixer.

6. Incubate at room temperature (ca. 22° C) for 3-6 h of its dark cycle.

7. After incubation, fix cells by adding 0.8 ml of 25% glutaraldehyde in 10 ml inoculum with fluorochrome. During all these procedures, subdued light condition in the laboratory is recommended.

8. Agitate well in a Vortex mixer and let sit for 10 min.

9. After 10 min, wash cells in sterile Euglena broth medium prior to microscopic examination in order to remove background fluorescence. Turn off room lights when observing cells under the fluorescence microscope.

10. Examine cells with blue-light fluorescence at a wavelength of 420 nm, with a high pressure mercury vapor lamp burner (Osram HBO-200) serving as a light source. Filter light beam through a BG-38 heat-absorbing filter and a BG-12 exciter filter. Insert a UG-1 barrier filter between the Wild Fluotar 100x oil immersion objective and a 10x ocular on the Wild Fluotar Heerburg M-20 binocular microscope.

11. For photomicrography, use Kodak High Speed Ektachrome (daylight) increased to ASA 400 (with special processing); Kodak High Speed Ektachrome (Tungsten 3200 K). Exposure times at the above setting range from 1, 2, 3, and

10 min depending on the objective used, concentration of fluorescing cells and the brightness of the fluorochrome used.

H. Benzpyrene Labelling for Lipid Fluorescence Microscopy (Chayen, et al. 1969)

1. Caffeine Solution

Saturate distilled water with caffeine (Sigma Co.) at room temperature for 2 days (ca. 1.5% caffeine). This solution #1 also serves as a control medium.

2. Benzpyrene Reaction Medium

3,4 Benzpyrene (Sigma Co.)	2 mg
Caffeine solution #1	100 ml

Dissolve benzpyrene in caffeine solution. Incubate for ca. 35-37° C for 2 days. Filter and dilute with equal volume of distilled water in order to prevent the caffeine from crystallizing out of the solution. Set aside for 2 h. Filter before use.

Procedure:

1. Distribute cells in carefully labelled test tubes.

2. Centrifuge cells at 2000 rpm for 2 min, room temperature.

3. Decant the supernatant growth medium and replace with respective reaction media #1 control and #2 treated. Wrap tubes in aluminum foil.

4. Agitate cells in a Vortex mixer for 1 min.

5. Set aside for 30 min to 1 h with intermittent agitation.

6. Fix cells by adding 0.8 ml of 25% glutaraldehyde in 10 ml of treated cells. Stir and let sit for 3 min.

7. Wash cells in distilled water prior to microscopic examination.

8. Examine cells with either blue-light fluorescence (420 nm) or UVL (365 nm). Lipids fluoresce silver blue, white to yellow depending on the concentration of benzpyrene taken up by lipids.

APPENDIX D

PREPARATION OF COMMON CHEMICALS USED IN ELECTRON MICROSCOPY

Fixative

1. Glutaraldehyde Stock Solution (6%)

Glutaraldehyde (Eastman, 25% in water)	6 ml
Distilled water	19 ml

Combine to make 6% stock solution.

2. Sodium Cacodylate buffer (0.2 M)

Sodium cacodylate (Polysciences, FW: 214)	20.45 g
Distilled water	500. ml

Dissolve sodium cacodylate in distilled water. Adjust to pH 7.2. To make 0.05 M buffer for use in rinsing, dilute 50 ml of the buffer stock solution (2) with 150 ml of distilled water.

3. Buffered glutaraldehyde fixative working solution

Combine glutaraldehyde fixative (1) and sodium cacodylate buffer (2) v/v to make 3% buffered glutaraldehyde. Adjust to the desired pH (e.g., 7.2).

Osmium Tetroxide Post-Fixative-Stain

4. Osmium Stock Solution (4%)

Osmium tetroxide (Engelhard Industry)	1 g
Distilled water (60° C)	25 ml

Use vinyl gloves and gas mask during this procedure. Measure 25 ml of warm glass distilled water (60° C)

into a glass reagent bottle. Under fume hood, dissolve premeasured osmium tetroxide crystals in vacuum-sealed vials by pouring hot water over vial. Wipe vial dry and wrap in weighing or glassine paper. With pliers or glass file, break vial over mouth of reagent bottle, and drop dissolved osmium. Fit bottle with ground glass stopper and swirl to dissolve osmium crystals. Wrap bottle with aluminum foil (or use brown glass bottle). Store in refrigerator.

5. Osmium Working Solution (1-2%)

   Dilute 1:1 osmium tetroxide stock solution (4) with either distilled water or with buffer to make 2% working solution. To make 1%, use a 1:4 dilution.

Embedding Medium

6. Mollenhauer's Embedding Medium (Mollenhauer, 1964)

Epon 812	25 ml
DDSA	55 ml
Araldite M	15 ml
Dibutyl phthalate	2-4 ml

   Add 8-14 drops of DMP-30 or BDMA accelerator from a Pasteur pipette for every 8 ml of above mixture during embedding and polymerization. Acetone may be used as a diluent during infiltration.

7. Spurr's Embedding Medium (Spurr, 1969)

ERL 4206 (Union Carbide Corp.)	10 g

DER 736 (Dow Chemical Co.)	6.0 g
NSA (Humphrey Chemical Co.)	26.0 g
S-1 (Conshohocken Chemical Corp.)	0.4 g

Measure all of the above chemicals gravimetrically. Either acetone or propylene oxide may be used during infiltration. For final embedding, use the above stock solution (7). Pre-heat Beem capsules in 60° C drying oven with desiccants to prevent moisture.

Poststains for Ultrathin Sections

8. Water-Base Uranyl Acetate (Pease, 1964)

Uranyl acetate (J. T. Baker Chem. Co.) MW: 424.194)	0.5 g
Distilled water	100.0 ml

Dissolve uranyl acetate crystals in distilled water. Store in refrigerator but warm solution to room temperature before use.

9. Reynold's Lead Citrate (Reynolds, 1963)

Lead nitrate (Use fresh salt)	0.666 g
Sodium citrate	0.880 g
Distilled water	15. ml

Combine above three ingredients by shaking mixture vigorously for 1 min in a 25 ml volumetric flask. Let stand with intermittent shaking to dissolve and insure complete conversion of lead nitrate to lead citrate. After 30 min, add 4 ml of 1.0 N sodium hydroxide (carbonate-free). Dilute mixture to 25 ml with distilled water. Mix by inversion to allow dissolution of lead

citrate. Adjust the pH to 12.0. Solution should clear. Discard as solution turns cloudy with time.

10. Saturated Methanolic Uranyl Acetate (Hayat, 1970)

    Saturate methanol with uranyl acetate by adding salt gradually. Stir solution in a Vortex mixer until solution is saturated. Methanol may be used straight (95-100%) or diluted to ca. 30-50% in water.

11. Lead Citrate (Venable and Coggeshall, 1969)

Lead citrate (K & K Lab.)	0.02 g
Distilled water	10 ml
Sodium hydroxide (10 N)	0.10 ml

    Shake above mixture until solution becomes clear. Before use, adjust pH to 12.0.

APPENDIX E

PROCEDURES FOR ULTRASTRUCTURAL CYTOCHEMISTRY

Acid Phosphatase Labelling of Lysosomes and Associated Organelles (Gomori, 1952; Berjak, 1968; Barka and Anderson, 1963a,b; Berjak and Villiers, 1970; Charvat, 1973 personal comm.)

Procedure: (cf. Appendix C, page 174)

1. Wash cells in Euglena broth BM medium at low speed. Suspend cells in a Vortex mixer (Lab Line) after rinsing. Rinse 3x for 15 min.
2. Fix cells in 3% glutaraldehyde (Eastman) buffered with 0.05 M sodium cacodylate, pH 7.2, v/v at room temperature for 1 h.
3. Wash cells in 0.02 M sodium cacodylate, pH 7.2, room temperature, 3x for a total of 45 min. Distribute cells in appropriately labelled tubes: (a) untreated and (b) Gomori-treated.

(a) Untreated

4. Post-fix cells in 1% osmium buffered with 0.05 M sodium cacodylate, pH 7.2 at room temperature for 2 hours or overnight in the refrigerator.
5. Wash cells in distilled water, 7x at room temperature for 45 min.
6. Dehydrate in graded ethanol followed by two quick changes

of dry acetone. Allow 5 min between each change. Room temperature.

7. Embed in Spurr's beginning with the last acetone change (cf. Appendix D). 1:1- for 2-4 h, room temperature.
8. Change to 3/4 plastic/acetone mixture. Suspend cells and leave overnight at room temperature.
9. Change to 100% plastic. Leave for 1 h at room temperature. Suspend cells occasionally.
10. Polymerize in pre-heated Beem capsules for 8-12 h in a drying oven, at 60-65° C.

(b) Gomori-Treated

4. Wash cells in acetate buffer 0.05 M, pH 5, 5x at room temperature.
5. Incubate at pH 5 in a water bath at 37° C or at room temperature for 15, 30, 45 min to 1-2 h with occasional agitation. Use Vortex mixer to shake the tubes. Examine condition of cells periodically under the microscope.
6. Wash cells in distilled water 3x at room temperature.
7. Saturate cells with 0.02% ammonium sulfide for 1 min.
8. Rinse cells in distilled water 1-2x, for 1 min.
9. Post-fix in 1% osmium buffered with 0.05 M sodium cacodylate as in step 4 of untreated cells. Follow procedure therein.

Succinate Dehydrogenase Labelling Using BSPT (Kalina, et al. 1972)

Procedure:

1. Fix cells in 2% depolymerized paraformaldehyde (Hayat, 1972) buffered with 0.05 M Tris buffer, pH 7.2, 4° C for 10 min.
2. Wash cells (ca. 4x) with Tris buffer, pH 7.2, 4° C for 1-2 h.
3. Incubate and label the following test tubes (pH 7.2, 37° C for 20-90 min)
   a. SDH medium at 37° C
   b. Control: with omission of succinate substrate
   c. Control: with malonate inhibitor (0.01 M)
   d. Control: cells previously heated in 95° C water bath for 10 min, then transferred to 37° C.
4. Wash the cells in Tris buffer, pH 7.2, room temperature for 15 min, 5x.
5. Treat with Tris buffered in 2% osmium. Shake intermittently for 3 h.
6. Rinse in distilled water, ca. 7x.
7. Dehydrate in ethanol series followed by two quick changes of propylene oxide (PO) (in fume hood), room temperature.
8. Infiltrate in Spurr's embedding media at room temperature (Appendix D).
   a. 50% plastic in PO - 12 h or overnight.

b. 75% plastic in PO - for 2-3 h.

c. 100% plastic mixture - 1 h

9. Embed and polymerized in 60° C oven for 12-48 h.

Preparation of Reagents for SDH Labelling Using BSPT

1. Paraformaldehyde Fixative Stock Solution (Hayat, 1972)

Paraformaldehyde powder (Eastman)	2 g
Distilled water	100 ml

Dissolve paraformaldehyde in distilled water with gentle stirring at ca. 60° C (use fume hood). Add 1 N sodium hydroxide, dropwise, until solution becomes clear. Use fresh preparation.

2. Tris (Trizma) Buffer, 0.05 M, pH 7.22, 37° C.

Trizma HCl (Sigma Chem. Co.)	6.35 g
Trizma Base (THAM) (Sigma Chem. Co.)	1.18 g

Bring to a final volume of 1000 ml with distilled water.

3. Tris (Trizma) Buffer, 0.05 M, pH 7.2, 25° C.

Trizma HCl (Sigma Chem. Co.)	7.02 g
Trizma Base (THAM)	0.67 g

Bring to a final volume of 1000 ml with distilled water.

4. Regular Tris (THAM) Buffer, 1.0 M

THAM (Tris hydroxymethyl) amino methane	121.1 g
Distilled water	1000.0 ml

Adjust pH with concentrated HCl according to specified temperature. Note: increasing the total concentration

of Tris buffer from 0.05 M to 0.1 M will increase the pH by ca. 0.05 pH unit (Sigma Chem. Co.).

5. Succinate Substrate (.6 H_2O), 1.0 M.

Succinate	13.5 g
Distilled water	50.0 ml

Store in refrigerator to keep fresh.

6. SDH-BSPT Reaction Medium

BSPT (Polysciences)	2.5 mg
DMF	0.25 ml
Succinate substrate (5)	0.50 ml
Sucrose (25%)	2.00 ml

Dissolve BSPT in 0.25 ml DMF. Combine all reagents and bring to a final volume of 10 ml reaction medium with distilled water. For controls, succinate substrate (5) may be omitted or replaced by malonate inhibitor. Adjust final pH to 7.2 before incubation.

7. Malonate Inhibitor, 0.05 M.

Malonate	0.37 g
SDH-BSPT reaction medium (6)	50 ml

Adjust to pH 7.2 before use.

Diaminobenzidine (DAB) Cytochemical Detection of Microbodies and Mitochondria (Novikoff and Goldfischer, 1969; Seligman, et al. 1972; Powell, 1973 personal comm.)

Procedure:

1. Wash cells in growth medium.

2. Fix cells in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.8, room temperature for 1-2 h.
3. Wash cells in 0.05 M sodium cacodylate buffer, pH 7.8, ca. 5x.
4. Stabilize cells in AMP buffer (0.05 M), pH 9, room temperature for 5 min.
5. Incubate in DAB reaction medium, 37° C for 1 h. Microbodies (for peroxisome catalase-peroxidase reaction, ca. pH 8; for catalase reaction, pH 9). Mitochondria (for cytochrome oxidase reaction, pH 7 - 7.8). Agitate cells occasionally.
6. Wash in AMP buffer, 0.1 M at respective pH's.
7. Wash in sodium cacodylate buffer, 0.05 M, pH 7.9, ca. 9x.
8. Post-osmicate in 2% osmium tetroxide buffered with 0.05 M sodium cacodylate, pH 7 and 9, for 1 h, room temperature.
9. Wash in distilled water 5x, room temperature.
10. Dehydrate in graded ethanol series, followed by two quick changes of 100% ethanol and dry acetone. Four-mesh drierite (anhydrous calcium sulfate) is added to acetone.
11. Infiltrate in Spurr's plastic (Appendix D)
    1/3 plastic - 2 h
    1/2 plastic - 3 to 8 h

2/3 plastic - overnight or 18 h

100% plastic for 2-4 h prior to polymerization

12. Polymerize for 24 h in 60° C oven.

Preparation of Reagents for DAB Labelling

1. DAB Stock Solution

DAB Tetra-HCl (Sigma Chem. Co.)	0.1 g
AMP buffer (5)	25.5 ml
Hydrogen peroxide (6% from 30% superoxol)	0.5 ml

Combine. This makes double strength DAB stock solution.

2. DAB Working Solution

DAB stock solution (1)	1 part
Distilled water or 0.1 M AMP buffer	1 part

Combine. Adjust to desired pH (pH 7.0, 7.8 and 8.9, 9.0).

3. AT Inhibitor Stock Solution

AT (amino triazole)	0.336 g
Distilled water	100 ml

Combine to make 0.04 M stock solution.

4. AT Inhibitor Working Solution

AT inhibitor stock solution (3)	1 part
DAB stock solution (1)	1 part

Adjust to pH 9 with concentrated 1 N HCl. Filter AT working solution if necessary (i.e., if solution is

cloudy, filter through a No. 1 Whatman qualitative filter paper).

5. AMP Buffer (Sigma Chem. Co.; MW: 105.1)

AMP powder (Sigma Chem. Co.)	4.20 g
Distilled water	100.00 ml

Combine to make 0.2 M AMP buffer stock solution.

6. Sodium Azide Heme Protein (Cytochrome Oxidase) Inhibitor (Eastman Organic Chem.)

Sodium azide powder (Eastman Organic Chem. Co.; MW: 65.02)	0.13 g
Distilled water	10.0

Combine to make 0.2 M cytochrome oxidase inhibitor. Dilute 1:1 with DAB stock solution (1 above).

7. Sodium Cyanide Heme Protein Inhibitor.

Sodium cyanide	0.49 g
Distilled water	50.00 ml

Combine to make 0.2 M stock solution.

8. DAB Working Solution for Mitochondria (Novikoff and Goldfischer, 1969)

DAB-tetra HCL (Sigma Chem. Co.)	20 mg
Acetate buffer (0.05 M)	8.9 ml

Adjust to pH 6 after thorough mixing. Then add

Manganese chloride (0.05 M)	1.0 ml
Hydrogen peroxide (1% prepared fresh from 30% superoxol)	0.1 ml

Filter with a No. 1 Whatman qualitative paper if solution is cloudy.

9. General Control-Denaturation of Enzymes

   Combine DAB stock solution (1) 1:1 with boiling glass-distilled water. Adjust to pH 9.0.

10. Control-Omission of Hydrogen Peroxide Substrate to Inhibit Endogenous Hydrogen Peroxide

    Boil cells in 100° C water for 5 min. Omit hydrogen peroxide substrate.

11. Control-Combination of Inhibitor and Denaturation

Inhibitor (3, 6, 7)	2 parts
Boiling distilled water	1 part
AMP buffer	1 part

Combine. Serves as control.

Ruthenium Red Labelling for Acid Mucopolysaccharides (Luft, 1971; Chambers, 1973)

Procedure:

1. Wash cells in growth medium.
2. Fix cells in solution containing 3% glutaraldehyde, buffered with 0.67 M sodium cacodylate, pH 7.2 and 0.1% ruthenium red (Dajac Lab.), room temperature for 1 h.
3. Wash cells in 0.67 M sodium cacodylate buffer, pH 7.2, ca. 7x.
4. Post-fix cells in 2% O_5O_4, 0.1% ruthenium red buffered

with 0.67 M sodium cacodylate for ca. 2.5 h, room temperature. Agitate intermittently.

5. Wash in distilled water for 5x, room temperature.
6. Dehydrate in graded ethanol series, followed by two rapid changes of 100% dry acetone with 4-mesh drierite added.
7. Infiltrate in Spurr's plastic (Appendix D)

   1/3 plastic for 2 h

   1/2 plastic for 3-8 h

   2/3 plastic overnight or 18 h

   100% plastic for 2-4 h prior to polymerization
8. Polymerize for 24 h in 60° C oven.

Preparation of Reagents for Ruthenium Red Labelling

1. Stock Solution of Fixative

Glutaraldehyde (Eastman Organic Chem., 25% in water)	5 ml
Sodium cacodylate (0.67 M)	20 ml

Adjust to pH 7.2.

2. Sodium Cacodylate Buffer (MW: 214)

Sodium cacodylate (Polysciences)	68.34 g
Distilled water	500.00 ml

Stir and adjust to pH 7.2.

3. Ruthenium Red Stock Solution

Ruthenium Red (Dajac Lab.)	0.5 g
Sodium cacodylate (1.34 M)	100.0 ml

Store in refrigerator.

4. Sodium Cacodylate Buffer

Sodium cacodylate	42.8 g
Distilled water	200.0 ml

Store in refrigerator

5. Buffered Ruthenium Red Working Solution (for Fixative)

Ruthenium stock solution (3)	5 ml
Distilled water	5 ml

Combine to make 0.25% ruthenium red in 0.67 M sodium cacodylate buffer.

6. Buffered Fixative Working Solution

Stock solution of fixative (1)	1 part
Buffered ruthenium red working solution (5)	1 part

Combine 1:1 (v/v) to make 1.5% glutaraldehyde, 0.12% RR in 0.67 M sodium cacodylate. Adjust pH to 7.2.

VITA

Maybelle P. Gomez was born May 5, 1941, in Manila, Philippines, the eldest daughter of Dr. Salvador L. Gomez, a physician-surgeon and Virginia P. Pansacola-Gomez, a pharmacist--both residents of Lucena City, Philippines. She attended grade school at Maryknoll Academy, Lucena City, and graduated from High School at Maryknoll College, Quezon City in 1958. She entered the University of the Philippines, Diliman, on a Preparatory Medicine study program after which she majored in Botany. During her senior year, she participated in a research project on the "Benthic Marine Algae of the Philippines" sponsored by the University of the Philippines and the National Science Development Board. She received her Bachelor of Science degree major in Botany with a minor in Anthropology in 1962. The same year she was appointed Graduate Teaching Assistant in the Department of Botany, where she pursued studies on a Master of Science program with Dr. Gregorio T. Velasquez as her Major Professor. Her research involved the marine algae of Cabalete Island and vicinity on the Pacific side of the Philippines. At the latter part of her Master of Science program in 1965, she was appointed to the faculty of the University of the Philippines.

In 1967, she was awarded a Rockefeller Foundation Predoctoral Fellowship. The same year she applied for admission at The University of Tennessee, Knoxville, upon the invitation of Dr. Aaron J. Sharp with whom she had met and worked with while he was investigating the bryophytes of the Philippines in 1965. She entered The Graduate School at The University of Tennessee, Knoxville, on January 1968, with Dr. Patricia L. Walne as her Major Professor. In 1970, she had the opportunity to participate in a Summer Institute of Advanced Study in Gerontology at the Ethel Percy Andrus Gerontology Center, University of Southern California, Los Angeles, as a special preparation for her dissertation research on cellular aging. Upon her return to The University of Tennessee, Knoxville, she trained in Electron Microscopy under Dr. Patricia L. Walne. During the latter part of her study program, she was awarded Graduate Assistantships and Graduate Teaching Assistantships from the Department of Botany, The University of Tennessee, Knoxville, during which time she participated in the teaching of General Biology, Cell Biology and Phycology.

Her membership in professional organizations includes: The Phycological Society of America, British Phycological Society, International Phycological Society, Botanical

Society of America, Association of Southeastern Biologists, The Society of Protozoologists and The Society of the Sigma Xi. Publications include two major papers and seven abstracts of papers presented at regional and national scientific meetings.