Electron Microscopical Studies of Frozen-Dried Yeast

IV. *Schizosaccharomyces*, *Nadsonia* and *Saccharomycodes*

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The periodate - S c h i f f reaction has been used to localize polysaccharides in frozen-dried specimens of several genera of the Endomycetales, of which three representatives are here described. No PAS reactive cell wall component was detectable in *Schizosaccharomyces*, in contrast to the distinctly reactive cell wall layer in *Nadsonia*, *Saccharomycodes* and *Saccharomyces*. These findings are in agreement with independent data from electron diffraction studies and analytical chemistry of these genera. These, and other observations suggest possibilities of phylogenetic research with the electron microscope in conjunction with cytochemical methods for comparative submicroscopic localization of cell wall polysaccharides and the study of morphological detail during budding or other types of cell division.

The structure of starch, the vacuole and its contents, and vesicular nuclei are described briefly.

Basophile staining of *Nadsonia*, in particular, disclosed bodies tentatively identified as mitochondria pending cytochemical localization of characterizing enzymes. These structures possessed internal lamellations, seen as negative images, seemingly corresponding to typical osmiophilic cristae. Dense, basophile particles measuring about 60 Å, hitherto not described in the mitochondrial literature, occurred in the spaces between the lamellations.

Part IV of these studies comprises brief descriptions of representatives of three genera of the Endomycetales. It forms a portion of a comparative study of yeasts, yeastlike and other fungi undertaken by the author with the view to exploring the usefulness of various cytochemical criteria in ascertaining phylogenetic relationships among these forms as well as others of more distant affinities. The type of information on which work of this nature depends is primarily physicochemical, such as X-ray diffraction and analytical chemistry of the organisms concerned, besides the guidance lent by information from the conventional methods of mycological taxonomy [1, 2]. With the exception of aid from the latter methods, which may not always prove to be inerrant, supporting data allowing definite conclusions are unorganized and too meager at present, and must be enlarged if the possibilities of an ultrastructural physicochemical approach to extensive taxonomic groups are to be fruitful. However, sufficient information exists on certain organisms which may be used as a basis for initial work. Results hitherto obtained from these studies suggest that generic differences or resemblances in cell composition in relation to structure, not otherwise resolvable by light microscopy or revealed by the usual methods of metallic staining for electron microscopy, may provide useful clues supplementing or opposing current ideas on evolutional relationship among microorganisms.

In the present work, only those salient aspects of fine structure which seem the most pertinent to a scheme of comparative morphology will be described. Of the several cytochemical criteria of potential value, two major ones – cell wall composition and basophile staining of protoplasm – have previously been studied by the author in sufficient detail in *Saccharomyces* [3, 4, 5, 6] to enable their extension to other genera of yeasts.

It was shown that satisfactory image contrast was obtainable in cells of *Saccharomyces* stained with organic compounds. When the staining was performed in conjunction with a series of essential controls to establish the significance of image density differences following various treatments necessary in interpreting the observations, certain cellular components could be localized both chemically and morphologically with the electron microscope. In this manner, the principally known yeast polysaccharides, in-

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[1] E. GAUMANN, Vergleichende Morphologie der Pilze. Jena 1925.

[2] E. GAUMANN, Die Pilze. Grundzüge ihrer Entwicklungsgeschichte und Morphologie. Verlag Birkhäuser, Basel 1950.

[3] B. MUNDKUR, Exp. Cell Res. **20**, 28 [1960].

[4] B. MUNDKUR, Exp. Cell Res. **21**, 201 [1960].

[5] B. MUNDUR, Exp. Cell Res. **25**, 1 [1961], **25**, 24 [1961].

[6] G. GARZULY-JANKE, Zbl. Bakteriol. Parasitenkunde, Abt. II, **102**, 361 [1940].

cluding mannan, glucan, acetylglucosamine and glycogen were identified by means of the periodate-Schiff reaction (PAS) which is specific for the 1:4 glycol groups of polysaccharides and related substances. An unknown polysaccharidic component of the bud scar plug, a morphological feature of the cell which appears to be of some diagnostic value, was also detected. In addition, high image densities resulting from staining with gallocyanin-chromalum were useful in studying the structure of nuclei and cytoplasm, including the disclosure of submicroscopic particles of DNA- and RNA-protein with enzyme and other methods directed specifically towards basophile components.

Methods

The organisms used in this study include *Schizosaccharomyces octosporus* (Y-854), *Nadsonia fulvescens* (Y-991) and *Saccharomycodes ludwigii* (Y-974); all three were kindly supplied by Dr. L. J. Wickerham. They were grown on Wickerham's synthetic medium (Difco) both on agar surfaces and in liquid culture at 30 °C. The p_H of the medium was adjusted to 4.8 and samples of aerobically growing as well as fermenting cells were obtained for freezing from vegetative and sporulating cultures.

Samples of yeast were frozen in liquefied propane at −175 °C and dried *in vacuo* at −53 °C. Details of experimental procedures and methods of staining for polysaccharides by the PAS reaction, and of basophilia by means of gallocyanin-chromalum have been described previously [3, 5]. In the present work, post-fixation subsequent to drying was carried out with alcohol vapors exclusively and only those controls relevant in localizing carbohydrates known to occur in *Schizosaccharomyces* have been used.

The material was embedded in methacrylate and polymerized in the presence of catalyst (Luperco CDB) at 55 °C. Ultra-thin sections cut with a Porter-Blum microtome were examined in a Philips electron microscope Model EM 100, at accelerating voltages of 40 and 60 KV.

Light microscope control sections (6 μ) of PAS stained material were cut from the same blocks that were used for ultrathin sectioning for electron microscopy. Living cells were observed with the phase and interference microscopes.

Observations and Discussion

Schizosaccharomyces

Cell Wall. The reduced intensity of PAS coloration of *Schizosaccharomyces*, as compared with similarly treated cells of *Saccharomyces*, is clearly distinguishable on macroscopic examination of pellets of whole cells. The intense brick-red appearance of PAS stained, frozen-dried cell pellets of *Saccharomyces* derives largely from the reactive outer mannan layer of the cell wall and partly from the deposits of glycogen in the cytoplasm. In contrast, cell pellets of *Schizosaccharomyces* show a weak red coloration. Control sections examined with the light microscope showed thin, continuous regions of PAS-positive material bordering prominent vacuoles. On the basis of light microscopy alone, these appearances could lead to their erroneous identification as cell wall material. However, at the level of the electron microscope, it is immediately apparent that this stained region is, in fact, protoplasm containing stainable polysaccharide, and not a cell wall.

In the most favorably thin sections examined in the electron microscope, the cell wall does not seem to be stainable by the PAS method (Figs. 1 *, 2). In thicker sections, however, the cell wall, though unstained, appears as an extremely faint, electron-translucent region of widths up to about 100 mμ (Fig. 12). The cell wall is also seen with advantage in relatively thicker sections of spores (Figs. 5, 6, 7) especially when it happens to be coated with the remains of epiplasm from sporogenesis or during stages of germination. These observations are consistent with data from analytical chemistry of *Schizosaccharomyces* by Garzuly-Janke [6] and Miller and Phaff [7], as well as X-ray diffraction studies by Houwink and Kreger [8], indicating the lack of a mannan moiety and the presence of glucan alone in the cell wall. If mannan were present, it would have been detectable by the PAS method owing to its free, adjacent aldehyde groups released by periodate oxidation and would have shown image contrast in the electron microscope; the β 1:3 glucopyranoside glucan is, in any case, not stainable by the PAS method as it has no free, adjacent aldehyde groups. The absence in *Schizosaccharomyces* of a PAS reactive, electron-dense outer wall analogous to that of *Saccharomyces* [3], *Nadsonia* and *Saccharomycodes* (see below), in addition to cytologically confirming these data from physicochemical methods relating to differences in wall composition in different yeast genera, establishes both the correctness of identification of

[7] H. W. Miller and H. J. Phaff, Antonie van Leeuwenhoek **24**, 225 [1958].

[8] A. L. Houwink and D. R. Kreger, Antonie van Leeuwenhoek **19**, 1 [1953].

* Figs. 1—26 see table p. 1080 a—h.

these components as well as the sensitivity of the electron microscope in registering a cytochemically specific reaction such as the PAS.

Starch. The increased electron density of cells after the PAS reaction, as compared with controls of unstained cells or cells subjected to Schiff's reagent without prior oxidation by periodate, corresponds to deposits of starch. These are lightly packed in cells from actively growing cultures (Figs. 1, 2, 11) but their occurrence is denser in cells from quiescent cultures. In either case, they occur as strikingly larger aggregates than the deposits of glycogen in frozen-dried *Saccharomyces* [3]. In contrast to the irregular aggregations prevalent in the latter, starch deposits in *Schizosaccharomyces* occur as dense, more or less separate groups measuring from 0.1 to 0.3 μ. High magnifications of each such group (Fig. 4) discloses extremely fine units measuring about 30 Å in length, recognizable on close examination to possess a certain periodicity in orientation within each aggregate which may be described as roughly circular. Often, several of these roughly circular units may be grouped together to form a larger aggregate. However, as photographic registration of these extremely fine internal structures is difficult owing to image contrast limitations with organic staining at these levels, a precise description of the internal organization of the smaller aggregates of starch cannot be attempted at present. It is suggested that the finest, approximately 30 Å units discernible in Fig. 4 represent the elementary, macromolecular units of starch. In the absence of further data on *Schizosaccharomyces* it is pertinent in this regard to cite some observations on the ultrastructural organization and dimensions of potato starch [9] as gathered from physico-chemical and replica methods. Lintnerized potato starch was found to show an organization of micellar strands, which, in cross section, was postulated to display a structure somewhat resembling that seen in *Schizosaccharomyces* in the present study. A greater portion of the starch grains comprised radially arranged microfibrils 220 – 320 Å in diameter and considerably greater length. The micellar strands making up the microfibrils were themselves about 80 Å – 90 Å in diameter and up to about 4000 Å in length, i. e. far greater than that observable in *Schizosaccharomyces* with direct staining by means of the PAS reaction. It was furthermore speculated on the basis of X-ray data that the molecules in a microfibril of starch were all oriented alike.

Vacuoles. In actively dividing cultures, the vacuoles frequently confine the protoplasm to a narrow zone except in the vicinity of the nucleus (Figs. 1 and 2). With the exception of mature spores, where they are generally absent, and in germinating spores where they are inconspicuous (Fig. 7), the vacuoles are a constant and prominent feature in all vegetatively dividing cells. Intra-vacuolar structures, often associated with amorphous matrices, are frequently observable in *Schizosaccharomyces* after staining with gallocyanine (Figs. 8, 9) or PAS (Fig. 11). Their identity and function are as yet undetermined, but it is possible that these amorphous bodies which possess a complex internal organization (Fig. 8) may correspond to the intra-vacuolar structures in living cells of this organism stained with fluorescent dye as described by Rustad [10].

Presumptive Mitochondria. Roundish bodies measuring about 0,5 μ were observed in the cytoplasm in both PAS treated (Figs. 1, 2) and gallocyanine stained cells (Fig. 3). The contents of these bodies were in each case granular, but the fact that the sizes and numbers of these granules are clearly discrepant would seem to indicate that they do not correspond. These granule-containing bodies, because of their similarity to structures observed in the cytoplasm of *Saccharomyces,* have been tentatively identified as mitochondria but a positive identification must await cytochemical localization of characterizing enzymes within them. The absence in *Schizosaccharomyces* of obvious internal lamellation, characterizing typical mitochondria after osmium fixation in many other cell types, contrasts with the clearly lamellated effect yielded by gallocyanine in the mitochondria of *Nadsonia,* discussed below.

The Nucleus. A feature of the nucleus of *Schizosaccharomyces,* which has not been noted in other members of the Endomycetales observed thus far, is its appearance after the PAS reaction. The nucleus stands apart fainter than, and clearly delineated from, the surrounding aggregations of starch, as in other yeasts storing glycogen. In addition, the nucleus contains fine, PAS reactive particles which

[9] C. Sterling and J. Pangborn, Amer. J. Bot. **47**, 577 [1960].

[10] R. C. Rustad, Exp. Cell Res. **15**, 444 [1958].

measure about 100 – 150 Å (Fig. 1). They are clearly different in size and organization from the nuclear particles seen after staining with gallocyanine (Fig. 10), and are, in addition, more numerous. However, it is not possible to state whether these PAS positive particles of the nucleus are of starch or a related polysaccharide, or even of some other more distantly related substance, such as mucin or mucopolysaccharide.

In gallocyanine stained specimens, the nucleus is seen as a typical vesicle much as that of *Saccharomyces* [5], sharing with the latter the general attributes of form and division by elongation and medial constriction. However, in vegetative cells, it is somewhat larger than the nucleus of diploid *Saccharomyces* and does not possess peripherally disposed, particulate clusters typical of the latter genus. Instead, the intra-nuclear clusters in *Schizosaccharomyces* are generally larger and centrally located (less frequently, eccentrically located) within the nucleus. The vegetative nucleus generally occupies a medial position in the cell in a spur of protoplasm which frequently separates the two prominent vacuoles of the elongate cell (Fig. 12).

Cell Division. Budding, such as occurs in *Saccharomyces* with the production of distinctive polysaccharidic scar plugs [3], is absent. Growth in liquid medium is of mycelial character and involves the separation of the short filaments into single-celled arthrospores which are similar to the single cells found on agar surface. These reproduce by the formation of a wall laid down amid the protoplasmic spur which usually separates the two vacuoles of the elongate cell. It is not certain whether the growth of the wall proceeds centripetally alone, i. e., at right angles to the long axis of the cell, to split the cell into two. The fission plate was frequently laid down *within* the protoplasmic spur following the division of the nucleus, in such a manner that the daughter nuclei were separated by the developing wall (Fig. 6). In germinating spores, nuclear division is followed exclusively by centripetal extensions of the wall material until the cells are completely separated (Fig. 7) followed by elongation and vegetative fission in the manner mentioned above.

It would appear relevant to consider the effects observed in specimens fixed with potassium permanganate in relation to those disclosed with the quite different methods used in this work, particularly in view of the investigations of Bradbury and Meek [11] and Amelunxen and Themann [12] on the attributes of permanganate as a fixative for electron microscopy.

The morphology of *Schizosaccharomyces octosporus* as revealed with permanganate has been discussed by Conti and Naylor [13, 14, 15]. These authors were able successfully to reveal the presence of membranes in this yeast with a clarity ordinarily associated with this fixative. Perhaps because of its extractive or transforming influences on basophilia [11, 12] they were unfortunately less successful in perserving the submicroscopic protoplasmic organization, the clearly particulate nature of which is demonstrable in yeast fixed by osmium in the usual manner as well as in frozen-dried material described herein and in previous studies in this series. Thus, in *Schizosaccharomyces* fixed with permanganate, prominent, dense ribosomes were not demonstrable. The densities and ultrastructural appearance of nuclear and cytoplasmic regions were so uniform that very frequently only the densely stained nuclear membrane enabled recognition of the nucleus. Furthermore, the vacuoles in vegetative *Schizosaccharomyces* are so voluminous and ubiquitous, as was observed in this work as well as by Rustad [10], that it seems very improbable that their absence or drastically small sizes in permanganate fixed specimens can in every case be attributed to unfavorable sectioning planes.

It must be realized, on the other hand, that the methods used in the present work are not intended for the visualization of cell membranes but for specific components such as polysaccharides and basophilia. It has been shown previously [4] that membranes in yeast can, however, be successfully demonstrated by freeze-substitution and post-fixation in alcoholic osmium tetroxide.

As the PAS method is capable of detecting the presence or absence of the mannan layer of cell walls in a specific manner, its application to frozen-dried cells would clearly be valuable in comparative morphological studies in which cell wall composition and cell division characteristics at the electron

[11] S. Bradbury and G. A. Meek, Quart. J. microscop. Sci. **101**, 241 [1960].
[12] F. Amelunxen and H. Themann, Mikroskopie [Wien] **14**, 276 [1960].
[13] S. F. Conti and H. B. Naylor, J. Bacteriol. **78**, 868 [1959].
[14] S. F. Conti and H. B. Naylor, J. Bacteriol. **79**, 331 [1960].
[15] S. F. Conti and H. B. Naylor, J. Bacteriol. **79**, 417 [1960].

microscopical level are likely to be valuable criteria. In this respect also, permanganate (and osmium) are not sufficiently suitable. Thus, CONTI and NAYLOR [13] reported lack of success in staining the cell wall of *Nadsonia*, which possesses a distinct, thick, PAS reactive outer wall layer (see description below), but produced photographs of *Schizosaccharomyces*, which lacks mannan, showing the outer surface of the cell wall lined with dense, probably fortuitous, matter.

Cell fission in relation to nuclear division has been discussed at length by CONTI and NAYLOR [13] and need not be repeated here, particularly as the more relevant aspects of the present work have been discussed above and illustrated with electronmicrographs. However, clarification might be sought on a point of morphological divergence. A comparison of frozen-dried *vegetative* cells and the cells described by these authors (their Figs. 2 to 5; 1959) indicates that nuclear and cell sizes in the latter instance are clearly smaller (even when allowance is made for possible, partly tangential sectioning planes) but agree in nuclear and total cell dimensions as well as appearance with ascospores retained within the ascus wall [14, 15]. The possibility must hence be reckoned that cell division as described in their earlier paper [13] pertained, at least in part, to free (released) ascospores in the culture used rather than (post-germination) vegetatively dividing cells.

This possibility is reinforced by three factors: 1. The circumstance that they saw no vacuoles in "young cells" with the phase microscope (vegetative cells in active division have very prominent vacuoles; spores frequently have none or have reduced ones). 2. Obvious differences in the size of the nuclei in ascospores and vegetative cells were observed in this study of the same yeast strain as used by CONTI and NAYLOR. This observation is consistent with the reduction in ploidy during ascosporogenesis and was strikingly apparent on the electron microscope screen. On the other hand, this effect is not mentioned by these authors nor is it apparent from their illustrations.

The vegetative nuclei (2 n) in Figs. 1 and 10, and the genetically reduced nuclei (n) in the ascospores in Figs. 5, 6 and 7 of the present work serve to indicate the clear distinctions in sizes as can readily be gauged from comparisons from the scales of magnification. Though random cutting planes in thin section studies preclude precise comparisons of nuclear dimensions, they are typical of the observations made during the intentional scanning of large numbers of vacuolated vegetative cells and ascorsporees to ascertain sizes of nuclei, and very probably reflect an approximately medial passage of the sectioning plane in the nucleus. Vegetative nuclei were about 2 μ and spore nuclei about 1 μ measured at the longer axis. The sequence of events as suggested by these observations may be summarized as follows: Ascospores formed by halving of ploidy of the vegetative cell initially possess a single (n) nucleus (Fig. 5). This nucleus splits equationally to yield two genetically identical nuclei (Fig. 6) which are later separated by compartmentation of the ascospore into two "triangular" parts (Fig. 7). It is each of these separated, "triangular" halves that will conjugate (not necessarily with each other) to reconstitute the 2 n vegetative cell as is so clearly apparent from the micrographs of CONTI and NAYLOR [14].

Nadsonia and Saccharomycodes

The cytology of *Nadsonia* both during the vegetative and sporulating cycles was described in an early work by NADSON and KONOKOTINE [16]. The following brief observations will not be concerned with details of nuclear events described by them, but have the same objectives, and are based on the same methods, as those considered above for *Schizosaccharomyces*. The observations pertain to both *Nadsonia fulvescens* and *Saccharomycodes ludwigii* which, owing to their being representatives of the apiculate group of yeasts, are not discussed separately. Both genera have been investigated by the analytical chemist. There is no previous report on the structure of *Saccharomycodes* as seen with the electron microscope.

Presumptive Mitochondria. The extravacuolar cytoplasm contains very numerous large bodies (Figs. 13, 14) whose shapes vary from elongated forms to spheroids, the latter measuring about 0.3 μ $\times$ 0.75 μ. Their general appearance and disposition very strongly suggest their mitochondrial nature, but it would appear preferable to identify them as mitochondria only tentatively pending specific cytochemical localization of enzymes within them. Their internal lamellations are very clearly disclosed as

[16] G. NADSON and A. KONOKOTINE, Ann. Sci. natur Bot. **8**, 165 [1926].

negative images against a gallocyanin-stainable background which is fainter than the density of staining of the cytoplasmic granules. The lamellations themselves appear to run in very closely parallel rows extending across the full length or cross-section of the mitochondrion. Occassionally the lamellations seem as though they have terminated abruptly towards the center of the mitochondrion, with the parallel rows issuing from opposite outer edges stopping short of meeting in the mid region of the mitochondrion. The uniform length and seeming orderliness of disposition of these parallel lamellations are very striking.

It should be pointed out that the gallocyanin-stainable, particulate mitochondrial contents are fewer in *Nadsonia* than in *Saccharomyces*, *Saccharomycodes*, *Schizosaccharomyces* and several other Endomycetales observed in preliminary studies. They are especially numerous in *Saccharomyces*, a circumstance which may have some relation to the well known fact that cells of this genus are exceptionally active in growth and metabolism, and chiefly for this, among other reasons, are favored in industry. The concentration of these basophile, mitochondrial particles in *Saccharomyces* have been illustrated elsewhere [5]. This density of packing in relatively small mitochondria suggests itself as the possible reason for the failure to detect negative-image lamellations clearly in frozen-dried specimens of this organism. But the more common methods of staining *Saccharomyces* with osmium or permanganate do reveal the familiar mitochondrial cristae, although such demonstrations have not always been consistent or as successful as those achieved by VITOLS et al. [17]. On the other hand, in no instance where these conventional methods were used was it possible to selectively demonstrate the occurence of basophile particles within the mitochondria as has been achieved by the methods used in the present studies.

It is not clear as to why no inner lamellations are visible after freezing-drying in *Saccharomyces* whereas they are so clearly evident in *Nadsonia* after identical treatments, but the reason for this may probably be that the relatively higher concentration and crowding of the particles in the former (in comparison to the few present in the mitochondria of the latter) preclude a clear visualization of the negatively imaged striae under these conditions. Negative images corresponding to the outer membranes of typical mitochondria as seen after metallic fixation are not evident in the organisms examined so far.

These fine intramitochondrial, basophile particles do not appear to be equivalent to the granulations of another type, seen in mitochondria following the more usual metallic fixation. These reveal the intercrastael matrix as being extremely closely packed with the same fine granulation (about 40 Å that comprise the background of the dense, 120 Å or more, ribosomal particles found outside the mitochondria. By contrast, the particles seen in *Nadsonia* and other yeast mitochondria are seemingly indistinguishable from the ribosomal particles of the cytoplasm stained by gallocyanin-chromalum.

The question remains, however, as to why particles of this type have hitherto evaded detection with the more common methods of metallic impregnation for electron microscopy in the case of a variety of organisms in which staining of extramitochondrial ribosomal particles has otherwise been successfully accomplished. In this regard too, the results of the present studies have been discrepant because the particles of the mitochondria are seen with the same clarity after non-aqueous osmium impregnation following alcoholic freeze-substitution (but without the osmiophilic cristae) as is possible with basic staining with aqueous solutions of gallocyanine-chromalum following freezing-dyring. Such particles are not visible in frozen-dried liver [18, 19], pancreas [20, 21] and adipose tissure [22] either.

Cell Walls and Budding. Comparative chemical studies of wall composition in several apiculate yeasts, including *Nadsonia* and *Saccharomycodes*, have been reported by GARZULY-JANKE [6] and MILLER and PHAFF [7]. These workers showed that walls in the latter genus were almost similar to those of *Saccharomyces* in possessing mannan, glucan, protein and acetylglucosamine (chitin) – the last occurring in trace amounts of 0.04 per cent. Walls of *Nadsonia*

[17] E. VITOLS, R. J. NORTH and A. W. LINNANE, J. biophys. biochem. Cytol. **9**, 689 [1961].
[18] H. FINCK, J. biophys. biochem. Cytol. **4**, 291 [1958].
[19] I. GERSH, J. VERGARA and G. ROSSI, Anatom. Rec. **138**, 445 [1960].
[20] V. HANZON and L. H. J. HEMODSSON, J. Ultrastructure Res. **4**, 332 [1960].
[21] F. SJOSTRAND and R. F. BAKER, J. Ultrastructure Res. **1**, 239 [1957].
[22] W. CHASE, J. Ultrastructure Res. **2**, 283 [1959].

on the other hand, were found to lack mannan but to possess glucan and unusally high amount of chitin – about 5 per cent.

Of the polysaccharides comprising the double wall of *Saccharomyces*, only the outer, mannan layer was shown to be electron dense following the PAS reaction [3]. This is a consequence of locally high electron scatter due to an increase in mass of the reactive glycol groups of mannan on exposure to sulphurous-leucofuchsin. Appropriate control tests showed also that the mannan layer was extractable from an associated chitinous framework whose image in the electron microscope could subsequently be detected under special conditions.

Figs. 15, 16, 18, 20 show PAS reactive sites in Nadsonia, and Figs. 22 to 25 in *Saccharomycodes*.

It will be noted in all cases that the outer wall layer appears densely reactive, while the inner region in electron transparent, i. e., not reactive with the PAS stain. While the pattern of this staining is compatible with data from analytical chemistry of *Saccharomycodes*, the electron density of the outer wall layer in *Nadsonia* must be attributed to polysaccharide other than mannan, which, as mentioned before, is known to be absent int his genus, or to related PAS positive compounds. The density of the outer cell wall of *Nadsonia* (Figs. 16, vegetative cell; and 20, ascus wall) in general appeared to be weaker in comparison to the outer wall in *Saccharomycodes* (Figs. 22 to 25). The weak density in the former genus may be due to the scatter of electrons from compacted concentrations of high amounts of chitin characterizing *Nadsonia*. However, as this substance is PAS non-reactive and as the walls in unstained controls were of distinctly lower density, it would appear that at least part of the image contrast must be attributed to stainable substance, on which data are not now available from the chemical literature. This suggestion finds support from light microscopical controls where the wall is observed to be very light red to pale red rather than deep purple as int he mannan possessing genera, *Saccharomyces* or *Saccharomycodes*.

The character of staining in the mature ascospore wall is of some interest owing to its conspicuously aculeate surface. Figs. 20 and 21 show the prominent protuberances in PAS and gallocyanine stained material, respectively. The image density of the spore coat following either of these treatments is far greater than might be anticipated on the basis of color intensities seen in corresponding (2 n) control sections examined with the light microscope. It will be noted, however, that the density of the chitinous ascus wall (previously the ascogenous, vegetative cell wall) in Figs. 18 and 20 is consistently less dense in comparison to the spore surface in PAS preparations and scarcely distinguishable in specimens stained with gallocyanine (Fig. 21). On the other hand, the inner spore wall appears to be weakly PAS reactive but unstained by gallocyanine-chromalum. Since no X-ray diffraction, qualitative or quantitative chemical data on *Nadsonia* spores occur in the literature, the unusually high electron density of the outer ascospore wall and its ornamentation may be surmised as due to: 1. very densely compacted, high amounts of chitin, perhaps in combination with components different from those with which it is combined in vegetative cell walls, 2. naturally occuring, brownish pigment in the spore coat as is strikingly seen in fresh mounts in the light microscope, 3. an hitherto undetected wall component synthesized during sporogenesis.

The character of budding in *Nadsonia* and *Saccharomycodes* is superficially similar in that both involve the production of buds in an exclusively terminal position and on a broad base, in contrast to the indiscriminate positions of bud origin in *Saccharomyces*. Bud scar plugs of the latter [3], and possibly other members of the Saccharomycetaceae, appear to be highly distinctive features and are not encountered in these two genera. Minor differences also appear to exist in the mode of fission. In *Nadsonia* there seems to occur an invagination of the outer cell wall simultaneously with the formation of the future scar area (Fig. 16), but such invaginations have been observed in *Saccharomycodes* (Figs. 22 to 25). The possible significance of scar plugs and budding mechanics in relation to "life spans" in yeast has been discussed previously [3].

Phylogenetic Considerations

Schemes of possible natural relationships among the fungi such as those proposed earlier by GAUMANN [1, 2] are based on comparative studies of life cycles and other morphological criteria. They provide an indispensable area of reference on which further work along physico-chemical lines, not commonly employed by mycologists, may be based in order to establish evolutionary affinities from wider, independent sources of data.

Abbreviations. — AsC — ascogenous cell; AsP — ascospore; AsW — ascus wall; CB — cytoplasmic basophilia; g — glucan; IW — inner cell wall; m = mitochondrion; N — nucleus; nc — nuclear cluster; OW — outer cell wall; S — starch; V — vacuole; vs — intravacuolar amorphous body.

Figs. 1 to 12. *Schizosaccharomyces octosporous.*

Fig. 1. Longitudinal section of a vegetative cell from a culture in active growth, showing aggregates of PAS reactive starch granules. The presence of minute, moderately dense particles within the nucleus (of undetermined nature but different from the basophile nuclear particles, see Fig. 10) is notable in this genus, 30,000 ×.

Fig. 2. Cross section of a vegetative cell showing the prominence of the vacuole, and the protoplasm which is restricted to an unusually thin peripheral band except in the region of the nucleus. A PAS reactive component of the wall, like that of *Saccharomycodes*, is absent; but the densely reactive material of the cytoplasm (starch) contrasts clearly with the less dense nucleus and other cytoplasmic bodies (mitochondria) as in Fig. 1. 15,000 ×.

Fig. 3. Cytoplasmic basophilia and mitochondria after staining with gallocyanine-chromalum. The mitochondria have tentatively been identified as such by analogy with those of *Nadsonia* which possess the well-known internal lamellations in addition to particulate entities seen here in the mitochondria of *Schizosaccharomyces*. The mitochondria contain many basophilic particles stained with gallocyanine-chromalum. 28,000 ×.

Fig. 4. High magnification of part of a PAS stained cell with heavy deposits of starch. The extremely fine, elementary units of starch measure approximately 25 Å to 30 Å and display a rough periodicity of orientation, seemingly circular, in each aggregate. 90,000 ×.

Fig. 5. Spore stained by the PAS method following digestion with salivary amylase. The previously heavy deposits of starch are partially digested and now yield reduced electron scatter. Remnants of the epiplasm of ascosporogenesis border the unstained spore wall which is constituted of PAS non-reactive glucan. 40,000 ×.

Figs. 6 and 7. Ascospores in two stages of development, (stained by PAS) in relatively thick sections to show the outer wall structure. Nuclear division precedes the formation of a cross-wall originating by centripetal growth of glucan material. An early stage is seen in Fig. 6 with the incipient cross-walls growing inwards to lead to the late stage in Fig. 7. Vacuolation of the ascospores is uncommon or at least not so prominent, as in the mature vegetative cell arising from the spores. 20,000 × and 25,000 ×, respectively.

Figs. 8 and 9. Intravacuolar bodies, stained with gallocyanine-chromalum. Both large, amorphous forms (Fig. 8) as well as smaller oval bodies containing basophile granules (Fig. 9) may be seen in the vacuole in contrast to the crystalline metaphosphate deposits in the vacuoles of *Saccharomyces* seen after similar treatment. The nature of these structure in *Schizosaccharomyces* is obscure. Both 55,000 ×.

Fig. 10. Part of cell stained by gallocyanine-chromalum. Note the dense structure (nc) which is often centrally located within the nucleus. It contrasts distinctly with an analogous, peripherally disposed, narrower structure seen in nuclei of *Saccharomyces* stained by the same method. The appearance of the basophile granules elsewhere in the nucleus is unlike that revealed by the PAS method. Note basophilic granules in the cytoplasm. 40,000 ×.

Fig. 11. Intravacuolar bodies in PAS stained material 38,000 ×.

Fig. 12. Longitudinal section through a typical vegetative cell showing its two vacuolar compartments due to a bridge of cytoplasm joining the shorter cell axis. Division of the cell takes place by the laying down of a wall within this bridge subsequent to nuclear division, but it is not certain that cell fission proceeds exclusively by centripetal extensions of new wall material as in the ascospores. Suggestions of the glucan cell wall seen in this figure are frequently absent in the most favorably thin sections such as those in Fig. 1 and 2. Staining by PAS. 24,000 ×.

Figs. 13 to 21. *Nadsonia fulvescens.*

Fig. 13. Mitochondria (m) and vesicular-type nucleus (N) of *Nadsonia*, amidst dense concentrations of cytoplasmic ribosomes. The mitochondria are larger than those of *Saccharomyces* or *Saccharomycodes* and possess clear internal lamellations. These are seen here as negative images owing to their indifference to basic dye. Each mitochondrion possesses basophile particles in the ground substance between the lamellations. Such particles have hitherto not been described in the literature on mitochondrial ultrastructure, except in the case of frozen-dried *Saccharomyces*, Gallocyanine-chromalum. 60,000 ×.

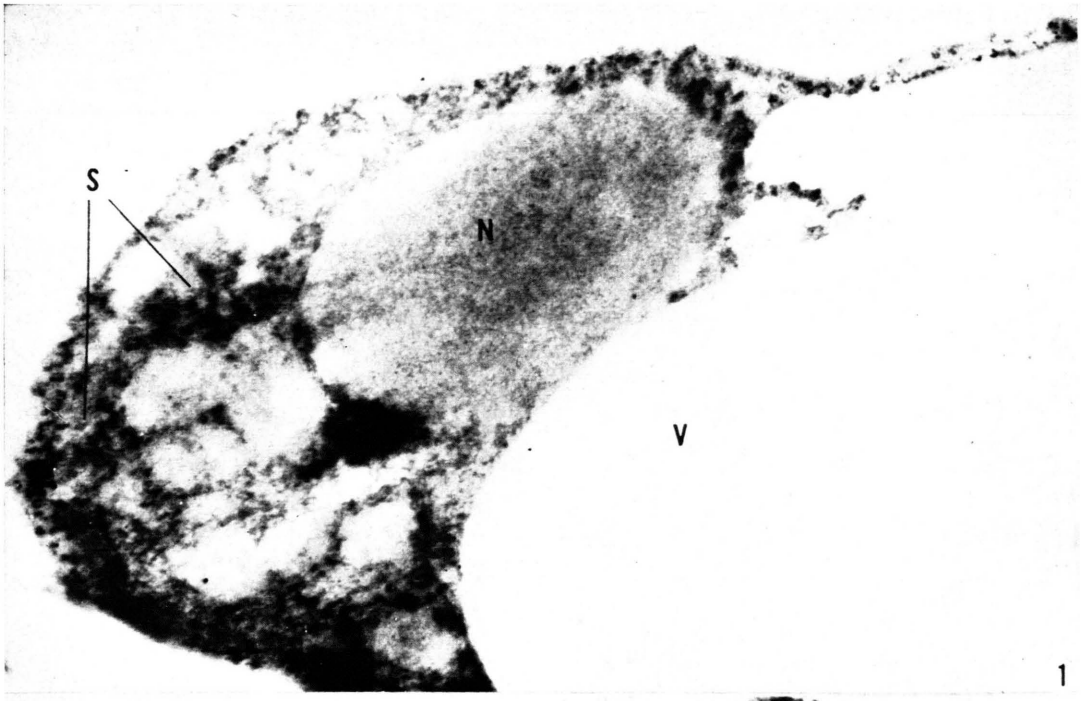
Fig. 14. Low power magnification of longitudinally sectioned *Nadsonia* stained with gallocyanine-chromalum, showing the profusion of mitochondria, many of which are elongate, and their relation to the vacuole. Note that the cell wall is unstained, in contrast to its clear revelation by the periodic-Schiff method as is evident in succeeding figures. 6,000 ×.

Fig. 15. Periodate - Schiff stained specimens. An unusual instance of an ascogenous cell of *Nadsonia* where more than one ascospore appear to be maturing from the nuclear tetrad. Normally, only one spore, as in the upper left corner, reaches maturity from among four genetically reduced nuclei. The possible evolutionary significance of this and other characters is discussed in the text. 25,000 ×.

Figs. 16 and 17. Constrasting effects in details of cell walls and site of bud origin (BA) in *Nadsonia* revealed with periodate - Schiff (Fig. 16) and gallocyanine-chromalum (Fig. 17). The outer wall layer of *Nadsonia* is stated in the literature to possess no PAS reactive mannan, but a weak stainability in comparison to *Saccharomycodes* (Figs. 22 to 25) is demonstrable with the PAS method. An hitherto uncharacterized pentosan or other substance amenable to the PAS reaction may be implicated. The complete absence of the wall and bud attachment detail in the gallocyanine stained specimen indicate that the differences cannot be attributed to an intrinsically electron-dense outer wall. Fig. 16, 24,000 ×; Fig. 17, 26,000 ×.

Fig. 18. Low magnification of a field showing characteristically elongate and empty (or emptying) forms associated with ascogenous cells (AsC) of *Nadsonia*. In the field also are mature asci (AsW) each with a single ascospore. Periodate - Schiff. 6,000 ×.

Fig. 19. Unusual departures from the single spore-ascus are sometimes seen, as in this figure. Note the mode of development of one of the future spore coats (arrow) around a protoplasmic mass, together with the presence of a sister mass and the exceptionally elongate, dense wall. The ascus wall (AsW) itself continues to remain unstained. Gallocyanine-chromalum. 25,000 ×.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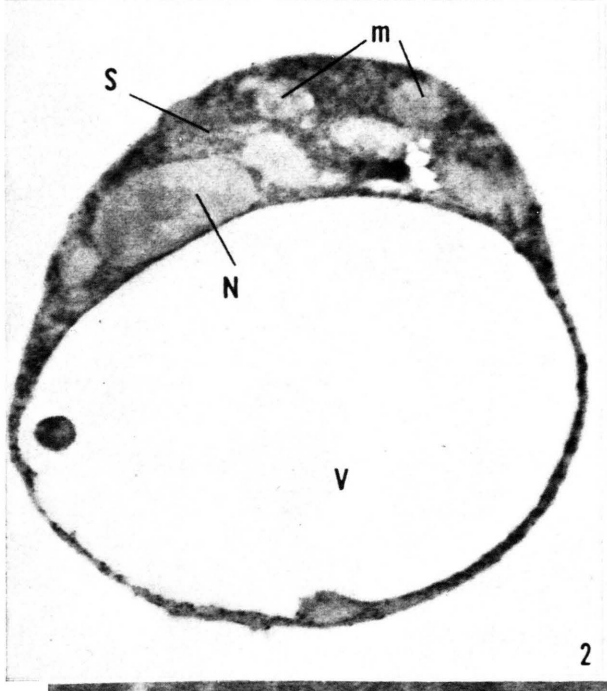
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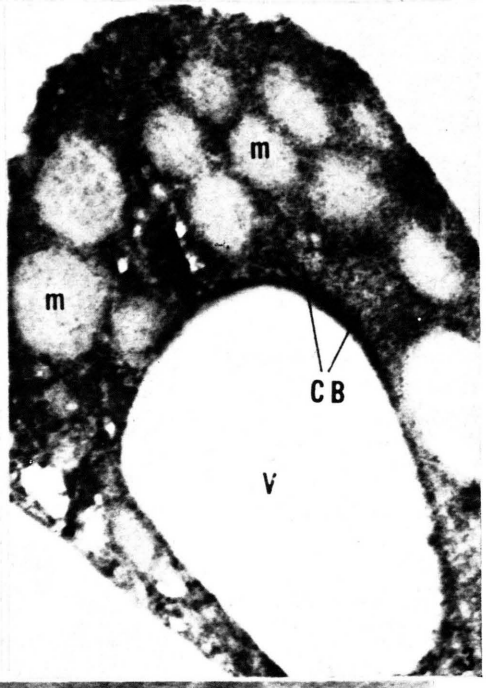
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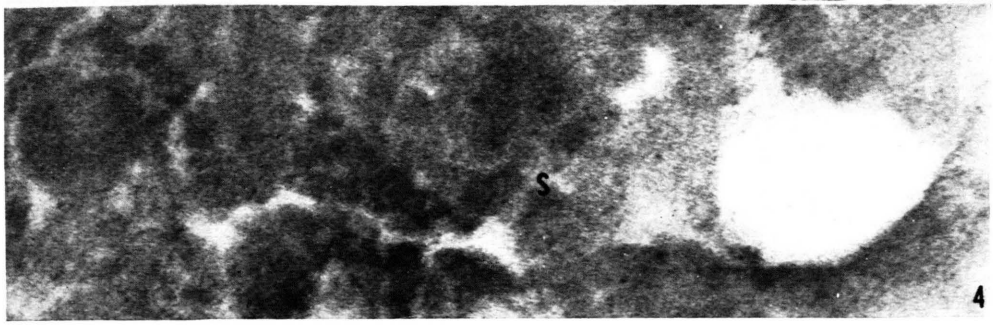
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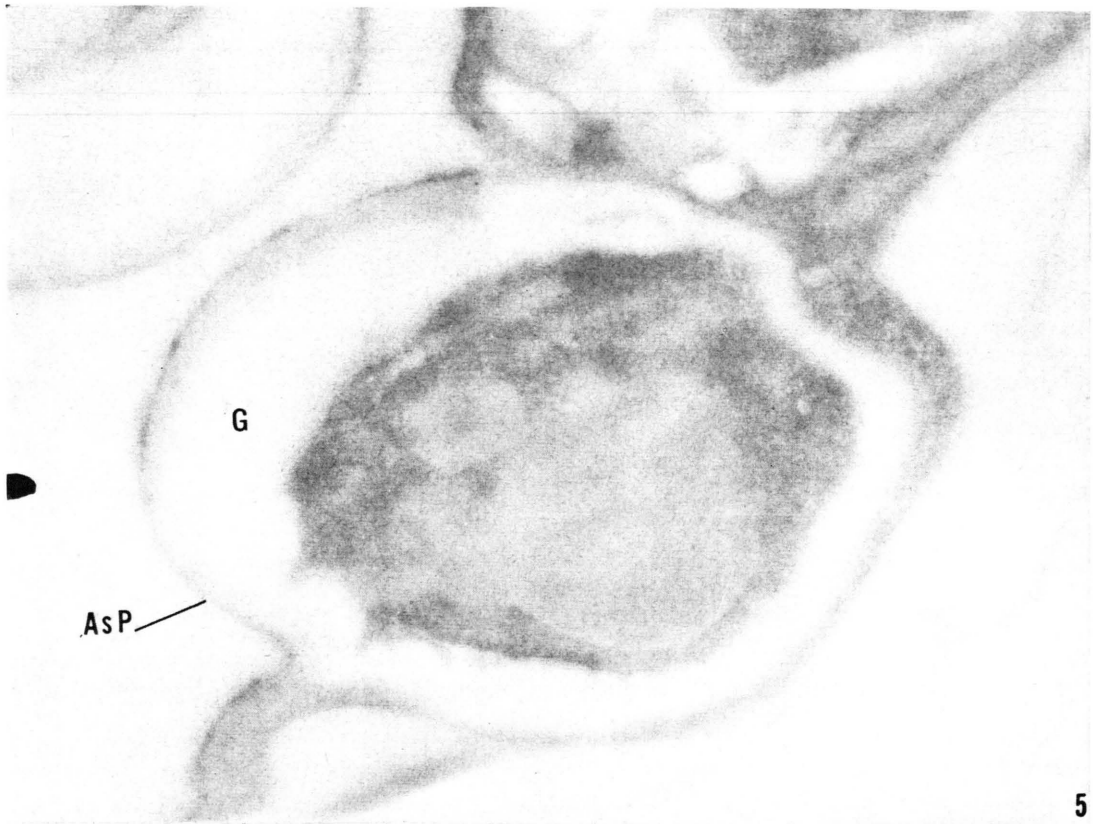

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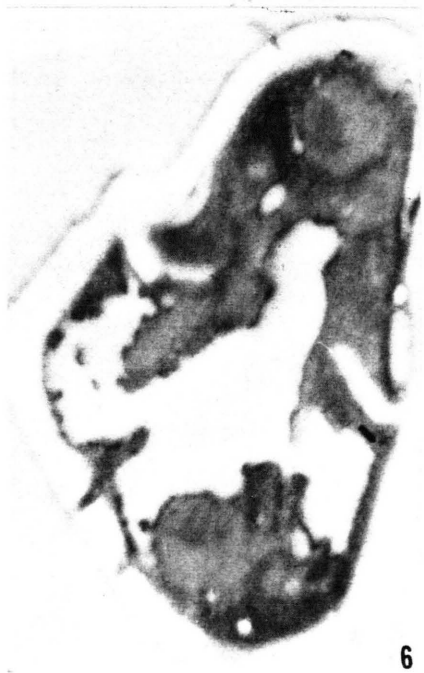

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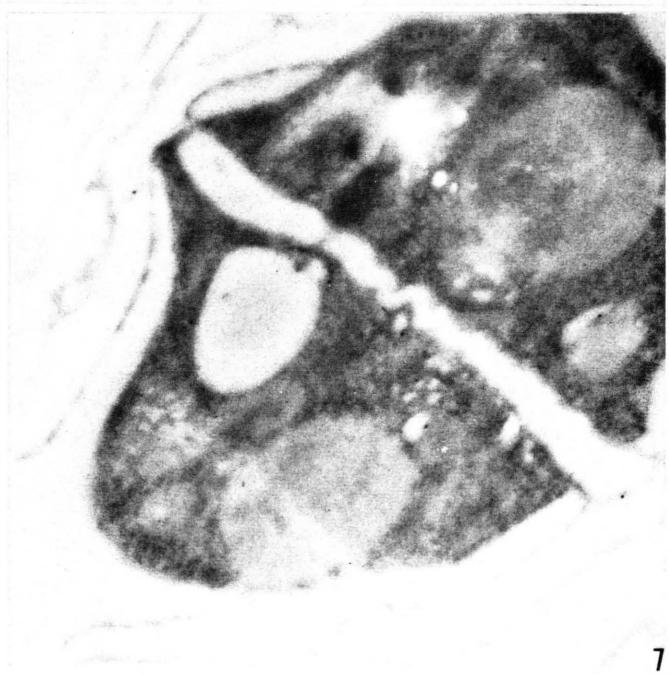

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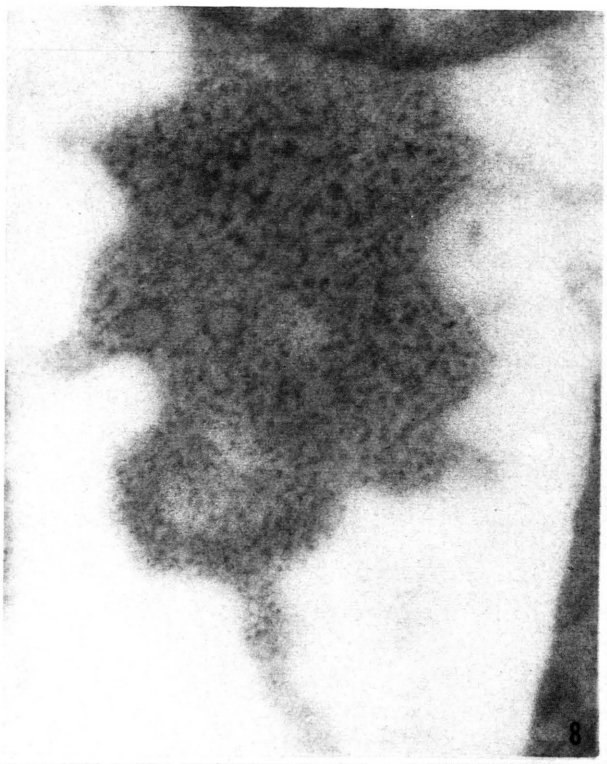

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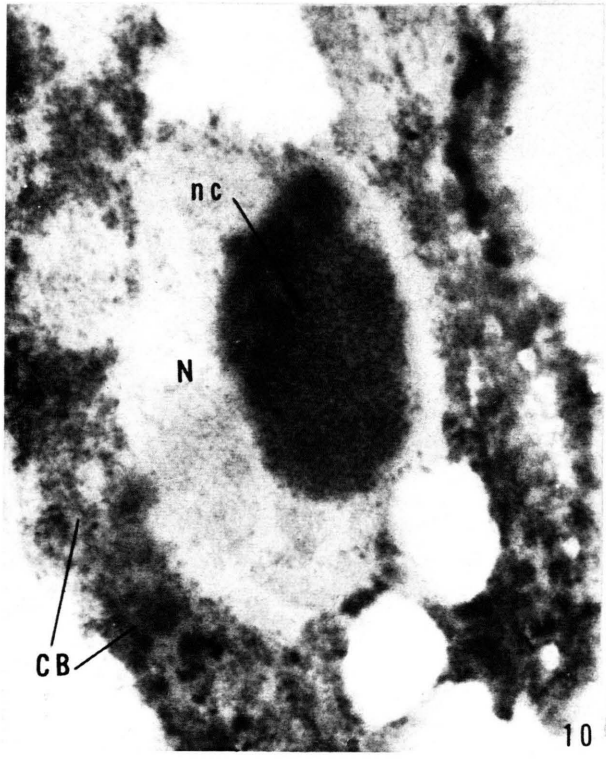

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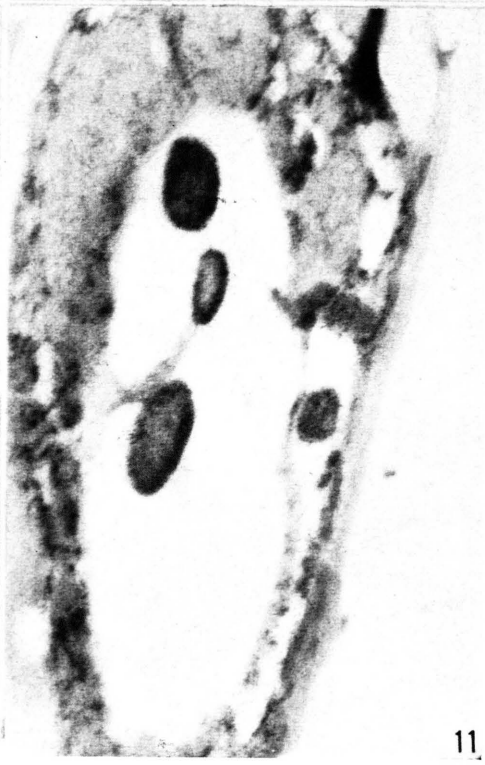

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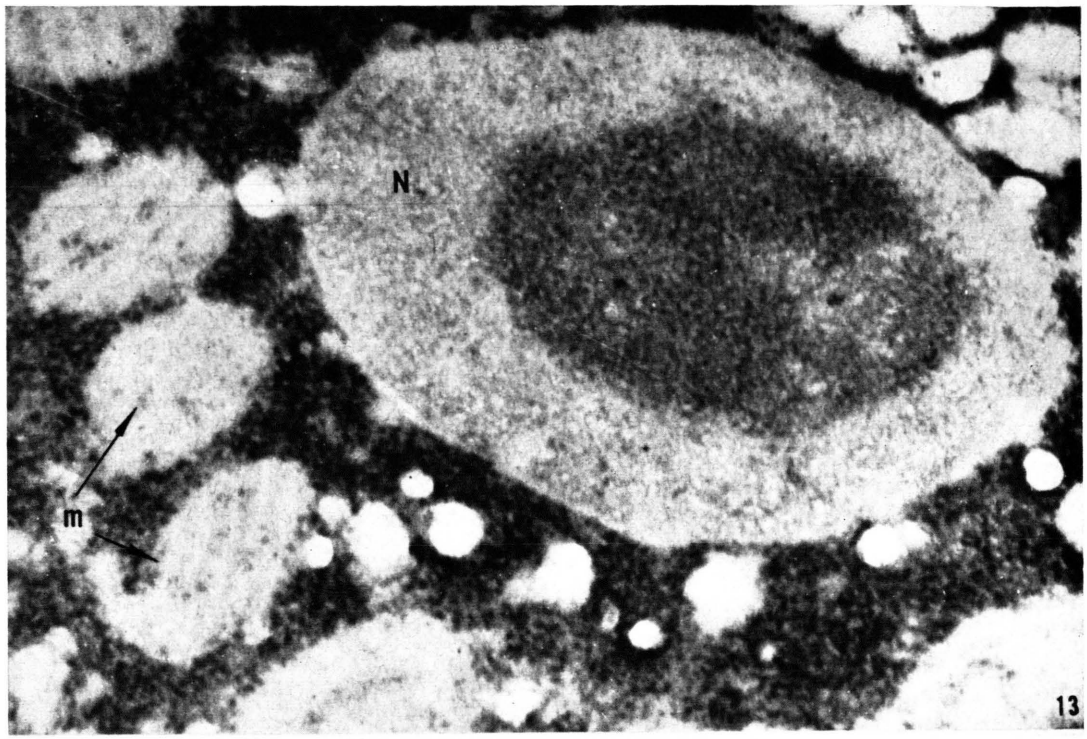
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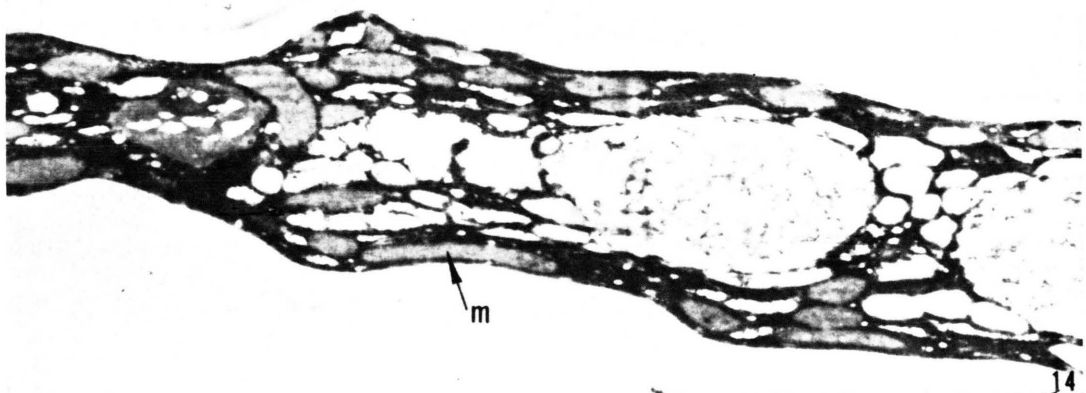
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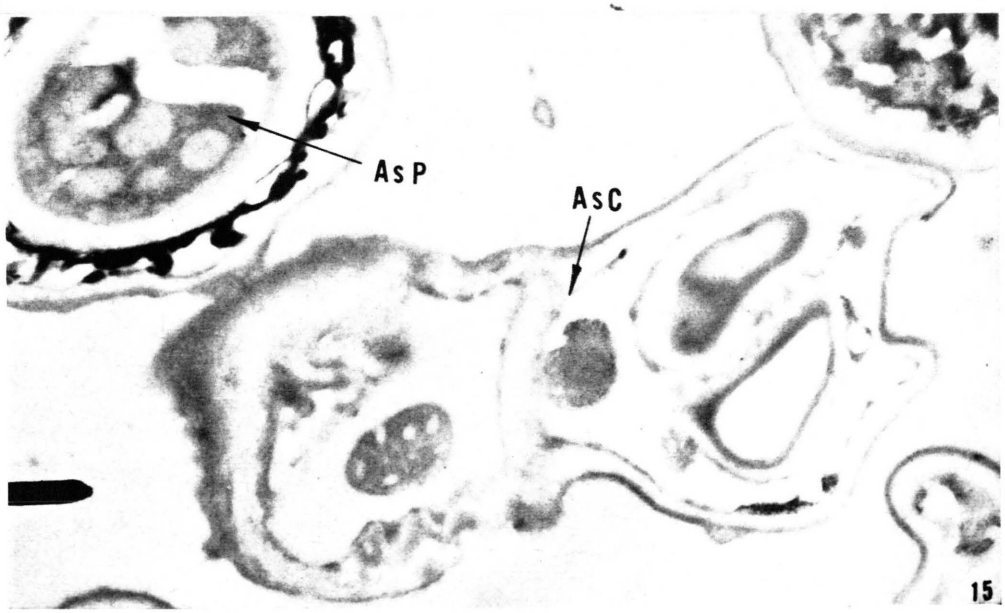
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AsC
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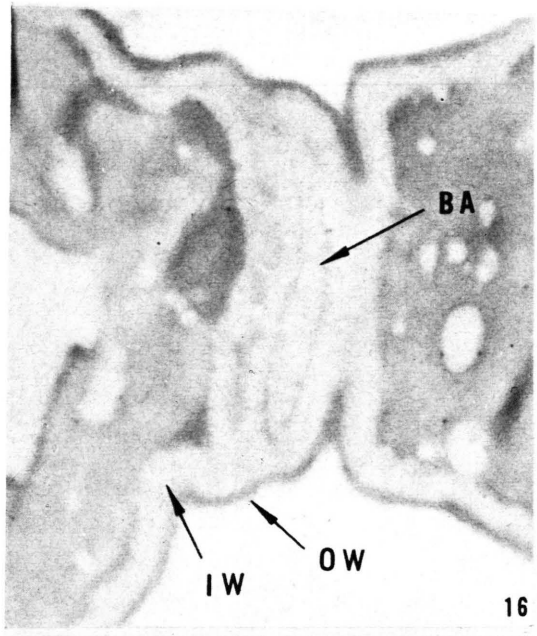
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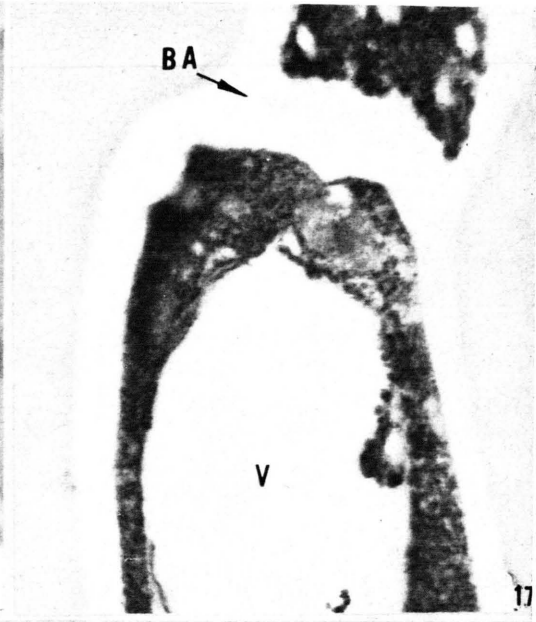
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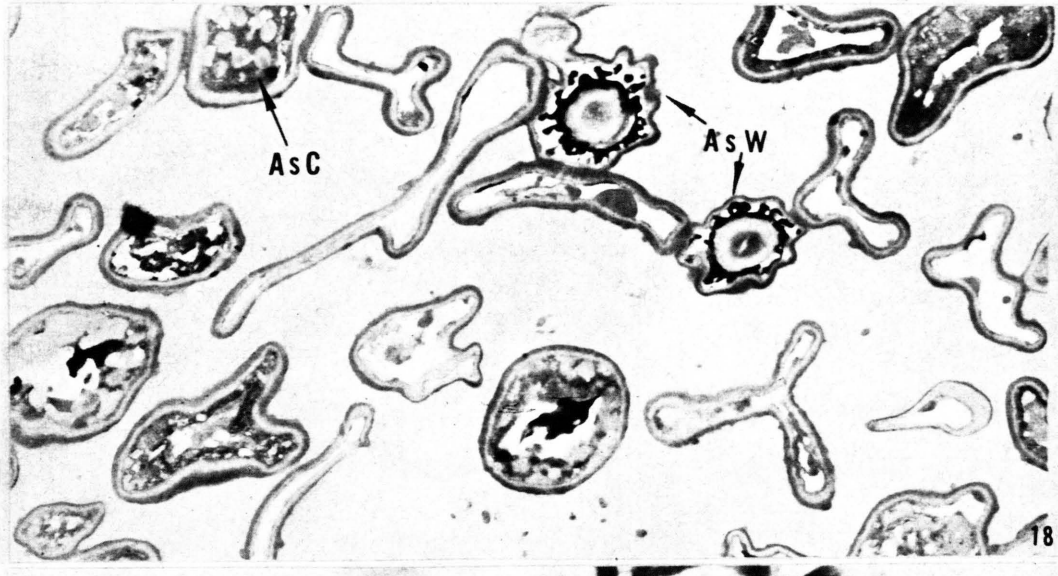
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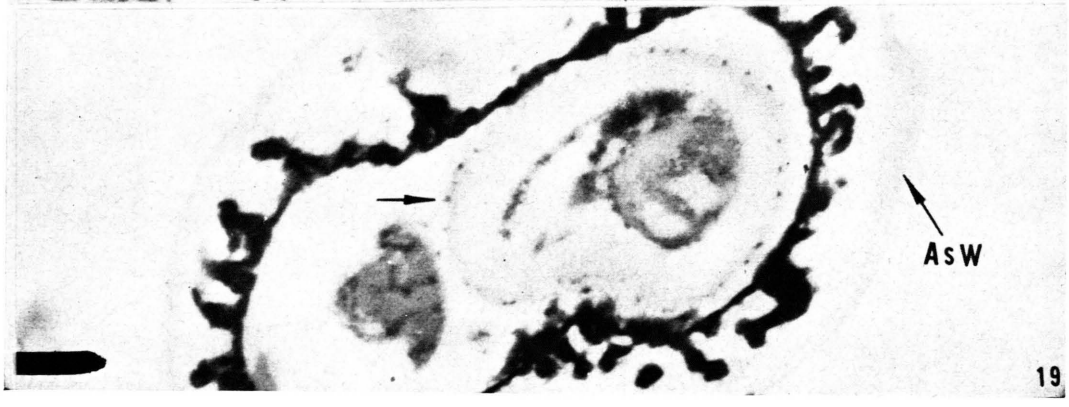
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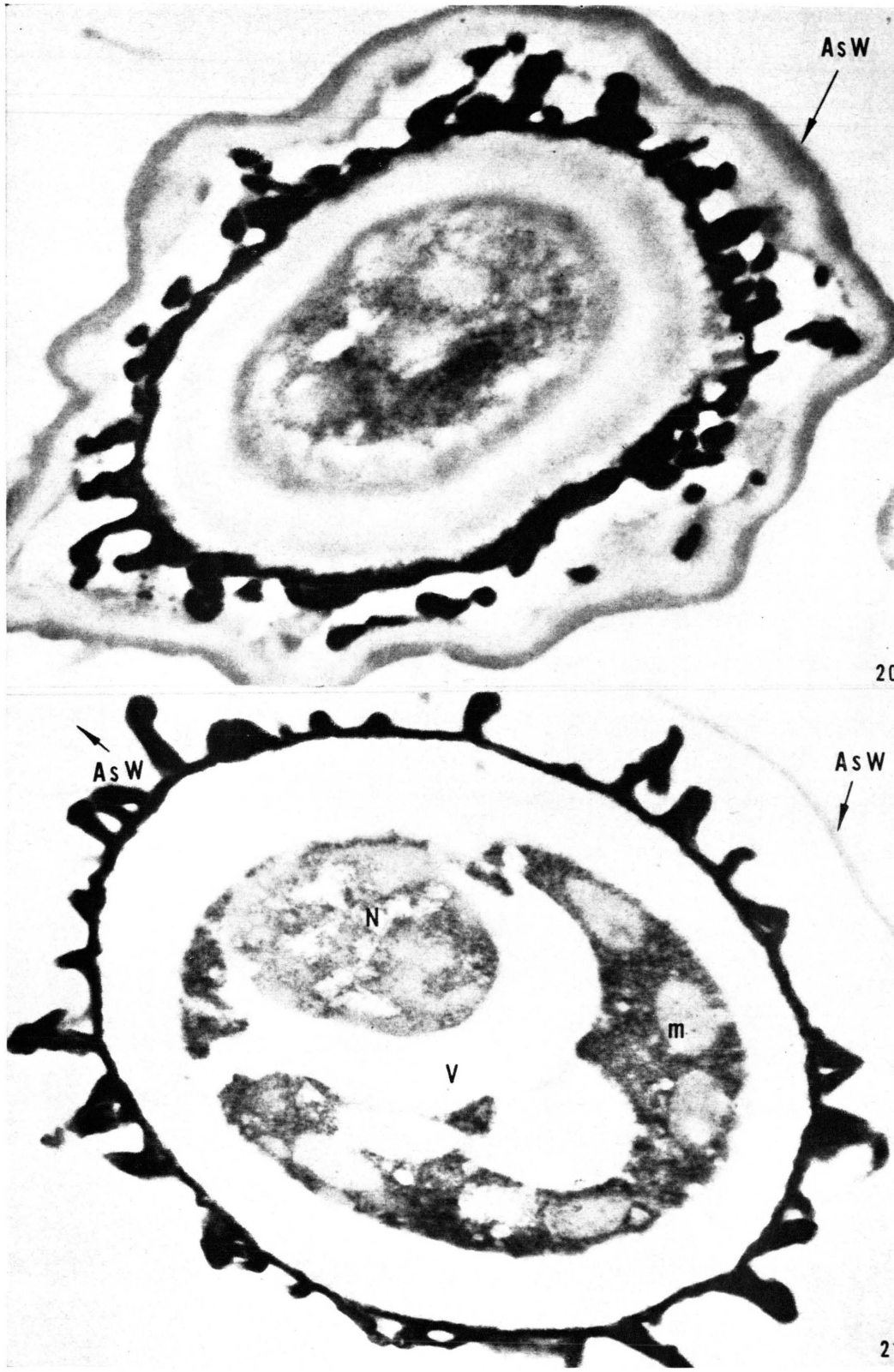

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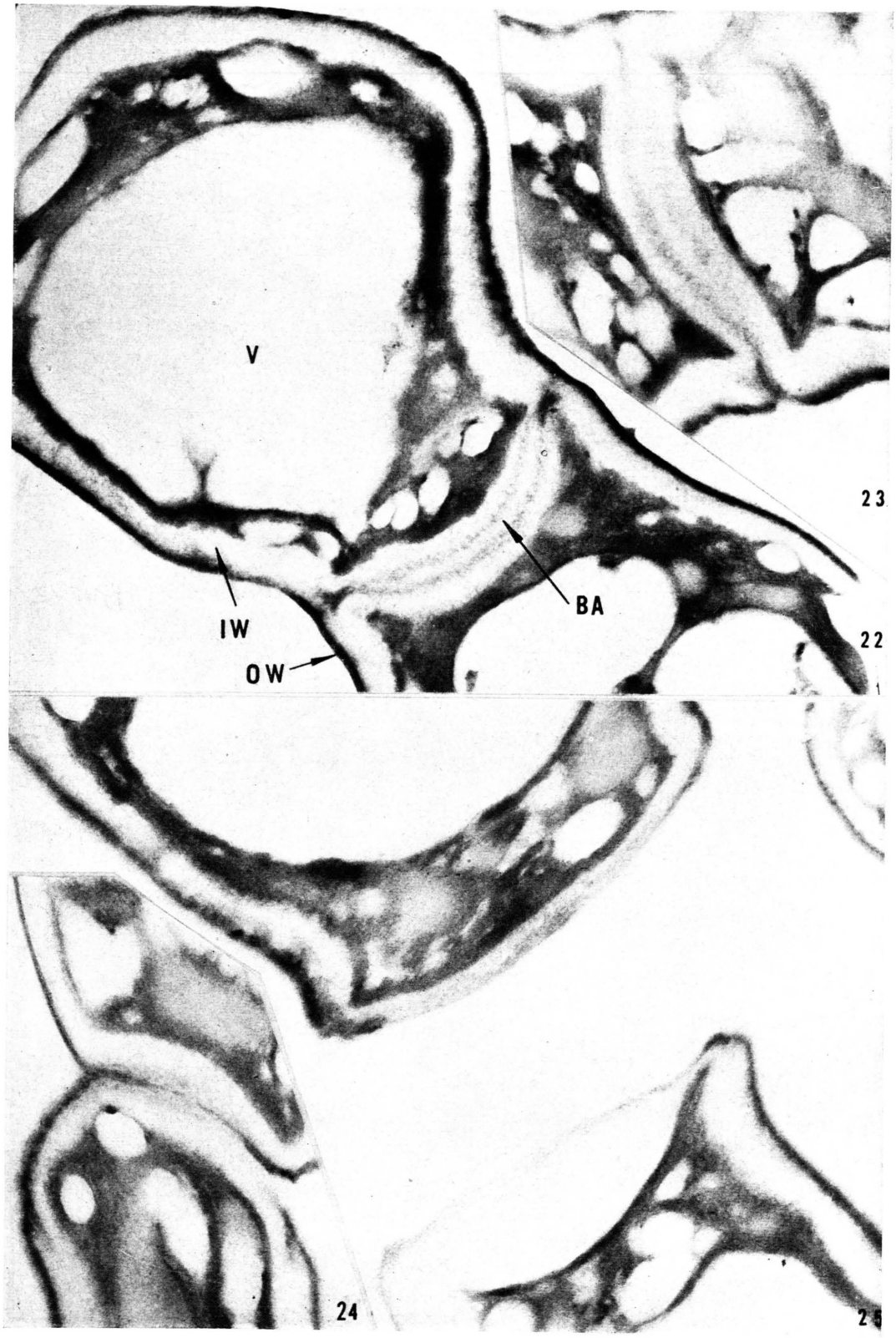
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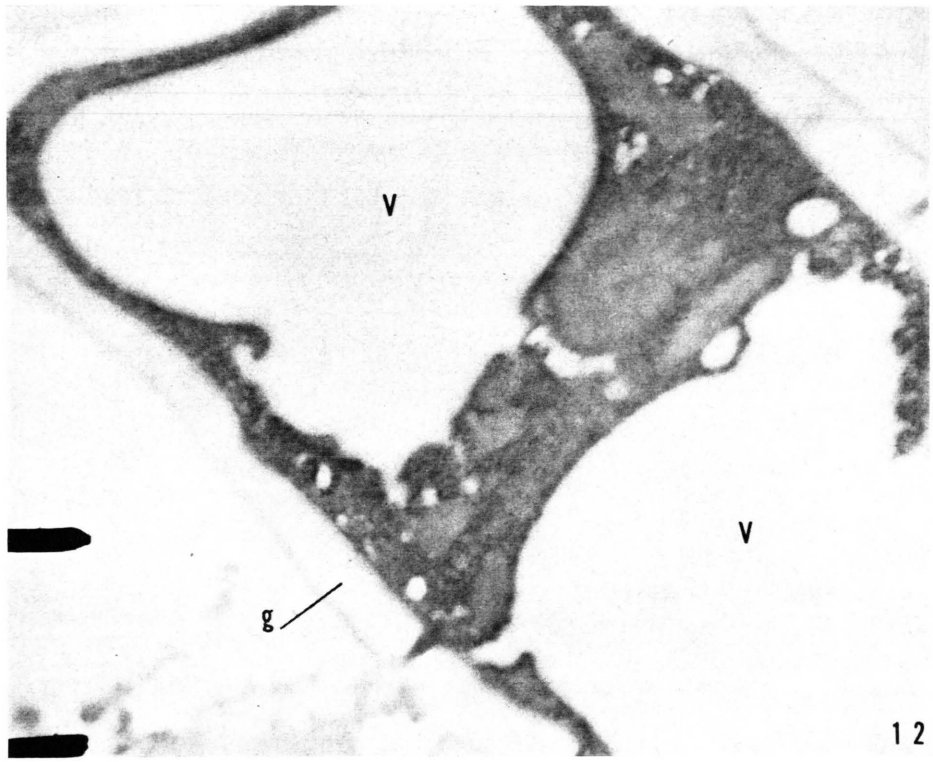
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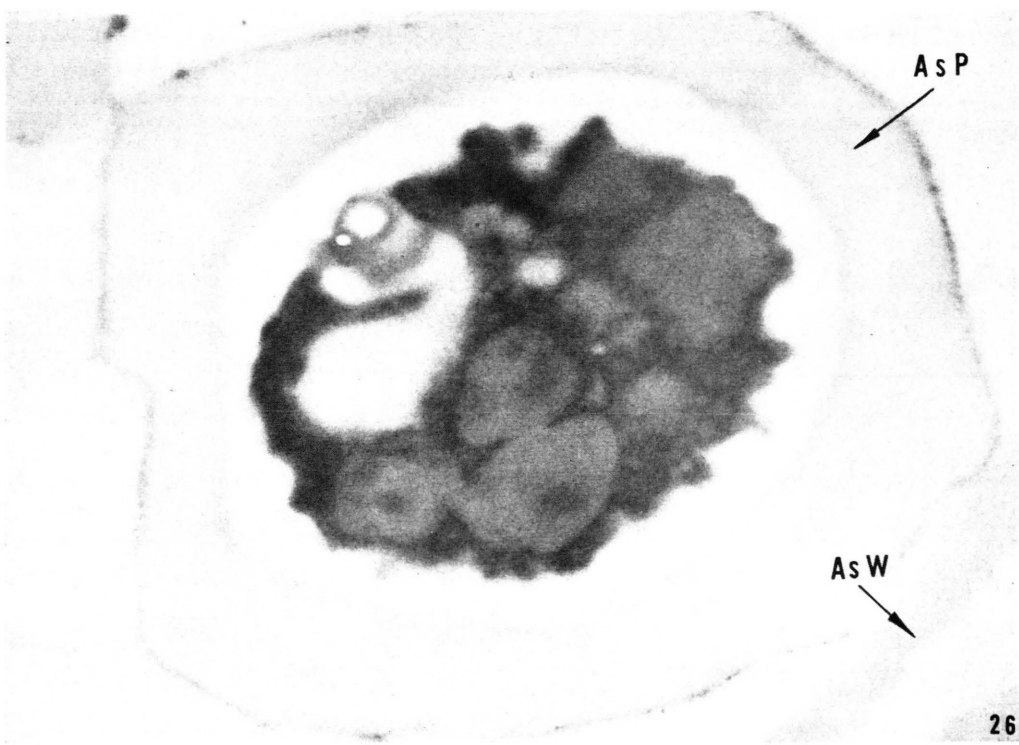
AsP
AsW
26

Figs. 20 and 21. Typical asci of *Nadsonia* stained with periodate - S c h i f f (Fig. 20) and with gallocyanine-chromalum (Fig. 21) to illustrate contrasts at higher magnification. The ascus wall (AsW) in the latter case is practically unstained. The composition of the outer, aculeate ascospore coat is unknown. It is far weaker in PAS reactivity as well as after staining with gallocyanine-chromalum in light microscope controls than might be suggested by its density in these electronmicrographs. Mitochondria within the spore (Fig. 21) show the usual basophile particles but the lamellations are not as clearly detectable as in Fig. 13. Both 42,000 ×.

Figs. 22 to 25. *Saccharomycodes ludwigii.*

Figs. 22, 23, 24 and 25. Wall structure and details of bud formation in *Saccharomycodes* seen after the periodate-S c h i f f reaction. Like *Saccharomyces*, cells of this genus possess both glucan and mannan in their inner (IW) and outer (OW) walls, respectively. The former is unreactive, the latter strongly reactive owing to the type of linkage of carbon atoms occuring in these two polysaccharides. The stainable region of the cytoplasm probably consists largely of glycogen or starch. Note that although a partial invagination of the stainable wall layer appears to form at the site of bud attachment, as in Fig. 24, distinctive bud scar plugs of PAS positive material such as occur in *Saccharomyces* are lacking in *Saccharomycodes*. Differences between these genera in the morphology of the bud attachment and details of the area of abstriction are clearly exemplified by the recently separated pair of cells shown in Fig. 25. The possible phylogenetic value of wall composition and morphology are discussed in the text. 20,000 ×; 24,000 ×; 24,000 × and 25,000 ×, respectively.

Fig. 26. *Eremascus fertilis.*

Fig. 26. Ascospore of *Eremascus* stained with the periodate-S c h i f f method, showing a lack of reaction in the spore as well as ascus (formerly vegetative cell) walls. The spores strikingly resemble those of *Schizosaccharomyces* owing to an absence of the dense outer wall. The faintly stained material on the periphery of the spore is not wall substance but remnants of the epiplasm of sporogenesis.

Considerable accurate and useful information on the composition of various yeasts has been obtained recently by such means as X-ray diffraction, moving boundry electrophoresis and analytical chemistry [7, 8, 23–27]. Although information of this nature could yield additional criteria in establishing phylogenetig relationships, in only one instance [7] has it been gathered purposefully with this objective in view. In any event, it seems necessary that many more genera in the Endomycetales and other fungi must be examined if such methods are to yield data of significance to phylogenetic research. But it is noteworthy in all these instances that the criterion most usefully exploited for comparative purposes was the variability in the polysaccharidic composition of cell walls – a character whose suitability for morphological observation and cytochemical localization at the ultrastructural level can, in addition, be demonstrated with frozen-dried specimens.

The methods used in the present work have, moreover, provided data enabling an extension, and sometimes correction, of conclusions based on results obtained through analytical chemistry. Thus, the presence of mannan is demonstrable cytochemically with the electron microscope in those genera (such as *Saccharomyces* and *Saccharomycodes*) where its occurrence has been previously established by independent means; the known absence of mannan in *Schizosaccharomyces* has been similarly confirmed. A weak PAS reaction, where none was expected on the basis of analytical data has, however, been detected in *Nadsonia*. And finally, the present methods have also strengthened the data based on analytical chemistry or examination of shadow-cast specimens of cell walls by showing that glucan occurs in the inner, and not the outer, wall layer in *Saccharomyces* [3]. Thus, the chief value of electron microscopical observation of frozen-dried material stained with the periodate - S c h i f f method is that of observing such characters of diagnostic importance as the nature of scar plugs, budding or fission, and walls. These are characters which have not so far lent themselves consistently to electron microscopical study in specimens impregnated with the usual metallic fixatives, nor to the light microscope owing to limitations in resolving detail.

These considerations prompt the belief that the electron microscope, used in conjunction with suitable methods of specimen preparation and cytochemistry, may prove valuable in studying phylogenetic relationships at structural and compositional levels hitherto unexploited by the comparative morphologist. It is superfluous to add that present

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