STUDIES IN THE DIFFERENTIATION OF COLONIAL FLAGELLATES

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INTRODUCTION

The bulk of the work to be reported in this thesis is concerned with the fine structural differentiation of one genus of the Volvocaceae, Eudorina. The aim of this section is to give a brief account of the position of the Volvocaceae amongst the algae and to show how certain evolutionary tendencies are represented in this group, together with a survey of the gross differentiation of all genera of Volvocaceae.

Classification of Algae

The assemblage of lower plants called algae is one of very great heterogeneity and it has become increasingly clear over the last half century that it includes several series, each with distinctive morphological and physiological traits. Among the morphological characteristics are the structure of the cell which has a striking constancy in its organisation, and especially in the number, arrangement and relative length of the flagella (Smith, 1950; Fott, 1964). The recent work of Manton (1964e) has shown that at the level of resolution of the electron microscope these morphological distinguishing characteristics are equally well defined. Significant differences in the fine structure of different groups are to be seen in the external coverings (walls, scales, etc.), in the structure and morphology of the flagella and other appendages, in the internal structure of mitochondria and plastids (see also Greenwood, 1964) and in the structure and distribution of endoplasmic reticulum and other cell inclusions. With respect to physiological differences there is, in each series, a constancy both in the plastid pigments present, and in the chemical nature of the food reserves formed through photosynthesis. There is a very strong suggestion that each series originated from separate pigmented ancestors.

The series of algae are classified as shown in Table 1.

Table 1

A Classification of Algae (Christensen, 1962)

PROCARYOTA	Cyanophyta	Cyanophyceae
EUCARYOTA		
Aconta	Rhodophyta	Rhodophyceae
Contophora	Chromophyta	Cryptophyceae
		Dinophyceae
		Rhaphidophyceae
		Chrysophyceae
		Haptophyceae
		Craspedophyceae
		Bacillariophyceae
		Xanthophyceae
		Phaeophyceae
	Chlorophyta	Euglenophyceae
		Loxophyceae
		Prasinophyceae
		Chlorophyceae

With the exception of the Chlorophyta they have also been described as separate plant-like kingdoms. The kingdom of grass-green plants consists of a number of divisions, of which the grass-green algae (Chlorophyta) are the most primitive and lead successively to the Bryophyta, Pteridophyta and Spermatophyta.

Evolutionary tendencies in the Algae

There is a marked parallelism in the evolution of plant body types in the various groups, which is explicable by the hypothesis that only four basic types of thallus construction can arise from a unicellular motile ancestral form. The main evolutionary tendencies seen are: (i) the volvocine tendency in which the individual cells become organised into a colony and retain their vegetative motility, the colonies showing increasing size and specialisation; (ii) the tetrasporine tendency in which there is a loss of motility except in reproductive stages, but a retention of the capacity to divide vegetatively; (iii) the chlorococcine or siphonaceous tendency also involving a loss of motility except in the reproductive stages, but with a loss of the ability to

divide vegetatively (Blackman, 1900). To these may be added (iv) the rhizopodal tendency where there is evolution to an amoeboid organisation.

The tetrasporine tendency is the most labile and is that leading to the higher plants. The earliest advance on this line of evolution is seen where motile cells become imprisoned within a gelatinous sheath temporarily as in Chlamydomonas or Carteria, or more or less permanently as in Tetraspora or Apiocystis. Colonies of the palmella type are next in their degree of integration as the cells within the gelatinous matrix normally lack flagella, but they retain the capacity to develop them at any time, to return to the unicellular motile condition. The cells possess the ability to divide vegetatively and colonies of amorphous shape and indefinite size or ones having a definite shape are produced. Loss of the ability to return directly to a motile condition gives either immobile unicells if separation occurs, or an unbranched filament if it does not, such as Tetraspora cylindrica, and this latter type is the forerunner of the various branched types.

None of the other three tendencies ~~are~~ is flexible enough to allow other than very limited development: thus the volvocine tendency, which has occurred in the Chrysophyceae (e.g. Dinobryon with dendroid colonies, Chrysosphaerella, Synura, etc. with globose colonies) but principally in the Volvocaceae, cannot give a colony of a reasonable size with individual cells retaining their motility. Nevertheless, the volvocine tendency, especially as illustrated in the Volvocaceae, is of great interest as a nicely graded series of forms exists showing successively an increase in the degree of differentiation of the colonial body. The principal manifestations of this are an increase in cell number, an advance from the condition where all cells are capable of reproduction, to one where only some possess this ability, and an advance from isogamy through anisogamy to oogamy.

Colonial Volvocales

The order Volvocales includes both solitary and colonial forms. Most of the colonial Volvocales belong to the family Volvocaceae in which there are seven genera: Gonium Müller, Pandorina Bory, Volvulina Playfair, Stephanoon Schwiakoff, Platydorina Kofoid, Eudorina Ehrenberg and Volvox Linnaeus. Three other families of the order have colonial members: the Spondylomoraceae has a number of genera with small colonies showing little differentiation; the Haematococcaceae has a single colonial genus Stephanosphaera which similarly shows little differentiation; the Astrephomenaceae comprises a single genus and species, Astrephomene gubernaculifera (Pocock, 1953) which is in itself of great interest, but being unique has not the comparative interest of the Volvocaceae. Astrephomene gubernaculifera consists of a coenobium of 16, 32, 64 or 128 cells, each surrounded by a distinct gelatinous matrix. Its main interest lies in the presence of several small cells at its posterior pole, usually four, but varying from two to seven, the apices of which are orientated so that their flagella form a directive apparatus, the "rudder". These cells are 6-10μ in diameter as compared with the remainder which are 26-45μ wide by 17-45μ long. Eyespots are present in all cells of the coenobium but tend to be smaller towards the posterior end and may even be absent from the "rudder" cells. All cells are capable of reproducing asexually to form daughter colonies, with the exception of the "rudder" cells. There is no inversion of the embryo during development, the cells attaining their normal orientation during the course of successive divisions. As will be seen below, inversion is typical during the development of Volvocaceae. Apparently all cells form gametes on sexual reproduction. These escape from the parent coenobium and fuse. There is a fortuitous appearance of anisogamy since in general colonies which give rise to gametes vary considerably in size. Close study shows that pairing takes place irrespective of size.

Structure and reproduction of the Volvocaceae

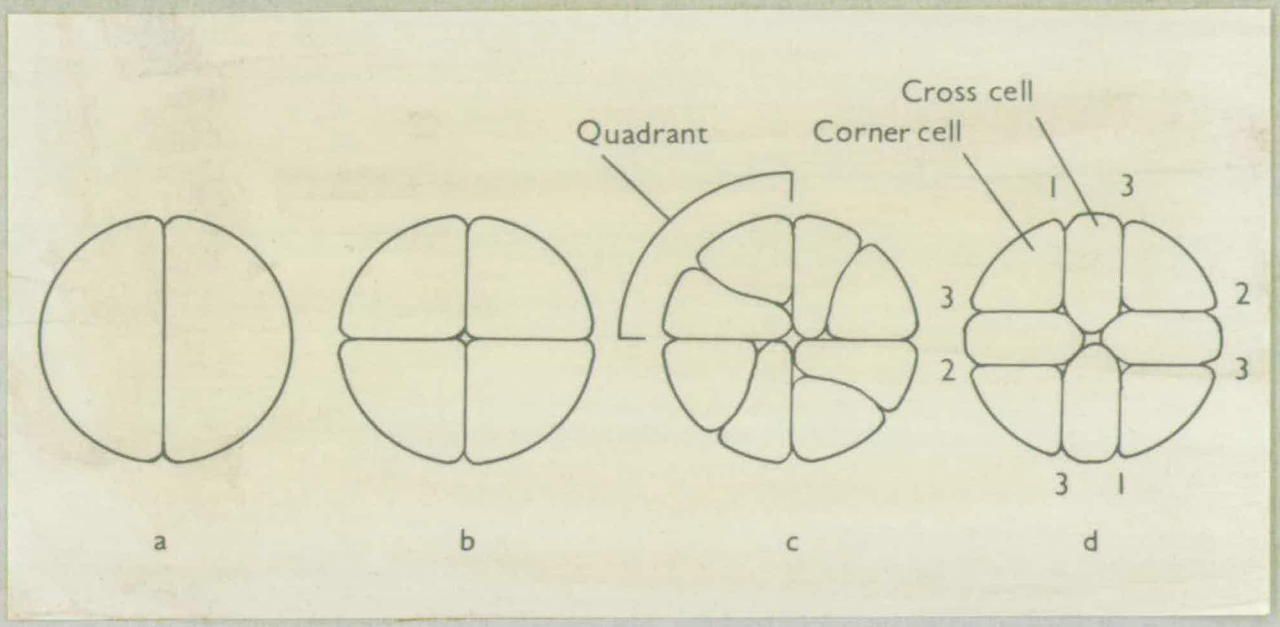
The Volvocaceae includes all the motile genera in which the cells are arranged in the form of a disc or hollow sphere (coenobium) and not in superimposed tiers. In many forms the envelopes of neighbouring cells are fused together to form a homogeneous colonial matrix, in which the cells are embedded (Fritsch, 1935; Smith, 1938; Smith, 1950). Colonies have definite anterior and posterior poles; this applies equally to forms with a flattened colonial envelope, such as Gonium, as to globose forms, such as Eudorina. During swimming the anterior pole is directed forwards.

The typical cell is ovoid to pyriform, with two flagella at the anterior end. Near the flagellar bases are two contractile vacuoles, and there is a single circular red eye spot in the anterior half of the cell in the longitudinal plane passing between the two basal bodies and perpendicular to them. There is no cytological evidence that the eyespot is part of a neuromotor apparatus as claimed by Mast (1928). All forms are positively phototropic except in strong illumination. The eyespot is a differentiated region of the chloroplast, which latter is the most conspicuous structure in the cell, being typically cup-shaped and occupying most of the space inside the plasma membrane. One or more pyrenoids are contained in it. These are of a proteinaceous nature (Czurda, 1928 - see Fritsch,1935, p.66) and are surrounded by starch plates. Young cells tend to have fewer pyrenoids than old cells. Another food reserve is commonly found in the form of oil droplets, and is considered to be a conversion product of starch, as it is especially common in old vegetative cells and zygotes. The nucleus is difficult to see as it occupies the depression in the chloroplast. In the interphase nucleus there is little material which is stained by the usual nuclear stains, except the nucleolus which stains deeply (Fritsch, 1935; Smith, 1950). From a study of a number of genera of Volvocaceae, Cave and Pocock (1951b) found, using a modified aceto-carmine technique

(Cave and Pocock, 1951a) that during mitosis the nucleolus disappeared in prophase and the chromosomes had become clearly defined by prometaphase.

In the "lower forms," such as Gonium sp., asexual reproduction takes place by the repeated division of all the cells in the colony to form daughter colonies (autocolonies). In the "higher forms," such as Volvox sp., only those cells specialised for reproduction, the gonidia, divide and form autocolonies. The ensuing account of the sequence of divisions is based upon the accounts of Goroshankin (1875), Pascher (1927), Doraiswami (1940), Gerisch (1959) and Goldstein (1964). All divisions are longitudinal and occur in a very regular sequence. The planes of the first and second divisions are at right angles to one another; that of the first division usually runs between the bases of the flagella. The plane of the third division is oblique, as seen in polar view, and runs from the circumference of each quadrant to cut either the first or second division plane at a point nearer to the circumference than the central axis. Considering each quadrant separately, the direction of the deflection of the third division plane is the same, but an examination of the figures given by different authors shows that they are not in agreement as to whether the deflection is to the right or left of the perpendicular drawn from the tangent to the circumference at the point where the third division plane originates. Chatton (1911), and Pascher of the authors mentioned previously, show it to the right whilst the remainder, Goroshankin, Doraiswami, Gerisch and Goldstein, all show it to the left. After the completion of the third division a re-arrangement of cells takes place to give the typical "cross stage" (Text-fig. 1).

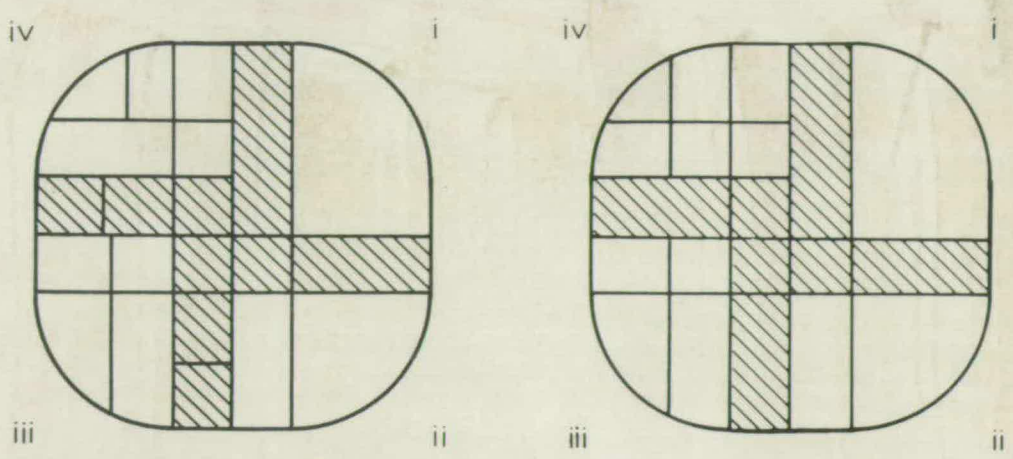
From the 8-cell stage the young colony is known as a plakea; it is in the form of a slightly concave plate. The apical (flagellar) pole of the former mother cell is represented at this stage by the concave surface. By the completion of the fourth division the curving of the plakea is often so



Text-fig. 1. Apical polar views of Volvocaceae division stages. a-c, divisions 1-3; d, rearrangement of cells after third division, to give the "cross" stage.

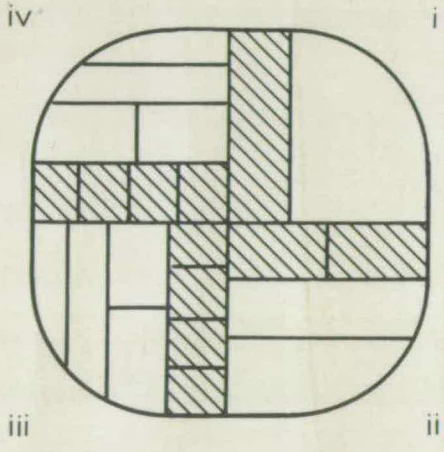
pronounced that the colony is in the form of a hollow sphere with an opening, the phialopore, at the outer pole. It is evident that, due to the inclination of the division planes, the normal polarity is reversed in the plakea. It is restored by the inversion of the colony through the phialopore during later stages of development. A detailed description of inversion in Volvox sp. is given by Pocock (1933).

Accounts of the course of divisions subsequent to the third differ. In Text-fig.2 these divisions are schematised following the accounts of Goroshankin (1875) based on Eudorina elegans, on the general account given by Pascher (1927), on the account of Gerisch (1959) which he states applies to Pandorina morum, E.elegans, E.californica, Volvox aureus and V.globator, and on that of Goldstein (1964) for Eudorina sp. It should perhaps be stressed that these divisions are difficult to follow and most accounts do not describe them in detail in the later stages. The terms cross cell, corner cell and quadrant as indicated in Text-fig. 1 have been adopted in the following account which describes the division sequence in a single quadrant. All four above-mentioned authors are agreed on the orientation of the first three divisions,

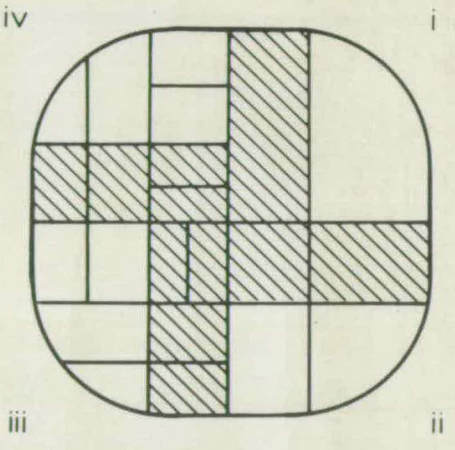


a. (After Goroshankin, 1895)

b. (After Pascher, 1927)



c. (After Gerisch, 1959)



d. (After Goldstein, 1964)

Text-fig. 2. Plakeal division sequence in the production of a 32-celled colony according to Goroshankin, Pascher, Gerisch and Goldstein. a and b, the four divisions subsequent to the second involved in Goroshankin's and Pascher's schemes respectively, are shown in quadrants i-iv. c and d, the three divisions subsequent to the second in Gerisch's and Goldstein's schemes respectively, are shown in quadrants i-iii. The cross cells and their derivatives are cross hatched.

but Gerisch reports that the fourth division of the corner cell is orientated parallel to the long axis of the cross cell, which itself divides across that axis. In Goroshankin's, Pascher's and Goldstein's accounts the fourth division is described as perpendicular to the third, both in the corner cell and in the cross cell. The fifth division, according to Gerisch, is parallel to the fourth in the cross cell derivatives and also in the adjacent derivative of the corner cell, but perpendicular to it in the remaining corner cell derivative. Goldstein indicates the fifth division in the inner cross cell derivative as being parallel to the third division. That of the other cross cell derivative is perpendicular to this, and the same is true for the two adjacent corner cell derivatives (this involves corner cell derivatives from adjacent quadrants). The schemes of Gerisch and Goldstein agree to the extent that on each successive division each cell produces two further cells. Doraiswami's account (1940) describing autocolony formation in Eudorina indica (= Eudorina californica (Shaw) Goldstein var.californica) is also ~~agreed~~ in agreement on this point. Pascher's scheme is at variance with this in that he indicates that the cross cell derivatives do not divide after the fourth division. A 32-celled colony is, nevertheless, produced as there are two subsequent divisions in the case of the corner cell derivatives. This results in an intermediate stage with 24 cells. The fifth division in this case is perpendicular to the plane of the fourth, and the sixth is parallel to the fourth but involving only the corner cell derivative adjacent to the cross cell derivatives. Goroshankin also describes the 32-celled plakea arising from a sequence involving six divisions. Like Pascher he maintains that the proximal cross cell derivative does not divide further, but at the fifth division, unlike Pascher, he records that the distal cross cell derivative does divide, the plane of division being parallel to the fourth. The corner cell derivatives divide in the plane perpendicular to the fourth. The sixth division involves only that corner cell derivative

not in contact with any cross cell derivative, the division plane being perpendicular to the previous division of the corner cell derivatives.

It is worthy of note that in the descriptions of the plakeal sequence, both of Pascher and of Goroshankin, the proximal cross cell derivatives arising at the fourth division do not divide further. It is these four cells which in a 32-celled colony comprise the anterior tier of cells which frequently have a diminished ability to form autocolonies. Where there is a conspicuous size difference between the cells of the anterior tier and the remaining tiers and the ability to form autocolonies is only very slight, they are commonly described as somatic or vegetative as opposed to the generative or gametic cells. It seems likely that the cessation of division in the proximal cross cell derivative is one of the first indications of the limitation of reproductive capacity, assuming the descriptions to be correct.

Only two divisions occur in some species of Gonium and Pandorina, which thus possess a 4-celled colony, but in the largest species of Volvox there are up to 16 cell generations. The theoretical cell number in each member of the family should be a factor of two, colonies being held to arise by simultaneous successive divisions of the mother cell. However, in the larger forms this postulated synchrony of division breaks down and certain cells, whilst remaining healthy, do not divide further and this results in colonies having considerably fewer cells than the theoretical number. There is no increase in cell number after the juvenile phase of development (Pocock, 1933).

In sexual reproduction the cells taking part are those concerned in asexual reproduction, but instead of producing autocolonies they form gametes. The tendency from isogamy to oogamy has been mentioned previously and will be discussed in detail later. The male gametes or antherozoids of anisogamous and oogamous species arise by a series of divisions such as occurs during autocolony formation. In the former type the gametes are released immediately but

in the latter the bundle of spindle-shaped gametes is released intact as a slightly curved plate (the antherozoid packet) and remains thus until in the vicinity of the female gametes when separation occurs. The female gametes of oogamous species are globose and they are never liberated from the gelatinous envelope of the parent colony where they have developed directly from the generative cells without division. Homothallism and heterothallism are found in different genera and sometimes within a single genus. In the case of heterothallic species protandry occurs. Unfertilised female gametes may possess the ability to develop without fertilisation forming parthenospores. This has been reported in Volvox aureus and Eudorina elegans (Mainx, 1929). Zygospores which are produced after fertilisation develop thick walls and must enter a resting phase before they are able to germinate. Division of the zygote is meiotic in some, if not all species. The resulting zoospores may escape singly as in Eudorina or united in the form of a 4-celled colony, each cell of which ultimately forms a colony, as in Gonium. In Volvox there is regularly a single zoospore and this is normal for Pandorina and Eudorina, only one of the meiotic products surviving. That vegetative cells are haploid is shown by the production of two "plus" and two "minus" strain zoospores in heterothallic species of Gonium. New colonies are formed by division following the normal plakeal sequence.

Differentiation in the Volvocaceae

The principle evolutionary tendencies within the Volvocaceae have already been mentioned: increase in cell number, limitation of the number of cells having the ability to reproduce and the advance from isogamy to oogamy. These aspects of differentiation and certain others - the form of the colony and the differentiation of individual cells, especially with respect to the eyespots, which are largest in the cells of the anterior pole and successively smaller in

more posterior cells, will be examined in detail. It is perhaps worth mention here that Gonium, the most primitive genus of the group, has the largest chromosome number, and in the most advanced genus, Volvox, there is a reduction in number from the most primitive to the most advanced species. This might well be of phylogenetic significance (Cave and Pocock, 1951b).

The colony of Gonium is saucer-shaped: the convex surface represents the "anterior pole". All the constituent cells are orientated with their flagella arising from the convex surface. The "anterior pole" which at rest is directed upwards, is directed forwards during swimming. The colony moves forwards on its edge along, and rotating about, the axis through the centre of the plate (Pocock, 1955).

The seven distinct species of Gonium show a progressive increase in cell number. G.sociale and G.sacculiferum have four cells, whilst G.octonarium has eight. Four or eight, but usually sixteen cells, are found in G.pectorale and sixteen cells are found in G.formosum. G.multicoccum has eight, sixteen or thirty-two cells, whilst G.discoideum usually has thirty-two, but rarely sixteen cells. Within the genus cell size does not vary much, cells being 7-13μ wide by 7-18μ long. Colony size depends upon cell number. The arrangement of cells in 4-cell forms is in a square; those of G.sacculiferum, unlike all other species, are not connected to one another by protuberances of the cell walls, but instead are held together by their attachment to the persistent parental cell wall and its gelatinous sheath (Stein, 1959). The eight cells of G.octonarium are arranged with two cells centrally surrounded by six marginal cells. However, in 8-celled forms of G.multicoccum this arrangement is never found. The commonest arrangement is one of three rows, two of three cells each, with a central row of two cells, and results basically from the arrangement of cells at the third division. In other cases the disposition of cells may be in rows of three, three, and two cells, and there is little difference between

such an arrangement and one where one cell assumes the central position with the remainder equally spaced around it (Pocock, 1955). The 16-celled forms of G.pectorale and G.formosum have four central cells surrounded by twelve marginal cells and the 32-celled forms of G.multicoccum and G.discoideum (Prescott, 1942) have 18 marginal cells surrounding 14 central cells. The symmetry is particularly striking, the colony being octagonal in outline, but not exactly isodiametric. The two sides parallel to the longer diameter consist of four cells whilst the remaining six sides all have three cells (the end cells being common to adjacent sides in each case). There are four central cells and ten intermediate cells.

The eye spots in members of the genus Gonium are usually distinct and of uniform size in all cells of the particular species; in G.discoideum, however, they have been described as small and inconspicuous.

In higher genera of Volvocaceae the pyrenoid number appears to be related to the degree of cellular differentiation, although only a slight indication of this tendency can be seen in Gonium as described at present. Single pyrenoids are present in G.sociale, G.pectorale (also rarely with two [Stein, 1958b]), G.formosum and G.sacculiferum (Pascher, 1927; Stein, 1959). G.discoideum is described as having two (Prescott, 1942). G.octonarium has none in young cells, but up to six are figured in old cells by Pocock (1955). The cells of a young colony of G.multicoccum contain a single basal pyrenoid, but as they age six to eight commonly develop, sometimes as many as 10-12, apparently de novo. Thus it seems that those species possessing higher cell numbers tend to have more pyrenoids in each cell. The exception is G.octonarium which, however, shows some signs of somatic differentiation in spite of its small cell number (vide infra).

Asexual reproduction normally takes place in Gonium by the simultaneous formation of autocolonies by all cells of the colony. G.octonarium is unique in that the onset of division in the two central cells is delayed to such an extent that, frequently, they are still undivided when the marginal cells have completed their division. Pocock (1955) recognises a new variety of G.pectorale, var.praecox, so-called because the formation of daughter colonies begins to take place in small colonies roughly half the size of the reproducing typical form (var.pectorale) which, however, shows no evidence of somatic differentiation. Inversion, which is typical of the family, results in the reversal of the curvature of the young saucer-shaped colony.

During sexual reproduction gametes are formed by a modified plakeal sequence and they separate on liberation from the parent colony. Gametic union is normally isogamous, although it may possibly be anisogamous in G.octonarium unless this is "false anisogamy". Early accounts report G.pectorale to be most frequently heterothallic (Schreiber, 1925; Stein, 1958b)but of 33 populations studied by Stein (1965) only one was heterothallic, showing that sexual isolation is not so prevalent as previously reported. It remains to be seen how generally heterothallism exists in other species. More recent work on G.pectorale (Stein, 1965a, 1966b) has shown that temperature and nutritional requirements are factors operating in sexual isolation. Both homothallic and heterothallic clones of G.sociale and G.sacculiferum have been isolated by Stein (1959) but with the former there is a varietal difference in that it is var. sociale which is homothallic and var.sacculum which is heterothallic. Some clones of G.sacculiferum readily produce thick-walled asexual spores (akinetes) resembling zygospores.

The form of the colony of Pandorina is subspherical to ellipsoidal, comprising 4, 8, 16 or 32 biflagellate cells embedded within a homogeneous

colonial envelope. The cells are arranged in a hollow sphere and are usually so close that they become flattened by mutual contact, and colony formation is comparable to that in Gonium, except that inversion is complete. The coenobium, being spherical, has true poles although these cannot be defined with respect to the colonial or gross cellular morphology alone. There is a difference, however, in the size of the eye spots, those of the anterior pole being larger and conspicuous whilst those of the posterior pole are small and inconspicuous or sometimes absent. P.morum, which has four tiers of four cells, lacks eye spots in the cells of the posterior tier but in successively anterior tiers the size of the eye spots is 1μ, 2μ and 3μ (Smith, 1920).

Besides the typical 16-celled P.morum, 4, 8 and 32-celled forms occur. P.charkowiensis usually has 32 cells, but 16-celled forms are also found.

Mature cells of P.morum possess one pyrenoid but none is present in the plakea stage (Coleman, 1959). P.charkowiensis has 3-6 pyrenoids in each cell, showing again the tendency for forms with a greater cell number to possess more pyrenoids, although with only two species no firm conclusion can be drawn in this respect.

Asexual reproduction takes place by the simultaneous division of all cells in the typical plakeal sequence.

Pandorina is held to be heterothallic normally, but Coleman (1959), from 60 clones representing 37 natural populations, was able to distinguish two homothallic clones, i.e. zygotes were produced within a single clone. Among the 49 clones which were definitely heterothallic, 15 separate pairs of mating type were found. [The remaining 9 clones were not classified]. Union of the gametes which are formed by a series of divisions identical to those in sexual reproduction is isogamous to weakly anisogamous in both P.charkowiensis (Coleman, 1959) and P.morum (Smith, 1920). The female gametes are distinguished

by their slightly larger size as compared with the male gametes and in that they are more sluggish.

The form of the colony in the genus Volvulina which contains the single species V.Steinii (Pocock, 1953; Stein, 1958a) shows an advance over Pandorina in that the cells, of which there are generally 16, lie separate from one another at the periphery of the slightly ellipsoidal colonial envelope. The cells are lenticular in shape as a result of the close appression to the edge of the envelope, and arranged in alternating tiers of four. They are more or less equal in size, being 11-13μ long by 16-18μ wide. Colonies measure 35-58μ wide by 44-60μ long. Colonies with 8 cells are sometimes found and also rarely ones with 4 or 32 cells. The eye spots are successively smaller in the cells of each tier from the anterior to the posterior pole where they may be absent. There are no pyrenoids.

Asexual reproduction takes place in the manner typical of the Volvocaceae, each cell producing autocolonies. There is an indication of somatic differentiation in the lagging in division of the cells of the anterior tier.

During sexual reproduction all cells function as gametes escaping through an apical pore in the gelatinous matrix. Union of gametes takes place between opposite strains (that is to say, Volvulina is heterothallic) and is isogamous to falsely anisogamous.

There are few descriptions of Stephanoon and these are incomplete (see Pascher, 1927). Two species are recognised, S.Wallichii and S.Askenasii. The form of the colonial envelope is spherical to oblately spherical, the cells being situated at the equator near the periphery in two alternating tiers. S.Wallichii has 8 cells of 15-22μ long by 10-17μ wide. The eye spots of the anterior tier are well formed. S.Askenasii has 16 cells of diameter 9μ, the equatorial diameter of the colony being 78μ and the polar diameter being 60μ. In common with Gonium the largest dimension is perpendicular to the direction

of movement during swimming and the colony also turns about its polar axis.

It is convenient to deal next with Platydorina, although it is commonly held to be derived from Eudorina (West, 1916; Smith, 1930). It more probably originated from an ancestral "Eudorina" type and developed in parallel but beyond primitive Eudorina species as now defined, as fertilisation is external to the colony which is closer in phylogeny to Pandorina. Also the female gametes are flagellated. Eudorina gametes retain their flagella but are not free-swimming. Furthermore in most colonies all but one or two cells, which remain vegetative, produce eggs (Taft, 1940). There is but one species, Platydorina caudata (Kofoid, 1900a).

The colony is flattened and horseshoe-shaped in outline, with a clockwise twist of about 45° when viewed edgeways along the polar axis. The anterior pole of the colony corresponds with the toe of a horseshoe. The envelope has three conical projections at the posterior pole. Cells number 16 or 32 and are disposed in a single layer. In 16-celled colonies there are 10 marginal cells in a U-shape surrounding a central plate of three ranks of two cells, and in the 32-celled form 12 marginal cells encompass four of the five ranks of four cells in the central plate. The marginal cells have their long axes in the plane of the colony whilst those of the central cells are perpendicular to this plane.

The cells are so arranged that the flagella of alternate cells emerge on opposite sides of the flattened colony. The means by which this arrangement is obtained may be seen during sexual reproduction. Normal inversion of the plakea occurs giving a colony of ellipsoidal form, which subsequently flattens the cells intercalating in the process. 32-celled colonies are on average 150μ in length, 130μ in width and 20μ in thickness. 16-celled colonies are narrower in proportion, having dimensions 70 x 43 x 16μ. All cells are

of approximately equal size, having in a full-grown colony an equatorial diameter of 15-20μ and a polar diameter of 15-18μ. One pyrenoid is present. No differentiation in eye spot size is reported although Kofoid (1900a) notes that in successively posterior ranks the eye spots are less bright.

All cells divide simultaneously in asexual reproduction.

Platydorina is heterothallic and gametic union anisogamous. Anisogamy results from the different modes of origin of the two sorts of gamete. In female colonies the constituent cells function directly as gametes. Only rarely do all the cells produce gametes, usually one or more remain vegetative and finally disintegrate. The gametes, which escape from the colony, are described as being of a green colour which is both darker and yellower than that of the non-reproductive cells. Male gametes are produced by a plakeal sequence of divisions, the mass of 32 gametes escaping in the form of a globose mass which does not dissociate until near to a female colony (Taft, 1940).

It is in Eudorina that the increased degree of differentiation of vegetative cells is most striking. Colonies are ellipsoidal and in various species consist of 16, 32, 64 or 128 cells arranged at the periphery of the envelope, frequently in tiers.

The status of this genus relative to Pleodorina, a genus erected by Shaw (1894) for a colonial type in which the anterior cells had lost the capacity to divide, relative especially to the type first described by Kofoid (1900b) as P.illinoisensis, has been discussed by many authors. Species showing a lower degree of somatic differentiation have been put in Eudorina without hesitation, but authors differ as to whether P.illinoiensis*, in which the number of somatic cells can vary from 1-12 in the 32-celled colony, can

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*As Grove (1915) remarks "The law of priority does not concern itself with grammatical errors. Kofoid wrote "illinoisensis" which is repugnant to the Latin method".

truly be put in Pleodorina,or rather in Eudorina, on the grounds that every gradation in size occurs between somatic and reproductive cells and the fate of any particular cell depends upon its size. In some forms the two cell types may be indiscriminately mixed (West, 1916). Grove (1915) and Kofoid (1900b) both found colonies of Pleodorina illinoiensis in which the anterior cells retained the capacity to divide and for this reason the species was transferred to Eudorina by Pascher (1927). Smith (1930) held that while such aberrant colonies did occur, it was still better to retain the species Pleodorina. There had been little doubt as to the validity of the genus Pleodorina as exemplified by the first described species P.californica Shaw 1894 (Pascher, 1927; Smith, 1950). Chodat (1902) had placed P.illinoiensis in synonymy with Eudorina, but only when Gerisch (1959) demonstrated experimentally that the small presumptive somatic anterior cells of P.californica had not lost their capacity to divide and were capable of asexual reproduction was there good evidence that this species might be regarded as a species of Eudorina. The small size alone of the anterior cells had not been considered diagnostic for Pleodorina (Iyengar, 1933; Smith, 1930). Iyengar (1933) described a new species, Eudorina indica with 32- and 64-celled colonies in which the first two tiers were of reduced size and presumably somatic. Thus he placed it between E.illinoiensis and P.californica on the basis of its vegetative morphology. However, identical forms have been observed in natural populations of Eudorina californica (P.californica sensu West and West, 1905 and Tiffany, 1935) and in clonal cultures (Goldstein, 1964). Doraiswami (1940) reported that the small anterior cells had not lost the capacity to divide. Thus Goldstein is inclined to place E.indica in E.californica.

The review of Goldstein (1964) is of particular value in two respects. Firstly vegetative morphology was studied in clonal cultures and all states of

a single form were thus observed, and by this means types previously described as species in randomly collected samples from nature could be recognised as variants of another species. Certain previously accepted taxonomic criteria were in this way shown to be unreliable, having been caused environmentally. Secondly a full study of reproduction and sexual isolation was possible. On this basis nine species of the genus Eudorina are now recognised: E.elegans, with three varieties, var.elegans, var.Carteri and var.synoica; E.unicocca var.unicocca and var.periferalis; E.cylindrica; E.Conradii; E.illinoiensis; E.californica var.californica and var.Tiffanyi; E.sphaerica (= E.illinoiensis or E.californica ?); E.interconnexa; E.echidna (= E.elegans?). There is some doubt as to the validity of the last three species, which were not examined by Goldstein and were possibly unhealthy specimens of other species. The genus Pleodorina has been dropped.

In the six good species the cells are arranged in tiers at the periphery of the spherical to ovoid colonial envelope. The usual cell number is 16 or 32 in all but E.californica, which tends to have 32, 64 or 128 cells. E.elegans and E.Conradii occasionally have 64 cells. Differentiation of the cells is most striking in E.californica where those of the anterior third to half are small, of the order of 12μ in diameter, relative to the posterior cells which are 24-36μ in diameter. The 32-celled colonies of other species tend to possess smaller cells in the anterior tier, but in 16-celled colonies this size differentiation is slight or not seen. In nature the cells of the anterior tier of E.illinoiensis measure 7.1-11.9μ and the remainder measure 11.9-19μ in diameter. The variation in cell size in species with no distinct differentiation is from 5.5-18μ in E.unicocca and E.cylindrica to 12-20μ in E.elegans and 12-25μ in E.Conradii.

Colony size tends to increase with cell size and number (measurements given quote equatorial diameter x polar diameter). E.unicocca and E.cylindrica, being the smallest, have dimensions 50-106μ x 60 -130μ, E.elegans 62-83μ x 83-156μ, E.Conradii 45-140μ x 55-170μ and E.illinoiensis 38-175μ x 46-200μ. E.californica is more nearly spherical, and measures 175-300μ in diameter.

In E.sphaerica (Pleodorina sphaerica Iyengar, 1933) small and large cells are interspersed in the 64 or 128 cell colonies. As in all other observed characters it agrees with E.californica, Goldstein (1964) is inclined to consider the species a physiological form of E.californica, but retains the species until there is conclusive evidence. It is perhaps relevant to mention here that somatic and reproductive cells may be mixed indiscriminately in E.illinoiensis (P.illinoiensis sensu West, 1916).

The number of pyrenoids in all species commonly increases with age by division of the original single pyrenoid or de novo although in E.californica and E.sphaerica the small cells tend not to have more than one pyrenoid. Both varieties of E.unicocca have one basal pyrenoid initially which later divides to give two or more; var.periferalis also develops one to several small pyrenoids de novo just prior to daughter colony formation near the periphery of the chloroplast. E.cylindrica also has one large basal pyrenoid and several peripheral ones associated with it. The number of pyrenoids in E.illinoiensis is one to several (8-13) and in E.elegans and E.Conradii several (5-10) to many.

The slight indications of anterior-posterior differentiation evident on cell size are emphasised on a consideration of asexual and sexual reproduction, both of which are typical for the order. There is also the differentiation of eye spot size previously mentioned for other genera, those of each successively more posterior tier being smaller until in the hindermost they may be absent.

During asexual reproduction, in all species lower than E.illinoiensis, the division of the anterior tier is delayed relative to that of the remainder, but all cells have an equal potential to produce 32-cell colonies. With E.illinoiensis the anterior tier may or may not divide to produce 4-, 8- or 16-celled colonies (Merton, 1908; Palik, 1958) but never 32-celled colonies (Iyengar, 1933). According to West (1916) whether or not a cell is somatic or non-somatic depends on size, and Gerisch (1959) has demonstrated experimentally that the anterior cells of E.californica have not lost their reproductive capacity and are able to divide. As mentioned previously, it was this lack of reproductive potential in the anterior cells which had led to the erection of the genus Pleodorina by Shaw (1894) rather than the reduction in size of the anterior cells which had been reported for other members of the genus Eudorina (Pascher, 1927; Smith, 1930; Iyengar, 1933).

Sexual reproduction is anisogamous in all species of Eudorina and in most species there is a heterothallic dioecious condition with (separate male and female colonies). In such species below E.illinoiensis all cells form gametes; in E.illinoiensis itself the cells of the anterior tier do not generally do so (Merton, 1908) but Grove (1915) reported male gamete production in all cells, and likewise Palik (1958) for female gametes. In E.californica the small cells never take part in sexual reproduction, and the same is true for E.spherica. In the latter case no gonidial cells are found in the anterior 1/4 - 1/5 of the colony (Iyengar, 1933), which it will be remembered has small and large cells interspersed. The two varieties, var.Carteri and var.synoica of E.elegans and E.californica var.Tiffanyi are homothallic and monoecious, gametic union occurring between eggs and sperm of the same colony. The homothallism of the last mentioned species approaches, however, a heterothallic condition in that the colonies are predominantly male with one or two female gametes, or predominantly female with one to four packets of antherozoids. The homothallism of

the other two types is more distinct. In E.elegans var.Carteri the four cells of the anterior tier form packets of male gametes and the tier posterior to it forms female gametes (Smith, 1930; Iyengar, 1933) and in the case of E.elegans var.synoica one to four packets of male gametes are formed at random in the colony, the remainder forming female gametes (Smith, 1930).

Within a heterothallic male clone sperm packets may surround and penetrate a vegetative male colony in a process simulating fertilisation, and although this does not normally lead to zygote formation it has been observed to do so in E.elegans, E.unicocca var.perferalis and E.cylindrica. Placed under conditions normally inducing the sexual process, such clones will produce a few eggs capable of fertilisation. Such "selfing males" are, however, best considered as heterothallic as typically thousands of zygotes result from a cross with compatible female colonies.

The anterior four cells of E.elegans var.Carteri sometimes give rise to between one and four 16-celled autocolonies instead of antheridial packets [Iyengar (1933) describing the species as E.illinoiensis]. When the posterior cells form 32-celled autocolonies the anterior cells form either antheridial packets or 16-celled autocolonies and occasionally when the posterior cells form antheridial packets the anterior cells either do not divide or form a few antheridia. In all cases the behaviour of the anterior cells is distinct from that of the posterior cells.

There is much freedom as to the course of development of female gametes, for unfertilised they are capable of forming autocolonies (Pascher, 1927; Doraiswami, 1940) or may form parthenospores as in E.Conradii and E.cylindrica. Some strains of E.elegans var.elegans and E.unicocca var.unicocca lack sexual reproduction and normally form parthenospores.

E.Conradii is unique in that there is evident an anterior-posterior cellular differentiation in the antheridial packets which are spherical rather than plate-like as in Eudorina, due to a complete inversion as described for Volvox Rousseletii and V.capensis (Pocock, 1933b). The cells of the anterior half of the packet are green, large and spherical with eye spots, whilst those of the posterior half are pale green, small and spindle-shaped lacking eye spots (Goldstein, 1959). There is no differentiation in the gametes of the two Volvox species, however.

A homothallic dioecious condition occurs in E.conradii and E.californica.

Goldstein (1959) studied 22 heterothallic pairs, 3 male strains and one female strain representing four species and one variety of Eudorina, and found five groups which were in almost complete sexual isolation. Of these grohps, three contained the single species E.elegans, the fourth contained E.elegans, E.cylindrica and E.illinoiensis and the fifth contained E.unicocca and E.unicocca var.periferalis. Within a group all types have the same zygote arrangement, either single or clumped, and are capable of intercrossing freely. Only rarely is a cross between members of different groups successful. In the two E.elegans groups with single zygote arrangement there was complete sexual isolation from strains possessing zygotes in clumps.

Intra-specific crosses indicated that a single pair of alleles controls the inheritance of mating type, a 1:1 segregation occurring. There is, however, sexual isolation within at least two single species of Eudorina, but compared with the condition in Gonium pectorale and Pandorina morum there is a greater tendency for outbreeding amongst strains as indicated by the smaller number of crossing groups (cf. Stein, 1958 and Coleman, 1959), within single morphological species. Although there is sexual compatibility within groups of Eudorina where strains of different species are included, there is high mortality in the products of the interspecific cross: thus gene flow is normally absent.

Cytological study of the products reveals polyploids and aneuploids in the F_1, in which there is an abnormal segregation of mating types and one new type, a selfing male. Some F_1 progeny of a cross between E.elegans females and E.illinoiensis males, of which there are six mating types, proved to be homothallic with the male phenotype predominant and these resembled selfing males from nature or homothallic strains of Eudorina. It is possible that natural hybridisation has produced both of these types, although the chromosome number is larger by a factor of two or more in the artificial hybrids.

The culmination of the volvocine tendency is seen in the species of Volvox. The spherical to ovoid colonies consist of 500-40,000 cells of the type typical of the order, arranged in a peripheral layer. Each cell has a separate gelatinous sheath which may be distinct or confluent with those of adjacent cells. In most species there are cytoplasmic connections between cells which are established early in development (Janet, 1912). The majority of the cells in the coenobium are vegetative and remain small, but some increase in size, ten or more times in diameter, develop many pyrenoids and form reproductive cells. During the period of bloom the tendency is for reproductive cells to be asexual in the early part and sexual later. Sexual reproduction is oogamous and colonies may be homothallic or heterothallic. The size of eye spots diminishes in successively posterior parts of the colony and they may be absent at the posterior pole. Reproductive cells lack an eye spot and flagella.

In discussing the differentiation seen in the 18 species of the genus Volvox, it is convenient to use the four sections recognised by Smith (1944, after Printz, 1927). These are, briefly:

1. Merrillosphaera (Shaw) Printz. This comprises eight species. They lack cytoplasmic connections between cells and have large gonidia (greater than 30μ

in diameter) which are differentiated very early in embryonal development. They are heterothallic and usually have male colonies markedly smaller than the female or asexual colonies.

2. Janetosphaera (Shaw) Printz. This comprises a single species. Individual sheaths round the cells are lacking. The cells are circular in polar view and have delicate cytoplasmic interconnecting strands. Gelatinous strands radiate to the colonial matrix from the central mass. Gonidia are of medium size, 18-22μ in diameter. Colonies are heterothallic with male colonies not markedly smaller than asexual or female colonies.

3. Copelandosphaera (Shaw) Printz. This also contains a single species. The cells are circular in polar view with delicate cytoplasmic connections and possess distinct gelatinous sheaths, but have no gelatinous material internal to the cellular sheaths, and ~~whose~~ the gonidia of which are of intermediate size, being 15-20μ in diameter. Colonies are homothallic with asexual and sexual colonies of approximately equal size.

4. Euvolvox Printz. The eight species in this section have cells stellate in polar view, connected by relatively coarse cytoplasmic strands and with gelatinous sheaths distinct from one another. The interior of the colony is completely filled with a homogeneous hyaline gelatinous substance. Gonidia are relatively small, being less than 15μ in diameter, and are not differentiated until after liberation of the daughter colonies. Homothallic and heterothallic types are found, with few antheridial packets in the former and many in the male colonies of the latter. Usually more than 50 eggs are found in female coenobia.

The range of size in Volvox is considerable within most species, the most vigorously growing asexual colonies being up to twice the size of the least, but the size of the members of the sections Merrillosphaera and Euvolvox is largely characteristic. The maximum size of the Merrillosphaera is usually

Table 2. Colony size and cell number in Volvox sp. [after Smith, 1944]

	Asexual colonies		Sexual colonies with mature zygotes		Male colonies	
	Size (in microns)	Number of cells	Size (in microns)	Number of cells	Size (in microns)	Number of cells
Merrillosphaera						
V.spermatosphaera	245-415x275-450	1100-2600	230-240x240-300	1200-1500	38-98x42-102	32,64 or 128
V.Carteri	231-730x264-890	2000-11000	370-690x390-730	2000-8000	200-360	440-1100
V.Weissmannia	330-385x340-396	1400-2800	275-340	2000-3000	110-154	270-450
V.obversus	374-407x440-520	2100-3300	253-360x275-385	2000-3800	up to 185	?
V.tertius	280-559x302-590	500-2000	259-409x311-480	500-2000	129-155x139-173	180-512
V.gigas	1615-2800x1700-3135	1000-3000	1679-2154x1723-2206	1000-3000	732-1141x794-1292	up to 1000
V.Powersi	1800-2500	?	?	?	?	?
V.africanus	240-430x275-520	2300-13700	220-430x250-475	1800-5200	200-320x230-400	910-5200
Janetosphaera						
V.aureus	300-475x340-510	1300-3200	330-385x340-415	2100-3000	300-320x330-360	1000-1500
Copelandosphaera						
V.dissipatrix	550-660x610-745	14700-31800	440-605x495-665	9000-27000	-	-
Euvolvox						
V.globator	380-540x400-575	8000-17000	350-480x370-510	9400-17500	-	-
V.capensis	667-1250x754-1357	9000-23000	495-1252x560-1313	?	-	-
V.Merrilli	670-890x710-925	9800-20100	375-650x390-690	12500-23000	-	-
V.Barberi	525-880x575-910	35000-47800	550-880x605-920	23200-53100	-	-
V.amboensis	up to 1200x1300	30000-50000	up to 2000	?	-	-
V.perglobator	550-980x610-1100	6500-12000	340-500x485-535	8500-12900	315-400x320-440	5300-9200
V.prolificus	400-1010	9000-25000	400-1090	?	410-800	?
V.Rousseletii	690-2058x754-2180	14000-42000	646-1052x710-1292	?	646-1058x710-1292	?

less than 500μ and the minimum size of the Euvolvox is usually greater than this, but, as may be seen from Table 2, there is a very great variation within the section. Likewise with cell numbers, in asexual colonies the Merrillosphaera tend to have fewer than 5,000 cells, only V.Carteri and V.africanus frequently having a greater number; with Euvolvox the minimum number approximates to 10,000 and the maximum number found is frequently over 25,000. V.africanus, it should be noted, has the smaller coenobium size typical of the Merrillosphaera, however.

The cell number in sexual colonies of homothallic species and male colonies of heterothallic species is approximately that of the asexual colonies. Male colonies of V.aureus and of heterothallic species of Euvolvox have roughly the same number of cells as asexual and female strains. In the Merrillosphaera the cell number in male colonies of V.africanus and V.gigas approaches that of the asexual and female colonies; male colonies of other species have a much smaller number of cells, in the case of V.spermatosphaera it is as few as 32, 64 or 128.

As mentioned above, in Volvox there is an extreme reduction in the number of reproductive cells relative to purely somatic cells. The number of gonidia may be constant within a species, or it may vary considerably. It is only constant enough in four species to be diagnostic. In V.gigas and V.Powersi the number is large, usually 30-60, V.africanus rarely has more than 6 gonidia and embryos, usually 2, 3 or 4, and the majority of V.Carteri have 8. Most other species have a number between 5 and 10 but V.spermatosphaera sometimes has 11 or 12 although most colonies have 8-10, and V.perglobator commonly has 3-5. (Table 3). Asexual reproduction is typical for the order by formation of autocolonies on the part of the gonidia.

In Volvox no reproductive cells are located at the anterior pole, and frequently they are found only in the posterior half of the colony. There is

Table 3. Size and number of gonidia in Volvox sp. [after Smith, 1944]

	Gonidia		
	Number	Size (in microns)	Arrangement
Merrillosphaera			
V.spermatosphaera	3-16 usually 8-10	33-36	Irregular
V.Carteri	6-25 usually 8	48-54	Two quadrats, one equatorial, one posterio
V.Weissmannia	6-11 usually 8-10	41-57	Two transverse tiers
V.obversus	5-8 usually 5-6	64-74	Two transverse tiers
V.tertius	2-12 usually 3-8	30-40	Irregular
V.gigas	up to 82 usually 30-60	35-49	Irregular
V.Powersii	30-78	?	Irregular
V.africanus	2-8 usually 2-4	49-60	2 equatorial, others posterior
Janetosphaera			
V.aureus	4-12 usually 5-9	18-22	Irregular
Copelandosphaera			
V.dissipatrix	4-10 frequently 8	15-20	4-5 equatorial, others posterior
Euvolvox			
V.globator	3-17 usually 4-7	10-13	Irregular
V.capensis	4-20 usually 6-10	11-13	Irregular
V.Merrilli	3-14 usually 4-7	12-14	Irregular
V.Barberi	3-9	12-15	Irregular
V.amboensis	1-14 frequently 7-8	?	Irregular
V.perglobator	3-9	12-14	Irregular
V.prolificus	4-20 usually 6-12	?	Irregular
V.Rousseletii	1-16 usually 6-10	13-15	3-5 equatorial others posterior

no definite arrangement of gonidia in V.aureus or in the Euvolvox (except in V.Rousseletii and V.capensis), whilst in V.dissipatrix and most Merrillosphaera there is. When V.dissipatrix has 8 or more gonidia, 4 or 5 lie at the equator and the remainder towards the posterior pole. V.africanus has equatorial gonidia and the others have posterior gonidia, whilst V.Carteri, V.obversus and V.Weissmannia of the Merrillosphaera and V.Rousseletii and V.capensis of the Euvolvox have an equatorial belt and a second belt midway between the first and the posterior pole. In V.Carteri the gonidia of the two tiers usually alternate and those of V.africanus and V.obversus frequently do so.

The maximum size of gonidia in the Merrillosphaera is regularly greater than 30μ in diameter, those in the Euvolvox are regularly under 15μ and those of the two remaining sections are 15-22μ (Table 3).

The time of differentiation of the gonidia varies markedly from section to section. In most species of Merrillosphaera they are clearly evident in embryos less than 100μ in diameter. In V.Powersi they are differentiated after birth, sometimes before the vegetative cells have ceased to divide, and in V.obversus specialisation is great although it is one of the smallest species - reproductive cells are differentiated at the 16-cell stage, the 8 posterior cells ceasing to divide whilst the 8 anterior cells continue through many divisions (Smith, 1944). In the case of V.Carteri (Metzner, 1945) and V.Weissmannia (Powers, 1908) differentiation is evident at the 64-cell stage. With V.gigas differentiation is evident shortly after inversion. Gonidia continue to enlarge after birth and do not divide until the colony has reached a large size (Pocock, 1933a).

In the section Euvolvox, gonidia are rarely evident in embryos, and liberated young colonies are usually over 250μ in diameter before they are recognisable, but Rich and Pocock (1933) report that in V.capensis differentiation of gonidia occurs before the completion of cell division and can be

seen clearly in daughter colonies which are about to invert. They remain undivided until after the birth of the daughter colony and are distinguishable by their greater size, relative to the somatic cells, due to a lag in division as shown by the presence of two pyrenoids compared with the single pyrenoid of the other cells. Similar observations apply to sexual reproductive cells. In V.Rousseletii the gonidia are differentiated at birth, probably on or just after the completion of cell division. They are evident soon after inversion, by their greater size, twice that of somatic cells, and by the possession of more than one pyrenoid. The daughter colonies at birth are, however, up to 473 x 539μ but may be as small as 182 x 204μ. Pocock (1933b) reports also that V.Rousseletii has gonidia twice as large as vegetative cells at birth. But male sexual cells in particular are smaller in early stages. The exact time of differentiation could not be determined, but in V.capensis it is before inversion and before the last cell division so that it may again be inferred that a reproductive cell is equivalent to at least two vegetative cells. This is also suggested by the large number of cytoplasmic connections with neighbouring cells, the number of cells surrounding a gonidia being in fact double that around a somatic cell. Only after liberation of daughter colonies is gonidial differentiation evident in V.Powersi, V.globator, V.Barberi (Smith, 1944), V.perglobator (Powers, 1908) and V.amboensis (Rich and Pocock, 1933).

With V.aureus and V.dissipatrix the time of differentiation of gonidia is intermediate between that in the Merrillosphaera and Euvolvox.

The differentiation may or may not be simultaneous. The latter case may be recognised where gonidia are of different sizes, where both gonidia and embryos are present and where embryos of different sizes are present. In V.africanus the two equatorial gonidia regularly differentiate into large embryos each with equatorial gonidia before those of the posterior part appear, and these gonidia may give yet another generation of embryos with gonidia by

the time the embryos of the posterior part of the colony show gonidia. In V.Weissmannia also the gonidia in the equatorial quadrant are first to develop, but with V.obversus those of the posterior quadrant are the first to develop and those in the equatorial quadrant lag.

Two species in the section Euvolvox, V.perglobator and V.prolificus regularly have embryos developing in an irregular sequence and have embryos differing widely in size. In all other species the development is simultaneous.

The most important aspects of differentiation, in distinguishing between sections, are the difference in size of gonidia, their number and arrangement and the sequence of development.

Sexual reproduction is oogamous; both male initials and oogonia closely resemble gonidia. In the case of the former, the antheridial mother cell, division takes place to give 16-512 products according to species, each of which metamorphoses into an antherozoid. Single oogonia develop from female initial cells directly. V.aureus and V.perglobator, V.prolificus and V.Rousseletii of the Euvolvox and all the Merrillosphaera, except some varieties of V.Carteri, are heterothallic. Usually the sexuality is distinct, but with V.aureus especially homothallic colonies may be found. Darnden (1966) has made an extensive study of the sexual differentiation of a homothallic dioecious strain of V.aureus and concludes that only three basic cell types occur - vegetative cells, gonidia and male initials; eggs he showed homologous with undivided gonidia. Gonidia are lacking in male colonies, but most cells of the posterior two-thirds of a colony function as male initials, giving packets of 32 bi-flagellate sperm after a rudimentary inversion. There is an anterior-posterior gradient in the initiation of division and maturation, the posterior cells starting first. There were no female colonies per se but newly released vegetative colonies with undivided gonidia function as such. As the number of

zygotes is always within the range of the number of gonidia, it would seem that divided gonidia are irreversibly committed to autocolony formation. Homothallic species of Volvox have a small number of antheridial packets, usually fewer than 10, compared with 50 or more commonly found in male colonies of heterothallic species. V.Weissmannia and V.tertius are exceptions; they regularly have fewer than 50 antheridial packets. V.gigas, V.africanus and V.aureus regularly have more than 150 and the remaining heterothallic species have an intermediate number. All homothallic species are protandrous, the antherozoids being liberated before the eggs are ready for fertilisation.

With the exception of V.africanus and V.gigas all Merrillosphaera have antheridial packets which develop to maturity whilst the male colonies are still embryonic and borne within the parent colony. These species with precocious development also have a smaller number of cells in the male colonies (Table 2) and a smaller size as compared with female and asexual colonies. In V.spermatosphaera all cells of the "dwarf" male colony develop into an antheridial packet, whereas in the other species with "dwarf" males less than a quarter do so.

Of the six heterothallic species in which male colonies develop their antherozoids after liberation, and which approach the dimensions of the female colonies, V.africanus, V.gigas and V.aureus have simultaneous development of the antheridial packet and V.perglobator, V.Rousseletii and V.prolificus do not, so that all stages of development of the antheridial packets may be found in a single colony.

In all species the maximum number of eggs is approximately three times the minimum number, but most counts show intermediate values. In the four species V.spermatosphaera, V.Weissmannia, V.tertius and V.aureus the number is usually less than 20, whilst the majority of colonies of V.Carteri, V.globator

Table 4. Sexuality and gamete numbers in Volvox sp. [after Smith, 1944]

	Sexuality of colonies	Antheridial packets: Number	Antheridial packets: Number of antherozoids	Number of eggs
Merrillosphaera				
V.spermatosphaera	heterothallic	32,64 or 128	32	6-16
V.Carteri	heterothallic	74-103	32 or 64	21-40
V.Weissmannia	heterothallic	8-45	128	8-22 usually 12-18
V.obversus	heterothallic	58-76	64 or 128	21-40
V.tertius	heterothallic	about 50	16 or 32	2-11 usually 3-8
V.gigas	heterothallic	up to 600-700	16-512 usually 32	300-400
V.Powersi	?	?	?	?
V.africanus	heterothallic	165-210	128	12-48 usually 20-35
Janetosphaera				
V.aureus	heterothallic or homothallic	600-1000	32	7-21 usually 10-14
Copelandosphaera				
V.dissipatrix	homothallic	3-7	256	26-126 usually 50-100
Euvolvox				
V.globator	homothallic	3-7	256	11-70 usually 20-30
V.capensis	homothallic	5-19	512	60-220
V.Merrilli	homothallic	5-8	256	45-174 usually 60-130
V.Barberi	homothallic	4-8	256	72-251 usually 100-200
V.amboensis	homothallic	?	?	20-700 usually over 200
V.perglobator	heterothallic	60-80	256	48-121
V.prolificus	heterothallic	25-55	256	80-200
V.Rousseletii	heterothallic	108-214	512	60-227 usually 160-170

V.obversus and V.africanus have between 20 and 40. V.dissipatrix and V.perglobator commonly have colonies with 50-100 eggs, those of V.Merrilli usually have 70-130 and those of V.capensis and V.prolificus have 60-220. V.Rousseletii has between 60 and 227, but usually 160-170. Of the remaining species about which the sexual characters are known, all have more than 100 eggs, the maximum numbers being 250 in V.Barberi, 300-400 in V.gigas and 700 in V.amboensis.

The sexuality of the colonies, the number of antheridial packets and the number of eggs all represent degrees of differentiation which are of systematic importance, and these are summarised in Table 4.

Of all the aspects of differentiation which have been considered in the introduction, that given particular attention in this study ~~was~~ is the variation in eye spot size in the coenobial form, Eudorina illinoiensis, at the level of resolution of the electron microscope. As will be seen, the eye spots vary greatly in their structure and congruity of development. These observations, which form part of a general account of the fine structure, will be correlated with the accounts of gross differentiation in the literature.

MATERIALS AND METHODS

Two species of the genus Eudorina were used in this study, E.illinoiensis (Kofoid) Pascher and E.californica (Shaw) Goldstein and were obtained from the Cambridge Culture Collection of Algae and Protozoa, Botany School, Downing Street, Cambridge, where they are listed as Pleodorina illinoisensis Kofoid, A and B, being respectively the male and female strains, and P.californica Shaw.

Culture of biological material

Cultures were maintained under controlled conditions. Illumination was by means of a series of 5 ft. 80W warm-white fluorescent tubes which were operated by a time switch set to give a 16-hour light period alternating with an 8-hour dark period daily. For rapid multiplication cultures were placed about one foot from the light source where they received illumination of approximately 300 ft.-c. intensity. Depending upon the condition of the inoculum, a dense culture was obtained in 1-2 weeks. After an initial period of rapid multiplication, stock cultures were shielded from the light source by thin paper, in which case they received illumination of about 75 ft.-c. intensity, or alternatively they were maintained in a North-facing window. Measurement of the intensity of illumination was carried out using a photographic light meter.

Absolute temperature regulation was not possible, but the cultures were kept in a room in which the temperature was thermostatically controlled, so that it never fell below 18°C. However, during the "light" period the temperature rose up to a maximum of 22°C as there was no refrigeration.

The glassware used in the preparation of culture media and in cultures was Pyrex exclusively. Cultures were kept in rimless test tubes (16 x 150 mm) stoppered with non-absorbent cotton wool.

The medium used for the bacteria-free cultures of E.illinoiensis is designated B.E.e+s, and that for E.californica, which was not kept bacteria-free, E+Ca, a soil-plus-water medium. Details of the preparation of these media are given in Appendix I.

Transfers of stocks, observing the usual precautions (Pringsheim, 1946), were made monthly in the case of E.illinoiensis and bi-monthly in the case of E.californica.

The sterility of new E.illinoiensis cultures was tested by transferring a small inoculum to a test medium (modified E6 after Provasoli, McLaughlin and Droop (1959) - see Appendix I) which promoted very rapid growth of bacteria if any were present. Only very infrequently was contamination found, although transfers were invariably performed in the open laboratory.

The growth rate of a culture growing under conditions for rapid multiplication is logarithmic for 4-5 days. Most material used in this study was harvested at the end of this period, although when larger colonies were required more slowly growing cultures were used.

In the following sections a certain amount of discussion of the methods used will be introduced, as being more appropriate to this rather than later chapters, since this thesis is not primarily concerned with methodology and techniques.

Light microscopy

The simplest investigation using the light microscope involved allowing the colonies to dry up on a slide under a coverslip, until the cells ruptured. This furnished some useful information, not otherwise available.

Other more specific investigations were made to elucidate certain features observed using the electron microscope. The material used was that embedded in Araldite after fixation in unbuffered osmium tetroxide for electron microscopy; various staining techniques and histochemical tests were applied to 'thick' sections cut in the usual manner on a Sorvall Porter-Blum ultramicrotome bringing the by-pass slide into operation on alternate revolutions of the hand wheel and with the maximum advance, to give sections of c. 1μ thickness. The sections were then expanded with xylol vapour and transferred from the trough to a clean glass slide using a hair loop. It was found advisable to mark the location of the sections on the underside of the slide using a diamond pencil, as even when stained they were not easily visible. The slide was then placed on a hot plate and as the water evaporated the sections adhered to the slide. No special adhesive was necessary.

Wanson (1964) has pointed out the recent adaptation for light microscope studies of the superior fixation and embedding methods used in electron microscopy, and has enumerated some thirty staining techniques which have been used with a variety of fixatives and embedding media. It is worthy of mention that one of the fixatives that he recommended from the results of his own investigations was the unbuffered 1% aqueous solution of osmium tetroxide introduced by Claude (1961).

Although the number of techniques open to use with Araldite embedded material is limited, it was found that the preservation was far superior to that of material embedded in paraffin wax. At best this fragile fresh water material is difficult to work with using the routine methods for light microscopy.

Delafield's haematoxylin (Johansen, 1940) and the metachromatic dye toluidine blue (Appendix II) were used with success. In the case of the

former, slides were stained for 30 min. at room temperature after having been soaked in xylol for one hour and then brought to water via absolute and 95% ethanol. Following a 5 min. wash in running tapwater the slides were transferred to distilled water acidified with a few drops of dilute hydrochloric acid for 1-2 min. and returned to tapwater until the sections turned purple. The sections were then dehydrated by being passed through 95% and absolute ethanol, cleared in xylol and mounted in Canada balsam. Resting nuclei and nucleoli, and chromosomes, mitochondria and other cytoplasmic particles appeared deep blue-black; the cytoplasm was blue-grey. Munger (1961) is of the opinion that the failure by earlier workers to use haematoxylin stains successfully with epoxy resin embedded material has been due to the fact that osmium tetroxide fixation was used, but in this work no difficulty was encountered using Delafield's haematoxylin.

Toluidine blue was used as follows: the stain was pipetted on to the sections and the slides were placed in an oven at 50°C for 1 hour, in a damp chamber to retard evaporation. After rinsing in distilled water the sections were dehydrated, cleared and mounted as described in the previous case. All nucleic acids are stained blue, unless the RNA has been removed by prior hydrolysis in 1N hydrochloric acid. Where, as in this case, hydrolysis was not performed, the effect is of a general staining because the cytoplasm has a high RNA content. Lipid droplets exhibit metachromasy and appear mauve.

Trump, Smuckler and Benditt (1961), using an 0.1% solution of toluidine blue in a 2-5% solution of sodium carbonate, pH 11.1, found that the staining pattern was not that usually associated with toluidine blue and tentatively ascribed the differences to the action of osmium in fixation. They found that mitochondria, nucleoli and basement membranes appeared lavender in colour, and lipids appeared green. Most cell granules showed metachromasia. This

material was Epon-embedded. The action of this stain is undoubtedly capricious, for McGee Russell and Smale (1963) were unable to stain Epon-embedded material.

The periodic acid-Schiff (P.A.S.) reaction was used for the detection of the total carbohydrates of insoluble polysaccharides, following the routine of Munger (1961), but either without counterstaining, or counterstaining with toluidine blue. Briefly the details of this procedure were as follows. After soaking for 1 hour in xylol, the sections were brought to water and treated with 0.5% periodic acid reagent for 10 min., washed in tapwater and then transferred to Schiff's reagent (Appendix II) for 30 minutes. After a further wash in tapwater, the sections were rinsed in peracetic acid reagent (Appendix II), which according to Munger facilitates nuclear staining, and then either mounted directly in balsam, or after counterstaining. Water-soluble saccharides and polysaccharides are not localised due to the use of aqueous solutions, but starch reacts very strongly.

Unsaturated lipids were detected by the peracetic acid Schiff (P.A.A.S.) reaction (after Jensen, 1962). Sections were brought to water and then placed in peracetic acid reagent for 2-5 min., washed in running water for an equal period, then left in Schiff's reagent for 30 minutes. After washing in running water for a further 10 min., the sections were mounted in glycerine jelly.

The last mentioned test provides a control for the periodic acid oxidation used in the P.A.S. reaction test for carbohydrates. The peracetic acid, which is a mild oxidant, attacks the double bonds present in the unsaturated lipids, resulting in the formation of an aldehyde which is then coloured by the Schiff's reagent, but it does not attack carbohydrates, whereas under some conditions the periodic acid used in the carbohydrate procedure will attack unsaturated lipids in addition to the carbohydrates (Jensen, 1962). According to Casselman (1959) this test may be considered conclusive only if the postulated

lipids also stain with Sudan Black or if prior bromination prevents the positive P.A.A.S. reaction.

Total lipids were detected by staining with a filtered saturated solution of Sudan Black B in 70% alcohol (Gomori, 1952; McGee-Russell and Smale, 1963) for 1 to 2 hours and mounting in glycerine-gelatin after a brief wash in 70% ethanol. It was not found necessary to stain for the long periods (up to 24 hours) as recommended by McGee-Russell (1963) for Araldite embedded material. The stain was taken up rapidly, especially by the resin, as noted by McGee-Russell and Smale (loc.cit.). Rather than view this negative image where the stainability reflects the amount of plastic in the material and thus presumably the density, the sections were then washed in 70% ethanol. This must be done rapidly as the stain is easily removed. However, as it is more easily removed from the densely stained plastic, the lipids remain stained. Lipids, including free fatty acids and phospholipids, appear blue-black. It should be noted that the material used was fixed with osmium tetroxide which itself reacts with lipids. The staining action of Sudan Black B depends upon the selective solubility of the dye in the lipids and this is not apparently affected by the previous reaction with the osmium tetroxide.

In this study all attempts to stain specifically the nucleic acids in Araldite-embedded material with gallocyanin-chromalum (Einarson, 1932), by the Feulgen reaction (Pearse, 1960) or with Azure A (De Lamater, 1951) proved unsuccessful. Gallocyanin-chromalum has been used with success by Munger (1961) using material which had been embedded in methacrylate which was dissolved before staining. His sections were stained for 4-5 days. With the Araldite-embedded material used in this study there was no trace of any stain being taken up after staining for over a week. Successful Feulgen staining of material embedded in Araldite has been reported by Huxley and Zubay (1961). Moses (1956) was also successful using material embedded in methacrylate.

In this study the methyl-green and pyronin test (Pearse, 1960) for nucleic acids gave a very successful demonstration of the presence of ribonucleic acid as indicated by the pyronin staining, but there was no staining of the desoxyribonucleic acid by methyl-green which agrees with the experience of Jennings, Farquhar and Moon (1959) for osmium-fixed methacrylate sections, who also found the method unreliable.

Electron microscopy

For an examination of the ultrastructure of whole flagella, negatively stained preparations were made using 2% phosphotungstic acid following the method of Bradley (1965, 1966), who has pointed out that this method reveals much structural detail using high resolution micrographs, which is not available from the study of ultrathin sections. Colonies were concentrated from a culture by light centrifugation and resuspended in 10 mls of distilled water to which was added one drop of 2% aqueous osmium tetroxide solution, and left overnight. The material was prepared for examination as follows: single drops of the material and of 2% neutral potassium phosphotungstate were mixed on a glass microscope slide and a collodion-carbon filmed grid floated on the surface. The slide was then sharply inverted and the colonies allowed to settle above the grid which was lowermost in the hanging drop. The grid was next drawn from the drop with watch-makers forceps. This left most colonies in the fluid remaining on the grid. Excess fluid was taken up using filter paper and the grid dried under an electric light bulb for 2 minutes. The optimal colony number was easily arrived at by adjusting the concentration in the fixative before mixing with the potassium phosphotungstate. Only few colonies were required as Eudorina is relatively so large.

The bulk of this study was carried out using sectioned material prepared using the standard methods of electron microscopy (Kay, 1965; Pease, 1964).

A number of fixation methods were tried: 2% and 5% $KMnO_4$, unbuffered, at room temperature (Luft, 1956; Mollenhauer, 1959) which gives rather poor general fixation and extracts the nucleus but, as is well known, gives good preservation of membranes, although the chloroplast is not well preserved. Palade's buffered osmium tetroxide fixative, a 1% solution buffered at pH 7.3 in veronal-acetate (Palade, 1952) was also used but not routinely, rather for a comparison of the results obtained by the method preferred, unbuffered osmium tetroxide in carbon tetrachloride, which proved a very satisfactory fixative early in this study. It was introduced by Afzelius (1959) to meet his requirement for an osmium tetroxide fixative of high concentration that would penetrate tissues fairly rapidly. He found that a 40% solution of osmium tetroxide in carbon tetrachloride gave good general preservation and a higher contrast than ordinary oxmium fixatives. The replacement of water as the solvent for the osmium tetroxide was necessary since the solubility is only about 6%. Although this fixative is at first sight crude, it does give good results. It is suggested that the increase in contrast is due to the high concentration of osmium tetroxide and the consequent rapidity of fixation before autocatalytic changes can occur, as might be the case where the concentration is much lower. Vapour pressure measurements suggest that in high concentration osmium tetroxide in carbon tetrachloride is in a polymerised state (Anderson and Yost, 1938); thus single reactive sites in the cell may be binding more than one osmium atom. It is also possible that a certain amount of extraction is taking place by the agency of the carbon tetrachloride. Claude (1961) has pointed out that this may be of practical importance in giving the differential contrast required for electron microscopy, but he associates this with slow penetration, a delay between death and fixation, or uneven fixation, which if correct suggests that only one or the other of the explanations put forward by Afzelius is correct, but not both.

The procedure adopted as routine was as follows: the culture selected was transferred to a centrifuge tube and spun gently (at about 400 r.p.m.) to concentrate the colonies. The supernatant liquid was then poured off, leaving the colonies in about 0.25 ml of liquid, and the colonies resuspended by gentle agitation. The fixative (Appendix III) was then added to the concentrated culture using a Pasteur pipette, and the tube stoppered. A two-phase system results, with the watery layer containing the colonies beneath the carbon tetrachloride layer in which the osmium tetroxide is dissolved. The colonies rapidly darken and sink to the bottom as the osmium tetroxide diffuses into the lower layer. Fixation was carried out at room temperature for 45 min., during which period the centrifuge tube was gently agitated at intervals to ensure uniform fixation.

Carbon tetrachloride is immiscible with lower grades of ethanol, but was removed during the dehydration in 70%, 95% and absolute ethanol. Three changes of absolute ethanol were used. Details of the embedding will be given later (p.46).

The fixative is essentially an unbuffered solution of osmium tetroxide in water with the carbon tetrachloride layer acting as the reservoir. Claude (1961) and Malhotra (1962) have questioned the need for buffer in osmium tetroxide fixatives, and Baker (1965) has pointed out that at the pH fixed upon by Palade for his fixative, pH 7.4, the sodium acetate in the Michaelis' buffer which was used by Palade is devoid of buffering capacity, its action being in the more acid range. Claude recommends that the fixative should be slightly acid (pH 6) and this may be attained by allowing the absorption of atmospheric CO_2 under normal pressure. He found that such a fixative gave better results than Palade's osmium tetroxide fixative buffered at pH 6, when tested by subjecting the material used, tumour cells, to distilled water treatment which accelerates and aggravates extraction and disintegration. Swelling and

extraction were found when buffered alkali fixation was used. Furthermore, Baker (1965) in his review of fine structure produced by fixatives remarks that unlike Palade (1952) he has found that unbuffered osmium tetroxide fixation produces the characteristic osmium picture, and this in spite of a difference in tonicity between that of the cells used, the exocrine pancreatic cell of the mouse, and that of the fixative, which contravenes the claims of Sjöstrand (1956) and Tahmisian (1964) that the solution should be isotonic with the material being used. Sjöstrand reports that good fixation was obtained with unbuffered osmium tetroxide which was made isotonic with the material by the addition of sodium chloride.

The method of fixation used in this study was selected on the grounds that the algal colonies should remain in the medium in which they have grown right up to the time of fixation, which is rapid in consequence of the high concentration of osmium tetroxide present in the fixative. Osmium tetroxide dissolves in neutral distilled water without change of pH (Claude, 1961), and it was found that no change in pH occurs in the water layer of the two-phase system when using the osmium tetroxide/carbon tetrachloride fixative.

Glutaraldehyde fixation was used for one investigation as being that aldehyde which gives the best general preservation of fine structure with little destruction of enzymatic activity, unlike osmium fixatives, unless they are used for very short periods only (Sabatini, Bensch and Barrnett, 1963; Sabatini, Miller and Barrnett, 1964). The aim of this particular investigation was the localisation of RNA widely found to be associated with the chloroplasts (Jacobson, Swift and Bogorad, 1963) by comparing material subjected to ribonuclease treatment with untreated material. The procedure adopted was as follows.

The fixative mixture was prepared at half the strength given by Ledbetter and Porter (1963), i.e. as a 3% solution buffered at pH 7 in 0.1M phosphate - see Appendix III. This was applied for 3 hours at 3°C. The material was then washed in three changes of buffer each applied for half an hour. The first two washes were at 3°C, but during the third the temperature was allowed to come to room temperature. The material was then divided into two portions. One of these was incubated for 2 hours in a water bath at 37°C with agitation every 15 min., in a solution of ribonuclease (Koch-Light 5 times crystallised salt-free ex-bovine pancreas) containing 1 mg/ml buffered in the same manner as the fixative; the other received exactly the same treatment except that the incubation was in buffer solution alone. Both portions were then washed in three changes of buffer, each for 20 min. and then treated with 1% osmium tetroxide in the same buffer for 45 minutes. These operations were all performed at room temperature. Osmium post-fixation was introduced because there is a better quality of preservation than when glutaraldehyde is used alone (Ledbetter and Porter, 1963; Sabatini et al, 1964).

Dehydration and embedding were carried out following standard procedures. As with fixation, the dehydration was done in the centrifuge tubes. For each change of ethanol, the material was spun down by light centrifugation and the supernatant fluid poured off; the colonies were then resuspended in the next fluid. By ensuring that the material never became compressed into the form of a pellet, distortion of the colonies was avoided. After the final changes of absolute ethanol, a 1:1 Araldite:absolute ethanol mixture was substituted and left for 30 minutes. The mixture is not viscous and there is no difficulty in concentrating the material for the final changes into Araldite (Glauert and Glauert, 1958) which has been used for all embeddings. For the first change to Araldite, the material was resuspended in the resin

in the centrifuge tube. This is not difficult but takes some time due to the very much greater viscosity of the undiluted resin mixture. After one hour the tubes were stoppered tightly with rubber bungs and heated in a water bath to 60°C, and when the Araldite had come to this temperature the tubes were centrifuged at about 2000 r.p.m. The Araldite is too viscous to allow any distortion of the colonies. After repeating this procedure a few times the material was concentrated at the bottom of the tube. Although the temperature of the water bath was greater than that commonly used to polymerise the Araldite, the value of this heating is to reduce the viscosity of the resin at a stage when its degree of polymerization is low.

For the second and final change to Araldite a Pasteur pipette with a wide bore was prepared for use in the following manner. The pipette was first allowed to take up fresh Araldite which was then slowly pipetted out so as to leave a thin coat of resin on the inner walls. The object of doing this is to minimise loss of material; if this precaution is not taken many colonies are lost in the layer of resin remaining in the pipette after the bulk has been pipetted out. Using the pipette the material was transferred from the centrifuge tube to a solid watch glass which was placed on a rotary shaker as devised by Jurand and Ireland (1965). The temperature was kept at about 45-50°C by means of a 60W light bulb placed immediately above the shaker, to decrease the viscosity of the Araldite and to facilitate a continuous flow over the material which leads to a more complete impregnation. After one hour of this treatment the material was allowed to stand overnight.

As the colonies tend to concentrate at the point of least disturbance in the shaker, it is an easy matter to transfer a sufficient number of them to give a dense pellet into several gelatin capsules, again using a pipette and taking the precaution previously described. The capsules are topped up with

fresh Araldite. Pellets are formed by placing the capsules in tightly stoppered centrifuge tubes and heating them in a water bath to 60°C in order to decrease the viscosity of the resin, and then spinning them at 2000 r.p.m. This operation has to be done 3-4 times to give a dense pellet. Flat embeddings in shallow aluminium foil trays for orientated re-embedding were made using the few colonies remaining in the watch glass. Polymerisation was carried out in an oven at 56°C. Sections were cut on a Sorvall Porter-Blum Microtome MT-1, using glass knives (Latta and Hartmann, 1950) and mounted on copper grids coated with collodion and carbon.

A major part of this investigation involved the serial sectioning of an orientated colony. The scale on which this was done was very large and many of the precautions taken by earlier workers who have attempted working with serially sectioned material on much smaller scales had to be ignored. Bang and Bang (1957) made their reconstructions from a series of only 11 sections and Sjöstrand (1958) worked from series of up to forty sections taken from a uniform structure; in these cases it was possible to study completely serial material. For this the serial sections should be mounted so that they can be seen without interruption by grid bars. To this end a two-step method involving mounting the ribbons on a support film and subsequently mounting this on grids with slits has been developed (Gay and Anderson, 1954). A special technique is adopted to orientate the ribbon along the slits. Alternatively ribbons can be mounted on grids with a single large hole (Sjöstrand, 1958; Dowell, 1959). Although for true serial sections these methods give the best results, both have the disadvantage of the instability of the support film. This applies especially to the case of the grid with a single hole, and for this reason such grids tend to be thicker than is otherwise normal. In addition, where films are unstable the contamination rate of the microscope is greatly increased, and it

was considered that such a degree of contamination was not permissible. As a compromise the grids selected for use had hexagonal holes of small dimensions: the chances of the same region being obscured by grid bars in consecutive sections are greatly reduced as compared with those using grids with square holes. Another important consideration was the time factor: the sectioning was done on such a large scale that had the elaborate two-stage mounting procedure been adopted, there would not have been time to complete it.

From a single colony 75 grids were collected and on many of these two ribbons were mounted. The number of sections cut was of the order of 700. In addition 49 sections of 0.5μ thickness were cut for investigation by light microscopy.

The grids collected were stored in order; it was, moreover, possible to determine the order in which the sections were cut by the expedient of trimming the block so that the block face was not a perfect square: the horizontal edges of the block as mounted on the microtome should be exactly parallel but the vertical edges are cut to be slightly divergent.

As it is not permissible to disturb the block or knife at any stage during sectioning, it is essential that the initial trimming be as accurate as possible and that the sides of the pyramid be relatively steep, so that as successive sections are cut the block face does not become unduly large. The success of the whole undertaking depends very greatly on the quality of the glass knife. Glauert and Phillips in Williams and Kallman (1955) emphasise that in serial sectioning it is the knife defects which limit success, and Kay (1965) quotes the figure 30 as being the number of sections which can be cut from each part of the knife, whereas it will be seen here that a number very greatly in excess of this was cut, with very little deterioration in the quality being evident. Undoubtedly there is a large element of luck in the choice of

a knife, and no rules can be given indicating the likelihood of any particular glass giving a good knife. The choice was made from a batch of knives which experience had shown to contain many good ones.

As the number of grids of serially sectioned material was so large, the period over which they were examined was inevitably protracted. It is worthy of mention that no deterioration was noted in the sections on grids which had been stored, in some cases, as long as five months. Storage was within a desiccator, and batches of grids were stained as required.

The stain used with the greatest success was that suggested by Mr. A. E. G. Dunn, late of the Department of Zoology, Edinburgh University. The method is a modified combination of the methods of Lawn (1960) and Watson (1958). The stain consists of a mixture of uranyl acetate and potassium permanganate solutions (Appendix III). Lead citrate (Reynolds, 1963) and 2% uranyl zinc acetate were also tried, but with less success.

A saturated solution of Sudan Black B in 70% ethanol was used for the detection of lipids. Grids were immersed in the stain from 30 min. - 2 hours and were then given a rapid wash in 70% ethanol and allowed to dry. The use of Sudan Black B as a lipid stain for electron microscopy was first reported by Casley-Smith (1963). The staining is at first sight remarkable in that no heavy metal ion is contained in the molecule, only hydrogen, carbon, nitrogen and oxygen, but the molecular weight is of the order of 500. The dense staining derives from the mean atomic mass per unit volume. As osmium tetroxide does not fix saturated lipids completely, they do not appear very electron dense, but subsequent staining with Sudan Black B renders the staining very intense.

Most specimens were examined using an A.E.I. electron microscope E.M.6 with a condenser aperture of 250μ and an objective aperture of 50μ operating at 60 kV, but a few were examined using a Philips E.M.75 instrument.

The photographic plates used in the E.M.6 were Ilford Special Lantern Contrasty and Ilford N.50 thin-film Halftone (no coated edges), both unbacked, and in the E.M.75 Ilford N.40 Process unbacked plates.

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RESULTS

The organisation and development of the envelope

The disposition of the 32 typical chlamydomonad cells in 5 ranks of 4, 8, 8, 8 and 4 cells at the surface of the colonial envelope may be seen clearly by light microscopy in intact colonies of Eudorina illinoiensis. From fixed and stained material it is evident that the cells lie in very close proximity to the envelope (Figs 121, 122, Plate XXXI).

The envelope, when viewed by electron microscopy, is seen to be bounded by two dense lines of 6-8 mμ thickness separated by a space of about 15 mμ. At high magnifications both dense lines are resolvable into two further dense lines of which those bordering the 15 mμ space of the low magnification picture tend to be very dense whilst the other two tend to be diffuse. Bearing in mind the structure in three dimensions it will be realised that each of these dense lines represents a sheet or membrane of material. Below the outer membranous layers is a very diffuse layer consisting of a loose fibrous meshwork, the thickness of which varies from 1-3.5μ in fully grown colonies. The individual cells of the colony are surrounded by a similar meshwork of thickness 0.5-1μ and occasional sections show a dense double membrane next to the cell. The meshwork surrounding the cells is in connection with that of the envelope at the apical or flagellar end of each cell in the region where the flagella pass to the exterior. The two flagella of each cell run in separate passages which show a characteristic differentiation. In transverse section the passages are seen to possess small tooth-like projections (Figs 15, 16, Plate VIII). Oblique longitudinal sections of the passages show that these represent ridges running the length of the passage (Fig. 22, Plate IX; Fig. 25, Plate X). The ridges, which number about 65, are 5.0-5.5 mμ wide and 17.5-18.5 mμ from centre to centre. The passages are 0.35-0.4μ in diameter. True longitudinal sections

also give evidence of the differentiation of the passages (Fig. 21, Plate IX): the margins present a compact structure.

At the point of emergence of a flagellum the envelope is frequently raised into a slight protuberance, appearing in the two-dimensional micrographs as a rim. In some micrographs of longitudinal sections through the exact centre of the passage the colonial envelope double membrane may be seen to turn over this rim and run a little way down the passage. A ring of very dense material is found surrounding the proximal end of the flagellar passages. It seems that the double membrane of the envelope is not in connection with that around the cells as there is no indication of such a membrane in the transverse sections through the ridged part of the passages.

The envelope of daughter colonies is evident by the time of the third division. Around the periphery of the daughter colony which is still in the mother cell envelope can be made out a thin fibrous network. Furthermore, between the just divided cells an even thinner fibrous layer having the appearance of a row of granules in section can be made out which is in connection with the daughter colony envelope (Fig. 6, Plate IV; Fig. 8, Plate V). The cells of daughter colonies separate whilst the colonies are still confined in the mother cell envelope. At this stage what is presumably the remnant of the fibrous layer between the cells in younger stages can be seen running midway between the widely separated cells which have acquired another thin fibrous envelope. In slightly older colonies which have escaped from the mother colony the only envelope parts evident are those around the cells and the colonial envelope.

Cell membrane

Each cell is bounded by a typical unit membrane of 8.0-9.0mμ thickness (Fig. 1, Plate I; Fig. 2, Plate II). At high resolution this resolves into

THE PLATES

Unless otherwise stated all figures are of material fixed in the osmium tetroxide/carbon tetrachloride fixative, embedded in Araldite and stained with the combined uranyl acetate and potassium permanganate stain.

Two magnifications are quoted for each figure, the first is the magnification of the print and the second, in brackets, is the magnification of the negative.

PLATE I

Fig. 1. General survey picture of a reproductive cell from a mature colony shewing: chloroplast (C) with pyrenoid (P), associated starch plates (sp) and isolated starch (s), a deep sited eyespot (E) and osmiophilic globules (arrow); Golgi bodies (G); Mitochondria (M); lipid droplets (l); unidentified vacuoles (V) and vesiculate system (Vs), ground cytoplasm and limiting cell membrane (CM). x 10,000 (x 10,000).

1

PLATE II

Fig. 2. General survey picture of two cells from a young colony. Seen, in addition to most of the organelles of fig. 1, are: the colonial envelope (EC), nucleus (N) and nuclear envelope (ne), flagellum (F). Upper cell has an additional basal body (arrow). For other abbreviations see fig. 1. x 13,000 (x 6,500).

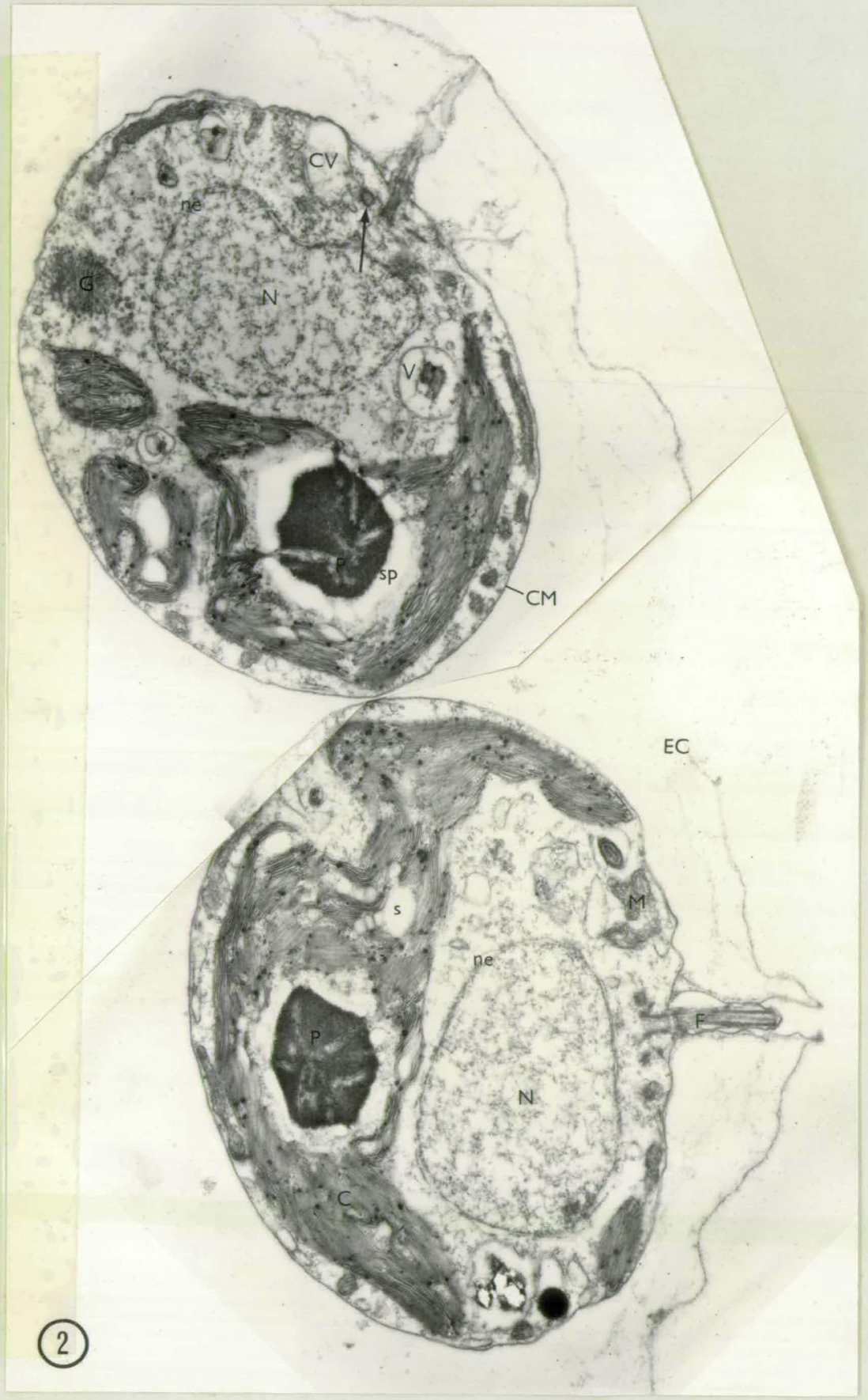

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PLATE III

Fig. 3. General survey picture of a cell from a young colony, with typically placed eyespot (E), contractile vacuole (CV) and grazing section at pyrenoid margin shewing starch plates (sp) and pyrenoid lamellar tubules (pt); osmiophilic globules (arrow). For other abbreviations see fig. 1. Tubular endoplasmic reticulum (TER). Chloroplast envelope (ce). x 32,000 (x 16,000).

3

PLATE IV

Fig. 4, Cell in division. The nuclear envelopes of the daughter cells have reformed and the nucleolus (nl) of the left hand cell is visible. Cytokinesis has not progressed far; the dividing cell membrane extends just under one third of the distance across the cell from one side only (arrow). The chloroplast is in the process of dividing by constriction. The cells contain much food reserve material both in the form of isolated starch grains (s) in the chloroplast and lipid droplets (l) in the cytoplasm. The lipid droplets are being consumed as evidenced by the vacuoles (V) with fragmentary membranous contents. Three contractile vacuoles (CV) appear in the plane of the section. Nucleus (N). Golgi body (G). x 8,000 (x 4,000).

Fig. 5. Detail of cell membranes forming between dividing cells. x 20,000 (x 5,330).

Fig. 6. Detail of the region of contact between two cells after the third division. The fine granular layer (arrow) between the cells represents part of the developing envelope and it is seen to be in connection with the colonial envelope of the daughter colony. x 30,000 (x 10,000).

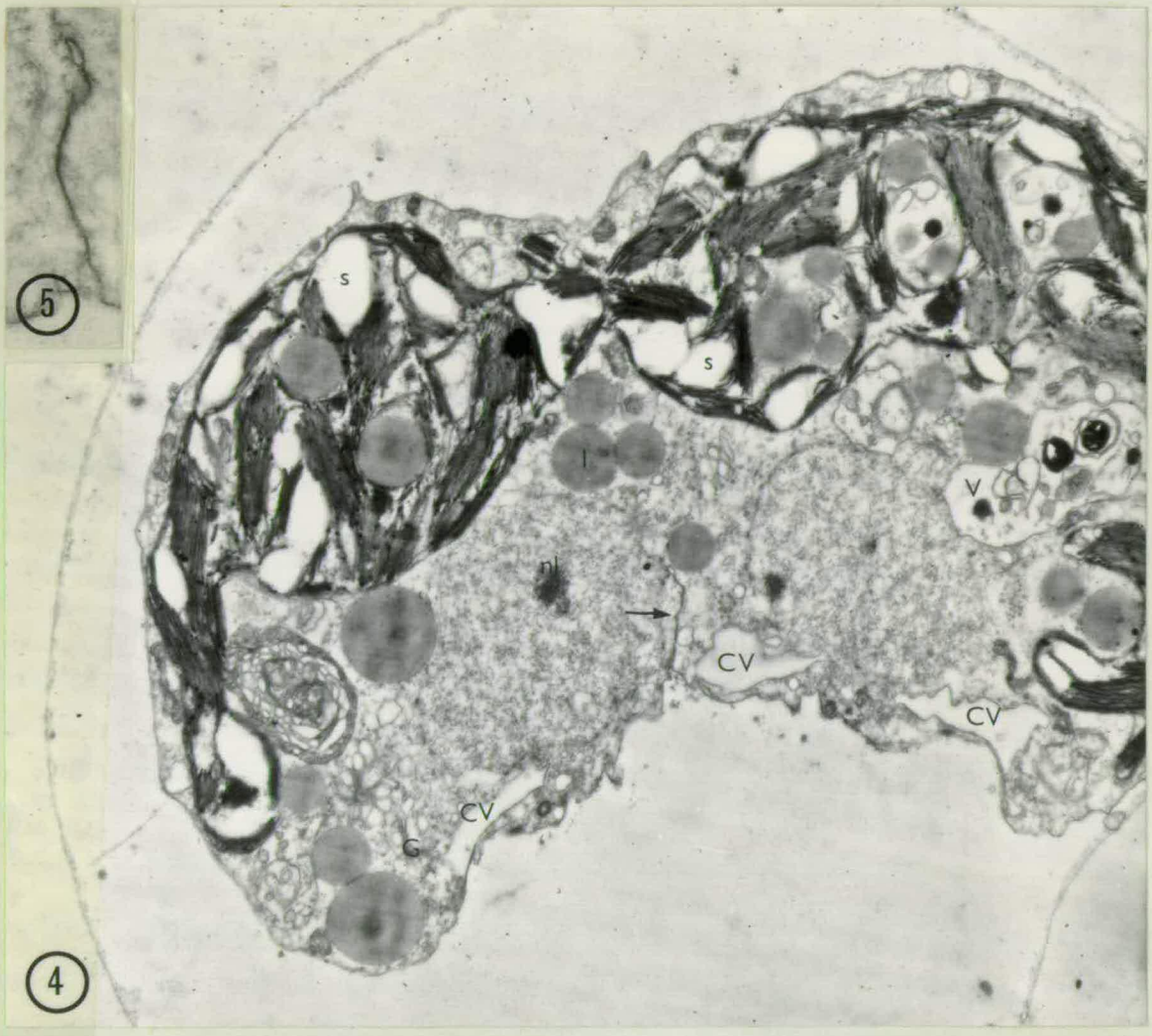
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PLATE V

Fig. 7. Daughter cells at the completion of the first or second division. Golgi bodies (G). Starch (s). Lipid droplets (l).
x 10,000 (x 4,000).

Fig. 8. Autocolony at the completion of the third division. Arrow shews contact of daughter colony and mother cell envelopes. x 8,000 (x 4,000).

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PLATE VI

Fig. 9. General survey picture of the nuclear region of a cell which has just completed a division. A number of mitochondrial (M) profiles appear in the vicinity of the nucleus (N) which has a contorted shape. The cytoplasm possesses extensive rough tubular endoplasmic reticulum (TER). The deep cytoplasmic microtubules (arrow) orientated towards the nucleus probably represent spindle fibres. A contractile vacuole (CV) in an early stage of formation appears at the top of the figure. Unidentified vacuole (V). Pyrenoid (P). x 20,000 (x 8,000).

9

a membrane of two dense layers with a light space between them (Fig. 45, Plate XV). The individual dimensions are difficult to measure as the profiles are not sharp, but the space is about 4.0-5.0 mμ wide and the dense layers 1.5-2.0 mμ. It was not noticed that the outer dense layer was more weakly contrasted with osmium tetroxide fixation than was the inner layer, as noted by Finean (1961, see Frey-Wyssling and Mühlethaler, 1965) and taken by him to be indicative of the polar structure of the cell membrane. Thus the cell membrane conforms rather with Robertson's (1959) symmetrical scheme for the unit membrane.

During cell division the new cell membrane forms asymmetrically, starting at the apical pole. In Fig. 4, Plate IV the new cell membrane extends almost one-third of the distance across the cell at the apical pole, but none has formed at the opposite pole. The new membrane of the prospective daughter cells is in very intimate contact except at the point of maximum constriction, a space occurring where the membrane folds back on itself (Fig. 5, Plate IV).

Flagella

Negative staining of whole flagella shows them to possess flagellar hairs. Pitelka (1963) reports that flagellar hairs have been observed frequently, but that nothing is known of their significance. They are not mastigonemes in the sense that such are found in heterokont algae and fungi. The flagella hairs are distributed uniformly along the length of flagella and seem to pass through the flagellar membrane which is continuous with the cell membrane (Fig. 21, Plate IX) to join the axoneme (the array of 9 + 2 fibrils characteristic of flagella) (Figs 10, 13, Plate VII). Some of these hairs approach 6μ in length and their diameter is 25 mμ. Certain details of their structure can be seen from high resolution micrographs (Fig. 11, Plate VII). The negative stain penetrates the hair to show a helical array with a single pitch of 45 mμ and a pitch angle of 16°. The image given is of a series of dense spots in two rows uniformly staggered. Light bars join the dots of each row in a zig-zag pattern.

Such a staining pattern results from a spiral space being filled with stain, the dense spots being where the stain is being viewed along the axis of the spiral and the light bars where viewed across the axis. The structure of the flagellar hair may be deduced from this to consist of a central filament around which is wound another filament in a tight spiral with successive windings in contact. The dimensions of the central filament and of the spiral filament are both $\sim$ 8 mμ.

The axoneme shows moderately distinctly in intact flagella (Figs 10, 12, Plate VII) but more so when the flagellar envelope has ruptured (Fig. 13, Plate VII), in which case the fibrils separate. The axoneme fibrils continue to the tip of the flagellum in a body and there is no indication from the negatively stained material that any terminate more than a very short distance from the tip.

Transverse sections of the flagellum distal to the point of its emergence from the envelope show the characteristic 9 outer double fibres and 2 central fibres (Fig. 14, Plate VIII). The centre fibres are nearly circular in cross section and approximately 25 mμ in diameter. The separation of the two fibres is 28 mμ from centre to centre. The width of the fibre wall is about 4.5 mμ. In many, but not all, sections a dark line connects the two fibres (Figs 14, 15, Plate VIII). It originates at a tangent from one fibre and follows a steep parabolic course to the other fibre. The separation between the parabolic lines at their midpoints is 35-40 mμ. In longitudinal section it can be seen that this line is a single spiral filament wound around the central filaments. It has a pitch of about 20 mμ and a pitch angle of 10°. The details of this can be best made out in sections in which the central fibres are in the plane perpendicular to that of the section. The spiral filament is then seen as a series of dots placed alternately on each side of the central fibres with a light line connecting them. The separation of the two rows of dots is about 40 mμ which

corresponds to the maximum separation of the parabolic lines seen in transverse section.

In longitudinal section the central fibres are seen to terminate just distal to the electron dense cylinder which occurs at the level at which the flagellum emerges from the cell (Figs 21, 23, Plate IX; Fig. 25, Plate X). Longitudinal sections of the flagellum also show an indication of fine connections between the central fibres and the outer fibres (Figs 21, 22, Plate IX).

Each of the outer double fibres which in transverse section is shaped in a figure of eight measures 25 by 36 mμ. The plane defined by the longitudinal axes of the two sub-fibres has been named the axial plane (Gibbons and Grimstone, 1960) and is inclined at a slight angle to the tangent to the flagellum at the membrane, on the radius passing through the centre of the double fibre. The sub-fibre which is closest to the centre of the flagellum is termed the A sub-fibre, and the other the B sub-fibre for descriptive purposes. The width of the fibre wall is 4.5 mμ. Arising at intervals from the A sub-fibres are pairs of arms measuring 15 mμ. These all point in the same direction and thus give the flagellum an asymmetry. In transverse sections at the level of the flagellar passage through the envelope adjacent double fibres are linked (Fig. 15, Plate VIII) apparently by the inner of each pair of arms just described. The staining reaction of these links is more intense than that of the other arms which are also seen, and corresponds more nearly to that of the fibres. Fixation with glutaraldehyde shows in this material one detail of the structure of the outer fibres which was not observed using osmium tetroxide ~~in this material~~, although it has been seen in the cilia of Paramecium fixed with Palade's osmium fixative (Jurand, personal communication). A fine single spiral winding around the outer double fibres is seen (Fig. 23, Plate IX) which has a pitch angle of 25° and a pitch of 12 mμ. The width of the fibril is about 4 mμ.

The space between the axoneme fibres is occupied by a loose matrix (Fig. 21, Plate IX).

The basal body is identified as that region in which the peripheral fibres have a triplet structure. However, there is a transitional region between this and the flagellum proper. In this region a number of differences in structure occur. The transverse section next proximal from that in which the outer fibres are linked is illustrated in Fig. 16, Plate IX. The centre fibres have terminated distal to this section and do not appear. The links between the adjacent outer fibres are absent, but the outer double fibres are now linked by a new and elaborate structure in the form of a 9-pointed star first described by Lang (1963b) in Polytoma. Fibrils run from the A sub-fibres of alternate peripheral fibres to meet near the centre in a widely obtuse angle. As the number of peripheral fibres is odd, starting from any one of them and tracing a course to the centre and then to the next fibre but one, two circuits are made around the central axis before the starting point is arrived at. The points at which the fibrils meet lie in a circle of diameter 50 mμ, the cylinder of longitudinal sections (Fig. 21, Plate IX; Fig. 25, Plate X). In transverse sections this has the appearance of a dense spot. Another form of the stellate structure in which a dense circle occupies the centre, as if the dense spots previously described had run together, which has been reported by Manton (1964b) and Ringo (1967) was not seen during this study.

In longitudinal section the cylinder shows a transverse diaphragm; the two-dimensional profile is H-shaped. Immediately proximal from the diaphragm the cylinder is frequently less intensely stained and it may be that there is a break here. The dimensions of the cylinder vary ~~between~~ from 124-180 mμ in length and 50-70 mμ in diameter. The relative dimensions of the parts of the cylinder proximal and distal to the diaphragm are constant, the distal part being 2.1

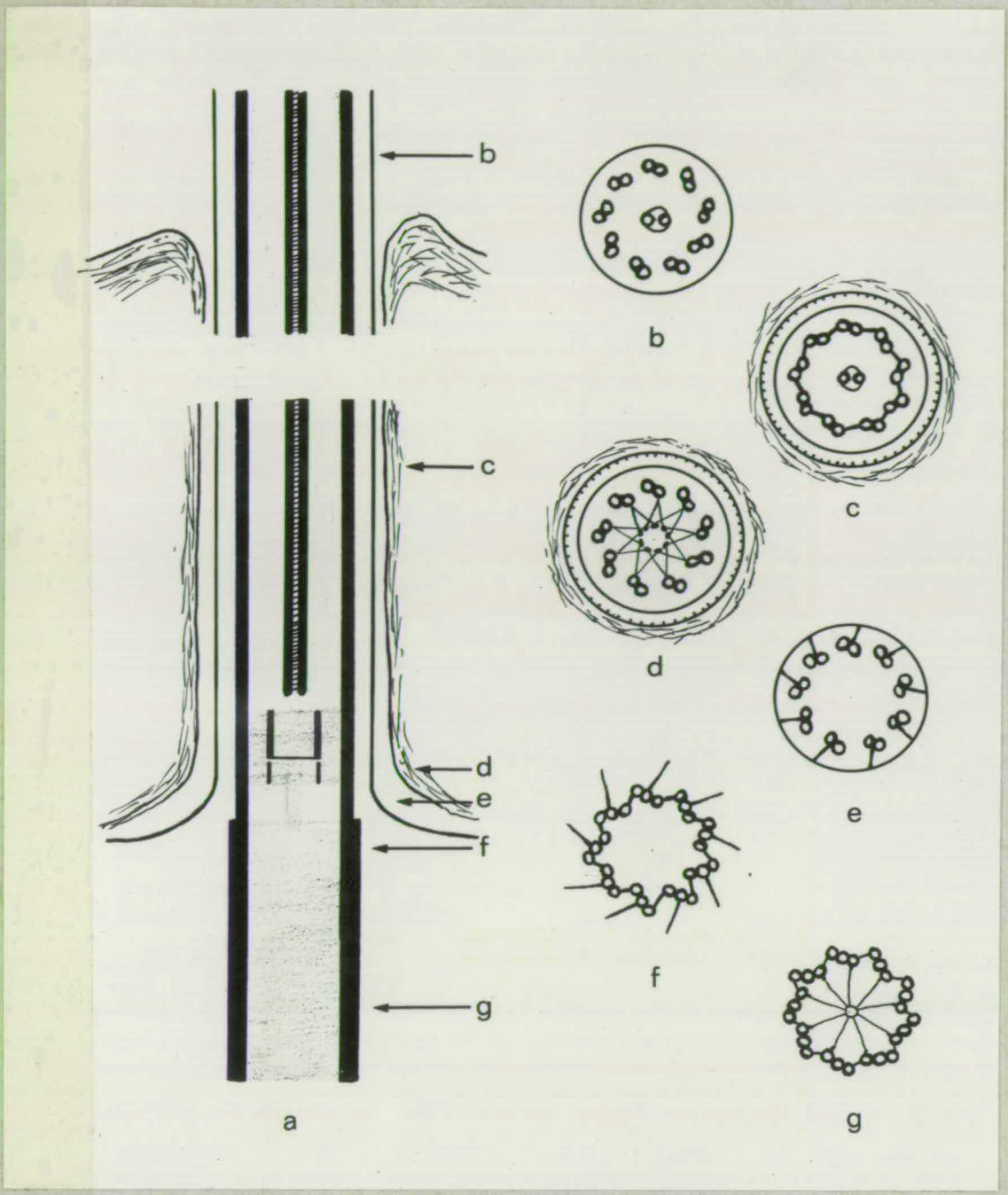
times the length of the proximal part. The width of the proximal part of the cylinder wall is about 5 mμ and that of the distal part about 12.5 mμ, although the staining reaction of this latter is intense only in the part continuous with the proximal region, and again measures 5 mμ. It is possible that the profiles showing a complete dark central circle represent sections through the distal region of the cylinder and those with the nine dense spots in a circle sections through the proximal region. There is a correspondence in the dimensions as measured from the published micrographs of Manton (1964b) and Ringo (1967). As in longitudinal sections the region within the cylinder appears of uniform density the 'fibrils' of the stellate structure must be, in fact, lamellae. The flagellar matrix between the cylinder and the flagellum membrane is of comparable density, although no structure is visible there in transverse sections.

Electron dense material occurs just internal to the peripheral fibres at the level of the lower two-thirds of the cylinder, and in some sections a band of electron dense material runs between this and the diaphragm. The density of this band is commonly, but not invariably, less than that of either of the parts it connects however (Fig. 2, Plate II; Fig. 21, Plate IX). The flagellar matrix external to the peripheral fibres at the level of the cylinder is also relatively denser than in the flagellum proper, and some sections show microtubules approaching this region. Microtubules are also rarely visible distal to the transitional region. Immediately proximal to the cylinder the flagellar matrix is conspicuously absent, but a narrow dense band runs from the centre of the cylinder to the matrix of the basal body region (Fig. 21, Plate IX) and in this region the peripheral double fibres have the less dense appearance of the flagellum proper region as none of the matrix surrounding them is dense, as in the cylinder region immediately distally.

The flagellar membrane in the transitional region has a greater integrity than elsewhere, and is not distorted by any of the fixation and embedding processes, as are more distal parts.

Slightly oblique transverse sections at the junction of the transitional region and basal body show that the peripheral double fibres have connections with the outer membrane, but these do not persist at the only very slightly deeper levels at which the peripheral fibres have become triple, and acquired the C sub-fibre (Fig. 17, Plate VIII). The C sub-fibre is about 350 mμ long. All sub-fibres terminate proximally at the same level. At the distal end filaments of 200-250 mμ thickness radiate obliquely from the central, i.e. the B, sub-fibres (Figs 17, 18, Plate VIII). More proximally these filaments are not seen in transverse section. They have not been recognised in any longitudinal section. The A and C sub-fibres of adjacent triplets are connected by a fine electron-dense strand (Fig. 19, Plate VIII). Some sections of the basal body show an elaborate cartwheel structure (Fig. 20, Plate VIII), but the only details which could be made out with certainty were the thin filaments running from the A sub-fibril, where they arise on a short (~ 3.8 mμ) dense arm which is placed to make an acute angle with the centre of the basal body, to a circular fibril of diameter 25 mμ. In text-figure 3 transverse sections of the flagellum and basal body are related to the longitudinal section.

The basal bodies of the flagella are in one plane and diverge at an angle of about 60°. Additional isolated basal bodies are frequently encountered (Fig. 2, Plate II; Fig. 20, Plate VIII; Fig. 28, Plate X) adjacent to the basal bodies which are a constituent part of the flagellum of the cell. The longitudinal axes of the adjacent basal bodies are mutually perpendicular in separate planes, but the site of the additional basal body is not constant relative to the normal flagellar bases and may be on any side except presumably between them,



Text-fig. 3. Schematic drawings of the flagellum. a. Longitudinal section of basal body and proximal part of flagellum. b-g. Transverse sections at levels indicated in a: b, flagellum proper; c, within 'toothed' passage through colonial envelope; d, transitional region through cylinder showing 9-pointed 'star'; e, transitional region below cylinder; f, distal part of basal body; g, proximal part of basal body showing 'cartwheel'.

as a system of fibres runs between the two basal bodies proximally, linking them (Fig. 31, Plate XI). These fibres are not very electron dense.

Associated with the flagella is a root system of microtubules, striated with a periodicity of 13.5 mμ (Fig. 24, Plate X) which in the apical region occur in compact groups (Fig. 29, Plate X) or in aligned groups a little removed from that region (Fig. 86, Plate XXIV), but which separate and run singly in parts distant from the flagellar bases. The root microtubules run just beneath the cell membrane. Where they are in the aligned group the separation of the individual microtubules is 32-34 mμ. The individual microtubules measure 25 mμ in diameter. This system has been observed only in sectioned material during this study. It might have been hoped that it could be found when observing dried specimens in which, by a spontaneous dismembering of the cells, the whole flagellar apparatus of the flagella and the associated roots had been torn from the cell intact. Such cases have been recorded by Manton (1964d; Ettl and Manton, 1964) for unicellular algae and their spermatozoids; it is considered that with colonial forms the chance of obtaining them is much reduced as the positioning would have to be particularly favourable if the dried up colonial envelope were not to obscure the parts of interest. Thus all information on the flagellar roots derives from sectioned material.

Close to the basal bodies sections of root are characterised by intense staining reactions, so much so that any detail of structure is difficult to see (Figs 27, 28, Plate X). A dense matrix found in the immediate proximity of the basal bodies further obscures the picture at the points of origin of the roots. The number of roots could not be determined from sectioned material, but there are at least two, for this number can be seen in single sections, and probably more (Fig. 28, Plate X). The roots are apparently attached to the proximal end of the basal bodies or at least run in very close proximity to them (Figs 25, 26, Plate X).

The root microtubules follow a long straight course from the basal body just beneath the cell membrane (Figs 26, 27, Plate X). In a number of cases the band of microtubules pass close to the eyespot or even run between the eyespot and the cell membrane, as shown in Fig. 86, Plate XXIV. This particular figure was taken from a series of five close sections. The grouping of the microtubules, 1 + 4, changed to a grouping of 1 + 3 where a gap in the series of 4 or 5 sections occurred. Four of the five sections of the group were serial and showed the 1 + 3 grouping, and in these the section of the microtubule slightly removed from the group of 3 gradually became oblique in successive sections. It may be inferred that the microtubules at the edge of the group have sharp bends, in that they disappear from the sections over a short interval. In another example transverse sections of two groups of microtubules, one of 4 and another of 3 a little removed from the first group, appeared close to an eyespot profile. It is not clear whether these represented a single group or whether separate roots were running together. Transverse sections of cells at deeper levels far removed from the flagella bases show that ultimately the root microtubules become widely separate and run singly (Fig. 54, Plate XVII).

The cytoplasmic microtubule system is very extensive. The individual microtubules are slightly smaller than the root microtubules and measure 20.0 mμ in diameter. They stain much less intensely than the root microtubules, have thinner walls and are not striated. The cytoplasmic microtubules radiate from both the flagellar bases and the two systems overlap so that microtubules from one flagellum base can be seen crossing those from the other (Figs 27, 28, Plate X). The cytoplasmic microtubules do not run in groups, as do the root microtubules, and they do not branch or anastemose. The exact region of origin of the cytoplasmic microtubules is difficult to make out, but it appears to be towards the distal rather than towards the proximal region of the basal body.

PLATE VII

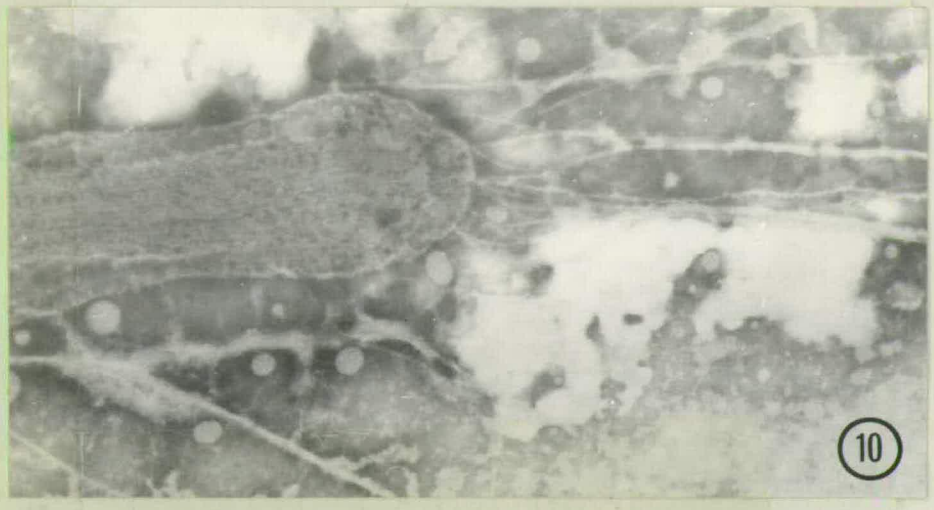
Negatively stained flagella of cells fixed in unbuffered osmium tetroxide.

Fig. 10. Tip of a flagellum shewing flagella hairs. x 40,000 (x 16,000).

Fig. 11. Detail of a single flagella hair shewing structure comprising a central filament around which another filament is wound in a tight spiral. x 120,000 (x 30,000).

Fig. 12. Intact flagella with axoneme fibres undisturbed. x 40,000 (x 20,000).

Fig. 13. Ruptured flagellum with separated axoneme fibres. x 40,000 (x 16,000).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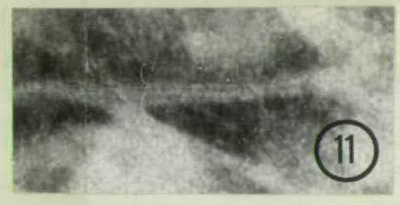
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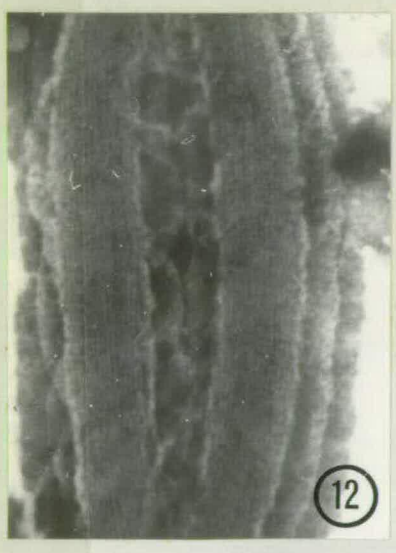
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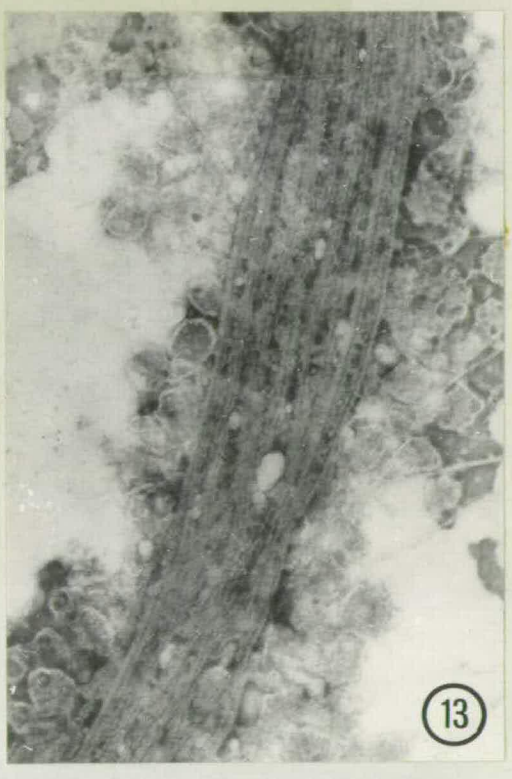
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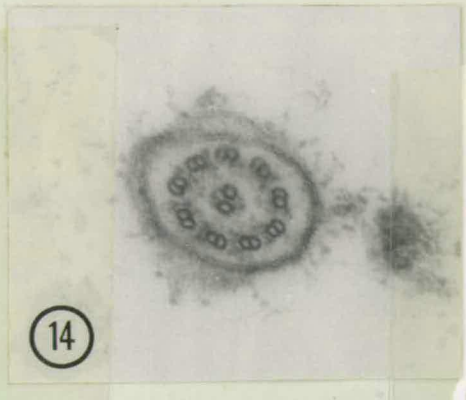
PLATE VIII

Figs. 14-19. Series of transverse sections at successively deeper levels through a flagellum. Fig. 14 is through the flagellum proper; fig. 15 sections the flagellum in its passage through the colonial envelope, and shews the tooth-like projections into the passage; fig. 16 shews the cylinder of the transitional region which is at the lower end of the toothed passage; figs. 17 and 18 differ only slightly in depth and section obliquely the lower limit of the toothed flagellar passage. In fig. 17 the teeth are still visible but in fig. 18 they are not whereas the double outer fibres of the axoneme of higher levels have become triple. Fig. 19 shews a deep section of the basal body.

Fig. 20. Longitudinal section of one basal body with an adjacent additional basal body in transverse section shewing 'cartwheel'.

All figs. x 80,000 (Initial magnifications x 40,000, x 30,000, x 16,000, x 16,000, x 20,000, x 20,000 and x 16,000 respectively).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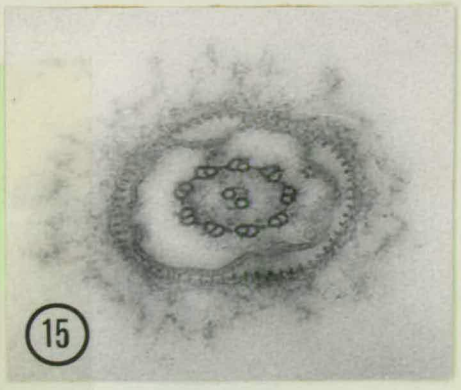
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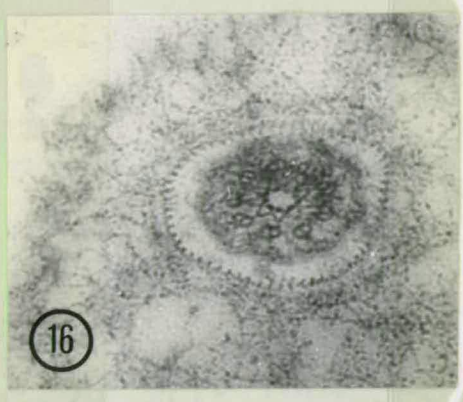
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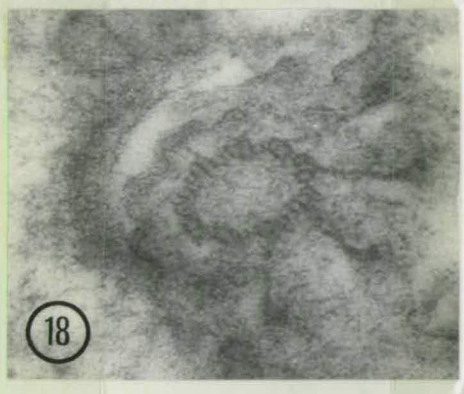
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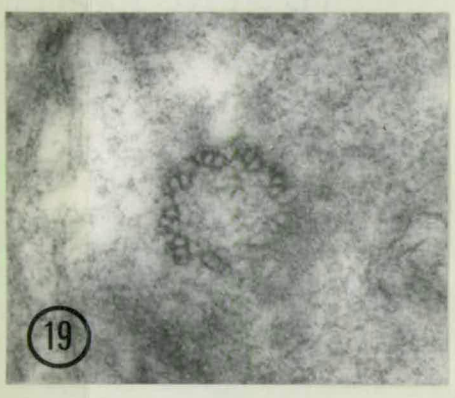
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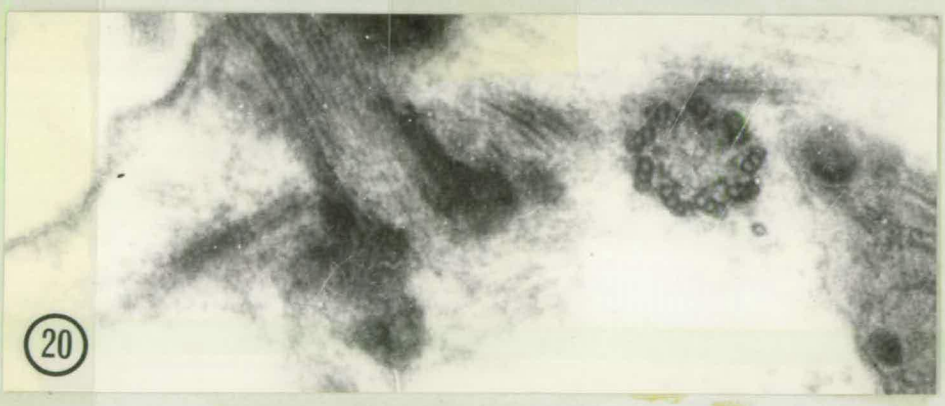
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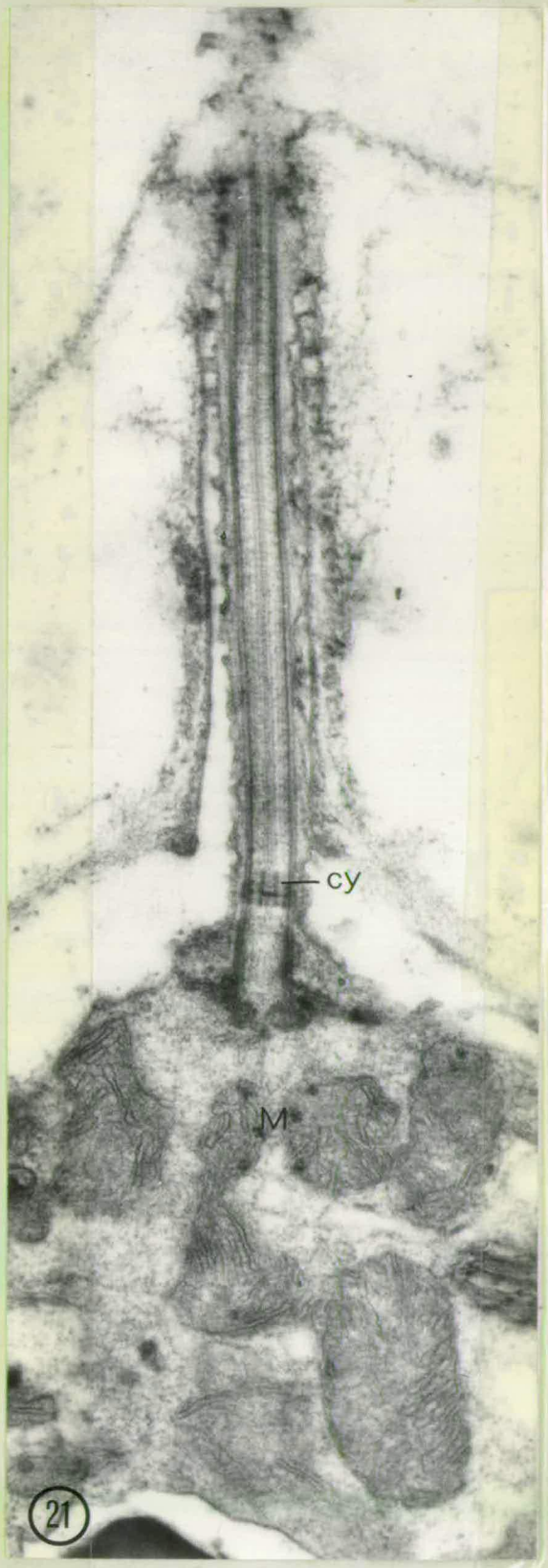
PLATE IX

Fig. 21. Median longitudinal section of flagellum. The central fibres of the axoneme appear in the entire length sectioned, terminating proximally just above the cylindr (cy) of the transitional region. The peripheral fibres continue into the basal body, the point at which they become triple being indicated by the thickening and increased electron density just proximal to the cylinder. Mitochondria (M) are concentrated in the apical region. x 40,000 (x 8,000).

Fig. 22. Grazing longitudinal section of flagellum and flagellar passage through the envelope. The tooth-like projections seen in transverse section are seen to be ridges extending the length of the passage. Cytoplasmic microtubules (cm) approach the basal body region. x 40,000 (x 16,000).

Fig. 23. Proximal end of flagellum and basal body region of cell fixed in glutaraldehyde. A spirally wound fibre (arrows) may be seen around the peripheral axoneme fibres. x 100,000 (x 40,000).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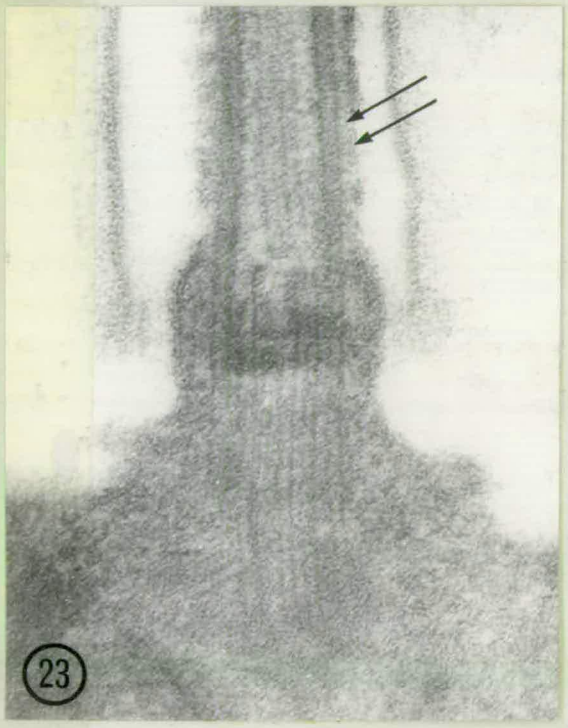
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PLATE X

Fig. 24. Grazing section of flagellum root shewing striations. x 40,000 (x20,000).

Fig. 25. Slightly oblique longitudinal section of flagellum and basal body shewing part of a root (R) and cytoplasmic microtubules (cm). Grooved flagellar passage (fp). Cylinder (cy). Contractile vacuole (CV). Mitochondria (M). Colonial envelope (EC). x 40,000 (x 16,000).

Fig. 26. Microtubules of flagellum root in longitudinal section. Mitochondrion (M). Chloroplast (C). x 30,000 (x 16,000).

Fig. 27. Oblique section of apical region with various profiles of cytoplasmic microtubules (cm) and flagellar roots (R) converging on the basal bodies. Mitochondria (M). x 30,000 (x 16,000).

Fig. 28. Section of apical region in which the double system of cytoplasmic microtubules (cm) related to the basal bodies are seen to cross. Root (R). x 30,000 (x 16,000).

Fig. 29. Flagellum root fibres in transverse section (arrow). x 40,000 (x 16,000).

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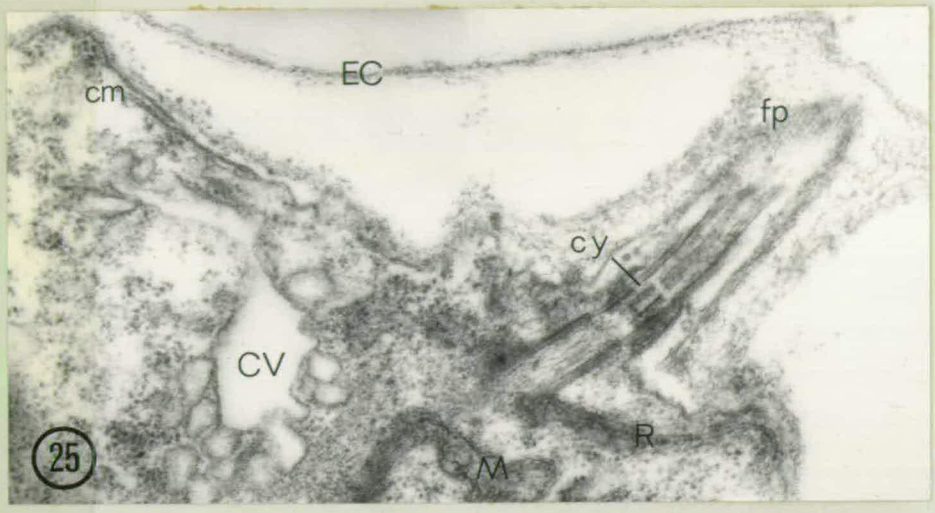
EC
cm
fp
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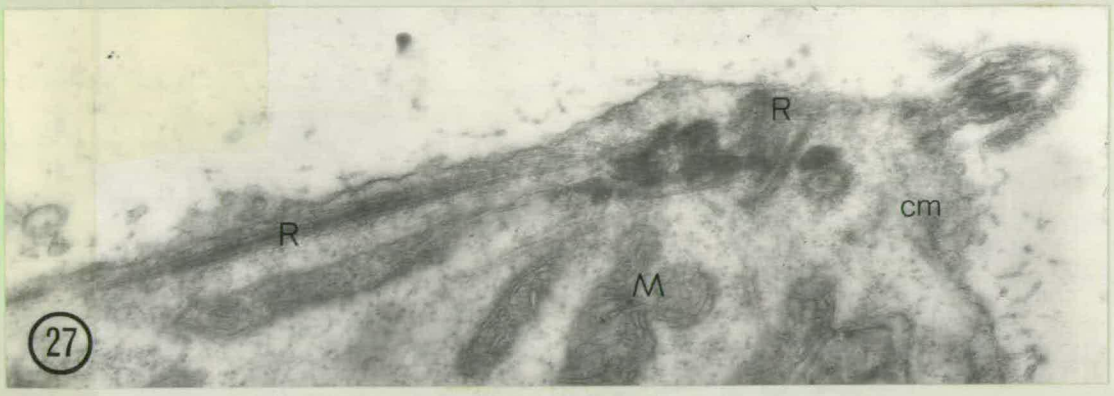
CV
R
M
25

26
M
C


R
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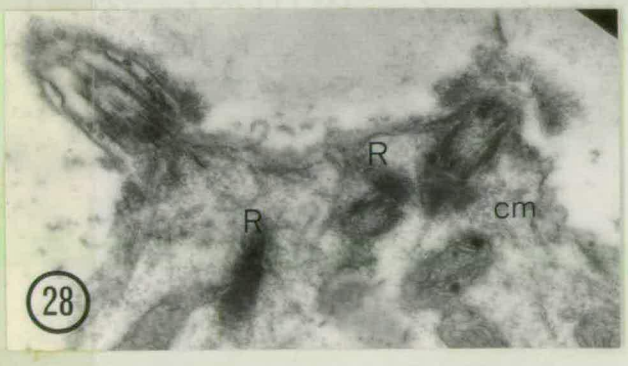
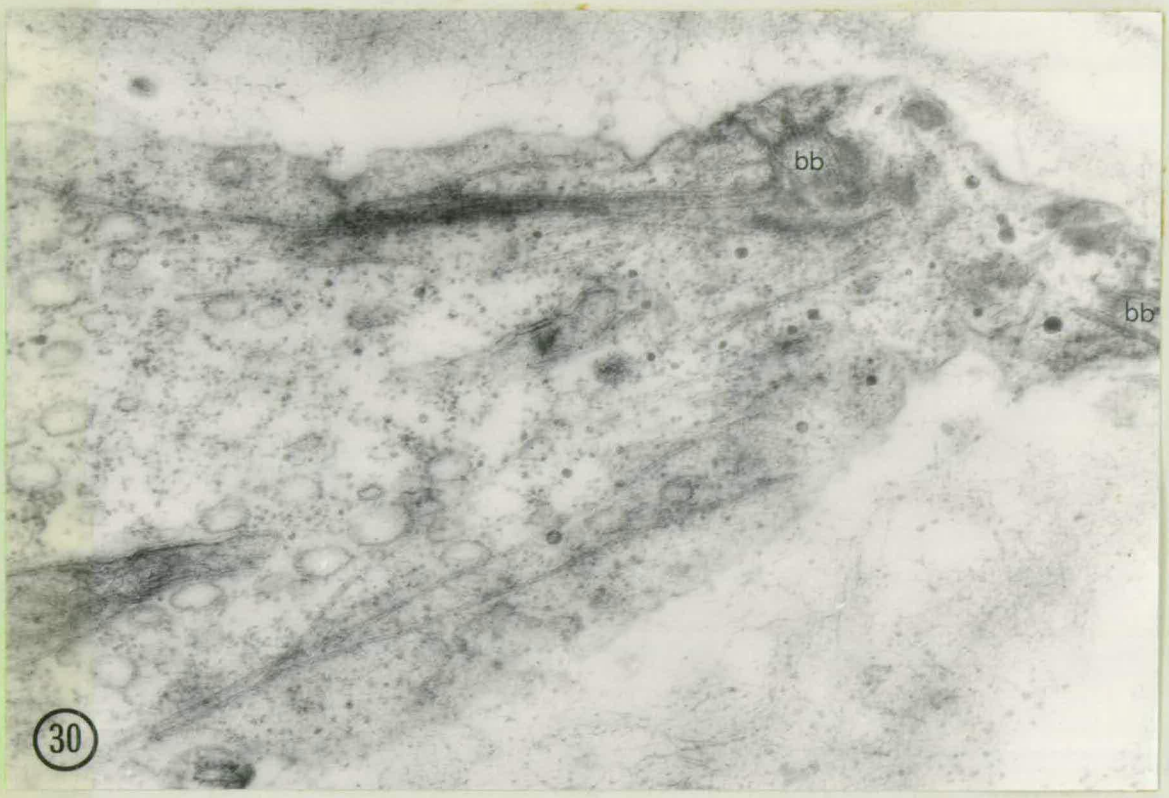

R
R
cm
28

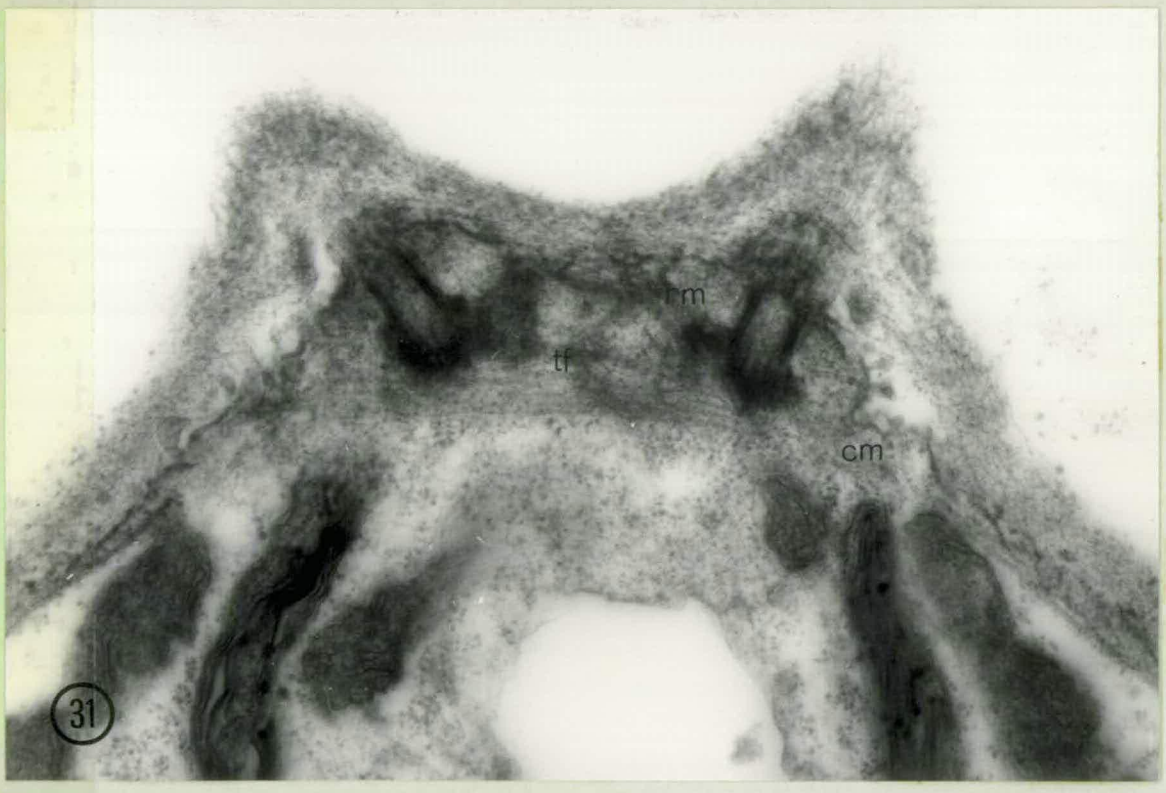
29

PLATE XI

Fig. 30. Grazing section of the cell at the level of the basal bodies (b.b.) shewing extensive system of cytoplasmic microtubules.
x 40,000 (x 13,000).

Fig. 31. Section including the transverse fibres (tf) between the basal bodies, cytoplasmic microtubules (cm) and root microtubules (rm).
40,000 (x 16,000).


bb
bb
30


rm
tf
cm
31

The microtubules are most conspicuous running immediately beneath the cell membrane. They tend not to be as straight or of such uniform diameter as the root microtubules, but nevertheless sections which include very long microtubule profiles are found (Fig. 30, Plate XI). The cytoplasmic microtubules and root microtubules are readily distinguished in cross-section by the difference in staining intensity (Fig. 86, Plate XXIV), and both are easily recognised even in oblique sections. Cytoplasmic microtubules are also located deep within the cell but are much less frequently encountered. They are readily visible if the section includes a sizeable part of their length, but are not easy to pick out when in transverse section.

Nucleus

The interphase nucleus of the mature cell occupies the depression in the cup-shaped chloroplast. In shape it is ovoid to sub-spherical. The nucleus is bounded by an envelope consisting of a double membrane with an intervening perinuclear space (Fig. 2, Plate II; Fig. 32, Plate XII). Many but not all sections show the outer of the two membranes to be covered with granules of 10-20 mμ which are presumably ribosomes (Fig. 32, Plate XII; Fig. 82, Plate XXIII). The nuclear envelope is pierced by many pores and at these the inner and outer membranes are continuous with one another. The pores are circular when seen in surface view and measure $\sim$ 50 mμ in diameter (Fig. 36, Plate XIII). The characteristic appearance of an electron dense staining reaction at the pores as seen in cross sections of the envelope (Fig. 35, Plate XIII) is ascribable to the fact that the thickness of a fine section is comparable to that of the diameter of the pores, and it is thus likely that most of any one pore sectioned will be within the thickness of the section. The grazing section shown in Fig. 36 shows that the pores are numerous and it can be seen that only very few sections could contain small parts of a pore rather than most of it.

Some authors have claimed to see diaphragms across the nuclear pores (e.g. Sager and Palade, 1957 in Chlamydomonas); this explanation is not thought to be satisfactory in accounting for the dense appearance. However, certain of the pores do show a central dense granule when seen in grazing section (Fig. 36, Plate XIII). A similar condition has been described in the nuclear envelope of Bradysia mycorum (Jacob and Jurand, 1963).

Fixation using potassium permanganate did not give the good preservation usually associated with this fixative.

A component of the endoplasmic reticulum was observed in connection with the nuclear envelope and this had a special relationship with the Golgi apparatus. It arises from the nuclear membrane to encompass completely the Golgi apparatus and rejoin the nuclear membrane on the other side (Fig. 37, Plate XIII). The term amplexus has been suggested for this endoplasmic reticulum (Lang, 1963a). No amplexi were seen in recently divided cells, but it cannot be conclusively stated that they do not occur in such cells. The amplexi are inevitably changed on cell division as the nuclear membrane breaks down.

Where the orientation of the Golgi bodies relative to the nucleus could be made out, it was most commonly found that the Golgi body cisternae with the largest diameters faced towards the nucleus and those with the smallest diameters were on the side distal from the nucleus and had the amplexus in close proximity.

With osmium tetroxide fixation the endoplasmic reticulum of the amplexi has a vesiculate appearance. There are wide spaces between the vesicles, so there is a free connection between the general cytoplasm and that within the amplexi.

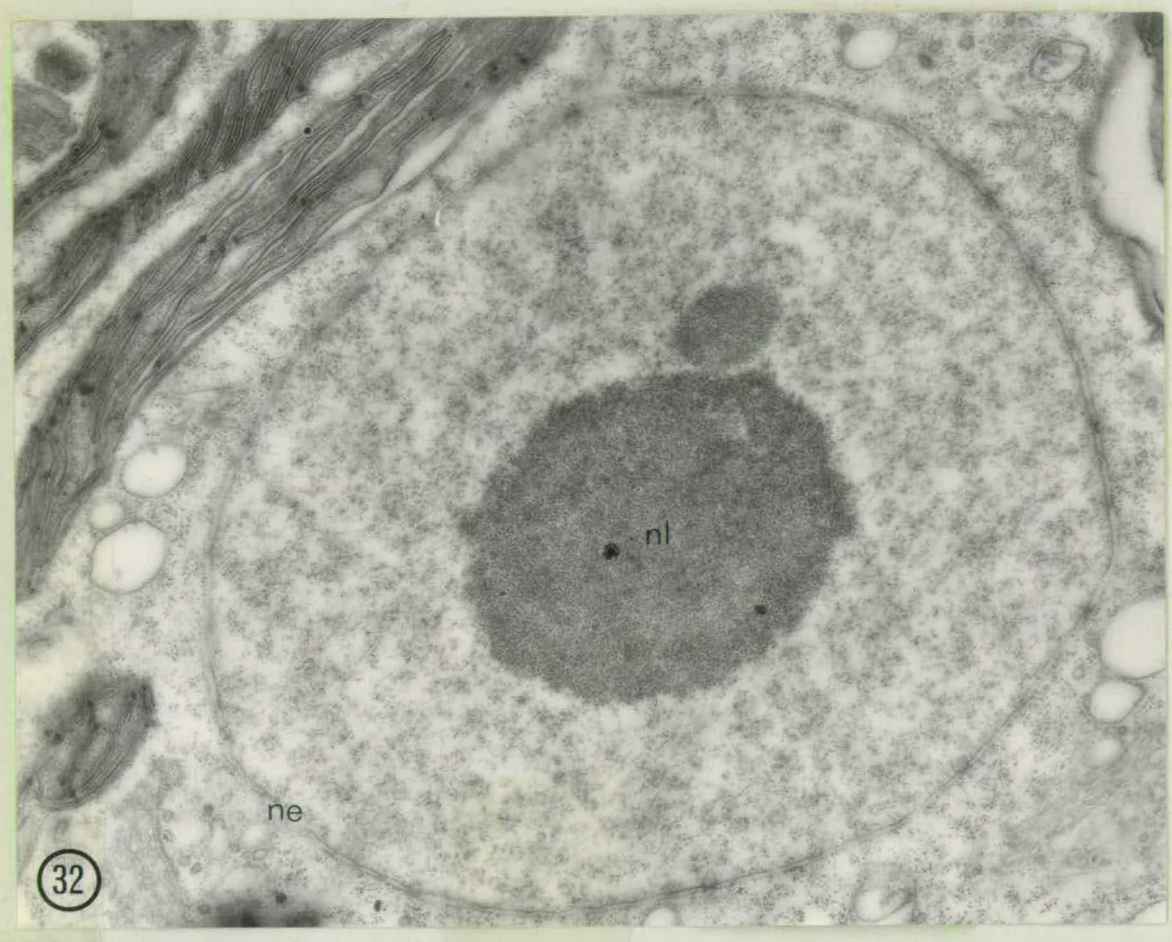
The nuclei of mature and young cells differ in the appearance of their nucleoplasm. In both cases this consists of clustered granules, but in young cells the density is greater relative to the cytoplasm, whilst in mature cells the reverse is true (Figs 38, 39, Plate XIII).

PLATE XII

Fig. 32. Nucleus with two nucleolar profiles (nl). The outer membrane of nuclear envelope (ne) is covered with ribosomal particles. x 20,000 (x, 10,000)

Fig. 33. Detail of the nucleolus shewing particles through which run fine fibrils (arrows). x 80,000 (x 40,000).

Fig. 34. Section of nucleolus with light core. The nuclear envelope has pores which appear dark in profile. x 20,000 (x 10,000).



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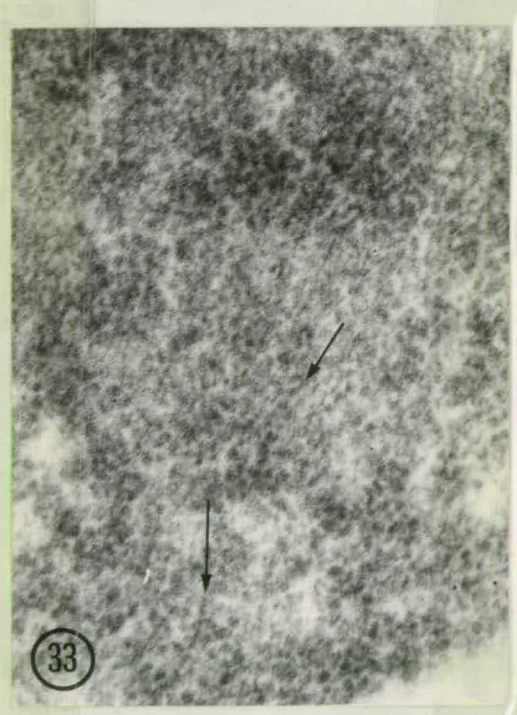

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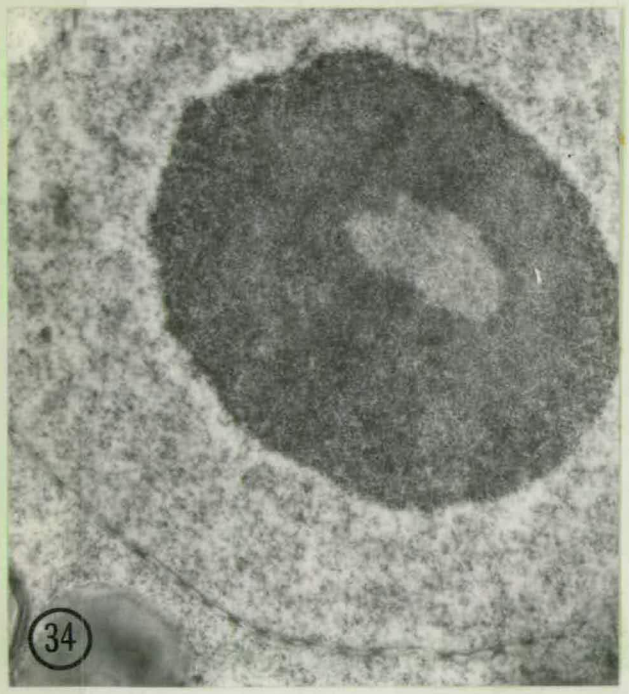

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PLATE XIII

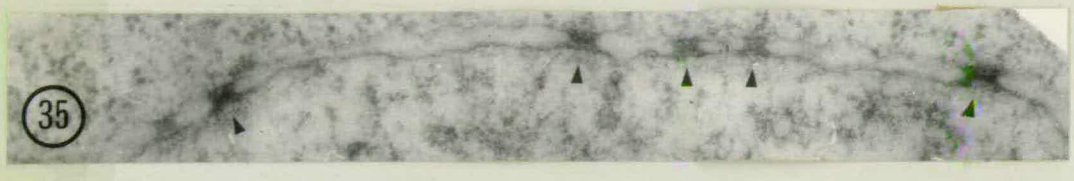
Fig. 35. Nuclear envelope in cross section shewing nuclear pores. (arrow heads). x 50,000 (x 13,000).

Fig. 36. Grazing section of nucleus shewing nuclear pores in surface view. A dense granule appears at the centre of some pores (arrows). x 40,000 (x 20,000).

Fig. 37. Nucleus with two adjacent Golgi bodies. Endoplasmic reticulum of the amplexus (AER) surrounds the Golgi bodies originating from the nuclear envelope. Vesiculate endoplasmic reticulum (VER). x 44,000 (x 30,000).

Fig. 38. Nucleus from a cell of a young colony still within the mother-cell space. The nucleoplasm is denser than the cytoplasm, c.f. fig. 39. x 20,000 (x 6,660).

Fig. 39. Nucleus from a mature cell shewing the lesser density of the nucleoplasm relative to the cytoplasm. x 20,000 (x 10,000).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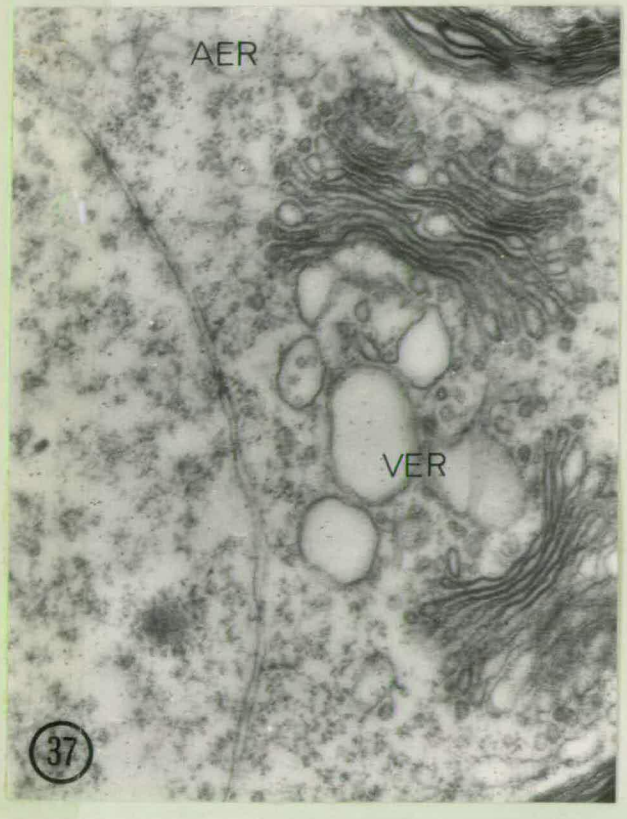

AER
VER
37

39

The most conspicuous differentiation within the nucleus is the centrally placed nucleolus. The dimensions vary between 1.75-2.5μ by 2.25-3.5μ. The nucleolus consists of granules of ~ 12 mμ in diameter which are probably ribosomal (Fig. 33, Plate XII). The intensity of staining varies greatly in different regions. From Fig. 34,Plate XII it might appear that the nucleolus has a light core, but other sections show the light part at the edge. Frequently two nucleolar profiles appear in one section (Fig. 32, Plate XII). It is not possible to say from this material whether this represents a single nucleolus with a finger-like extension or, as is considered more likely, two separate nucleoli. High resolution micrographs show that the nucleolus is penetrated by many fine fibrils of width ~ 5 mμ (Fig. 33, Plate XII).

Running towards the nuclear region of dividing cells microtubules are seen more frequently than is general deep in the cytoplasm. These may represent spindle fibres (Fig. 9, Plate VI).

Cytoplasm

The ground cytoplasm appears uniformly granular and comprises largely what are presumed to be ribosomal particles of 17.5-20 mμ diameter (Frey-Wyssling and Mühlethaler, 1965). The appearance is very much dependent upon the fixation employed. With osmium fixation the granules appear clustered (Fig. 40, Plate XIV); after glutaraldehyde fixation they are also clustered but appear relatively much denser (Fig. 111, Plate XXIX). In addition to the Golgi apparatus, mitochondria, contractile vacuoles and chloroplast, all of which will be discussed in separate sections later, a number of other components are usually to be seen. Short lengths of tubular rough endoplasmic reticulum (ER) appear in many sections, especially in young cells (Fig. 3, Plate III; Fig. 9, Plate VI). The ribosomes of rough ER are ~~very~~ evenly distributed. Vesiculate ER is found particularly in the region of the Golgi apparatus and is of the smooth type (Figs 40, 41,

Plate XIV). The other ER principally associated with the Golgi apparatus, that of the amplexi referred to above, is rough ER.

Two other membrane systems are found. One seems to represent an invagination of the cell membrane and comprises an irregularly and sparsely branching system of smooth tubular membranes. The other system was not observed to have its origin from the cell membrane, but its site was always at the surface suggesting that this might be the case. This system consists of a dense agglomeration of vesicles (and tubules ?) having an amorphous content (Fig. 1, Plate I; Fig. 51, Plate XVI).

In addition to the vesicles conspicuously of the ER system are others which are larger and either lack contents or contain a condensation of amorphous material (Fig. 2, Plate II; Fig. 3, Plate III) or membranes (Fig. 4, Plate IV). The latter possibly represent the membranes of the lipid droplets of the cytoplasm, referred to in the next paragraph, after the lipid has been utilised.

The cytoplasm of mature cells, and especially cells of old cultures, contains lipid droplets ~~of~~ 0.5-1.5μ in diameter. In cells from old cultures they can be present in such numbers that the other cell contents are all but obliterated. Cells of young colonies in which divisions have just been completed do not possess these droplets (Fig. 2, Plate II) but they are found in dividing cells (Fig. 4, Plate IV; Fig. 7, Plate V). They have a uniform light staining reaction when fixed in osmium tetroxide and stain with the combined uranyl acetate/potassium permanganate stain, and are limited by a membrane with more intense staining reactions, as may be seen in favourable sections, and inferred from the fact that droplets in contact do not coalesce (Fig. 4, Plate IV). The identification as lipid depends upon the following observations with the light microscope. The droplets are metachromatic when treated with toluidine blue (Fig. 121, Plate XXXI) and give a positive reaction using the PAAS test

(Fig. 124, Plate XXXI). They stain intensely with Sudan black B. Sections prepared for electron microscopy show this staining very strikingly (Fig. 114, Plate XXIX). The explanation for this reaction with lipids which have been fixed using osmium tetroxide has been given in the Materials and Methods section (p.50).

Golgi apparatus

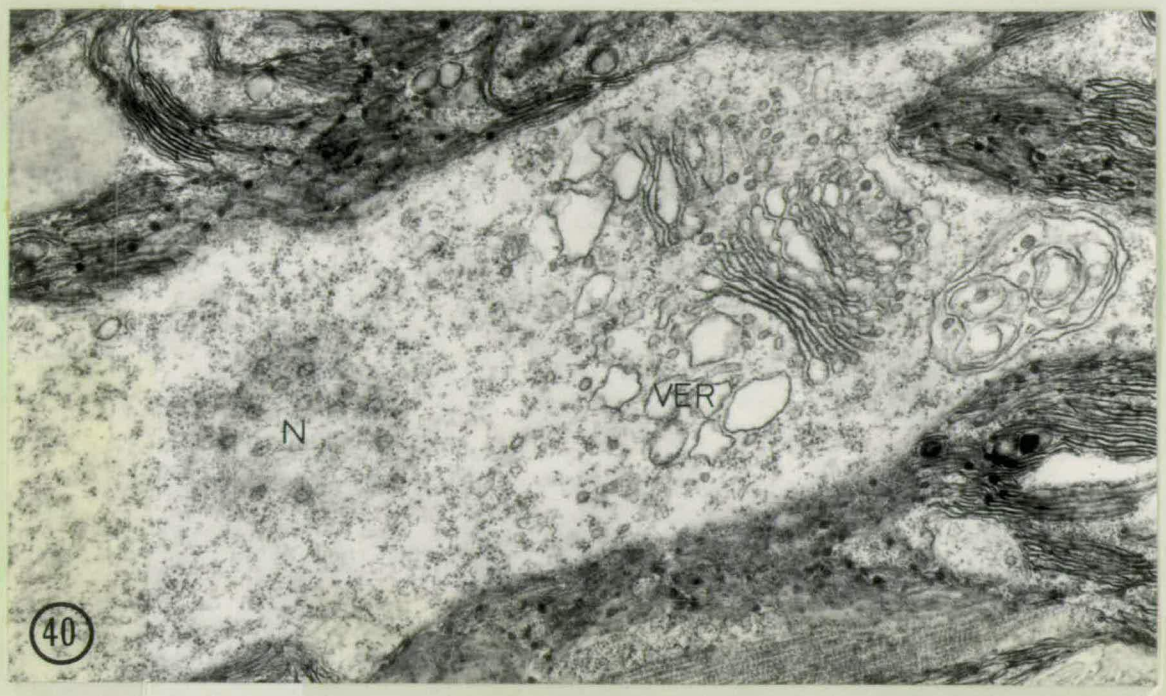
The Golgi bodies of the mature cell show the typical arrangement associated with this organelle (Figs 40, 41, Plate XIV). Each one consists of a trunkated conical stack of up to 15 flattened saucer-shaped cisternae, the two membranes of which are commonly apposed and appear as a single electron dense line of about 14 mμ. The membranes do not come into contact at the extremities; in terms of three dimensions this means that each cisterna has an expanded rim. The curvature of the cisternae is such that the widest end of the cone of cisternae presents a concave surface, and this is most frequently the surface facing the nucleus, with the encompassing amplexus being in proximity to the narrow end of the cone. The overall dimensions of the Golgi bodies in mature cells are 0.6-0.9μ by 0.8-1.3μ, but the diameter of the cisternae at the narrow end of the stack may measure as little as half that of those at the wide end. In close proximity to the stacks a vesiculate component of the Golgi body is found. The vesicles measure ~ 50 mμ in diameter and are apparently derived from the edge of the cisternae.

The appearance of Golgi bodies in cells which are in division and in cells which are the immediate products of division differs from that of the Golgi bodies in mature cells. The gross shape is more nearly cylindrical with overall dimensions of about 0.6μ wide by 0.8μ deep. In dividing cells the staining reaction is not intense and the cisternae are expanded so that the pairs of membranes are not in contact (Fig. 7, Plate V; Fig. 9, Plate VI). These two things combine to make the Golgi bodies rather inconspicuous.

PLATE XIV

Fig. 40. Three Golgi bodies situated near the nucleus (N) which appears in grazing section. The Golgi bodies are sectioned in two planes. Vesiculate endoplasmic reticulum (VER). x 32,000 (x 13,000).

Fig. 41. Four Golgi bodies, one sectioned in the plane of the cysternae, the remainder sectioned perpendicular to them. Rough endoplasmic reticulum of the amplexus (AER) is seen encompassing the latter three Golgi bodies. x 32,000 (x 16,000).



40

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The Golgi bodies of young cells, on the other hand, are very conspicuous and are densely stained. The membranes appear to be covered with some amorphous material which gives them a woolly outline (Fig. 2, Plate II; Fig. 3, Plate III). This may be taken as a representation of the increased activity of the Golgi apparatus of growing cells.

Mitochondria

The mature cell of Eudorina possesses an extensive mitochondrial system. Sections show the mitochondria to be distributed at the periphery of the cell between the cell membrane and the chloroplast. There is also a dense agglomeration of mitochondria in the apical region in which the flagellar bases are situated (Fig. 1, Plate I). Sections from young cells show few mitochondria, and the apical concentration of mitochondria is conspicuously absent (Fig. 2, Plate II). In dividing cells the mitochondria are few in number but are, however, found at deeper levels in the cell, as illustrated in Fig. 9 (Plate VI), where they may be seen between the nucleus and the chloroplast. In older cells the occasional profile is encountered in one of the cavernous spaces in the divided chloroplast.

Mitochondria are commonly described as being short rod-shaped organelles of dimensions 0.5-2.0μ. Small profiles are found in this material (Fig. 44, Plate XV) but it is considered that these are likely to be transverse sections of much elongated mitochondria. A remarkable alignment of mitochondria can be seen in grazing sections, and it is noteworthy that these are parallel to the microtubules also appearing in the sections (Fig. 46, Plate XV). It can be stated as a general rule that where microtubules appear in transverse section, the mitochondria will there present their smallest profile; the profiles enlarge with increased obliquity of section. This rule does not apply with respect to the apical region where special conditions obtain. In favourable sections very long profiles of mitochondria may be seen, as in Fig. 42, Plate XV which shows

a 7μ length.

The apical region has a great concentration of mitochondria and these are of a very elaborate structure. It appears that the mitochondria form a reticulum in three dimensions and of considerable complexity. Single sections can show much of this (Fig. 43, Plate XV). The longitudinal axes of the parts of this reticulum tend to be directed towards the basal bodies (Fig. 27, Plate X) but many cross-linking parts occur. This accumulation of mitochondria is to be expected at a region of high oxidative activity. Another, though less dense concentration, occurs around the contractile vacuole.

The profiles of the mitochondria are smooth, and the limiting membrane double. The cristae which are infoldings of the inner membrane do not interdigitate but tend to appear aligned with the limiting membrane (Fig. 45, Plate XV). In length the cristae profiles are rather larger than the width of the mitochondria which measure 0.5μ. Surface views of the cristae were difficult to make out but it seems likely that they are basically disc-shaped to oval, being attached at a single point on the rim.

Using osmium tetroxide in carbon tetrachloride as a fixative, the outer mitochondrial membrane presents a much less clear profile than the inner membrane. It is frequently reduced to an indistinct fuzz. In this connection it will be noted that the outer membrane of the chloroplast double envelope is also only slightly electron dense (Figs 52-55, Plate XVII). The inner unit membrane seen at high resolution consists of two dark lines with a light space between them and has an overall width of $\sim$ 8 mμ (Fig. 45, Plate XV). The space between the unit membranes forming the cristae varies between 5 and 15 mμ with the space at the end distal to the point of origin at the inner membrane even more expanded.

PLATE XV

Fig. 42. Longitudinal section of a 7μ length of mitochondrion. x 30,000 (x 10,000).

Fig. 43. Section of mitochondrion from the apical region. One of the basal bodies is visible near the surface of the cell at the extreme right of the figure. Sections serial with the one presented shew that the complicated profile forms part of a mitochondrial reticulum. x 30,000 (x 10,000).

Fig. 44. Mitochondria seen in transverse section. x 30,000 (x 10,000).

Fig. 45. Detail of transverse sections of mitochondria shewing the origin of the cristae from the inner mitochondrial membrane. Cell membrane (CM). x 100,000 (x 40,000).

Fig. 46. Oblique longitudinal section of mitochondria on the side distal to the apical region. The profiles of microtubules (arrows) are parallel to the mitochondria. x 30,000 (x 20,000).

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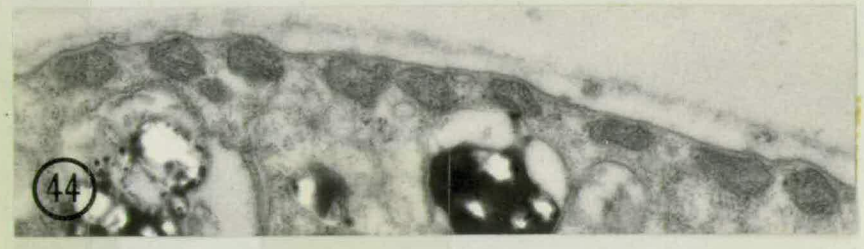

44

CM
45

46

Contractile vacuoles

The typical chlamydomonad cell such as is found in the Volvocales has two contractile vacuoles which may be recognised in living material by their regular formation and discharge. In material examined by electron microscopy these vacuoles are identifiable at various stages of their development with moderate certainty. The contractile vacuole which is limited by a unit membrane forms by the fusion of small vacuoles with the collapsed vacuole, with the subsequent addition of the contents of further small vacuoles to the larger vacuole so formed (Figs 47, 48, Plate XVI). In the early stages when the vacuole is yet small, the profile in section is very irregular with long tubular canals extending from the collapsed vacuole up to 700 mμ, but in late stages the enlarged vacuole has a smooth profile (Fig. 50, Plate XVI). At this stage the contractile vacuole is in very close proximity with the cell membrane. No section was encountered at the time of discharge of the vacuole so the configuration of the cell membrane relative to the membrane of the contractile vacuole is unknown. The largest vacuoles seen in micrographs measured 2.5μ.

Surrounding the contractile vacuole in early stages are vesicles which may be identified with Porter's alveolate vesicles (see Leedale, Meeuse and Pringsheim, 1965). They measure 40-100 mμ in diameter, the majority being over 80 mμ. In favourable sections these vesicles appear to have a network of hairs (Figs 47, 49, Plate XVI). Leedale (loc.cit.) considers that such vesicles which he found in the region of the contractile vacuole of Euglena and which Manton (1964a) has described in Stigeoclonium are concerned in osmoregulation fusing with the contractile vacuole rather than being responsible for recovery of material from it by pinocytosis. Manton observed that the vesicles vanished when the contractile vacuole ceased to function and thus they also must be concerned with the water relations of the naked cell. In this material also the alveolate

PLATE XVI

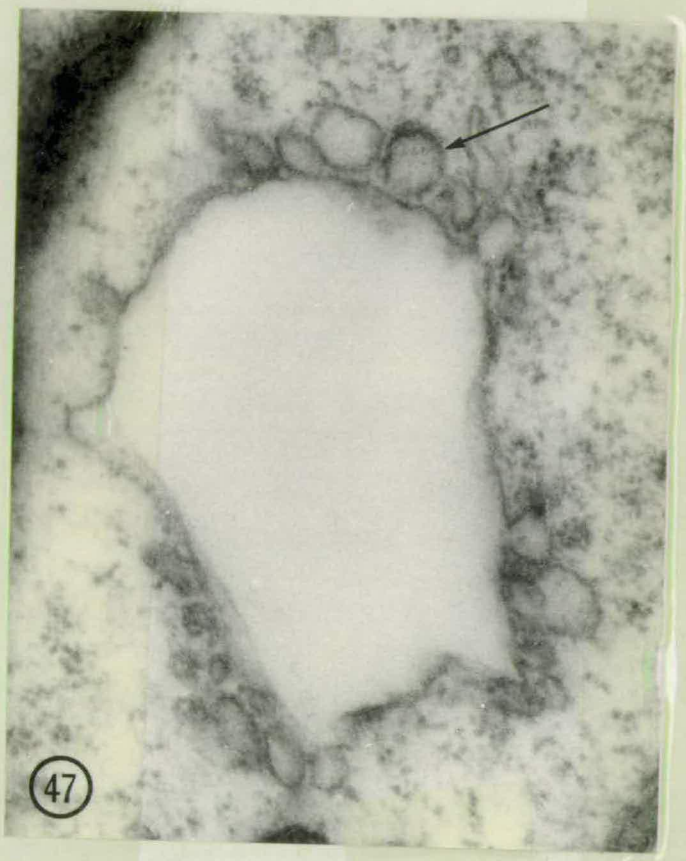
Fig. 47. Cross section of the contractile vacuole at the stage of enlargement. Alveolate vesicles surround the main vacuole and join it. (arrow) x 60,000 (x 16,000).

Fig. 48. Section of contractile vacuole normal to the cell surface. Channels extending into the cytoplasm join the vacuole and alveolate vesicles surround it. x 30,000 (x 16,000).

Fig. 49. Detail of alveolate vesicles shewing contained network of hairs. (arrow). x 60,000 (x 16,000).

Fig. 50. Fully expanded contractile vacuole. x 30000 (x 20,000).

Fig. 51. Vesiculate system at cell surface. x 30,000 (x 16,000).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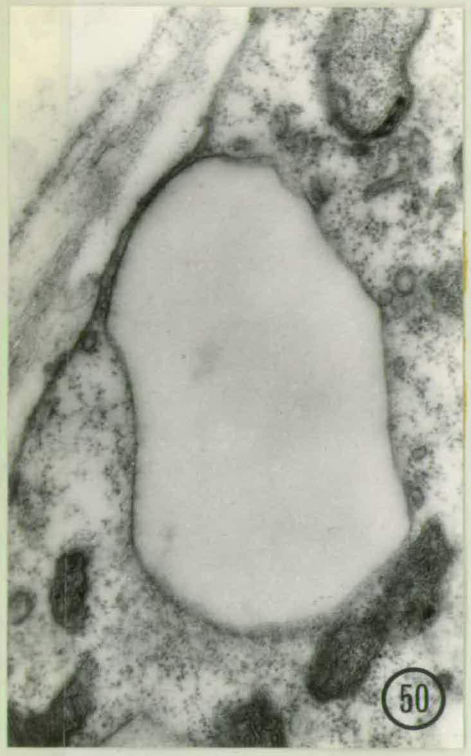
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51

vesicles may be seen fusing with the contractile vacuole (Fig. 47, Plate XVI).

The vesiculate system with the amorphous content mentioned on p. 79 is reminiscent of the so-called nephridial tubules surrounding the radial canals in the contractile vacuole of Paramecium (Pitelka, 1963), but as no special study was made of this organelle it is possible only to note this resemblance which conceivably represents one of the first stages in the formation of the contractile vacuole.

Chloroplasts

From light microscope studies it has been generally agreed that the chlamydomonad cell possesses a single chloroplast, the gross form of which is cup-shaped. It is impossible to confirm or deny this using the electron microscope without a most elaborate serial sectioning study. Assuming it to be so, it can be stated that the chloroplast has a very irregular shape, for many isolated profiles occur in sections of mature cells (Fig. 1, Plate I). In the cells of young colonies the chloroplast tends to be more compact, although even here some lobulation is evident (Fig. 2, Plate II; Fig. 8, Plate V). During cell division the chloroplast constricts so that more or less equal parts go to the daughter cells (Fig. 4, Plate IV).

The chloroplast, which is surrounded by a double envelope, comprises a densely granular stroma in which the chloroplast lamellae run. It possesses regions of special differentiation: the pyrenoids, of which there is one in young cells but which appear in increasing numbers in older cells, and the usually single eyespot, which is made up of a variable number of layers of pigment globules, each layer being underlain by a single lamellar unit or thylakoid (Menke, 1962). Globules of similar appearance to those in the layers of the eyespot are also to be found scattered irregularly throughout the chloroplast, singly or in small groups (Fig. 1, Plate I; Fig. 3, Plate III). Starch plates are associated with the pyrenoid and isolated starch grains are found throughout

the chloroplast in mature cells especially (Fig. 1, Plate I).

The chloroplast envelope is comprised of two unit membranes and has an overall width of ~ 17.5 mμ. As mentioned earlier the outer of these membranes tends to be indistinct after fixation with the osmium tetroxide in carbon tetrachloride fixative. This is not invariably so (Fig. 80, Plate XXII), and in such cases the dimensions of the membranes and the intervening space can be measured. The membranes measure ~ 7.5 mμ and the space ~ 5.0 mμ. The envelope surrounds the chloroplast entirely without interruption. The outer membrane of the envelope is seen quite frequently to be thrown into folds very locally (Figs 52, 55, Plate XVII; Figs 56, 57, 59, Plate XVIII). As the profiles are of continuous double lines with the characteristic weak staining reaction of the outer membrane, it seems that the extension has a flattened rather than a tubular form. Fig. 59 shows a simple extension, but the more characteristic profile is much more elaborate with the extension so folded about itself to appear rather 'onion-like'.

A more conspicuous differentiation of the chloroplast envelope is the development of long extensions in which both membranes participate. In many of these extensions there is very little or no stroma and the two inner membranes come into close proximity or into contact. The figures of Plate XVII show this feature. From the fact that long sections appear (1.8μ in Fig. 52, Plate XVII) it is apparent that the extensions are sheet-like rather than tubular, although tubular cross-sections do occur. Not only do flat sheets occur, but also ones apparently bowl-shaped which may be seen in two mutually perpendicular planes of sectioning in Figs 54 and 55 (Plate XVII). The extremities of the extensions are sometimes dilated and possess a ribosomal content (Fig. 53, Plate XVII) or a small group of chloroplast lamellae (Fig. 79, Plate XXII). A few thylakoids may penetrate a short distance at the proximal end of the extension (Fig. 54, Plate XVII). In rare cases the two double membranes were seen to be separated

by a large space, but still stroma was lacking from between them. It is thought unlikely that this was a fixation artefact as it has been observed in circular profiles in which the circumferences of the two profiles were markedly different and in general no obvious disruption has been observed using this fixation. It is tentatively suggested that these profiles represent a stage in the elaboration of the chloroplast of the young cell to give the extensively lobulated condition of the mature cell, for they are encountered principally in what are judged to be young cells. Rather shorter extensions of similar appearance may be seen to connect major chloroplast lobes in mature cells.

With osmium fixation the stroma is seen to contain ribosomes at a density far higher than in the ground cytoplasm (Fig. 54, Plate XVII), but fixation with glutaraldehyde gives the impression that there is little difference in density between the two, although there is a difference in granule size (Fig. 111, Plate XXIX).

The chloroplast lamellae usually run parallel to the cell surface. The lamellae consist of bands of 2-3 thylakoids which come together at intervals to form stacks of up to 16 thylakoids and which are grana-like. However, stacks of more than six thylakoids are uncommon; the numbers most frequently encountered being 2-4. In the stacks some thylakoids may be continuous with those of the lamellae but others may possess no connection with any other thylakoid in the plane of the section. Stacks persist as such over only short distances.

In the stacks it can be seen that thylakoids may be folded back upon themselves so that the spaces of adjacent thylakoids in a stack are in connection at its edge. This shows most clearly in glutaraldehyde fixed material (Fig. 111, Plate XXIX), but may be seen in favourable osmium fixed material also (Fig. 72, Plate XX). It is possible that, as shown by Heslop-Harrison (1963) for higher plants, the spaces of all thylakoids are in connection. This would mean that

the entire lamellar system is separated from the stroma by a single membrane. In this connection another observation is of relevance. In the vicinity of the pyrenoids there are frequently to be seen profiles in which a light membrane surrounds a series of parallel dark membranes (Fig. 71, Plate XX). Such profiles have been observed in which 2-5 dark lines occur. These represent respectively groups of from three to six thylakoids, the spaces of which are all in connection at one end. Such a profile is illustrated in text-fig. 4f. Another example in which the thylakoid spaces are seen to be in connection which is occasionally encountered shows the dark line which represents the apposed membranes of adjacent thylakoids having small gaps.

With the osmium fixation used there is some distortion of the lamellae and the thylakoid spaces become enlarged. Glutaraldehyde gives a very regular fixation and enables the dimensions to be measured (Fig. 118, Plate XXX). A single thylakoid is 13.5-15 mμ wide and the central space measures ~ 2.5 mμ.

Thylakoids occur singly in the stroma and in such cases they may frequently be seen to be passing from one lamella to another. Leport (1963) observed sinuous double thylakoid profiles in Pandorina morum. Similar single thylakoid profiles were observed in this study, but only in some of the material fixed using glutaraldehyde (Fig. 112, Plate XXIX). Whether this is a regular chloroplast structure under certain conditions or a fixation artefact is not certain.

At the edge of a thylakoid after osmium fixation a small dense granule (15 mμ) representing a thickened rim is seen (Fig. 59, Plate XVIII). In addition much larger diffusely dense granules of up to 60 mμ are often found at the same site. As these obscure any detail at the point where they occur, it is not possible to say whether they represent another form of the small dense granules or whether they represent something different. They occur also on the lamellae which penetrate the pyrenoid (Fig. 63, Plate XVIII). Leedale (1965, in Leedale, Meeuse and Pringsheim) is of the opinion that similar osmiophilic

PLATE XVII

Fig. 52. Empty extensions of the chloroplast envelope seen in various planes of section. The outer membrane of the long profile is thrown into folds at the foot of the figure. x 20,000 (x 8,000).

Fig. 53. Chloroplast envelope with distal end expanded and filled with stroma. x 20,000 (20,000).

Fig. 54. Section of a curved chloroplast envelope profile, suggested to represent a bowl shape (c.f. fig. 55). A small number of thylakoids penetrate the proximal end for a short distance. The chloroplast can be seen clearly. It consists of a dense stroma in which run lamellae of 2 - 3 thylakoids, and which contains isolated starch granules. Root microtubules are seen in transverse section (arrows). x 20,000 (x 10,000).

Fig. 55. Circular profiles of chloroplast envelope extensions. On the right of the figure the outer membrane is extended. x 20,000 (x 10,000).

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particles typical of the edges of thylakoids in Euglena represent lipid stripped from the membranes during fixation. As in the present material the dense granules were missing if glutaraldehyde was used for fixation some corroborative evidence is given.

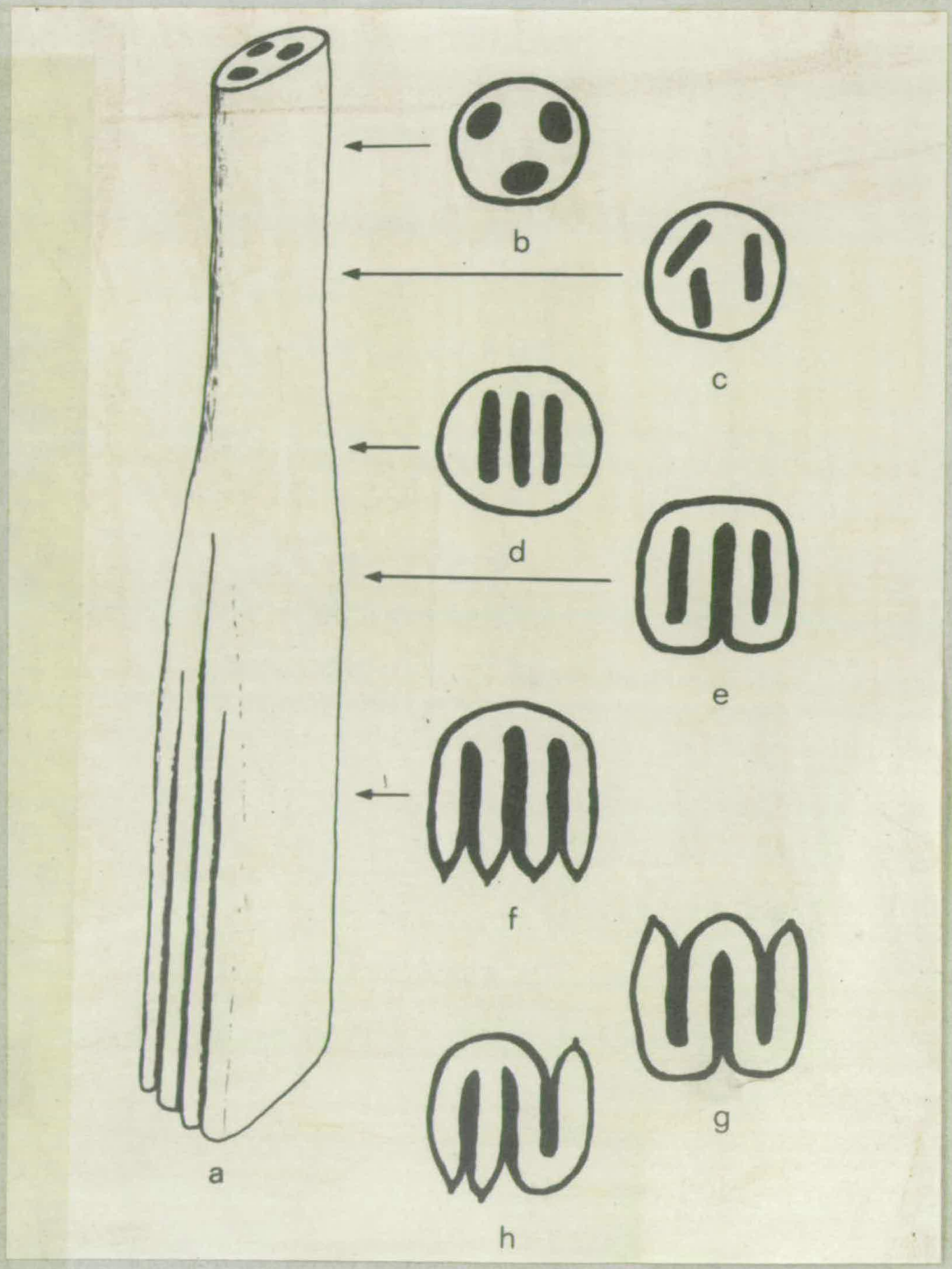
The typical pyrenoid consists of a sub-spherical to ovoid body having a finely granular dense matrix which is surrounded by starch plates and penetrated by a branching system of tubular extensions of the chloroplast lamellae (Figs 61-63, Plate XVIII). Pyrenoid sections show the tubular lamellae sections either predominantly in longitudinal or transverse section suggesting that the bulk run parallel and that the interconnections are relatively few. The diameter of the lamellar tubules is about 80-90 mμ. From transverse sections they are seen to be limited by a membrane and to contain from 1-6 round dense profiles. In longitudinal section these profiles appear as thick parallel lines running within the tubules.

In addition to the profiles showing thylakoids stacked with their spaces in connection at the edge of the stack, two other types of configuration are found near the pyrenoid edge. One shows compact yet irregular and much folded profiles of varied but obviously related types (Fig. 62, Plate XVIII; Figs 72, 73, Plate XX). In one of these, thick dark lines run alternately from one or the other side of the profile which is limited by a membrane, whereas although the other has basically the same appearance some of the dark lines are isolated from both sides. The former type may be considered as a thylakoid which is folded back upon itself repeatedly, the thick dark line representing the membranes of adjacent folds in contact and the latter as a section at a level where a connection has been made between the spaces of adjacent thylakoids on the free side of the stack. This means that the isolated dark lines represent intuckings and consist of two closely apposed membranes. Where all connections between the limiting membrane and the dark lines have been broken and the profile has a

circular outline and contains a rank of dark lines (Figs. 72, 73, Plate XX), this may be visualised in a reconstruction as an enlarged tubule containing strap-like extensions. If the membranes comprising these extensions were to separate, the space they contained would be continuous with the stroma. The tubules penetrate the pyrenoid and their diameter diminishes to 75-85 mμ and at the same time the strap-like intuckings taper until they have assumed a near cylindrical shape and measure 15-25 mμ (Fig. 63, Plate XVIII). The arrangement of the profiles within the tubule alters, as the tapering occurs, from a condition in which the strap-like profiles are parallel to one in which the cylindrical ones are arranged at the circumference of the tubule. Text-fig. 4 presents this interpretation of the changes in thylakoid structure in the pyrenoid. If the interpretation offered is correct the lumen of the tubule within the pyrenoid corresponds in theory with the spaces of one more thylakoid than the number of contained profiles. There is obviously a lot of variation in the thylakoid configurations as they approach the pyrenoid and form the pyrenoid tubules; nevertheless, only one type of change is required to reduce them to a common form.

In very rare instances profiles such as are seen only at the margin of the pyrehoid, and not within it, have been seen deep in the pyrenoid. Some pyrenoids do show a number of thylakoids of the lamellar type entering the margin but these were never observed to penetrate far.

The pyrenoid tubules enter the pyrenoid after passing through the stroma separating the starch plates (Figs 64-67, Plate XIX; Figs 68-70, Plate XX). Grazing sections of the pyrenoid show that the starch is indeed in plates with intervening spaces into which the stroma penetrates extensively rather than being in the form of a shell pierced only at the points of entry of the pyrenoid tubules (Fig. 3, Plate III; Fig. 68, Plate XX). Dumb-bell shaped profiles of the pyrenoid, which are seen occasionally, suggest that the increase in pyrenoid numbers comes about by the constriction of pre-existing pyrenoids.



Text-fig. 4. a. Reconstruction of chloroplast lamellae as entry into the pyrenoid is made. b-f. Transverse sections of the pyrenoid tubule at the levels indicated. b shows the round profiles within the tubule which are considered to be derived by the compacting of the profiles seen in c and d. These are intuckings of the wall into the thylakoid space. In d they retain their original disposition but in c they are becoming re-arranged so that they occupy ultimately a position at the circumference of the tubule. g and h show other profiles from which the series e through b could be

Besides the starch found around the pyrenoid, grains are found isolated in the chloroplast (Fig. 1, Plate I). It may be identified as such by the good positive reaction with the P.A.S. test (Fig. 123, Plate XXXI).

Tubular profiles are encountered in groups of up to 10 in the stroma (Fig. 58, Plate XVIII). It was not possible to trace them through more than a few serial sections, suggesting that they are either very short or very tortuous in the course they follow. These tubules were never seen in the close proximity of the pyrenoid and cannot be related with the round profiles within the pyrenoid tubules. They are, moreover, larger, measuring up to 27 mμ in diameter with a lumen of ~ 10 mμ. The walls of the tubules are of comparable thickness to the limiting membrane of the thylakoids and it seems likely that these are indeed tubular extensions of the thylakoid system. Although they are found widely dispersed throughout the chloroplast the greatest concentrations occur near such regions as illustrated in Figs 74-78 (Plate XXI). This series of sections is taken to be in a plane perpendicular to that of the figures of Plate XIX and to be through such a region just below the chloroplast lamellae which surround the pyrenoid in these figures (Figs 64-67, Plate XIx). The structure sectioned apparently represents columnar-like links between two large lobes of the chloroplast. Of interest is the fact that thylakoids traverse these links assuming a tubular form in so doing, as may be seen in Plate XXI. The profiles within the cross-sections of the columns are not as uniform as the tubular profiles which have been mentioned previously, however. The appearance alters greatly from section to section in the series. In other cases the linking columns are larger and the thylakoids passing through them are of the usual flattened form. The whole complex presents a sponge-like system of reticulate chloroplast and space. The spaces within the reticulum are clear and not granular as the ground cytoplasm with which they are in connection.

PLATE XVIII

Fig. 56. Detail of folds of outer chloroplast membrane taken from Fig. 9. x 40,000 (x 8,000).

Fig. 57. Tubular extension of inner chloroplast membrane with outer membrane thrown into folds. x 30,000 (x 16,000).

Fig. 58. Tubular thylakoid profiles (arrow) in chloroplast. x 60,000 (x 16,000).

Fig. 59. Detail of the point of extension of the outer chloroplast membrane (arrow 1). The thylakoid rims are seen to be thicker, the thickening appearing as a granule in section (arrow 2). x 30,000 (x 16,000).

Fig. 60. Unidentified differentiation within chloroplast. x 60,000 (x 16,000).

Fig. 61. Section of pyrenoid edge with pyrenoid tubules passing between starch plates to join in the pyrenoid. Dense osmiophilic globule (arrow) on pyrenoid tubule thylakoid. x 30,000 (x 16,000).

Fig. 62. General survey picture of pyrenoid surrounded by starch plates showing characteristic thylakoid profiles at the margin and pyrenoid tubules. Many of the chloroplast thylakoids have associated dense osmiophilic globules (arrows). x 30,000 (x 16,000).

Fig. 63. Section of pyrenoid showing pyrenoid tubules in longitudinal and transverse section. x 30,000 (x 20,000).

Inset. Enlargement of pyrenoid tubule in transverse section as indicated (arrow). x 100,000 (x 50,000).

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PLATE XIX

Figs. 64-67. Sections through a pyrenoid; respectively the 1st, 3rd, 6th and 7th sections of a series. x 25,000 (x 16,000).

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PLATE XX

Figs. 68-70. Sections through the edge of a pyrenoid, respectively the 1st, 2nd and 4th of a series shewing the passage of the pyrenoid tubules between the starch plates (fig. 68) and their subsequent changing profiles within the pyrenoid. x 30,000 (x 16,000).

Figs. 71. Grazing section shewing characteristic thylakoid profiles around the pyrenoid. Arrow shews a 5-thylakoid stack with the spaces in continuity at one margin. x 30,000 (x 16,000).

Figs. 72, 73. Sections grazing the pyrenoid, the 1st and 4th of a series, shewing rearrangement of tubule profiles (arrow 1). Arrow 2 in fig. 72 shews thylakoid bent back upon itself. x 30,000 (x 16,000).

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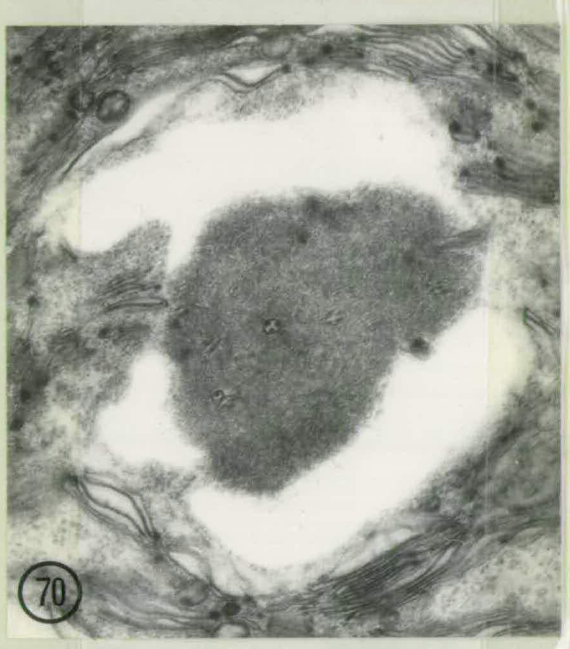
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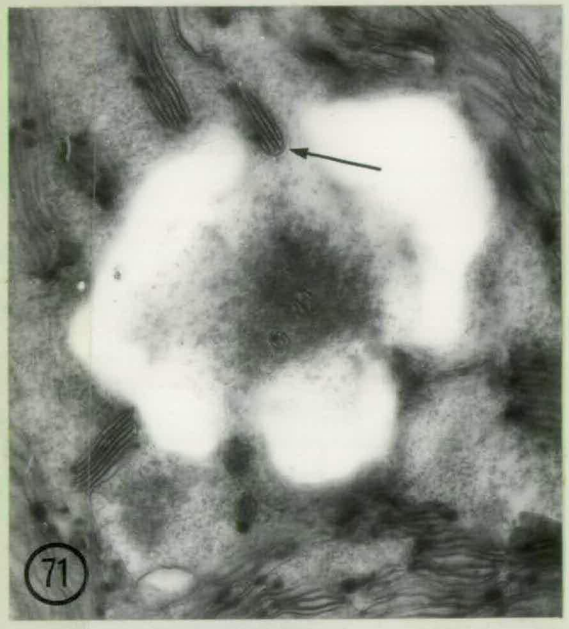
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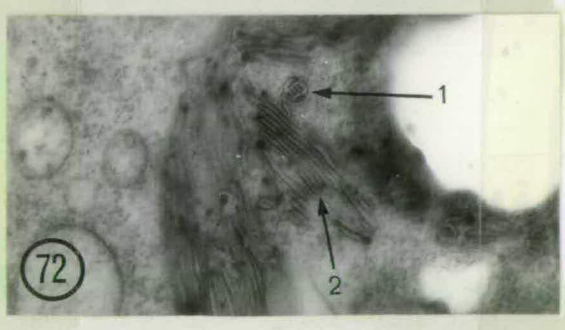
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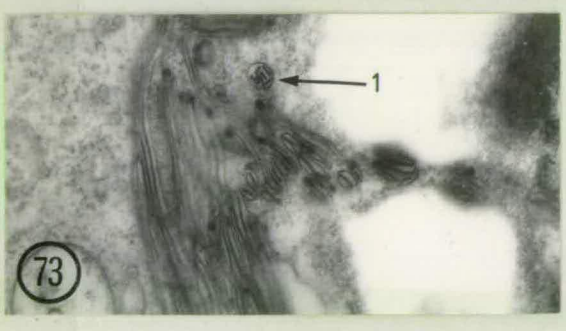
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PLATE XXI

Figs. 74-78. Serial sections of chloroplast through such a region as seen below the pyrenoid in figs. 64-67, shewing tubular form of thylakoids. The 1st, 3rd, 4th, 5th and 7th sections form the series. All x 30,000 (x 16,000).

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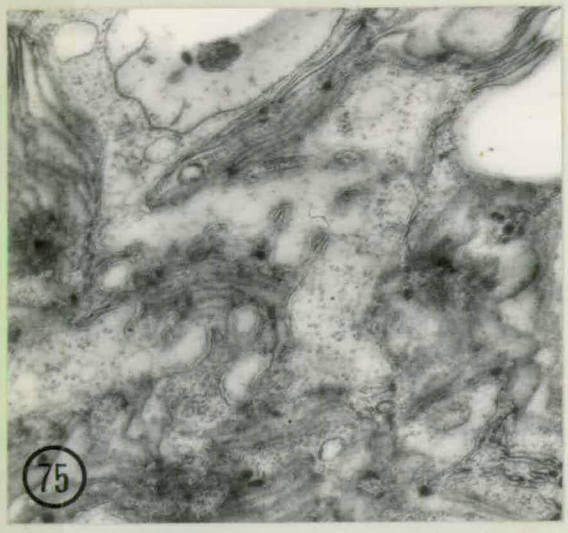
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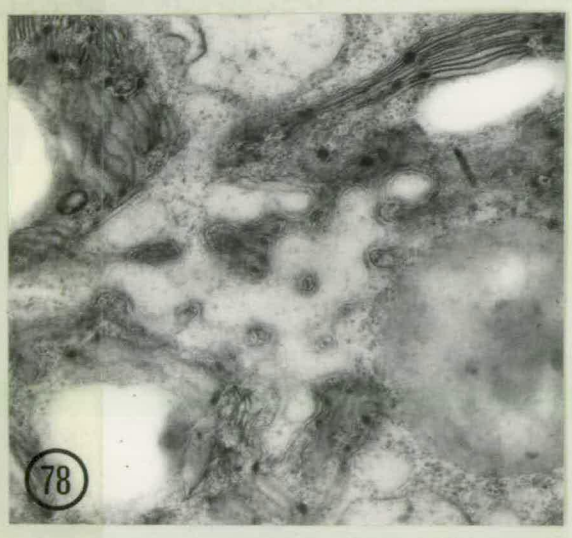
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Another differentiation within the chloroplast which was only very rarely seen, and which remains unexplained, is illustrated in Fig. 60 (Plate XVIII).

The second region of major differentiation in the chloroplast is the eyespot. As recorded in the introduction, certain variation has been noted in the size of the eyespots in the various cells of colonial forms. In a word, the colonies have a definite polarity and cells at the anterior pole have larger eyespots than those of the posterior pole, from which the eyespots may be absent. Where the colonies have all cells in contact in a close sphere or where the cells are disposed in a plate no great variation of eyespot size is evident.

It is only rarely that the site of the eyespot may be discovered by electron microscopy as the exact plane of sectioning is usually difficult to fix. Certain indications do exist, however, concerning the orientation of a section, such as the plane of sectioning of the microtubules and of the flagellum root microtubules. The eyespot is normally found in the apical region of the chloroplast and near the flagellar bases (Fig. 87, Plate XXV; Fig. 92, Plate XXVI), but not bearing a constant relationship to them (Gerisch, 1959). Another indication of the proximity to the basal bodies is given where the flagellum root microtubules appear in a section as a group (Fig. 86, Plate XXIV) rather than widely separated as in more posterior regions. Typically where an eyespot is differentiated the chloroplast comes into close proximity with the cell membrane so that the chloroplast envelope which limits the eyespot lies directly underneath it (Fig. 3, Plate III; Fig. 79, Plate XXII). The structure of the eyespot generally assumed to be typical comprises a series of curved plates of osmiophilic globules of 125-150 mμ packed in single layers, each of which is underlain by a single thylakoid. The globules are considered to be the likely sites for the accumulation of lipid-soluble pigments. The plates of globules number up to nine and are usually curved with the concavity directed outwards, although occasional sections

show the reverse arrangement (Fig. 85, Plate XXIV). Sections in the plane of the globule layers and oblique sections show that they are packed uniformly in a hexagonal array which may be slightly irregular due to small variations in globule size (Figs 87, 90, Plate XXV; Fig. 92, Plate XXVI). There is, however, less variation in globule size than it seems from cross-sections in which only a proportion of the globules can be sectioned through their centres. It is unusual for any substantial structure to lie between the chloroplast envelope limiting the eyespot and the cell membrane. The diameters of the globule layers nearest the cell membrane are the largest whilst at deeper levels layers of rather smaller diameter occur, so that they may not appear in sections through the edge of the eyespot. Nevertheless, serial sections show that in a majority of sections through an eyespot all the globule layers occur or are otherwise indicated (Plate XXVIII). The eyespot possesses considerable integrity for when cells are ruptured by drying up and the cell contents extruded, the eyespot may be seen to remain intact although from the structure as seen using the electron microscope there is little apparent reason for this integrity.

The relationship of the eyespot to the rest of the chloroplast is not constant. Most frequently it appears to occupy a single lobe, being in connection with the rest of the chloroplast on one side only. When this is so the eyespot thylakoids which are extended from the lamellae follow their general orientation. It is not to be expected that the lamellae should approach from underneath the eyespot and have to change their course sharply to give off the eyespot thylakoids. The frequent occurrence of eyespots with no connections with the rest of the chloroplast in the plane of the section is another indication of the fact that they often occupy single lobes. In the case of small eyespots the connections may be on all sides (Fig. 92, Plate XXVI); transverse sections in such cases show connections on opposite sides of the eyespot (Fig. 82, Plate XXIII).

The thylakoids underlying the globule layers arise from the lamellae which approach the edge of the eyespot where most of the thylakoids terminate. Most of these lamellae have 2 or 3 thylakoids. One lamella contributes the eyespot thylakoid to one or two globule layers. In some cases the thylakoid from one layer may be seen to run back under a second layer (Fig. 80, Plate XXII). The more usual condition is for the thylakoids to end on the side opposite their entry;even where contact with the rest of the chloroplast occurs on both sides the tendency is for all the eyespot thylakoids to be continuous with lamellar thylakoids of a single side. Micrographs in which the eyespot thylakoids are continuous with lamellar thylakoids from either side in the plane of the section suggest that in three dimensions they are tongue-like and free round three parts of their circumference. The thylakoids underlying the innermost layer of globules show most variations from the uniform arrangements of the other layers. The underlying layer may comprise two thylakoids entering from opposite sides. This interpretation is offered rather than that a large gap appears in a single thylakoid, on the grounds that where they terminate they are frequently reflected back on themselves for a short distance (Fig. 80, Plate XXII). This may be contrasted with the condition which is seen in the other layers in which the breaks occurring in the underlying thylakoids prove on examination of serial sections to be pores (see Figs 93-96, Plate XXVII). Also the innermost globule layer may be underlain by a lamella of two thylakoids (Fig. 110, Plate XXVIII).

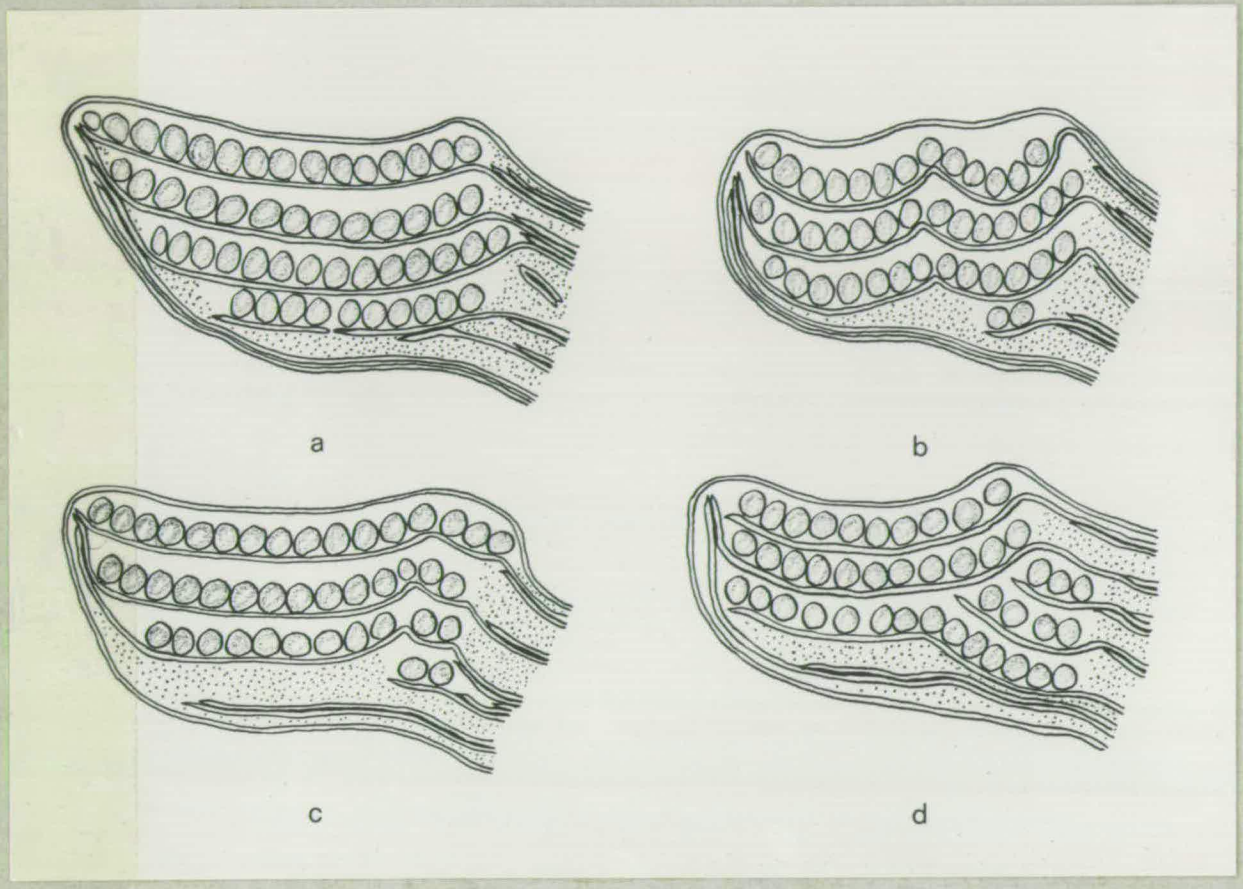
The appearance of the thylakoids at the edges of the globule layers is often similar to that at the pyrenoid margin. Stacks of short thylakoids are found in various orientations, sometimes perpendicular to, and sometimes in the plane of the globules (Fig. 94, Plate XXVII). Both arrangements noted in conjunction with the pyrenoids occur: stacks consisting in essence of one thylakoid repeatedly reflected back upon itself (Fig. 95, Plate XXVIII) and stacks in which

all the thylakoid spaces are in connection at one margin of the stack (Fig. 93, Plate XXVIII). The eyespot thylakoids in some cases are seen to join or form these stacks (Fig. 84, Plate XXIV). The granular stroma of the chloroplast ends at the margin of the eyespot and is not found between the pigment globules. Large stromal areas, triangular in section, tend to be found at the base of the eyespots and in some cases round aggregations of particles which have the general appearance of the nucleolus are found in these areas.

There is frequently a space between the globules of one layer and the thylakoid of the layer above it, or in the case of the outermost layer between the globules and chloroplast envelope. This has been identified by Lang (1963a) as a hyaline space in the latter case, which she believes may correspond with the refractive lens which earlier workers claimed to see using the light microscope. However, the occurrence of this space is far from uniform. It may appear only between the chloroplast envelope and outer layer of globules as reported by Lang and as illustrated here in Fig. 3 (Plate III), also between the first and second layers of globules (Fig. 81, Plate XXIII) and to varying extents between deeper layers (Fig. 83, Plate XXIV). No structure whatsoever can be made out in these spaces, but it does seem that they have some integrity as in some cases where they are not bounded by the granule layer of thylakoids and the stroma comes into contact with them there is no encroachment in the region (Fig. 84, Plate XXIV).

The dimensions of eyespots having what is considered to be congruent organisation vary considerably according to the number of layers of globules they possess. The smallest eyespots measure 0.5μ deep by 1.0μ across and the largest measure 2.25μ deep by 3.0μ across. These measurements illustrate the proportional change in the eyespots. The largest eyespots with the most layers of pigment globules are more nearly spherical in cross sectional outline than those with few layers which are more flattened. The increase in depth is more than proportionally greater than the increase in diameter.

The extent to which variations in the site and structure of the eyespots occur can be quite small or very considerable. Eyespots are found which have a completely typical organisation but of which the site is not the usual one. Fig. 91 (Plate XXVI) illustrates such a case in which mitochondria and lobes of the chloroplast are interposed between the eyespot, whose orientation is normal, and the cell membrane. This particular example is unusual in that the section includes a normal eyespot within the same chloroplast lobe. In other examples seen the eyespot was nearer the cell membrane with only mitochondria between it and the cell membrane, but in yet others it was still deeper. Another condition found in which the eyespot structure is not very irregular is illustrated in Fig. 89 (Plate XXV). In this the eyespot, although sited just beneath the cell membrane, is orientated with the layers of eyespot globules perpendicular to the cell membrane. Five layers of globules of short diameter are apparent. Slight irregularities in the structure of normal eyespots may occur: globule layers may be incomplete and pass only part of the way across the diameter of the eyespot. The organisation is not seriously disrupted. However, in eyespots of intermediate size having 4-6 layers of globules the structure is frequently irregular. The profiles seen most often may be visualised as being derived from the fusion of two regular eyespots of equal size but slightly differing orientation so that the layers of globules instead of having a smooth concave profile facing the exterior appear W-shaped (text-fig. 5b). The variant of this structure figured in text-fig. 5c may be considered as derived from the fusion of two eyespots of differing size and orientation. The irregular form figured in text-fig. 5d is such that the thylakoids of the two parts are not in continuity and it represents a more extreme condition. Globule layers and their thylakoids abut the thylakoids of the next to lowest layer of the other part of the eyespot. The lowermost thylakoid, however, underlies the globules of both parts of the eyespot. Eyespots



Text-fig. 5. Eyespots in Eudorina illinoiensis. Typical (a) and variant (b - d) organisation of the pigment globule layers. See text for further explanation.

having this form are found both at the surface and also deep within the cell (Fig. 1, Plate I). A more extreme condition of similar appearance to text-fig. 5d has the globules beneath the thylakoids abutting the main part. This must be derived from the fusion of parts with the difference in axes being 180° greater than the example shown in text-fig. 5d. The irregularities in structure which have been described may be quite local so that in serial sections many of the profiles will be completely normal.

The osmiophilic globules which are found in the eyespots are also widely distributed throughout the chloroplast, occurring singly or in small groups (Fig. 1, Plate I; Fig. 3, Plate III). When in groups they tend to be aligned with respect to a single thylakoid as in the eyespots. Such configurations occur in many places and it is a moot point whether they should be considered as small eyespots of abnormal site or merely chance combinations which show an incipient eyespot structure. It is perhaps significant that single thylakoids underlie the rows of globules rather than that they occur between lamellae. In Fig. 88, Plate XXV, there is an example of this eyespot differentiation in which the eyespot faces away from the cell membrane which appears at the top of the figure, and which consists of three layers of globules.

The study of the eyespots of a single colony by serial sectioning proved difficult, as the size of the colony was too great for the most effective observation. In spite of the use of hexagonal grids so that the chance of obscuring consecutive sections was lessened, it was rare to find runs of serial sections in which were found more than a few of the total number of sections which would be expected to contain any one eyespot. Bearing in mind the average size of the eyespot (1-3μ in diameter) and the thickness of the sections ($\sim$ 60 mμ) and their number per grid ($\sim$ 10), where an eyespot has been sectioned normally to the cell surface one might in theory be expected to find sections of one eyespot

over 2-5 grids, in 20-50 sections. In practice this was not obtained. The best results gave series of 16 and 19 near-to-serial sections. In the former group 9 sections came from a sequence of 20 sections (all references to the numbers of sections must of necessity be approximate) and the remainder were further apart, and in the second group the 19 sections were taken from a sequence estimated to contain 24 sections, and of these 14 are presented in Plate XXVIII. The average number of sections in any of the series obtained was eight.

The colony, which was serially-sectioned, was of an equatorial diameter $\sim$ 80μ and fine sections were cut of three-fifths of it. The colony was orientated with its poles in the plane of sectioning. It is possible to make the working assumption that 18 of the 32 cells were sectioned. In this study the series of sections obtained were placed tentatively in 20 groups and of these 15 represented, quite definitely, separate eyespots. The justification for this is as follows: the eyespot series can be arranged in groups which cannot possibly be overlapping on a consideration of the maximum number of grids over which sections of an eyespot could appear; this information is then considered together with that concerning the position occupied by the cell with the eyespot in question. A number of isolated sections and small groups of eyespot sections were obtained which could not be related to any series.

Although in theory it should be easy to identify the approximate site of the separate cells of the colony, having a knowledge of the polarity, in practice it was far from easy due principally to obstruction by grid bars. Aids in the identification of eyespots without full knowledge of the relative position of the cell in the colony include: the site either at the surface or at varying depths from the surface in the chloroplast; the orientation where this is in some way abnormal; the structure where this is abnormal (this is not a good criterion as irregularities of structure can be very local); relationships with other

organelles. Concerning the last mentioned point, the orientation relative to the tubules of the flagellar roots and the microtubules was of especial assistance.

A further difficulty in this work was the problem of the location of cells relative to their ranks, the anterior or posterior ranks of 4 cells or the three intervening ranks of 8 cells. It is not possible to say dogmatically that any cell comes from a specific rank but only that a particular cell is from an anterior rather than a posterior rank or in other cases that it is from a middle rank rather than from the anterior or posterior rank. This gives an overlapping definition visualised as follows: the anterior rank of 4 cells and the next rank of 8 cells are defined as 'anterior' and correspondingly for the 'posterior' cells. The three ranks of 8 cells may be defined as 'median' but the only rank which is never likely to be included in another class is the middle of these three ranks.

Having established the meaning of 'anterior' and 'posterior' cells in the context of work on serial sections it is of interest to examine the number of layers of pigment globules in the 20 groups of eyespot sections. These are presented in Table 5.

Table 5

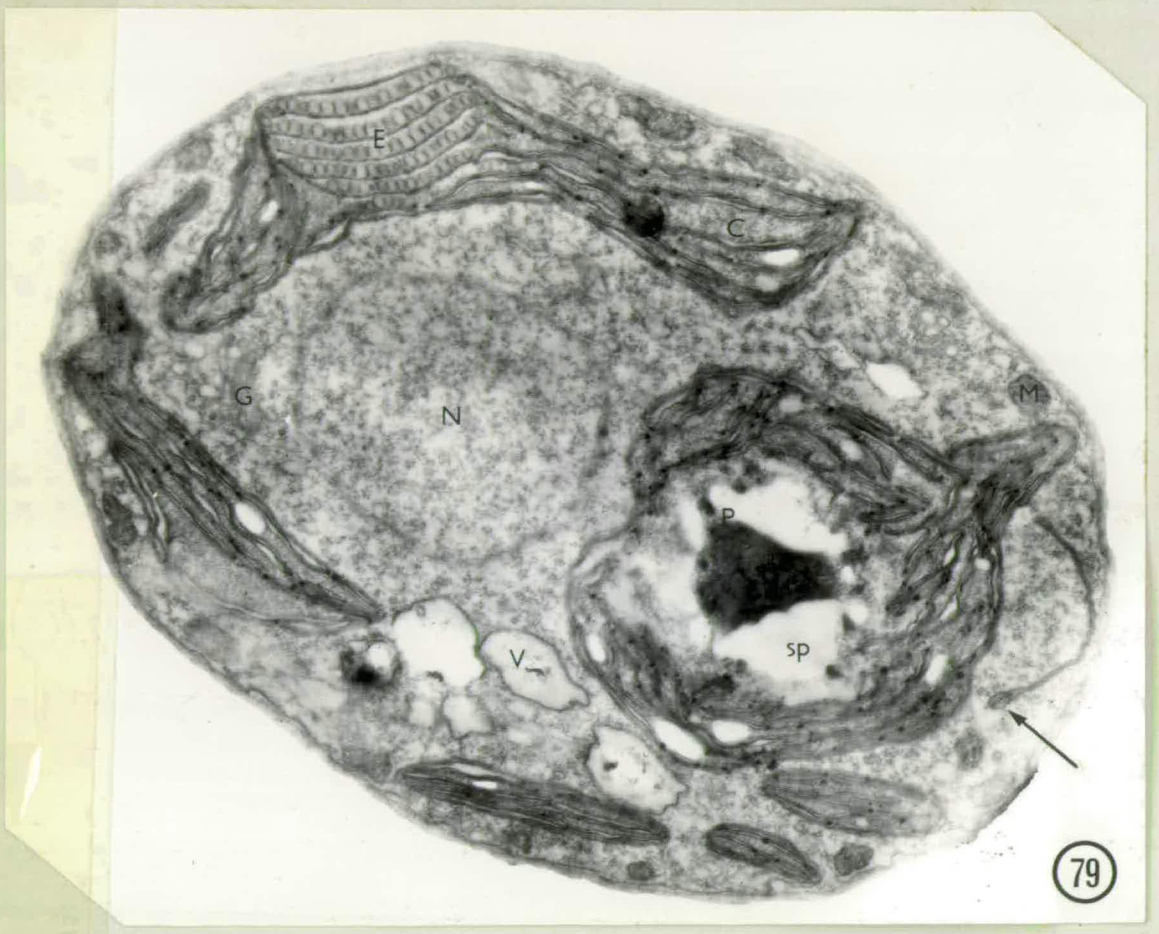
No.of globule layers	3	4	5	6	7	8(+)
Frequency	2	4	5	5	1	3

The three eyespots with 8 layers of globules were all from 'anterior' cells and that with 7 layers was defined as median, and is thus tentatively identified as occupying an anterior position. The eyespots with 3 layers of globules and 2 of those with 4 were identified as 'posterior'. Another of the eyespots with 4 layers was abnormal in its siting, being deep placed, and in its orientation which was perpendicular to the usual. All remaining eyespots were classified as median. Certain generalisations can be drawn: no 'anterior' eyespot has fewer than 7 globule layers and no 'posterior' eyespot has more than 4 layers;

PLATE XXII

Fig. 79. General survey picture of a cell shewing typical position of eyespot (E) relative to the other organelles. Note the chloroplast envelope extension, at the pole opposite from the eyespot, which contains a 2-thylakoid lamella at one end (arrow). Nucleus (N). Pyrenoid (P) with starch plates (sp) Chloroplast (C). Golgi body (G). Mitochondrion (M). Unidentified vacuole (V). x 16,000 (x 8,000).

Fig. 80. Detail of the eyespot from fig. 79. The single thylakoids underlying the eyespot globules are continuous with some of the lamellar thylakoids at the right of the micrograph most of which terminate at the edge of the eyespot. The thylakoid underlying the layer of globules 5th from the exterior is reflected back to underly the 4th row also. The chloroplast envelope (ce) limits the eyespot to the exterior and lies in close proximity with the cell membrane. A space occurs between the envelope and first globule layer, and between the first and second layers, thought to be hyaline. x 40,000 (x 20,000).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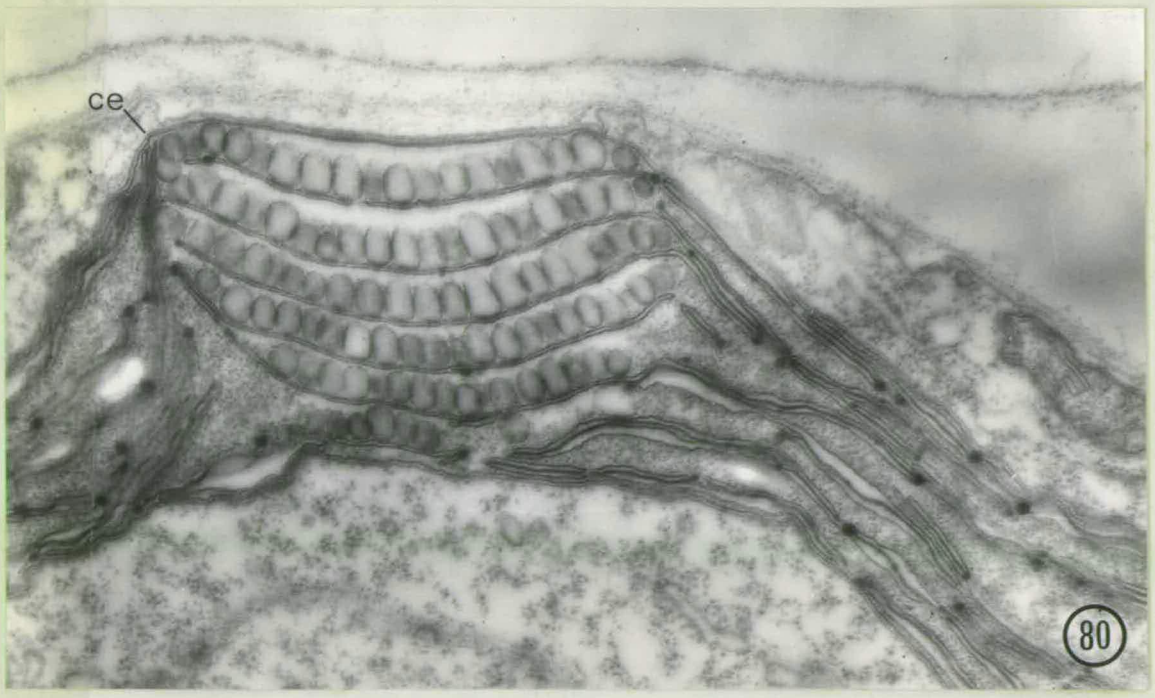
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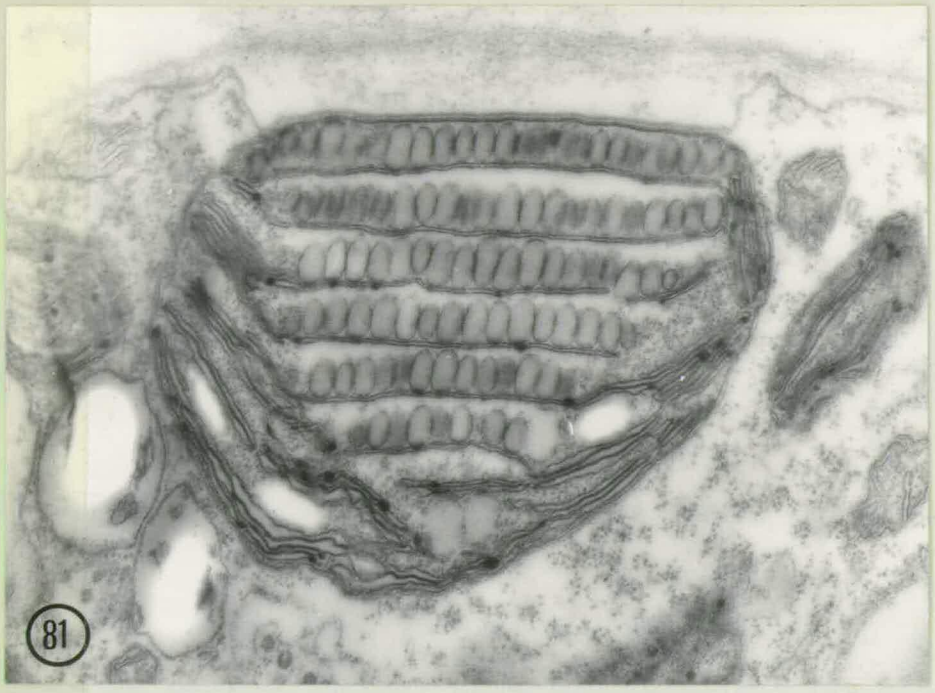
PLATE XXIII

Fig. 81. Eyespot isolated from the chloroplast in the plane of sectioning having 6 (?7) layers of globules. A putative hyaline space occurs between the first and second layer of globules. x 32,000 (x 16,000).

Fig. 82. Eyespot from a young cell shewing 3 layers of globules. This section shews also the relative electron densities of the nucleoplasm and cytoplasm and tubular endoplasmic reticulum (TER) characteristic of the young cell. The outer membrane of the nuclear envelope (ne) is covered in ribosomes. Golgi body (G). Vesiculate endoplasmic reticulum (VER). x 20,000 (x 13,000).



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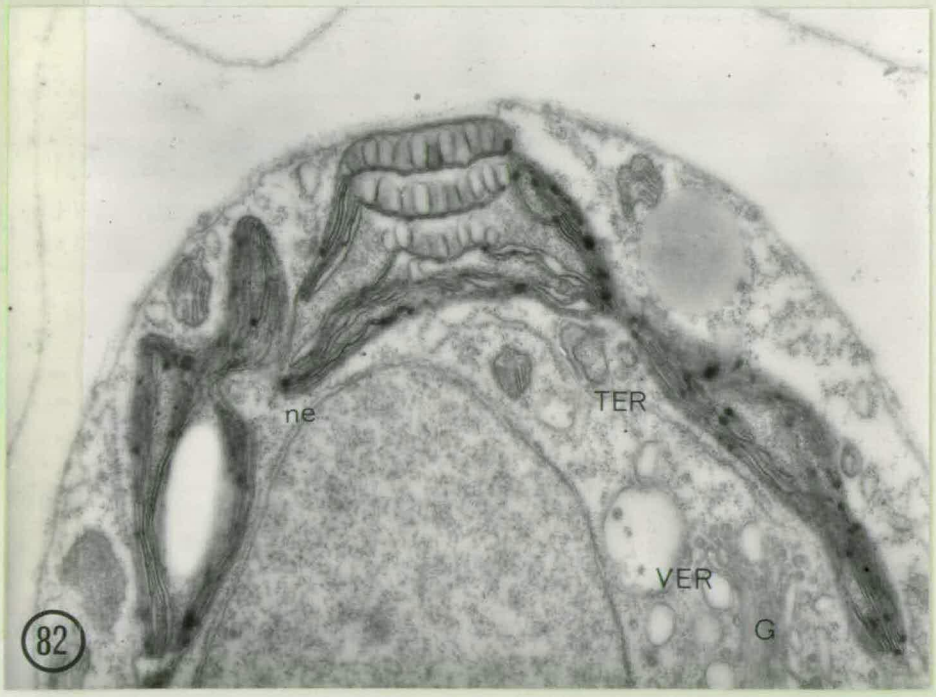

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PLATE XXIV

Fig. 83. Eyespot from a somatic cell shewing 8 layers of pigment globules. There is a slight irregularity in organisation in that the second layer from the exterior does not extend across the entire diameter of the eyespot. The majority of the eyespot thylakoids underlying the pigment globules make connection with the lamella thylakoids out of the plane of sectioning. The putative hyaline spaces are conspicuous, between the outer fine layers of globules, especially that between the first and second. Cytoplasmic microtubules (arrow heads). x 40,000 (x 16,000).

Fig. 84. Oblique section of eyespot with 3 pigment globule layers. Note the integrity of the structureless space between the second and the deepest layer. One eyespot thylakoid joins stack (arrow). x 32,000 (x 16,000).

Fig. 85. Eyespot with layers having convex curvature outerwards. Palade's osmium. x 20,000 (x 6,000).

Fig. 86. Eyespot from a median rank of cells shewing fine microtubules of the flagellum root (arrow) in transverse section between the eyespot and the cell membrane and scattered transverse sections of cytoplasmic microtubules (arrow heads) beneath the cell membrane. x 40,000 (x 16,000).

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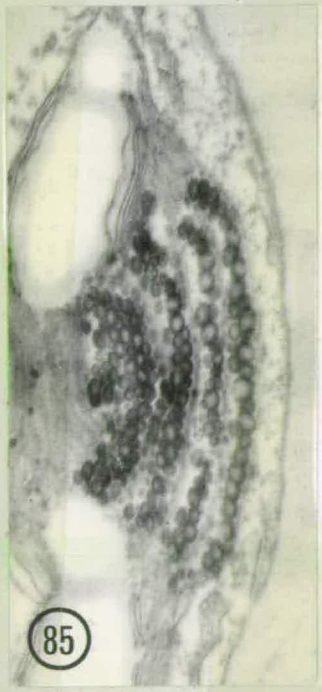
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PLATE XXV

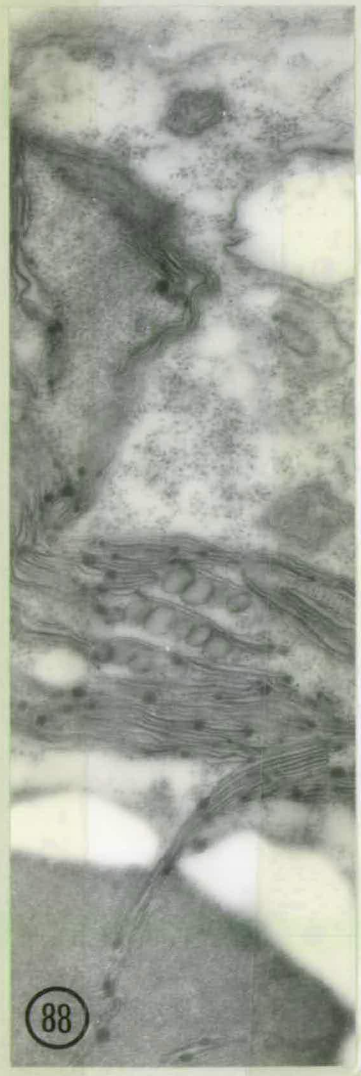
Fig. 87. Grazing section of the apical region including a section of the eyespot in the plane of the globule layers, one of the basal bodies (bb) and between these the contractile vacuole (CV) and a mitochondrial profile (M). Numerous cytoplasmic microtubules (cm) diverge from the basal body region. Two segments of flagellum root (R) are seen, one of which makes connection with the basal body. x 40,000 (x16,000).

Fig. 88. Deep sited eyespot differentiation of abnormal orientation. It faces the pyrenoid, not the cell membrane which appears in oblique section at the top of the figure. x 32,000 (x 16,000).

Fig. 89. Eyespot of 5 globule layers sited at the surface but of abnormal orientation. The normal axis of orientation is displaced 90°.
x 32,000 (x 16,000).

Fig. 90. Oblique section of an eyespot shewing the hexagonal array of globules x 40,000 (x 16,000).

87



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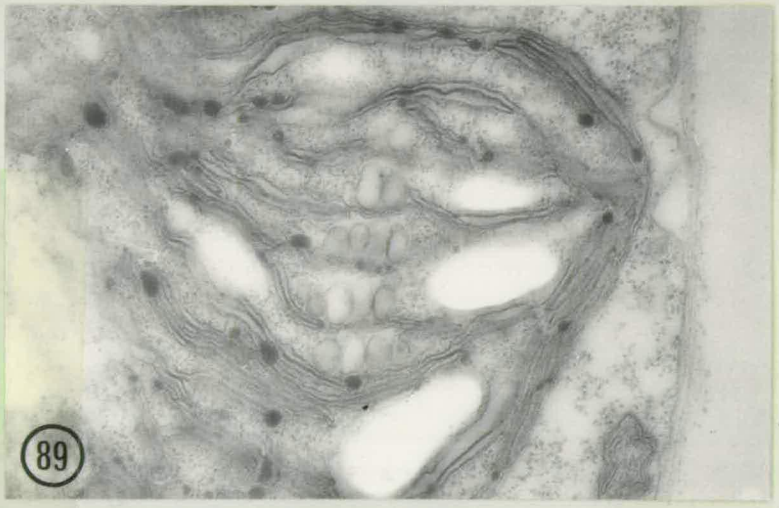

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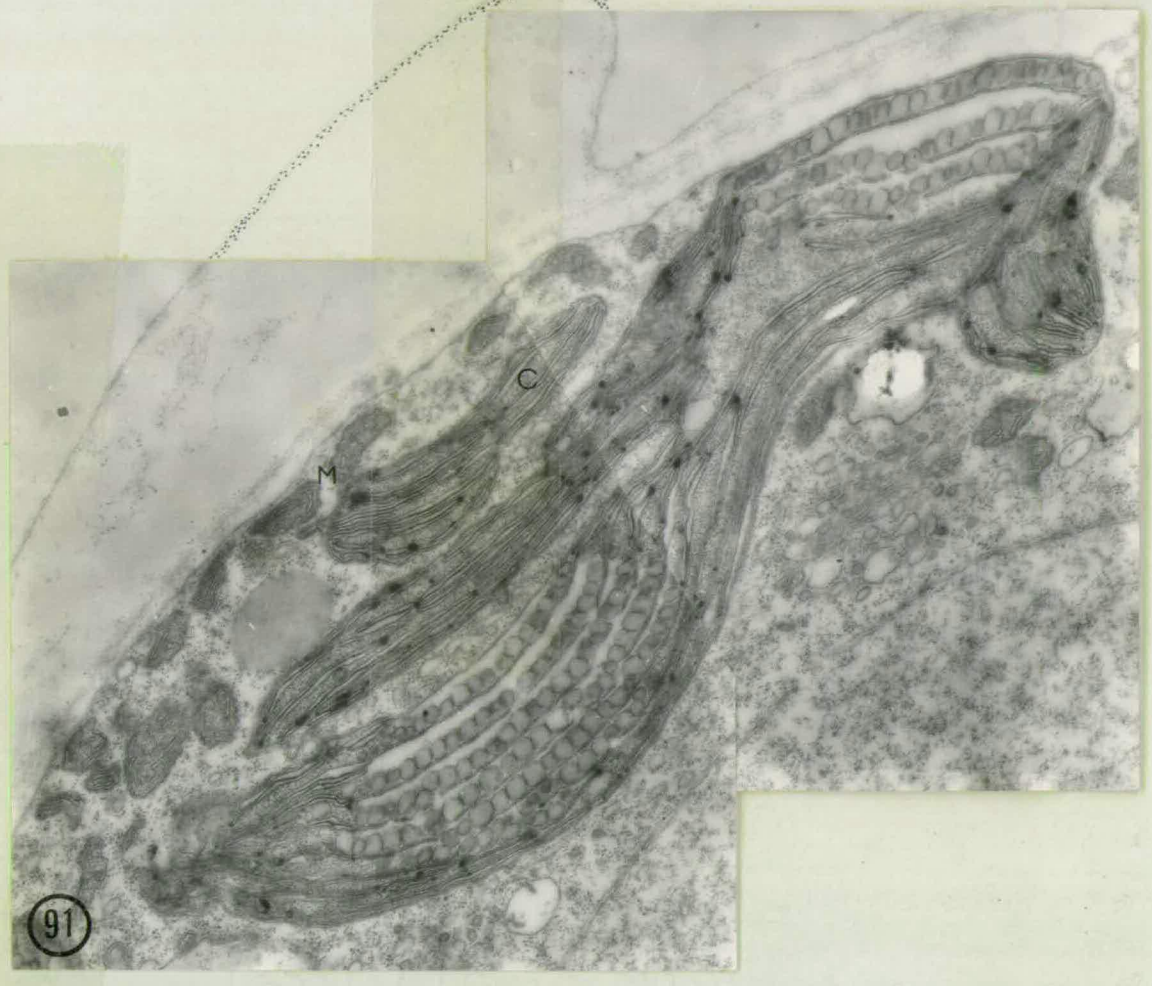
PLATE XXVI

Fig. 91. Section of a chloroplast lobe containing two eyespots, one quite typical in site and orientation, the other of typical organisation and orientation but placed deep in the cell with mitochondria (M) and chloroplast lobes (C) between it and the cell membrane. The regularity of the deep sited eyespot leads to the suggestion that this is the eyespot of the mother cell from which the colony was derived and that the eyespot at the surface is a new differentiation. x 20,000 (x 16,000).

Fig. 92. Section in the plane of the eyespot globules cutting also the flagella as they pass through the envelope. The contractile vacuole (CV) is seen between the eyespot and flagella. x 20,000 (x 16,000).



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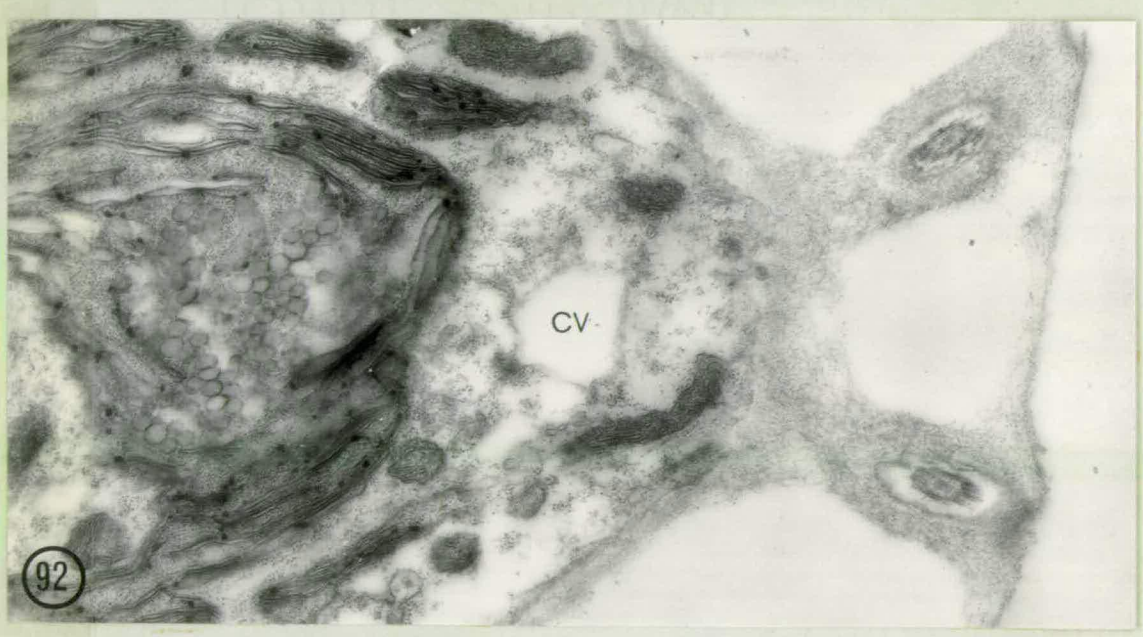

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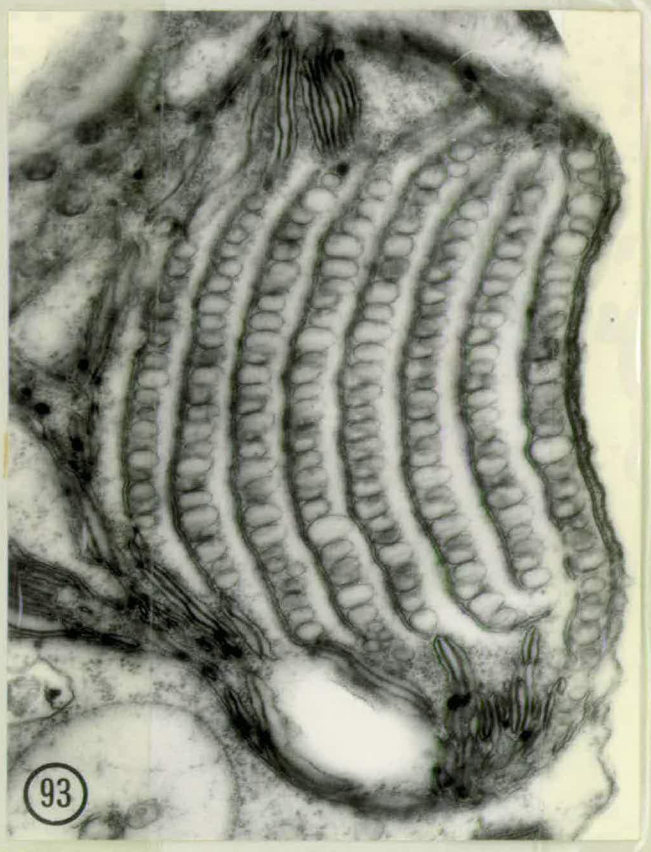
PLATE XXVII

Figs. 93-96. Sections through a somatic cell having 9 layers of pigment globules. Characteristic stacks of thylakoids are seen at the margins. The sections are respectively the 1st, 2nd, 5th and 7th of a series. Pore in thylakoid (arrow). x 30,000 (x 16,000).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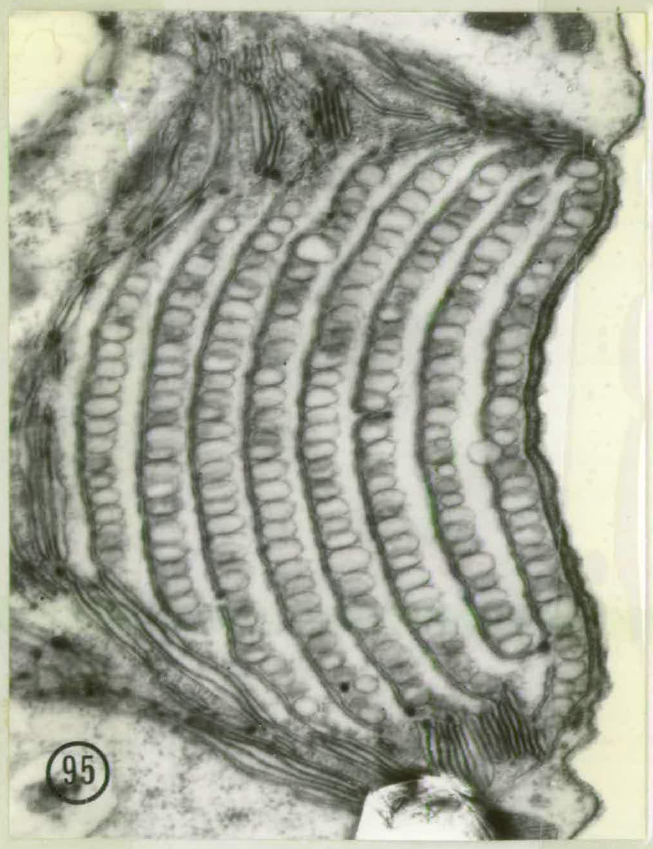
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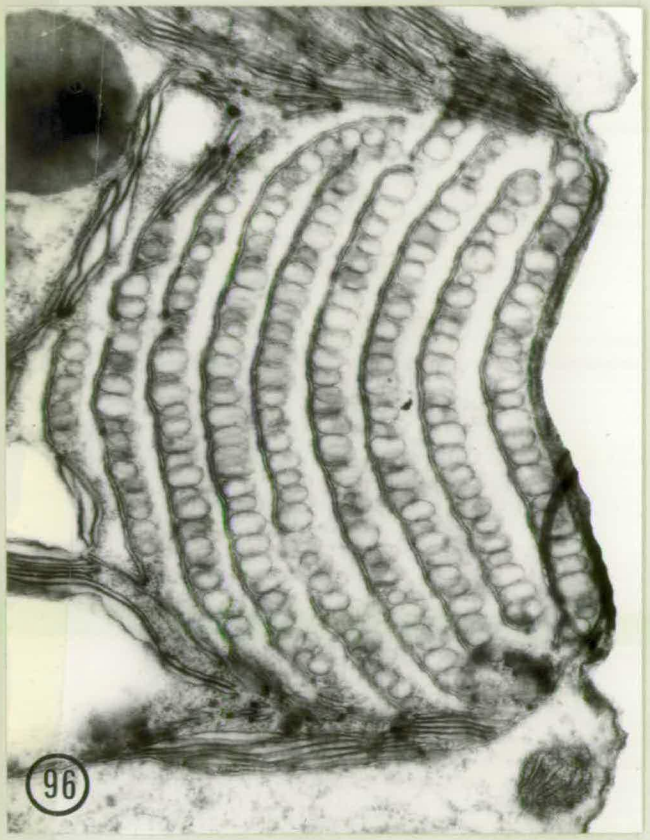
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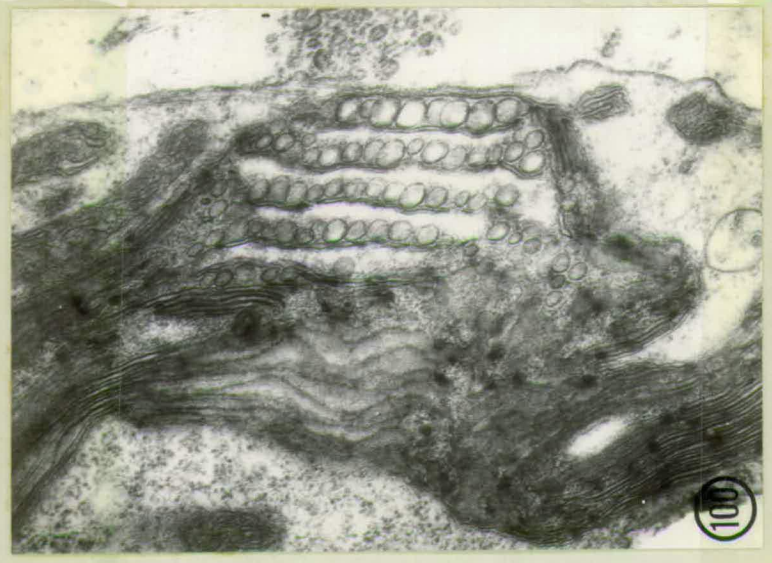
PLATE XXVIII

Figs. 97-110. Serial sections through an eyespot. In this series an entire eyespot has been almost completely traversed and most micrographs have been presented in spite of their variable quality. The plane of sectioning is approximately longitudinal as may be deduced from the fact that the microtubules visible appear in longitudinal section. The first five sections figs. 97-101, are completely serial after which a gap of an unknown number of sections occurs and the relationship of the succeeding eight sections to one another is 1, 3, 5, 6, 7, 10, 12, 13 after which another gap occurs and the final section follows. Cytoplasmic microtubules (arrows).
x 30,000 (x 16,000).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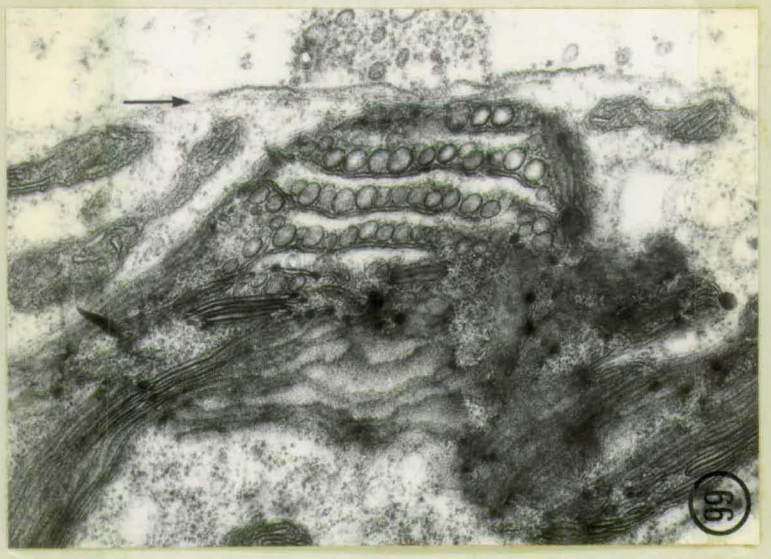
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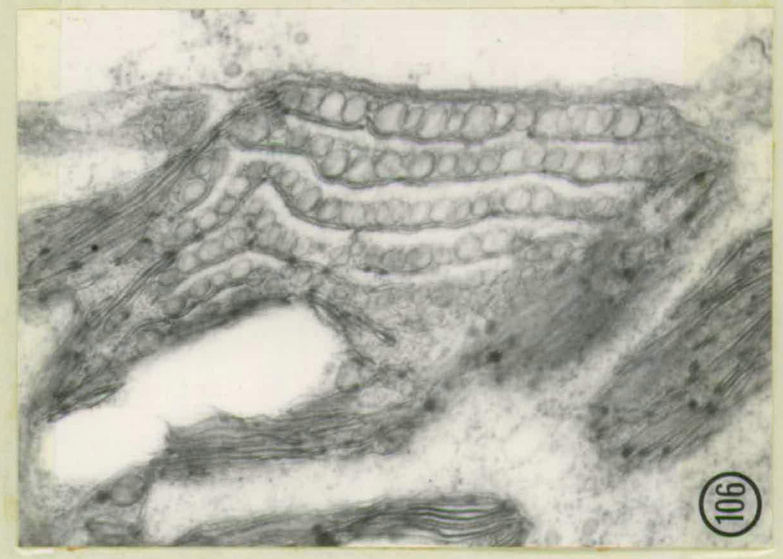
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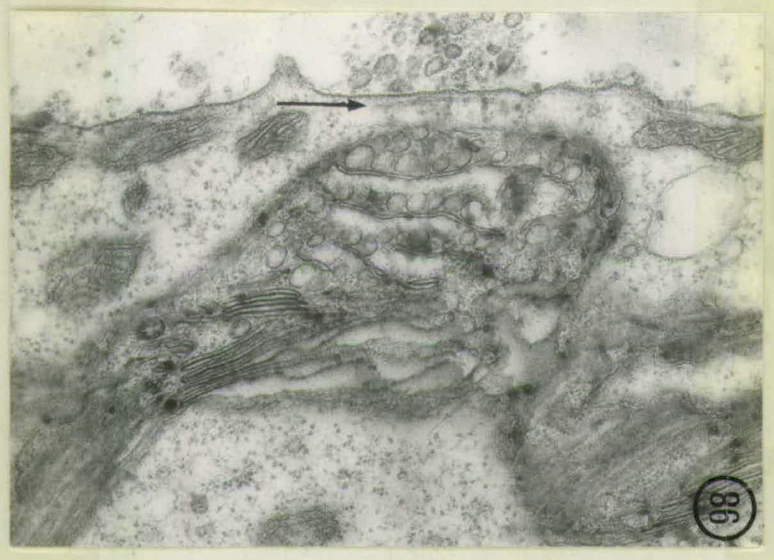
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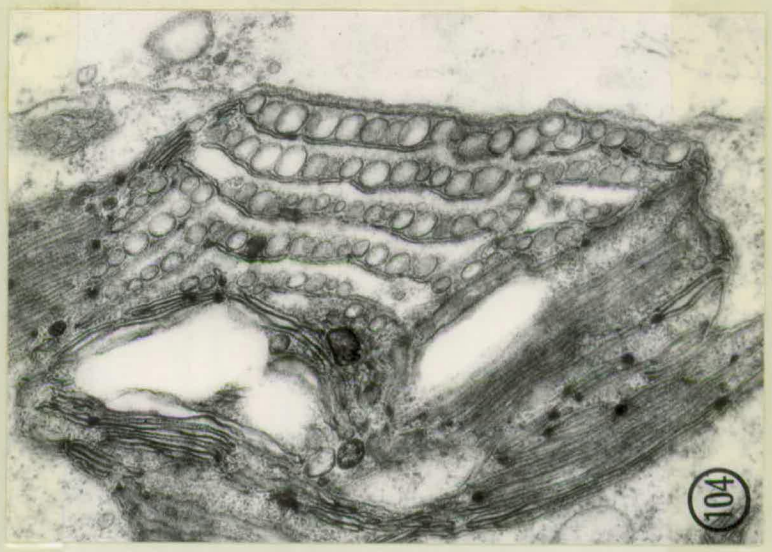
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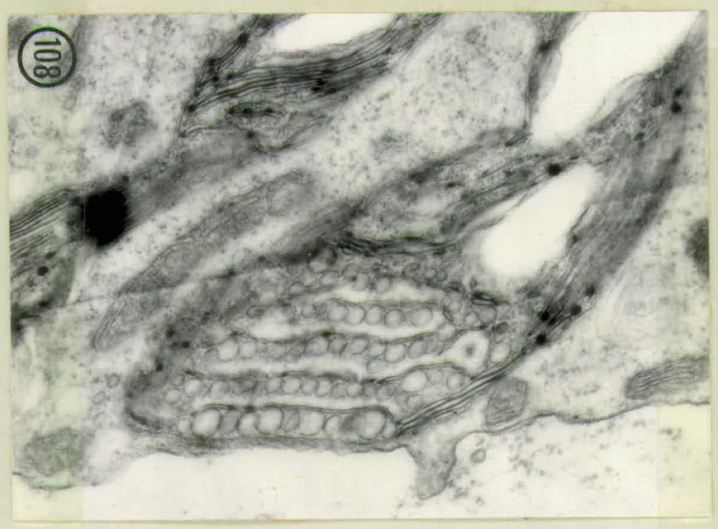
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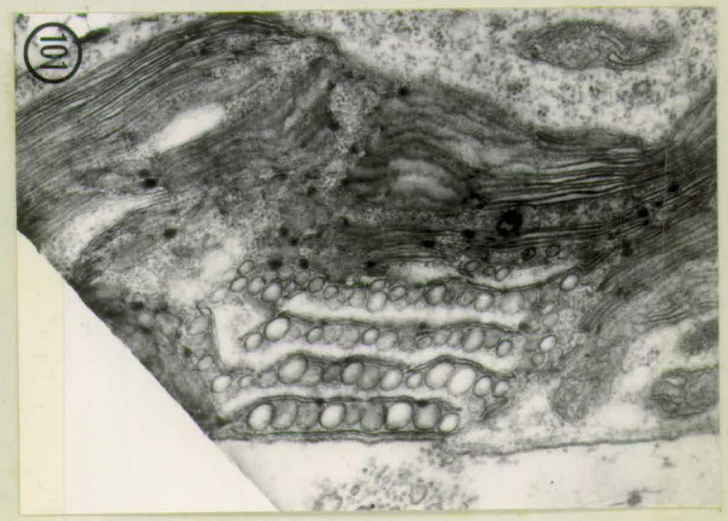
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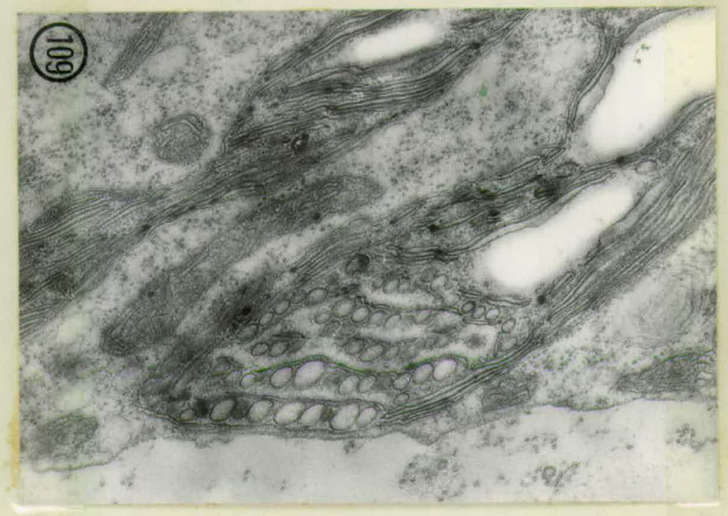
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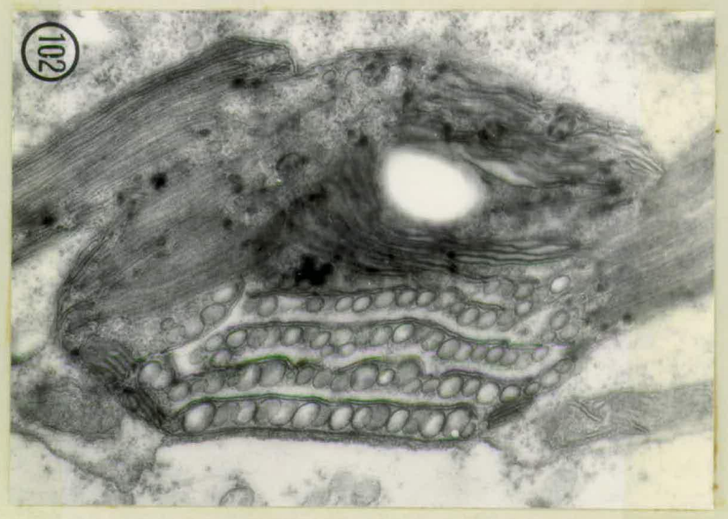
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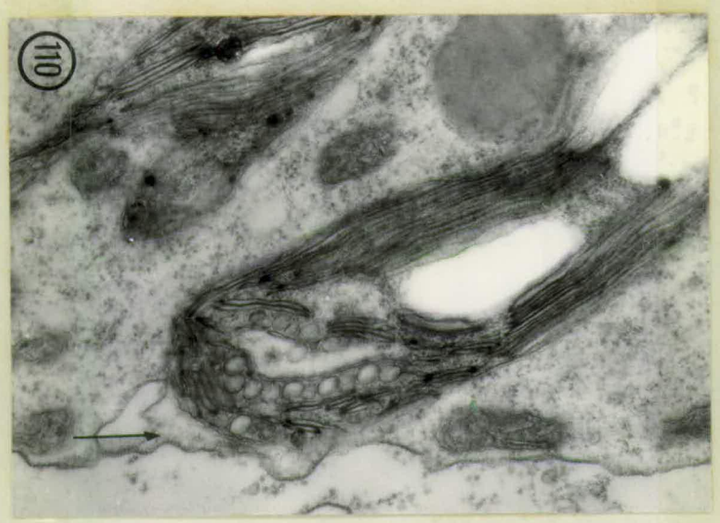
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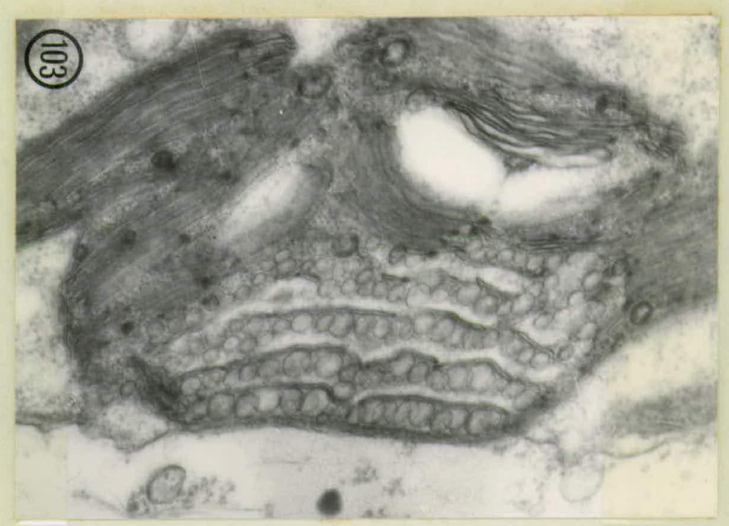

103

the median ranks possess 5-6 layers. It is tempting to equate the group of eyespots having more than 7 layers with the group of 4 somatic cells occupying the anterior rank in Eudorina illinoiensis in that the eyespot noted as having 7 layers of globules may, have in fact, had 8 in a more favourable plane of sectioning, and this would leave a significant gap in the distribution between eyespots having 6 layers of globules and eyespots having 8. Even as the figures stand this irregularity in the distribution is quite striking. Of the group having 5 and 6 layers of globules, one and two eyespots respectively had an abnormal site.

The organisation of the 'anterior' eyespot illustrated (Figs 93-96, Plate XXVII) is striking in its regularity, in no way departing from the typical structure described previously. Eight complete layers of globules of approximately equal diameter are seen, together with part of a smaller ninth layer; thus most layers will appear in all but the obliquest sections of such an eyespot.

In a 'median' eyespot the organisation may be less regular (Figs 97-110, Plate XXVIII). In the particular eyespot illustrated the globule layers are arranged as in text-fig. 5c. It can be seen from the sections at the margins that cytoplasmic microtubules run in close proximity to the eyespot (Figs 97-99, and Fig. 110). The majority of the sections show 5 layers of globules or indications that there are 5 layers. This is of considerable relevance when any conclusions are drawn based on a limited number of sections from a series concerning the spatial relationship of a cell in the colony relative to the globule layer number.

Glutaraldehyde fixation and ribonuclease treatment

The information derived from material fixed using glutaraldehyde has been mentioned above where appropriate; some general observations are now offered. There was apparently far less extraction of material after fixation in glutaraldehyde compared with fixation in osmium tetroxide, so that the granular content

of the cytoplasm and the chloroplast and mitochondrial stroma appeared very dense. The granulation of the cytoplasm appears coarser, but this proves to be due to a clustering of the individual granules. The mitochondria were difficult to see and could be made out only vaguely as the cristae show moderately clearly (Fig.111, Plate XXIX). The Golgi apparatus was strikingly preserved with the cysternae having a very regular shape; the vesiculate component was, however, not at all clear (Fig.113, Plate XXIX). The chloroplast lamellae appeared much more regular than when fixed with osmium tetroxide as the thylakoid spaces were not at all expanded, but the details of pyrenoid structure were much less clear (Figs 111, 112, Plate XXIX).

The effect of ribonuclease treatment was difficult to explain. Material was either very little affected (Figs 115, 116, Plate XXX) or was very strikingly extracted (Figs 117, 119, 120, Plate XXX). In the former case the only obvious difference between control material and treated material was the form taken by the mitochondria. The membranes and cristae were more clearly visible and the mitochondria as a whole were swollen. The cristae also appeared to have contorted into an elaborate system. In the latter case where the extraction was complete, the granular stroma of the chloroplast and mitochondria, the ground cytoplasm and the pyrenoid were removed leaving only a ghost of the chloroplast lamellae and mitochondria recognisable. The extraction of the pyrenoid did not, unfortunately, serve to elucidate the structure of that organelle further.

PLATE XXIX

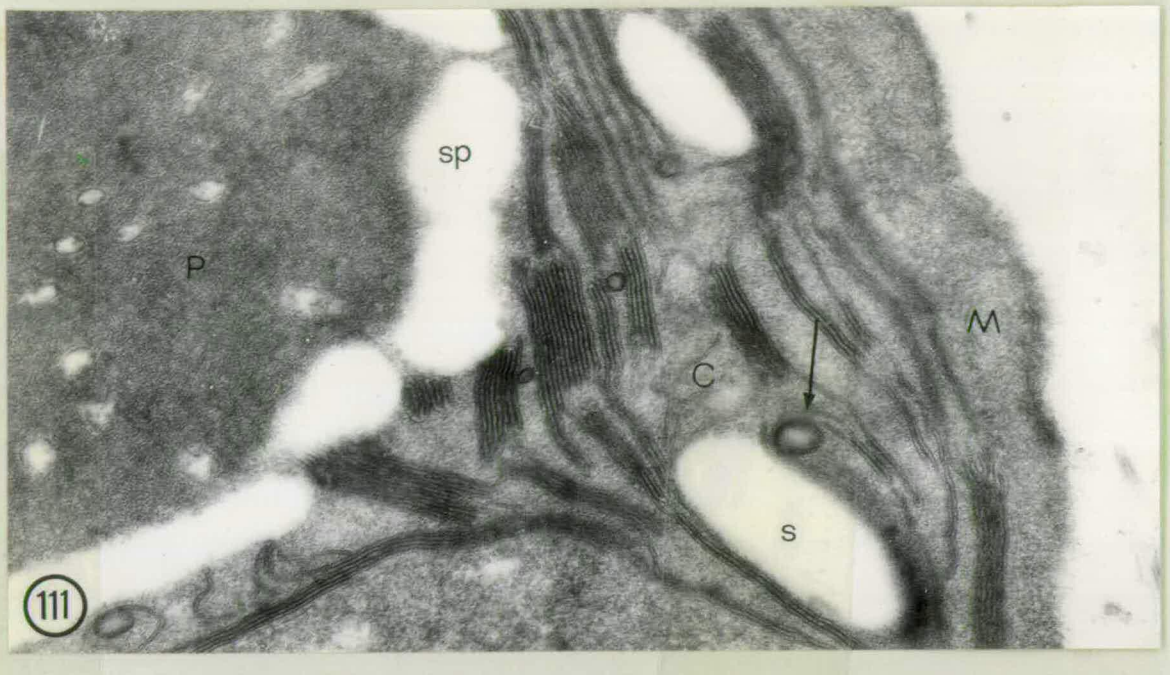
Figs. 111-113 are of material fixed in glutaraldehyde.

Fig. 111. General survey picture of part of a cell including chloroplast (C) and pyrenoid (P) and mitochondrial profiles (M). The preservation of the chloroplast lamellae is particularly uniform but no pyrenoid tubules are clearly visible. Mitochondria are visible mainly on account of their cristae; their limiting membranes are not visible. The granulation of the cytoplasm is coarser than that of the chloroplast or mitchondrial stroma. Starch plate (sp). Starch (s). Osmiophilic globule (arrow).
x 40,000 (x 16,000).

Fig. 112. Eyespot. The preservation is such that much detail seen using the routine fixation is obscured. x 40,000 (x 16,000).

Fig. 113. Golgi body cyternae. Vesiculate component obscure.
x 40,000 (x 16,000).

Fig. 114. Lipid droplets stained in 70% alcoholic solution of Sudan black B.
x 20,000 (x 8,000)



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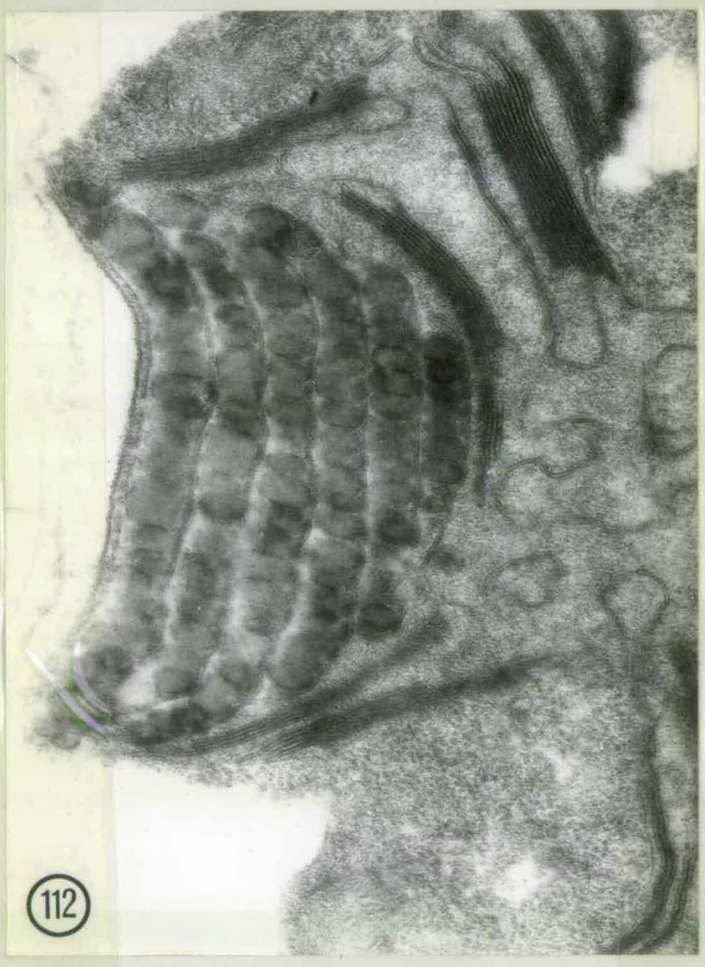

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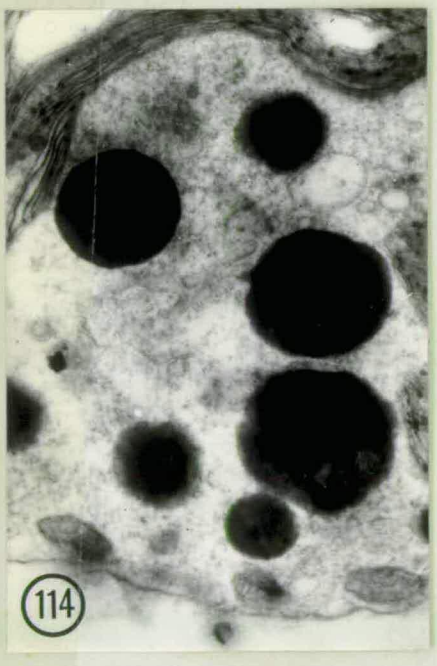

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PLATE XXX

Micrographs of material subjected to ribonuclease treatment.

Fig. 115. General survey picture. The mitochondria (M) are much enlarged and the cristae have an irregular form. The chloroplast (C) appears unaffected. Starch (s). Lipid droplet (l). x 40,000 (x 16,000).

Fig. 116. Chloroplast lamellae are apparently unaffected. Mitochondria swollen. x 40,000 (x 16,000).

Fig. 117. Completely extracted mitochondria. x 40,000 (x 16,000).

Fig. 118. Detail of chloroplast lamellae of 2 and 3 thylakoids. x 120,000 (x 50,000).

Fig. 119. Section of pyrenoid. Extraction has been complete. The chloroplast lamellae at the top of the figure are distorted. Pyrenoid tubule ghosts pass from the lamellae into the starch plate region. The formerly dense pyrenoid appears at the bottom of the figure. x 40,000 (x 16,000).

Fig. 120. Section shewing pyrenoid tubules crossing the starch plate region to enter the pyrenoid (top right). x 40,000 (x 16,000).

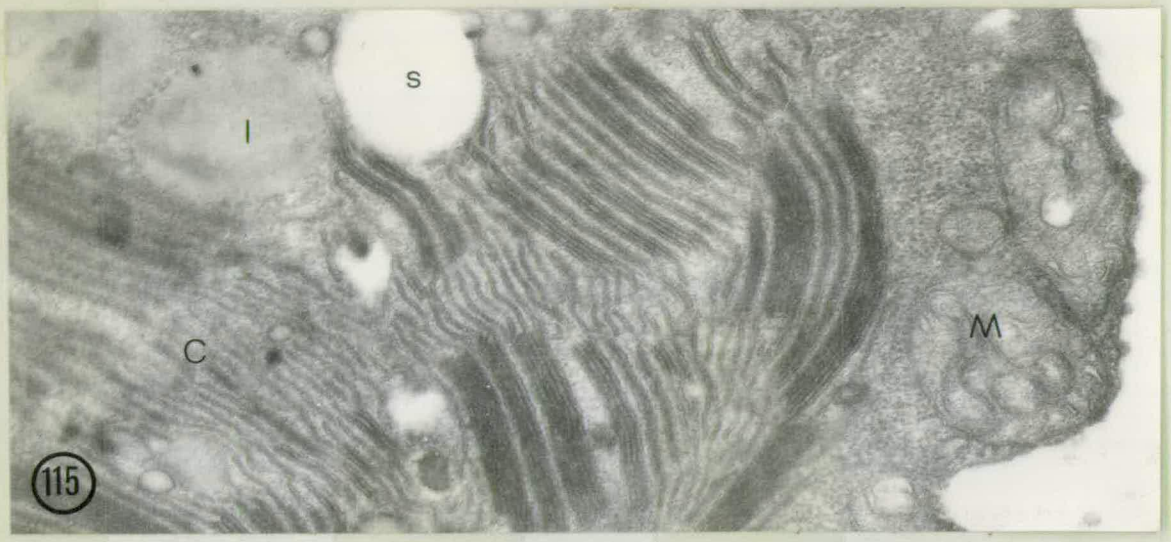
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PLATE XXXI

Light microscope pictures. x 600.

Fig. 121. Toluidine blue. Staining is general, but the nucleus is stained particularly deeply.

Fig. 122. Delafield's haematoxylin.

Fig. 123. P.A.S. test. The starch plates around the pyrenoids and isolated elongated starch granules are strikingly visible.

Fig. 124. P.A.A.S. test. Small round deeply stained profiles represent lipid droplets. The outer layer of the pyrenoid apparently possesses some lipid content.

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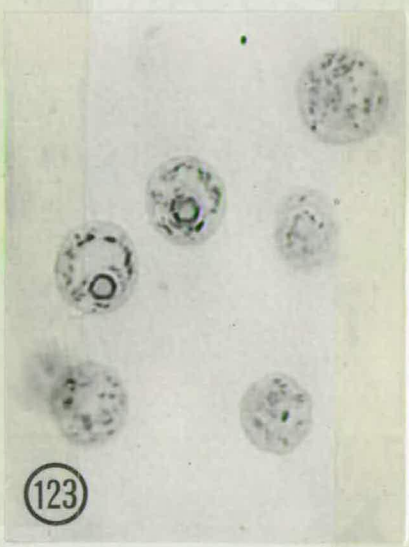

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DISCUSSION

Although much work has been done on the fine structure of the phytoflagellates as a whole, to an extent such that it is now possible to recognise, with reasonable certainty, taxonomic groupings from observations on their fine structure, there has been little detailed work within the Order Volvocales (see Pitelka, 1963). This is somewhat surprising in that the differentiation of the colonial forms of these algae has been thought at various times to offer a conceptual bridge with studies on early Metazoan development. The colonial Volvocales can be arranged in a series in which there is an increasing complexity of organisation and which has been considered by some authors to illustrate in living forms certain of the possible intermediate stages in the origin of the Metazoa (Hyman, 1940). This is not the place to review the present status of these theories, but the fact remains that little work has been done on the fine structure of any of the Volvocales.

The earliest comprehensive accounts on volvocacean fine structure are those of Sager and Palade (1954, 1957) for Chlamydomonas reinhardi. The flagellar apparatus of C.moewusii has been described by Gibbs, Lewin and Philpott (1958) with especial reference to the structure of flagellar mutants. Subsequently a number of workers have published papers on various aspects of the structure of the Volvocales, of which the more comprehensive are those of de Haller and Rouiller (1961), Ris and Plaut (1962), Joyon (1963), Lang (1963a), Lefort (1963) and Joyon and Fott (1964).

From the present study a picture of the structure of the Eudorina cell can be built up which in general conforms with that derivable from the literature as typical of the volvocacean cell. In detail a number of points worthy of mention emerge which have not been dwelt upon by previous authors, and it is these which will be considered in the discussion.

Concerning the flagellar apparatus the structure conforms in general to that described by Gibbons and Grimstone (1960) which varies little in a very wide variety of types (Sleigh, 1962). More specifically the additional features which have been shown characteristic of the flagella of green algae are present.

The peculiarity of the flagella of the green algae resides in the transitional region where the so-called cylinder is found (Lang, 1963b; Manton, 1963b; Ringo, 1967). Transverse sections of this region show a characteristic stellate pattern in which fine fibrils link the A sub-fibres of the axoneme outer double fibres with a circle of dense material (text-fig. 3d). Manton (1964b) illustrates a transverse section of the transitional region of Stigeoclonium (Chlorophyceae) in which the dense material is made up of a circle of nine granules and another transverse section of the transitional region of Heteromastix (Prasinophyceae, non sensu Christensen, 1962, see Manton, Rayns, Ettl and Parke, 1965) in which the dense circle is complete. It is considered that although not identical these stellate patterns may be readily recognised as having essentially the same structure. It is thus of interest that Ringo (1967) found both these types of profile in transverse sections of the transitional region of Chlamydomonas reinhardi. In a text-figure he indicates the 'Heteromastix'-like profile above the diaphragm which divides the cylinder two-thirds of the way down and the 'Stigeoclonium'-like profile below the diaphragm, and with this interpretation the present author agrees on the basis of measurements on his material and on the published micrographs in question.

Additional basal bodies which appear frequently in close proximity to the regular basal bodies have been described by a number of workers and seem to herald the division of the cell. One additional basal body was found in the uniflagellate Chromulina pusilla (Manton, 1959; subsequently re-named Micromonas pusilla, Manton and Parke, 1960) and two were found in the biflagellate Prymnesium parvum (Manton, 1964c). In both cases the appearance of these basal bodies was associated

with the beginning of cell division. Ringo (1967), working on Chlamydomonas reinhardi, and the present author both encountered additional basal bodies. In Eudorina these appear even in young cells, in which case it seems that in this genus replication of the basal bodies may take place long before cell division. In a detailed study of replication of the flagellar apparatus in Chlamydomonas reinhardi, Cavalier-Smith (personal communication) found more than two additional basal bodies in the cell just prior to division. In an asexual cycle where there are four division products ultimately the theoretically expected number of additional basal bodies is six.

The relationship of the regular basal bodies in Eudorina is at variance with the condition described for Chlamydomonas reinhardi (Sager and Palade, 1957; Ringo, 1967), C.moewusii (Gibbs et al, 1958), Chlorogonium elongatum (de Haller and Rouiller, 1961) and Stephanosphaera pluvialis (Joyon, 1963) amongst the green algae. In all these cases the basal bodies of the two flagella are placed with the proximal ends very close indeed, and they are connected by sets of fibres. The orientation is such that the angle between the longitudinal axes of the two flagella as they emerge is greater than 90°. Only the description of Ringo (1967) goes into any great detail. Two sets of fibres are described, one at the proximal end of the basal body and one at the distal end. Underneath the latter lies a flattened plate at which the flagellar root microtubules originate. Unfortunately Joyon's description (1963) of the basal body region of the colonial form Volvox aureus is not sufficiently detailed for comparison with Eudorina studied here. The differences between the structure which appears common to the unicellular forms of Volvocaceae reside in the proximity and orientation of the basal bodies and in the structure of the linking-fibre system. Whereas in the published micrographs of the apical region of Chlamydomonas reinhardi (Sager and Palade, 1957; Ringo, 1967) the basal bodies are in virtual contact, in the present study they

are about 0.09μ apart at their closest approach to one another, and the angle of intersection of their longitudinal axes is about 65°. The fibre system linking the basal bodies is much less elaborate and consists of a single band at the proximal end. No trace of the plate which occurs beneath the distal transverse fibres in Chlamydomonas was seen in the present study with Eudorina.

Information concerning the flagellar root system comes from incidental observations, as no special study of it was made. Flagellar roots are found widely in motile algal cells, varying in the numbers and arrangement of the root microtubules. Thus there are alternately roots having two and three microtubules in the four-root systems of the uniflagellate Pedinomas minor (Ettl and Manton, 1964) and of the biflagellate green alga Chaetomorpha (Manton, Clarke and Greenwood, 1955). In the zoospores of Draparnaldia and Stigeoclonium, which also have four roots, roots having two and five microtubules alternate (Manton, Clarke and Greenwood, 1955; Manton, 1964a). Chlamydomonas reinhardi has four roots with four microtubules (Ringo, 1967). Nothing conclusive can be said about Eudorina concerning the number of root microtubules, but if the interpretation of the micrograph in Fig. 86 is correct it would seem that one at least has five tubules. In another series of sections seven root microtubules could be traced through a series of sections estimated at 20, but these were in two adjacent groups of three and four which maintained the spacing within groups, and it may well be that the profiles represented two roots which were running parallel to one another. The arrangement of the root microtubules at the apical end is not known, but it was possible to detect the separation of microtubules from the group and to detect single microtubules in parts of the cell far distant from the apical region.

The cytoplasmic microtubule system is made up of microtubules of about 20 mμ diameter and unknown length running beneath the cell membrane. Similar microtubules are found in a wide variety of animal and plant cells. Described

originally in animal cells, they were subsequently described in detail in plant cells by Ledbetter and Porter (1963) but earlier accounts had been given by Manton (1957, 1959). The occurrence of microtubules has been extensively reviewed recently by Pitelka (1966), and by Behnke and Forer (1967) who recognise four classes of microtubules by their reaction to such treatment as negative staining and pepsin digestion. It had been generally considered that osmium tetroxide fixation did not preserve cytoplasmic microtubules and that they were to be expected only after glutaraldehyde fixation, but in fact Manton (loc.cit.) used osmium fixation, and so did the present author. Behnke and Forer point out that the temperature of application of the fixative is likely to be a relevance, for in the cold the cytoplasmic microtubules disappear as a cellular response; osmium tetroxide is commonly applied in the cold. In the present study it was applied at room temperature, as it was by Manton.

Various propositions have been made concerning the function of the cytoplasmic microtubules including involvement in cell motility and in cytoplasmic streaming, that of skeletal support and function as spindle fibres and for the transport of water and small ions. The class of cytoplasmic microtubules is certainly more diverse than the other three classes which are all microtubules of the flagellum. Behnke and Forer, it should be noted, do not mention root microtubules in their classification. It will be remembered that the electron density of these and the cytoplasmic microtubules differs strikingly. Ringo (1967) has ascribed to the flagellum root microtubules the function of distributing the stresses incurred during flagellar beating, but makes no mention of a cytoplasmic microtubule system. Subpellicular microtubules attached to kinetosomes are common in ciliates and flagellates and this apparently includes all phytoflagellates (Pitelka, 1966). Organised microtubules are rare in flagellates but common in ciliates where their function is thus considered to be the maintenance of cortical

rigidity as in Paramecium. In Opalina, however, Wessenburgh (1966) argued that they have the function of maintaining the cortical relationships. Where there is attachment it is perhaps more likely that there is a functional relationship as with the postulated transport system function which has been ascribed to the microtubules found in association with the Golgi apparatus in Hydra (Slautterback, 1963). Furthermore it is suggested that to give skeletal support the microtubules would have to be much more numerous and ordered as in the ciliates. Where, as with Eudorina, a diverging system spreading from the basal bodies occurs, some other function such as that of a transport system seems more likely. It is conceivable that some control could be exerted on the flagellar beating by the mediation of such a system, but it will be remembered that the root microtubules are also observed to bear a relationship to the eyespot in some cases. In the Stigeoclonium zoospore a similar relationship exists (Manton, 1964a). As the eyespot is considered to be the photoreceptor and Eudorina is positively phototropic, it could be that a root so placed could provide an equally good transport system, if not a better one, as in the root a number of microtubules run in parallel whilst the cytoplasmic microtubules are more widely spread out. However, it appears that the root comes into a relationship with the eyespot only infrequently - in 2 of the 20 series of eyespots resulting from the serial sectioning work - and thus it is probably a chance occurrence and the cytoplasmic microtubule system must still be considered as the primary transport system if indeed any such function is being carried out by the microtubules.

Closely associated with the flagellar system is the mitochondrial system. The point of interest here lies in the elaborate branching form observed in the mitochondria of the apical region. This region is one of high expected oxidative activity and this would provide an explanation for the concentration of mitochondria

in it. Previous descriptions of the fine-structure of Volvocaceae give few details of the mitochondrial system, but Sager and Palade (1957) do mention that 'branching mitochondria are sometimes encountered'. In Euglena spirogyra two states of the mitochondrial complex occur. Cells taken in the dark period of a day-night culture cycle have a mitochondrial system in which many rod-shaped mitochondria are found dispersed generally in the cytoplasm, but cells taken in the light period have a reticulate mitochondrial system in the superficial part of the cytoplasm; however, the system is one of considerable lability in which branches are continually being formed and broken (Leedale, Meeuse and Pringsheim, 1965). In the present study colonies were harvested during the light period so by comparison with Euglena branched mitochondria might be expected.

No detailed study of the nucleus was undertaken but certain new observations have been made concerning the nucleolus, which has fine fibrils of diameter 5 mμ running through it. Godward and Jordan (1965) failed to see any such structure in the nucleolus of Spirogyra sp., reporting only 15 mμ 'fibrils', but in the nucleolus of Bradysia (Sciaridae) and Smittia (Chironomidae) (Jacob and Sirlin, 1963, 1964) similar fine fibrils have been found, although in both these cases the fibrils were associated in pairs and one explanation suggested was that they represented chromosome fibrils which had become incorporated in the nucleolus, having originally initiated its formation. A second explanation offered was that the fibrils consisted of newly synthesised R.N.A. on the basis of their D.N.A.ase resistance.

The very extensive corpus of literature concerning work which has been performed on chloroplast structure has been fully reviewed by Kirk and Tilney-Bassett (1967). The structure found in this study on Eudorina conforms, in general, with that previously described in other Volvocaceae (Sager and Palade,

1954, 1957; Gibbs, 1962b, 1962c; Joyon, 1963; Lefort, 1963). However, the condition of the outer membrane of the chloroplast envelope is unusual in the way it is found to be extended over a small area and thrown into folds. No reference to such a condition is known to the present author.

A second differentiation of the chloroplast envelope which also appears to be a new observation is that of the extensive folded or tubular extensions involving both the membranes. These extensions were most frequently empty of contents in which case the two inner membrane profiles were in close proximity, or contained a little stroma or a few thylakoids. It is thought that the folded extensions are most common as circular profiles were rare and long profiles frequently appeared in sections. Unfortunately this observation has not been related to any stage in the life history of the cell, and it is impossible to say from the electron micrographs whether a growth or regression of the chloroplast is involved. However, it will be remembered that the chloroplast of mature cells is much divided, but that this is not the case in young cells. The extensions are found in what are judged to be young cells, so it is considered that the extensions represent a growth of the chloroplast. This means that there is asynchronous and local growth of the parts of the chloroplast: firstly the envelope extends and secondly the stromal substance increases and thylakoids enlarge, to invade the extension of the envelope. Such a means of growth of the chloroplast would explain the lobulation; in many cases major lobes in the chloroplasts of mature cells are seen to be connected by bridges formed by the close apposition of two envelope profiles and having only a little stroma between them. Invasive growth of the thylakoids can be inferred from the occurrence of isolated envelope profiles which are empty except for a few thylakoids and from profiles in which thylakoids extend into the proximal part of an extension.

The most complete recent work devoted to the ultrastructure of the pyrenoid is that of Gibbs (1962a, 1962b), but many incidental references are to be found in general studies of the chloroplast (Leynon, 1954; Ueda, 1961; Gibbs, 1962c). Although in many of these descriptions tubules which were considered to be derived from the chloroplast lamellae are described as penetrating the pyrenoid, no attempt has been made to elucidate their structure or to relate them to the lamellae. More frequently pyrenoids are traversed by lamellae consisting of a small number of thylakoids, but in other cases such as in Scenedesmus quadricauda (Bisalputra and Weier, 1964), Chlorogonium elongatum (de Haller and Rouiller, 1961) and Micromonas squamata (Manton and Parke, 1960) no lamellar structures enter the pyrenoid.

The green algae show a greater variation in the ultrastructure of the pyrenoid than any other division. Gibbs (1962b) traces a series in which there is a progressive reduction in the lamellar structures crossing the pyrenoid. Examples of species having the extreme condition in which no lamellae cross the pyrenoid have just been given. The pyrenoid of Pyramimonas is traversed by lamellae which at the periphery consist of 2 to 4 thylakoids, but these are reduced to single thylakoids near the centre. A similar condition was reported in Spirogyra The next stage in the series of reductions involves the number of lamellae entering and crossing the pyrenoid which may be reduced to such an extent that only a single lamella crosses the pyrenoid as in Chaetomorpha linum and Rhizoclonium tortuosum. In Stichochrysis (Chrysophyceae) the single thylakoid may be reduced to a tubule at the side opposite its entry. Gibbs (1962b) reports that in Chlamydomonas moewusii lamellae with two thylakoids enter the pyrenoid and are reduced in width rather than in number. This accounts for the occurrence of ovoid profiles. In Gibbs' terminology the lamellar extensions comprise double discs (i.e. two thylakoids); however, it is reported that the central dark line of the profiles

is frequently not parallel to the limiting membranes but shows undulations, which is suggestive of a condition in which the pyrenoid is penetrated by a tubule in which a free rod-like invagination runs, as is suggested to be the case in Eudorina in the present study. The undulating course of the dark central profile described by Gibbs could be a fixation effect, but it would be easily explicable if it did indeed represent a free extension (intucking) of the lamella which was not in connection with the pyrenoid tubule wall at the level of sectioning, but only at the level of the starch plates surrounding the pyrenoid. Unfortunately Gibbs does not publish any micrograph which shows a clearly transverse section of a pyrenoid tubule. The present study has shown that a large number of pyrenoids have to be sectioned in order to obtain such sections. Chlamydomonas reinhardi has tubular profiles in the pyrenoid which contain dense profiles as described here (Cavalier-Smith, personal communication). Indications of this structure are easy to detect in retrospect from the work of Sager and Palade (1954, 1957) on Chlamydomonas reinhardi, Joyon (1963) on Volvox aureus, Lang (1963a) on Gonium sociale, Volvulina pringsheimii and Volvox aureus and Leynon (1963) on Pandorina morum. Sager and Palade, and Lang found branching tubules and Joyon, although he was unable to find branches, assumed them to exist; Leynon makes no mention of branches. The published micrographs show circular, elliptical or ovoid profiles with what are apparently thickenings spaced fairly regularly; the better preservation of this structure achieved here is a measure of the advances in fixation and embedding techniques over the past few years. The condition found in Chrysochromulina strobilis (Chrysophyceae) is of interest, for here the lamellar extensions of the pyrenoid are tubular and run in pairs (Parke, Manton and Clarke, 1959). In oblique section they give a profile of two light lines with a thick dense line between them; this condition is, of course, that postulated by Gibbs (1962b) to occur in Chlamydomonas moewusii. The volvocacean Dunaniella has a

similar structure with paired tubules and Rhodosorus marinus (Rhodophyceae) also has a pyrenoid penetrated by a branching system of apparently empty tubules which in this case arise from the chloroplast lamella which completely surrounds the pyrenoid (Giraud, 1962). Usually, however, the Rhodophyceae have pyrenoids which are traversed by lamellae (Gibbs, 1962a; Giraud, 1962).

It is evident that tubular configurations of the lamellae are not uncommon, but the pyrenoid tubule structure described here is one of considerably greater complexity than has been described by any previous author. It is difficult to decide whether to consider it conceptually as comprising a single thylakoid of unusual shape (i.e. cylindrical, not flattened) which contains intuckings of the limiting membrane, or as a stack of thylakoids in which the spaces have become confluent at both sides and the formerly prominent apposed membranes have been much reduced to leave only remnants (the compact dense profiles within the tubule). The tubule would thus represent a number of thylakoids one greater than the number of contained profiles. The author favours the second suggestion.

Although it has been claimed that pyrenoid structure bears no relation to systematic position (Ueda, 1961) it does seem that in the chlamydomonad cell it has a characteristic structure. Gibbs (1962a) is able to propose characteristic structures for the pyrenoids of a number of divisions of non-green algae which, as with the green algae, differ in the nature and extent of the lamellar system. In most the pyrenoid is crossed by a lamella or lamellae of a small number of thylakoids; however, tubules again occur as in Nitzschia dissipata (Bacillariophyceae).

Eyespots are found commonly in motile algal cells including both the simple motile forms and the gametes and zoospores of non-motile forms (Smith, 1950). The eyespot is usually situated at the anterior end of the cell and appears as a

red structure when viewed with the light microscope. It can be a separate differentiation as in Euglena (Gibbs, 1960: Leedale et al, 1965) but is more frequently a differentiated region of the chloroplast lying just beneath the cell membrane as in the majority of green and brown algae. The Chrysophyceae have a characteristic eyespot in which a single layer of pigment globules bear a special relationship to an expansion of the flagellum. In Chromulina psammobia which had been considered as uniflagellate a second expanded flagellum was discovered completely enclosed in an invagination of the cell surface and lying above the eyespot which comprised a single layer of loosely packed globules not underlain by a thylakoid (Rouiller and Fauré-Fremiet, 1958). In Dinobryon divergans (Chrysophyceae) one flagellum is conspicuous and the second is not, being short and lying in a deep groove in the cell surface above the eyespot which encompasses the groove and is thus trough-shaped. Immediately distal to the basal body this flagellum is expanded on the side of the eyespot. The eyespot globules are underlain by chloroplast lamellae (Joyon, 1963; personal communication). A flagellar swelling is also associated with the eyespot in Euglena, but in this case it is the locomotory flagellum which is swollen whilst still in the reservoir as it passes the level of the eyespot which has one or two layers of pigment globules.

Brief descriptions of the eyespots of a number of families can be found in the literature. In general eyespots consist of one or a few layers of globule underlain by chloroplast lamellae, although this is variable. The globule layers of non-colonial forms tend not to have a pronounded curvature but where they are curved the convex surface is often presented to the exterior. Chlorogonium elongatum has an eyespot with a single layer of globules (de Haller and Rouiller, 1961) as does the zoospore of Stigeoclonium (Manton, 1964a). The largest eyespots occur in the Volvocaceae (thus Chlamydomonas reinhardi has an eyespot with 2 to 3 layers of globules), but more especially in the colonial volvocales.

The eyespots of the different cells within the colonial form Eudorina illinoiensis have been shown to vary considerably in their structure and in the congruity of their development. The structure comprises a number of curved layers (often deeply so) of pigment globules with the concave surface directed to the exterior, each of the layers being underlain by a single chloroplast thylakoid. The variation in structure exists mainly in the number of layers of pigment globules which make up the eyespot. The number varies from 2 to 9 and it is clear that the variation in the size of the eyespots relative to the position of the cell within the colony (which has been extensively reviewed in the introduction) is a reflection of this variation in the number of globule layers. The observation that the eyespots with fewer globule layers are not proportionately less in diameter implies that the size distinction is clearest when the electron microscope is used to view the eyespots in cross-section, whereas it is correspondingly more difficult to observe the difference in size when the light microscope is used to view the eyespots in living material. It is believed by the author that, given an electron micrograph which shows a near median section through an eyespot, it is possible to identify the relative position of the cell from which it came, but probably not the actual rank occupied. Although the amount of information on globule layer numbers derived from some 20 of the eyespots of a single colony is small if any generalisations are to be made, it is perhaps significant that there is a break in the distribution curve of eyespot globule numbers plotted against frequency between 6 and 8 layers which might possibly represent the difference between the condition in the somatic cells of the anteriormost rank of 4 cells and the gametic cells which have fewer globule layers. The numbers of eyespots with 4, 5 and 6 globule layers are approximately equal whilst the number with only 3 layers was small. This might be thought indicative of the organisation of the eyespots in the ranks of gametic cells: one might speculate that the tendency was for the anterior rank of eight gametic cells to have 6 globule layers, the next 5 layers, the posterior

rank of 8 gametic cells, 4 layers and the posterior rank of 4 gametic cells, 3 layers. This can, on the present information, be taken only as an indication of what might be the eyespot structure relative to cell rank.

Unfortunately no other similar study of a colonial form has been made, so all references to eyespot structure are incidental. The eyespot of Pandorina morum is reported to have from 3 to 5 globule layers (Leynon, 1963) but no information exists on whether this variation may be related to cell position. Smith (1920) states that in the posterior of the four tiers of cells in P.morum the eyespot is absent and that in successively anterior tiers they measure 1μ, 2μ and 3μ. It is conceivable that the three size groups correspond with the three globule layer groups of 3, 4 and 5 layers. The present author would expect that rudimentary or misorientated eyespots will be found in the posterior rank of cells on a closer examination by electron microscopy, which would not be visible by light microscopy. Lang (1963a) describes the eyespot as consisting of from 3 to 8 layers of globules, but as she does not state whether this is general for all the genera of Volvocaceae and the unique genus of Astrephomenaceae studied the information is of little value in the present context. The eyespot of Volvox aureus has been described by Joyon (1963) and Dolzman and Dolzman (1964) as having up to five layers of globules. Sphaerellaceae are described by Joyon (1963) as having eyespots with 3 to 4 layers of globules. In the colonial form Stephanosphaera fluvialis, in which the 8 cells are arranged equatorially, the curvature of the globule layers is pronounced with the convex surface presented to the exterior. The curvature is such as to raise the eyespot above the general surface level in a prominence. It would be of great interest to learn whether in such a form all the eyespots do, in fact, have an identical structure as the author suspects. Similarly it is not expected that the eyespot structure in Gonium, where the colony has the form of a curved plate, will vary greatly, but in Stephanoon where the cells alternate in two equatorial tiers it might be expected that the

eyespots of the anteriormost tier will tend to be larger and to possess more globule layers. The condition in Volvulina might be expected to be similar to that in Pandorina, but more striking differences are expected in the structure and size of the eyespots of the colonial forms where the colony does not have a compact form, the cells are further apart, and in which a greater degree of somatic differentiation is shown. This is especially true in Volvox sp. where further investivation is called for. Also of great interest will be to examine the condition of Eudorina sp. considered to be lower and higher in the degree of specialisation than E.illinoiensis ás judged by the criterion of their somatic differentiation.

The deep-sited eyespots are thought by the author to be of two kinds. The first is considered to be a regular eyespot derived from the mother-cell. Although deep-sited these appear at the margin of the chloroplast and are well organised. It is common in light microscope observations to find one cell of a young colony having a very large eyespot and the reasonable assumption is that this is the eyespot of the mother-cell. The disorganised deep-sited eyespots could also be derived from the mother-cell but as they tend to be situated deep within the chloroplast it is thought that they represent more probably de novo accumulations of pigment globules. There is certainly a tendency for eyespot-like differentiations to organise in the chloroplast. Small groups of globules become aligned and are underlain by single thylakoids. The orientation of these 'eyespots' is frequently such that they face into the cell.

It is open to question whether deep-sited eyespots have any photoreceptive ability. A functional relationship undoubtedly exists between the eyespot and the flagella but in the absence of a physical connection presumably some transport system is required for the relationship to operate; the suggestion has already been made that the cytoplasmic microtubule system might serve such a function. If this is so it may well be that a deep-sited eyespot does not act as a

photoreceptor or acts with reduced efficiency, as the cytoplasmic microtubule system is much less extensive deep in the cytoplasm.

The globules which form part of the eyespot are ubiquitous in algae and higher plants, but it is only in the algae that they are organised into photoreceptors. They are thought to be the sites for the accumulation of lipid soluble pigments (see Greenwood, Leech and Williams, 1963). The variation in globule layer number would seem to indicate a quantitative reaction on the part of the photoreceptor in response to light. Gross changes in orientation would presumably affect this reaction as well, and it is thus not surprising that misorientated eyespots are to be found in posterior parts of the colonial forms.

Gerisch (1959) made a light microscope study of Eudorina californica (Pleodorina sensu Gerisch) in an effort to find the means whereby this coenobial form could swim 'purposefully' and came to the conclusion that this depended upon the relative positioning of the eyespot and the flagellar bases. The flagellar beat he described as parallel to that plane joining the eyespot with the midpoint between the basal bodies, the position of the eyespot being variable relative to the basal bodies. It is considered likely from the various studies on flagellar roots that the position they occupy relative to the basal bodies is constant; this would give further weight to the argument that the cytoplasmic microtubules provide the transport system for inhibitory or stimulatory substances derived from the eyespot, if such is the mode of action of the eyespot, for these microtubules radiate evenly from both the basal bodies. The relative positions of the flagella and the eyespot would not be of relevance with the widespread cytoplasmic microtubule system, but would affect one in which there was a localised distribution as with the root microtubule system, at the level of the eyespot. For the working of the scheme Gerisch has proposed in which the relative positioning of the eyespot and basal bodies varies in the different tiers of

cells, a genetic determination or cellular differentiation is implied. The plakeal division sequences described by Goroshankin (1875) and Pascher (1927) in the formation of a 32-cell colony give indications of cellular differentiation in that the central cross cell derivatives formed at the fourth division (the 16-cell stage) divide no further and the number of cells is made up to 32 by additional divisions of the corner cells (text-fig. 2). It is the central cross-cell derivatives which in a 32-cell colony form the anterior rank. The mechanism for such a differentiation becomes increasingly difficult to visualise in colonial types with large numbers of cells. It may be significant in terms of the co-ordination of the largest Volvox species that these are members of the sections possessing cytoplasmic connections between the cells and it is the generally smaller Merrillosphaera which lack cytoplasmic connections.

In conclusion it must be admitted that this study has raised more problems than it has answered, and these are problems of great complexity. It will be very instructive in the first instance to investigate how the globule layer number varies with the position a cell occupies in the colony in other colonial forms. Following this the mode of action of the eyespot requires investigation with especial consideration of the putative transport system, as does the means of determination of the combined eyespot and flagellar system.

APPENDIX I

Preparation of soil-extract cultures [after Cambridge Culture Collection notes]

The basic medium, e, is made by putting garden loam (free from artificial fertilisers and weed-killers) into a flask, adding water until the volume of supernatant water is twice that of the soil, and steaming for a period of 3 hours. This may be done conveniently in a domestic pressure cooker, leaving the valve open. After allowing the soil to settle the supernatant liquid is filtered and stored with preservative (about 2% of the volume of the extract).

The preservative (Droop, M.R., personal communication) consists of a mixture of

o-fluorotoluene	1 pt.
1 chlorobutane	1 pt.
1:2 dichloroethane	2 pts.

Medium B.E.e+s consists of a mixture of beef extract, soil extract and salts dissolved in distilled water

Beef extract (Oxoid 'Lab-Lemco' L30)	0.1%
Soil extract, e	10.0%
KNO_3, Analar	0.02%
K_2HPO_4, Analar	0.002%
$MgSO_4$ 7 H_2O, Analar	0.002%

The salts may be kept in a stock solution, s, 100x concentration in medium. Preservative is also added to this solution.

The medium is best made up in small quantities as required, and sterilised by light autoclaving. Five min. at 15 lb was found invariably to be sufficient to ensure sterility. All traces of the preservative are removed during autoclaving.

Soil-plus-water medium used for non-sterile culture is made by placing 1 cm of garden loam as specified above, together with a marble chip, in test tubes then adding distilled water until there is about 8 cm of supernatant, stoppering with non-absorbent cotton wool and steaming for a period of one hour on two consecutive days. The medium, the pH of which is about 7, should be allowed to stand a further day before inoculation.

Sterility test medium [after Provasoli, McLaughlin and Droop, 1957]

Soil extract, e	2.5 ml
KNO_3, Analar	5.0 mg
K_2HPO_4, Analar	0.5 mg
Liver infusion (Oxoid L25)	25.0 mg
Bacto tryptone	25.0 mg
Glucose, Analar	25.0 mg
Distilled water to 100 ml.	

APPENDIX II

Recipes for light microscopical stains and reagents

Toluidine blue stain

Dissolve 0.5 g toluidine blue and 1.0 g $Na_2B_4O_7 \cdot 10H_2O$ (Borax) in 100 ml aq.dist.

This recipe happens to be half the strength of that given by Meek in discussion of a paper by Mercer (1963). Charles (ibid) emphasises the need for using toluidine blue at a high pH for staining Araldite embedded material, but an objection to this is the instability of the stain under such conditions, and Pease (1964) recommends in general that stains be made alkaline by being dissolved in dilute borax solution rather than in water, for more effective staining of resin embedded material.

Delafield's Haematoxylin (Johansen, 1940)

Add 4.0 g haematoxylin to 25 ml 95% ethanol and add this drop by drop to 400 ml of a standard aqueous solution of ammonium aluminium sulphate. Allow to stand exposed to air and light for 4 days to 1 week. Add 10 ml glycerine and 100 ml methanol. Allow to stand exposed to air for several months until the colour is a dark wine shade. Filter before use.

Schiff's Reagent (after de Tomasi, 1936)

Dissolve 1.0 g basic fuchsin in 200 ml of boiling distilled water. Shake well and cool to 50°C. Filter and add 20.0 ml NHCl. Cool to 25°C and add 1.0 g sodium metabisulphite ($Na_2S_2O_5$). This solution should be allowed to bleach for 24 hours in the dark in a tight stoppered bottle. It was not found necessary to use the decolorising carbon usually advocated. Store the solution in the dark at 4°C, but allow it to assume room temperature before use.

Peracetic acid reagent (Lillie, 1954)

To 95.6 ml glacial acetic acid add 259.0 ml 30% H_2O_2 and 2.2 ml conc. H_2SO_4. Allow to stand for 1-3 days. Add 40 mg di-sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$) as a stabiliser. Store at 0-5°C.

APPENDIX III

Reagents and stains for electron microscopy

Unbuffered osmium tetroxide fixative

Dissolve 0.1 gm OsO_4 in 0.2 ml carbon tetrachloride; osmium tetroxide dissolves readily in carbon tetrachloride.

The specific gravity of carbon tetrachloride with respect to water is 1.595, so the weight of 0.2 ml is 0.319 g: thus the fixative mixture contains approximately 30% osmium tetroxide by weight.

Combined uranyl acetate and potassium permanganate stain

Dissolve in a 1% $KMnO_4$ aqueous stock solution 0.25 g uranyl acetate per 10 ml, one hour before required. Filter before use.

Staining procedure: stain for 20 minutes. Rinse in two changes of distilled water. Wash in a weak citric acid solution (1 drop of a 5% solution per ml of distilled water) for 30 seconds. Rinse in two changes of distilled water.

All chemicals used should be of reagent grade.

Staining and washing is done by floating the grids face downwards on the liquid surface. The stain and other liquids are covered to keep the surface free of dust, the covers being removed only as necessary to transfer grids. This may conveniently be done using a small spatula; the grids are picked up on small drops of liquid, held by surface tension on both the upper and lower surfaces of the spatula. When it is lowered vertically on to the next liquid surface, the grids float off. Only very rarely are they wetted, causing them to sink.

All glassware and implements used in staining must be scrupulously clean.

Serially-sectioned material was stained in series of cavity slides having triple depressions so that the grids were kept in order.

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