

FINE STRUCTURE IN FROZEN-ETCHED YEAST CELLS

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ABSTRACT

The freeze-etching technique, which is a special kind of freeze-drying, allows electron microscopic investigation of cells and tissues in the frozen state. In regard to yeast cells (*Saccharomyces cerevisiae*) a freeze-fixation technique has been developed which does not kill the object. The electron micrographs therefore are considered to impart an image of high fidelity. The cutting of the frozen object, which actually consists of a fine splintering, produces not only cross-sectional views (cross-fractures) of the structures but also surface views of the membranes and organelles. Many surface structures are described which have not been shown by the usual sectioning techniques. The cytoplasmic membrane contains hexagonal arrangements of particles which are apparently involved in the production of the glucan fibrils of the cell wall. Alterations of the distribution of nuclear pores are shown in cells of different ages. Freeze-etching enables a clear distinction of endoplasmic reticulum and vacuoles in yeast cells. The membranes of the vesicular systems are covered by ribosomes arranged in circular patterns. The mitochondrial envelope shows small perforations which could allow the exchange of macromolecules. The storage granules consist of concentric layers of lipid, presumably phosphatide. A Golgi apparatus has been detected which may be involved in the storage of lipid. The structure of the unit membrane and the membrane structures of all organelles as revealed by chemical fixation are confirmed in principle. Glycogen agglomerations are identified in the ground plasma of older cells. Insight into artifacts introduced by common chemical fixation and embedding techniques is obtained and discussed.

INTRODUCTION

Several authors have investigated the electron microscopic structure of yeasts (especially *Saccharomyces cerevisiae*) using the common fixatives (2, 19, 20, 57). They have shown a variety of organelles comparable to those found in higher plant cells. Two exceptions should however be mentioned: neither plastids nor Golgi apparatus was detected. It is somewhat surprising that the quality of the published micrographs does not reach a high level in many cases. The comparison of the reported results with some of our own gives some evidence of a great sensitivity of baker's

yeast to chemical fixation. Important structures often are distorted or totally blurred, presumably owing to shrinkage and washing out of material. These artifacts are the unavoidable consequence of chemical treatment.

To overcome this problem Mundkur (38-41) has applied a freeze-drying technique to yeast cells. His results, although having interesting cytochemical aspects, show even less morphological detail than do those obtained with the former chemical procedures. Membranes, for instance, are nearly invisible. This fact can be explained if

we consider the effect of the combined physical and chemical treatments that Mundkur utilizes. The probability of producing artifacts is greatly multiplied by such combinations.

Up to now the only really productive alternative to the common fixation and embedding techniques is freeze-etching (51). This method is a special kind of freeze-drying which allows the investigation of objects in the frozen state. There is no chemical treatment of the object during the whole procedure until the replica is formed on the etched fracture plane through the frozen specimen. How-

Microtome

The description of our freezing ultramicrotome can be found in an earlier publication (34).

Freezing

Several techniques were used. (a) A small droplet (about 2 mm³) of a very concentrated suspension of yeast cells is placed on the object table. The quick attachment of the table to the precooled stage ("conducting rod") at -160°C results in a fairly rapid lowering of temperature (-10°C/sec). (b) A smaller droplet (about 0.2 mm³) is placed on a supporting

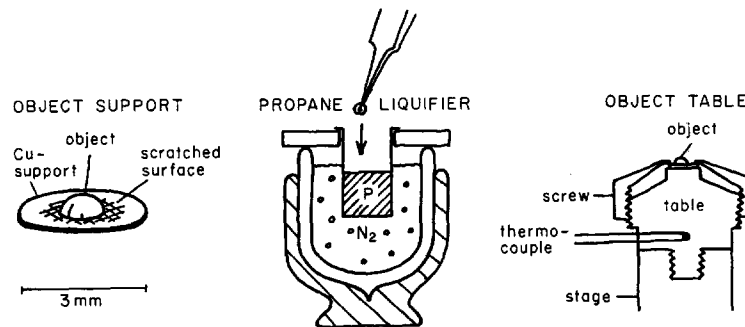


FIGURE 1

Diagram of the snap-freezing procedure (left and center) and of the device which enables the attachment of the frozen object to the table of the freezing ultramicrotome (right).

ever, the great advantages of this method depend on a suitable freeze-fixation.

By means of snap-freezing and/or glycerol impregnation, yeast cells have been frozen-fixed so as to preserve the life of the organisms. The utilization of this "ideal" fixation in conjunction with an improved freeze-etching technique (34) has yielded a great deal of new information about fine structure in baker's yeast.

MATERIALS AND METHODS

Object

Baker's yeast (a strain of *Saccharomyces cerevisiae*) was grown on agar plates containing 2 per cent malt extract. The time of cultivation varied from 0 hours to several days, and the temperature usually was 30°C. Some of the cultures were given a starvation treatment in a refrigerator at 4°C; some were suspended in 20 per cent glycerol for at least 4 hours. Both treatments made the yeasts more resistant to freezing damage.

copper disc (3 mm in diameter and 0.05 mm in thickness) the surface of which is scratched for better adherence of the object. The object then is frozen by throwing this support into liquid propane at -190°C, producing a presumed temperature drop of -100°C/sec. After freezing, the support must be attached with a screw to a special precooled object table (Fig. 1) and mounted on the stage. (c) Technique b can be adapted to liquid helium II (see Fernández-Morán, 12, 13) which may freeze the object more than ten times faster.

Sectioning

The frozen object, mounted on the object table, is cut, etched (that is, the cut surface is freeze-dried to a depth of a few hundred Angstroms) and covered with a platinum-carbon replica in the high vacuum of our freezing ultramicrotome. The object temperature is -100°C during the whole procedure; the vacuum reaches 2 to 3 × 10⁻⁶ mm Hg.

Detachment

The vacuum is broken after the deposition of the replica on the frozen-etched face of the object. The object table is detached from the stage and warmed

to room temperature. The table is dipped into water and the replica floated off. Adhering yeast cells are dissolved away from the floating replica by washing in turn with *eau de Javelle* (a commercial product containing sodium hydroxide and sodium hypochlorite) and then with 70 per cent sulfuric acid. After washing with distilled water, the replica is taken up on a Formvar-covered grid.

Survival Rates

The survival rates of the frozen yeast cells were determined in order to check the effectiveness of the different freeze-fixations. The frozen droplets were thawed as quickly as possible by placing them in water at 40°C. An adequately diluted suspension was spread on agar discs and incubated at 30°C for 7 hours. After incubation, living cells could be recognized since by division they formed colonies of at least 4 to 8 cells. They were counted under a phase contrast microscope equipped with an eyepiece which contained a graduated grid.

Electron Microscopy

All micrographs were taken with a Siemens Elmiskop I.

Shadows

The micrographs are generally mounted in such a way that the incidence of shadow casting descends from the upper right or left. Planes exposed to the beam of shadow casting appear dark while the shadows are bright.

ORIGIN AND QUALITY OF THE ELECTRON MICROSCOPIC IMAGE

The first and decisive problem of the freeze-etching technique is the perfection of the freeze-fixation (33). We expect that an object which can recover from freezing will not show any significant artifacts in the frozen state. We have therefore determined the survival rates of frozen-fixed yeast cells in order to test the efficiency of our freezing techniques and certain pretreatments. The results are tabulated below:

Pretreatment	Survival rates		
	Survivors at different freezing speeds (objects frozen to at least -160°C)		
	Quick -10°/sec	Snap -100°/sec	He II -10,000°/sec
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
None	1	30	~100
Starvation		60	
20 per cent glycerol	70	100	100

Further studies (to be published later) show that a temporary rise up to -50°C of the object temperature does not alter these results. We can conclude that at -100°C (the temperature during cutting, etching, and shadow casting) the object is more than 50°C below the zone of actual ice crystal growth and therefore alterations caused by devitrification do not have to be taken into account.

The fixation is considered "ideal" in all cases in which the survival rate exceeds 50 per cent. This statement is verified by the electron microscopic image. Only in these cases are the holes in the cytoplasm (produced by ice crystals) absent or so small (100 Å or less) that their identity is not evident.

A comparison of glycerol impregnated and non-impregnated cells indicates some masking but no hiding of fine structure by the antifreeze agent. The ability of baker's yeast to grow and ferment in nutrient solutions containing 20 per cent glycerol proves that no toxic effect need be taken into account.

Next it must be shown that the subsequent steps of the freeze-etching procedure do not introduce artifacts. The "cutting," executed on our freezing ultramicrotome, is actually a fine splintering of the deep-frozen material. The production of fracture planes instead of cut faces has two great advantages. (a) A great deal of the object field does not come into contact with the knife and therefore no artifacts are introduced by superficial warming or shifting of material which might cause recrystallization. (b) The fracture plane may penetrate the structures thereby yielding cross-sections, but also it may follow limiting membranes thereby revealing surface views never seen by the usual sectioning techniques (Fig. 2). Even without etching, a replica of the relief produced by "cutting" may show certain details of the object.

The etching (consisting of a very brief freeze-drying) reveals the fine structure in the fracture planes by a superficial removing of the masking ice (Fig. 3). The evaporation of free water from the solid is a physically and chemically harmless process which does not deform the structures.

The last preparational steps, which may affect the object, involve the evaporation of the replica materials. The possible introduction of artifacts derived from heat radiation cannot be excluded in principle, but it is very unlikely that this is a factor. The sources of evaporation are quite dis-

tant from the object surface (at least 12 cm), the evaporation time is short (3 to 6 sec) and the object is perfectly cooled. Recrystallization is excluded from the dried part of the object, which is the only part recorded by the replica. In confirmation of these considerations, we were never able to detect any indication of a burning effect on the fine structure of our objects.

Some limiting factors inherent in replica techniques have to be taken into account. The very uneven relief produced by the splintering is responsible for the great variability in resolution obtained by a replica. From place to place the exposure to the evaporation beam may suddenly change, improving or hindering the visualization

of fine structure. Under the most favourable conditions the resolving power is directly dependent on the thickness of the shadowing layer and may be 20 to 30 Å (32). These facts lead to the conclusion that a replica and, accordingly, an electron microscopic image of high fidelity are produced by freeze-etching. There is only one reservation. This image imparts purely morphological information on the natural state. Specificity cannot be introduced by any differential staining or similar techniques. Nevertheless, freeze-etching should be able to give some insight into the chemical nature by tracing modifications in the specimen after previous physiological or chemical manipulations.

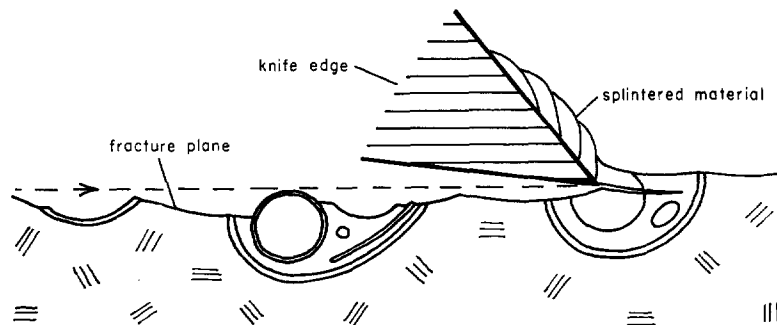


FIGURE 2

Diagram of the "cutting" procedure which actually consists of a fine splintering of the deep-frozen object (yeast cells).

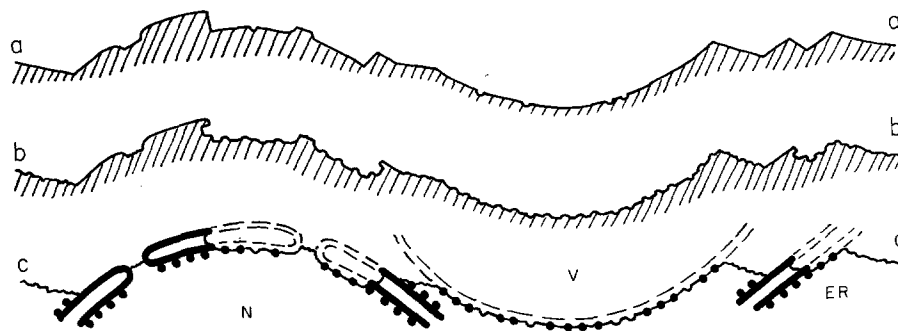


FIGURE 3

Diagram of how the splintering and the etching reveal the fine structure of a frozen object. *a*: cross-section through the fracture plane (enlarged area of the central yeast cell in Fig. 2). *b*: etched fracture plane, showing the fine structure. *c*: the reconstructed structural details of the recorded object. *N*: nucleus, showing a partially removed envelope. *V*: totally removed vacuole, rendering possible a surface view of the adjacent cytoplasmic ground substance. *ER*: endoplasmic reticulum, fractured at a low angle.

RESULTS AND CONCLUSIONS

Cell Wall and Cytoplasmic Membrane

Earlier communications (2, 26, 57) based on chemical fixation reported that in the yeast the cell wall and cytoplasmic membrane could be distinguished. In the present work, many cases have been found in which these structural units are clearly separated by the splintering process (Figs. 7 to 9). A fracture of the cell wall into subunits never occurred. The fracture plane is found either to follow the surface of the cell wall or to penetrate the whole wall perpendicularly. A "cross-fractured" wall shows a fine granular structure which may grow coarser from the very smooth outer side to the inner surface (Figs. 5 and 6). Only very rarely have we detected a sharp change in the granularity in the middle of the wall, indicating the presence of two layers. However, there is no evidence of the two "membranes" which are claimed by Bartholomew and Levin (3). The wall has an over-all thickness of 700 A. In very young cells it can average 100 A less, in starved cells 100 A more.

The structure of the bud scars, which produce the only relief on the outer surface of the cell wall, is the same as that reported in Bradley's paper (6, see Fig. 5). The inner surface shows the characteristic invaginations created by the cytoplasmic membrane (Fig. 6). The existence of such invaginations has been demonstrated in earlier papers (26, 57), but the authors were not able to distinguish between real structure and shrinkage artifacts. In our specimens, artifacts of this kind do not have to be taken into account.

If we combine surface views (Figs. 6 and 7) and cross-fracture views (Fig. 8), we are able to get a 3 dimensional image of the invaginations of the cytoplasmic membrane. These invaginations have an average length of 3000 A, a width of 200 to 300 A, and a depth of 500 A. In older cells there is an average of 15 invaginations per square micron, in younger cells less or none. From these data one can calculate that a sculptured membrane shows a surface enlargement of at least 50 per cent compared with a completely flat one.

The examination of the internal structure of the cytoplasmic membrane and indeed of all membranes present in the cell of *Saccharomyces cerevisiae* has led to an unexpected result. Cross-fractured membranes give an image that is in

every detail, the same as that shown by membranes in sections of permanganate-fixed material. The membranes consist of three subunits, each of which is about 25 A thick, which is in agreement with the structure of the unit membrane found by Robertson (47, 48). The structure of the unit membranes revealed by freeze-etching can be seen most clearly by examination of the nuclear envelope (Figs. 10, 12).

The cytoplasmic membrane could never be separated from the adjacent ground plasm by fracturing the frozen object. Therefore we are able to show only the outer surface which is found to be covered by a variety of particles. A portion of these particles is concentrated in hexagonal arrangements containing 20 to 50 units and having a lattice period of 180 A. These groups of particles are situated between the invaginations. An average of 10 arrangements can be found per square micron, but they may go up to 40 in certain areas, totally covering the flat part of the cytoplasmic membrane. In any case, the invaginations are free of such particles (Fig. 7).

A closer examination of the hexagonal arrangements reveals that they are penetrated by fibrils of short length (Figs. 7, 9). In regions of the cytoplasmic membrane that are close to a perpendicularly fractured wall (Fig. 9), the fibrils can be observed to disappear into the innermost layer of the adjacent wall. These fibrils, which are about 50 A in diameter, correspond in size with the glucan fibrils found by Houwink and Kreger (22) in hydrolyzed yeast cell walls. These authors did not determine whether the fibrils are produced by hydrolysis or are a natural structure. Our findings suggest that at least a part of the glucan is not amorphous and that there are specialized centers in the cytoplasmic membrane that play a prominent role in the production of fibrils.

Between the hexagonal arrays, a great number of single particles are spread over the entire flat surface of the cytoplasmic membrane. A comparison of the caps produced by the shadowing layer on the grouped and on the single particles shows that they are identical in size. Single particles are present in a concentration of about 1000 units per square micron on every cytoplasmic membrane, while the arrays of particles may be entirely absent. The absence of arrays or lattices goes hand in hand with the absence of fibrils. One might suggest now that the glucose is polymerized and crystallized to glucan fibrils at the

surface of the arranged particles. At the same time it is evident that the particles do not have to be arranged and that the glucans, which are always present, do not have to be crystallized (22). The combination of these facts permits the following hypothesis. (a) The function of single, randomly distributed particles, is solely the polymerization of glucose to amorphous glucan. (b) The particles concentrated in patterns are responsible for the production of fibrils.—This leads to the consideration that the texture of the glucans would be a function of the arrangement of the particles. Only about one-fourth of all particles are found to be concentrated in arrangements, a fact which coincides with the observation that only a small part of the glucans may be crystallized (22).

In the cytoplasmic membrane, holes of the size of single particles can be identified (arrow in Fig. 9). They indicate the places where the particles have been plucked out by the preparative procedure. The same phenomenon is usual with whole arrangements in very young cells, confirming that the particles not only adhere at the surface of the membrane but are a part of it. Inter-

ruptions of the unit membrane, having the diameter of the arrangements (500 to 1000 Å), should be visible in sections of chemically fixed material. In fact, breaks of that size can be recognized in the published literature; e.g., Fig. 5 on page 694 of Vitols' paper (57). The cytoplasmic membrane is clearly visible around the invaginations, but in the flat portions it suddenly disappears. The invisibility of the single or arrayed particles in chemically fixed material is easy to explain. Like the ribosomes of the cytoplasm, these particles are probably hidden by the superposition of fine structure which is unavoidable in sections having a thickness of at least 400 to 500 Å.

Nucleus

As in the cells of higher plants, the nucleus of *Saccharomyces cerevisiae* is surrounded by two unit membranes (57), which are clearly visible in our specimens (Figs. 10, 12). Another observation, confirming the identity of the nucleus, is that perforations (*i.e.* the pores) are present in this membrane complex (Figs. 11 to 15).

The resting nucleus generally has a globular

FIGURE 4

Total view of a cross-fractured yeast cell, revealing a large vacuole (in surface view), a nucleus (cross-fractured), and several mitochondria close to the cell wall. The "protuberances of the cell wall" are artefacts created by the freezing of the glycerol-containing medium which surrounds the cell. $\times 14,000$.

FIGURE 5

Surface view of the cell wall, showing two bud scars. $\times 20,000$.

FIGURE 6

Inside view of a portion of the cell wall showing rod-like (elongated) invagination. $\times 20,000$.

FIGURE 7

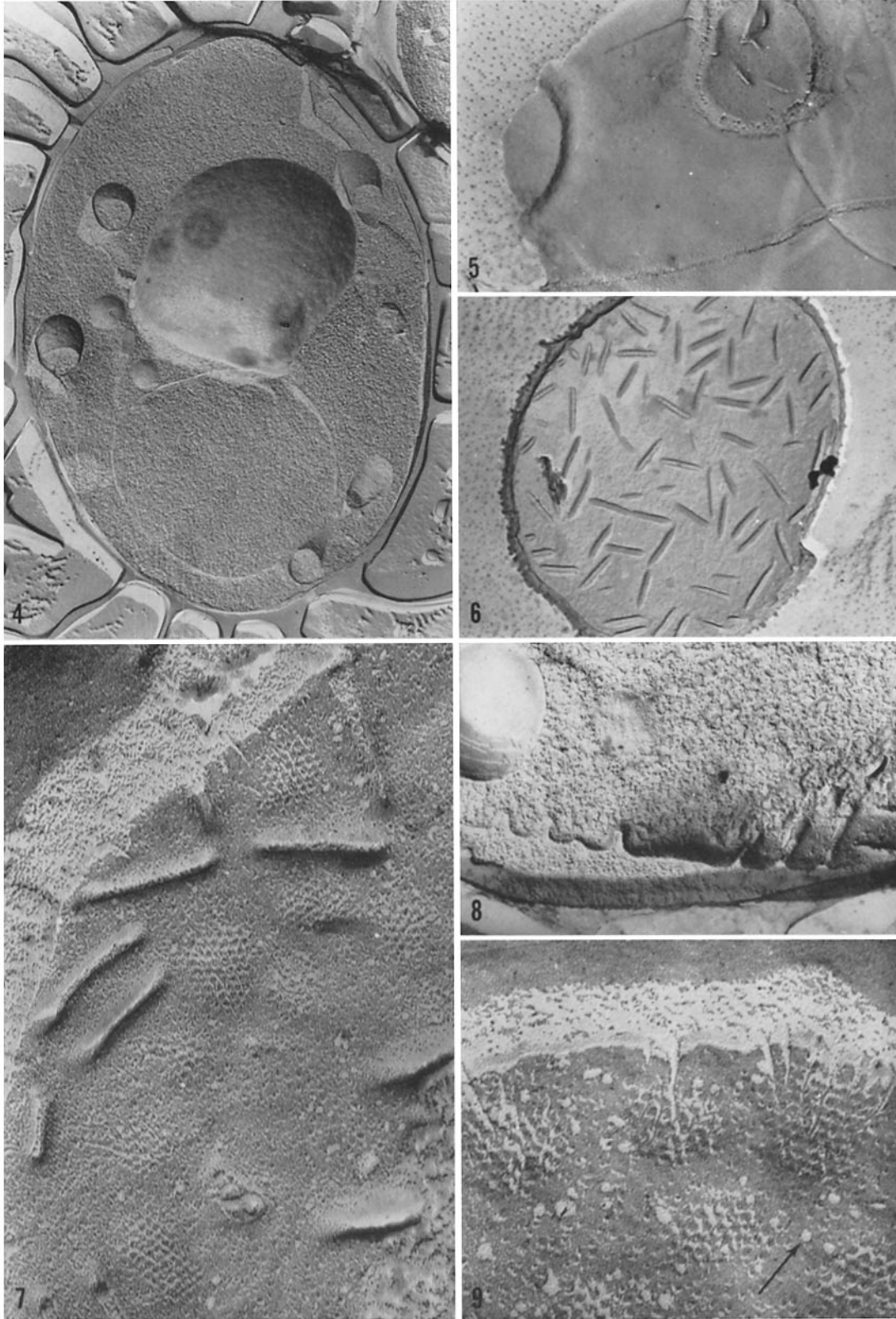
Surface view of the cytoplasmic membrane. The hexagonal arrangements of particles are situated between the invaginations. A cross-fractured cell wall is visible on the upper left. $\times 90,000$.

FIGURE 8

Invaginations of the cytoplasmic membrane; left, cross-fractured; right, surface view. $\times 40,000$.

FIGURE 9

The hexagonal arrangements of particles. The glucan fibrils, which penetrate these areas, extend to the innermost layer of the adjacent cross-fractured cell wall (top). The arrow marks a hole in the cytoplasmic membrane where a single particle has been plucked out by the splintering procedure. $\times 120,000$.



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shape. Irregularly indented forms, which are common in chemically fixed and embedded material, never occur. These deformations are obviously shrinkage artifacts probably produced by the alcohol or acetone dehydration. A natural alteration of the nuclear shape is caused by vacuoles or lipid granules, which may be pressed against the nuclear envelope resulting in a local concavity.

The nucleus has a very constant diameter of about 2.3 microns. Alterations in size are caused only by polyploidy or occur during nuclear division.

The circular pores, 800 to 900 Å in diameter, are spread over the whole nuclear envelope. They generally occur in a density of 10 to 15 units per square micron, occupying 6 to 8 per cent of the surface. If we take into account the whole surface, one nuclear envelope of baker's yeast is perforated by about 200 pores. Mainly in old cells (derived from starved cultures), the pores may be concentrated in areas containing 20 and more (Fig.

11). In these cases the total number of pores is not altered for, in the regions of high pore concentration, only about two-thirds of the nuclear surface is covered by membranes. (The calculation of pore numbers was carried out on 10 surface views of different nuclei.) Several authors (1, 58, 61) have described certain structures present in nuclear pores of animal cells. In yeast cells, cross-fractured (Fig. 12) and cross-sectioned (57) pores show neither locking membranes nor the so called "annuli." The pores contain nothing but ground substance.

For understanding the surface views of the nucleus, an explanation is needed of how the nuclear membranes are made visible by the splintering process. An examination of the surface views present on Figs. 13 to 15, showing the nuclear membranes from the outer (Fig. 13) and from the inner side (Fig. 14), reveals in both cases a similar image. There is a smooth piece of membrane partially covering a rough background. On the smooth surfaces the pores have the appearance

FIGURE 10

A cross-fractured nuclear envelope, showing the structure of the unit membranes. The nucleoplasm is visible on the lower right, the cytoplasmic ground substance on the upper left. $\times 90,000$.

FIGURE 11

Surface view of a nucleus derived from an old cell. The pores are concentrated in certain areas. $\times 30,000$.

FIGURE 12

Cross-fracture through a nuclear envelope which is perforated by a pore. The structure of the unit membranes is partially visible. $\times 135,000$.

FIGURE 13

Outside view of a nuclear envelope. In the lower part of the figure it is splintered away, unveiling the adjacent surface of the nuclear content. $\times 80,000$.

FIGURE 14

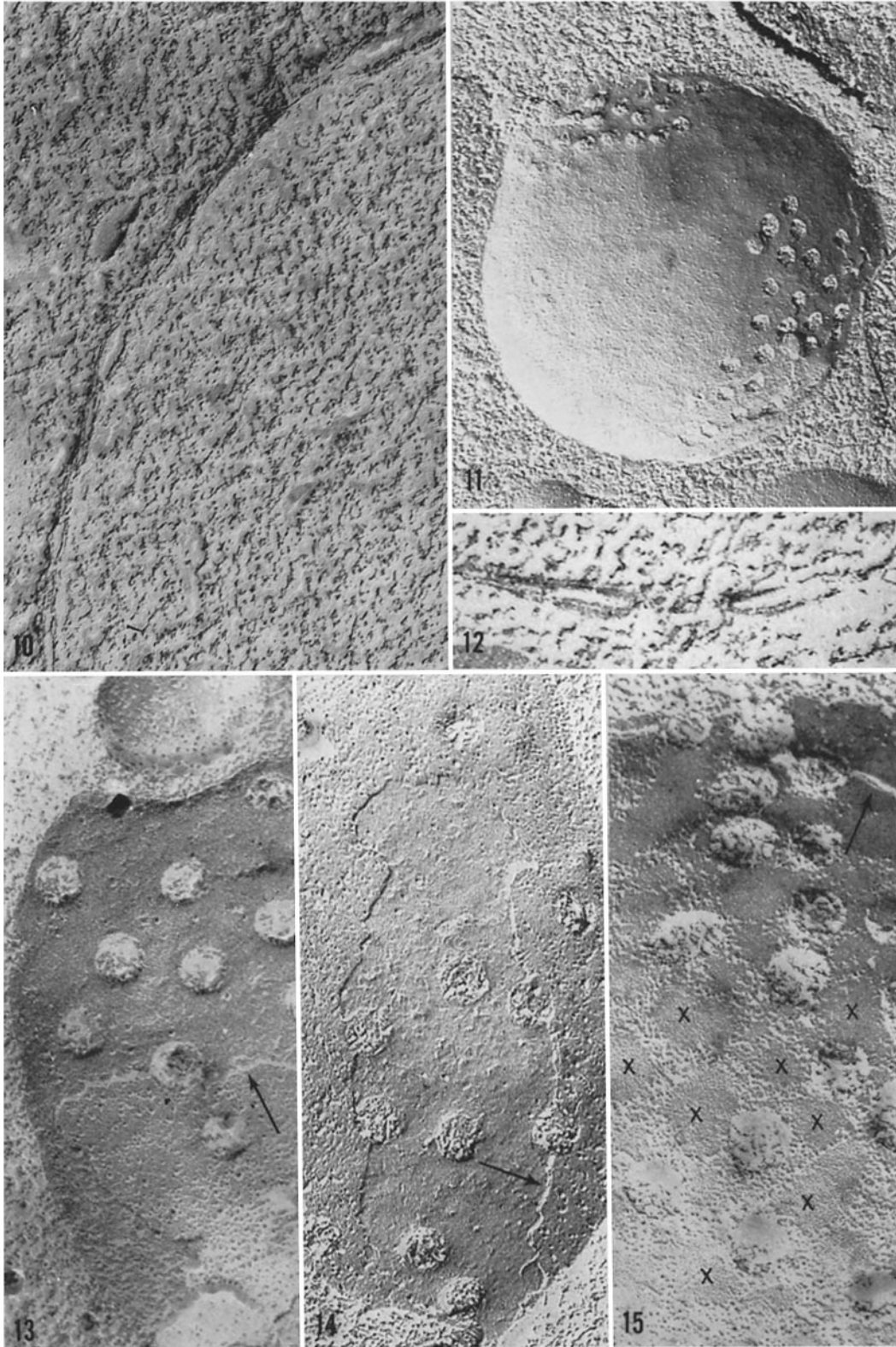
Inside view of a piece of the nuclear envelope left by the splintering on the surface of the adjacent ground plasm. $\times 80,000$.

FIGURE 15

Surface view of the content of an old nucleus. The envelope is totally removed by the splintering. The position of the pores is indicated by circular depressions or elevations. The pattern on the flat part of the surface ("closed pores") (x) is created by circularly arranged particles (ribosomes). $\times 80,000$.

FIGURES 13 TO 15

The arrows indicate the fracture edge of the nuclear double membranes.



of holes, while on the rough ones they may be raised above the surrounding area. These facts are explainable if we assume that a fracture plane follows the inner or outer surface of the nuclear envelope. The rough background would correspond to a surface view of the cyto- or caryoplasm, respectively, which is adjacent to the nuclear membranes. The smooth pieces would have to be fragments of the double membrane left on the background by the splintering procedure. An examination of the border of these membrane pieces (arrow in Figs. 13 to 15) justifies the above-mentioned assumption. The replicas show in each case the presence of two simultaneously fractured membranes; *i.e.*, the totally cross-fractured double membrane (see Fig. 3). The different shapes of

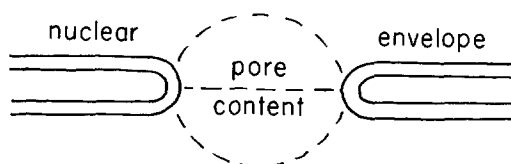


FIGURE 16

Diagram of a cross-section through a nuclear pore. The interrupted lines indicate the possible courses of the fracture plane.

the pores can also be explained. On the double membranes the pores really look like holes, while the adjacent plasmatic surface has to show the opposite relief; *i.e.* short stalks above the surrounding area. However, certain exceptions have to be mentioned: they are caused by pore contents which have not been broken apart in the plane of the smallest diameter (Fig. 16).

The particles, which cause the roughness of the "background," have an average diameter of 80 to 120 A. Their position on the surface of the caryo- and cytoplasm indicates that originally they must have been associated with the nuclear membranes. In other words, the "background" shows the particle pattern of the nuclear envelope. In young cells the particles are uniformly spread over the whole surface, while in old ones they may be arranged in circular groups which have the same diameter as the pores. The appearance of such a surface (Fig. 15) suggests that these circular areas represent "closed pores." Since we know that the pores in young and old cells (*i.e.* growing and starved cells) have different positions, a mechanism has to be considered which enables a

dislocation of the perforations. To assume the occurrence of a "pore-drift" in the double membrane complex is rather inadequate. However, there is enough evidence that a nuclear envelope can be fractured and reconstituted (31, 35, 59). From this point of view a closure of old pores and an opening of new ones, *i.e.* an interruption of a membrane connection and a construction of new membranes, is more likely. The coincidence of a pore dislocation in starving cells and the appearance of "closed pores" on old nuclei confirms the supposed character of these structures. The concentration of the particles around the pore scars, the structure which makes these areas visible, indicates that the particles can be correlated with the closure of the pores. Assuming a functional relation, we may consider that the particles have a membrane-fracturing and/or -synthesizing task.

The perinuclear space has an average width of 100 to 300 A. In cross-fractured nuclear membranes (*e.g.* Fig. 12, helium frozen) it appears to be completely empty. This fact eliminates any suspicion that the particles could be situated on the membrane surfaces that face each other across the interlamellar space. It confirms the position already proposed for the particles and validates our interpretation of micrographs showing the nuclear envelope after the splintering. In specimens frozen at a slower cooling rate the space is remarkably enlarged (Fig. 18, propane-frozen). This fact can be explained only by a larger growth of the ice crystals pushing the membranes apart. We can conclude that the perinuclear space is mainly filled with water, which may contain only dissolved substances in a very low concentration.

The nuclear content is known to be differentiated into chromatin particles (chromosomes), a nucleolus, and caryolymph. These data originate from light microscopic investigations of stained yeast cells (*e.g.* references 11, 49). The electron microscopic image of osmium- and permanganate-fixed cells is less informative, since only diffuse spots of higher or lower electron opacity are shown. The electron microscopic identification of the intranuclear structure has been questionable in any case. It is generally supposed that this lack of information is due to the fact that osmium tetroxide and potassium permanganate do not preserve nuclear structures well. Our specimens indicate this conclusion to be erroneous. In frozen-etched yeast cells no structure is visible at all, either in resting nuclei, or in nuclei having under-

gone mitosis and meiosis, except for a uniform background granularity which is scarcely distinguishable from the cytoplasmic ground substance (Figs. 10, 12). These facts lead to the following conclusions. Neither the chromosomes nor the nucleolus of *Saccharomyces* are separated by any kind of membrane from the remaining content. All constituents of the caryoplasm seem to be uniformly hydrated (also during division), resulting in the appearance of a uniform distribution. No component can be larger than about 100 Å (background granularity), except in one direction. In other words, the structural units are granules or meandering threads having approximately this diameter. But the presence of a great number of rather large perforations in the nuclear envelope suggests that the caryoplasm contains "linked groups" which hinder a loss of nuclear material through the pores. This is probably intermolecular linkage (*e.g.* present in chromosomes and nucleolus) which does not result in fixed and (in our conditions) resolvable structure.

In usual preparation procedures (chemical fixation) these groups can therefore be made visible only by differential staining and/or by the introduction of shrinkage artifacts that alter the original distribution of the nuclear constituents.

Vacuoles and Endoplasmic Reticulum

Two clearly distinguishable organelles are included in the cytoplasmic ground substance of *Saccharomyces cerevisiae*, both of which are surrounded by a single unit membrane. A globular system can be identified as vacuoles (Figs. 4, 17, 24, 25). The appearance of ice crystals indicates the high water content of these vesicles (Fig. 25). Throughout a given cell the vacuoles generally have a similar size. The smallest vesicles, having a diameter of about 0.3 micron, usually are aggregated around the nucleus in groups of 5 to 6, while the largest vacuoles, having a diameter of about 3 microns, occur singly. The second or "flat" system is a reduced kind of endoplasmic reticulum (Figs. 17 to 19, 21). Its identity is verified by its occasional connections to the outer nuclear membrane (Figs. 18, 21). Cross-fractured cells show either one large piece of paired membranes, extending through the whole cytoplasm, or several smaller units. In chemically fixed and embedded material (57) the difference between these two systems may be heavily obscured. The vacuoles are often remarkably shrunken, creating struc-

tures which may look like "diverticula extending into the cytoplasm." Vitols (57) concludes therefore that probably any kind of endoplasmic reticulum is an appendix of the vacuoles. In our unshrunken specimens the vacuoles are fully expanded structures, in any case, and do not show any protuberances.

The splintering procedure exposes these membranes in the same way it does the nuclear envelope as already described. The fracture planes show either the outer side of the unit membrane or the adjacent ground plasm. A piece of endoplasmic reticulum, fractured under a low angle, can be seen in Fig. 19. The small fragments of the double membrane show a smooth surface (arrow), while the adjacent ground plasm is rough. At a vacuolar surface two different views have to be distinguished. A form rising above the surrounding area shows the outer side of the vacuolar membrane (Figs. 4, 17, 24) while a depression indicates a surface view of the adjacent ground plasm (Fig. 19). A distinction between these views based on roughness is hindered because the particles cover both sides. Fig. 3 represents a diagram of the structures discussed and their development by freeze-etching.

Particles of the same size as those demonstrated for the nuclear envelope are in contact with the membranes of both vesicular systems. They are mostly recognizable on the ground plasm adjacent to the organelles, causing the mentioned roughness of the fracture plane. At the vacuoles separation of the particles from the membrane is not complete, and some of the particles are left in the original position (*e.g.* Fig. 24). If we count the particles on both sides, a total amount of 2000 to 3000 units per μ^2 can be calculated. Circular, uncovered areas, distributed uniformly over the whole surface, are recognizable on the vacuoles (Fig. 24) and on the surface of the ground plasm adjacent to the endoplasmic reticulum (Fig. 19). These areas, mostly occurring in growing and dividing cells, do not exceed a diameter of about 1000 Å.

The properties of the described population of particles lead to the following considerations. (a) In fresh cultures of yeast the organelles generally are growing and dividing like the whole cell, resulting in a general expansion of the vesicular membranes. (b) The well defined pattern suggests that the particles have a fixed position on the membranes. (c) The size of the uncovered

areas, having an upper limit, indicates that these regions do not undergo any further enlargement. It can be assumed therefore that the membranes grow only in the particle-covered region. As in the case of nuclear membranes, we may assume that there is a functional relation between the particles and the production of membrane material. The similarity of the vesicular and nuclear membranes is increased by the appearance of

perforations in the endoplasmic reticulum which have the same size as the circular arrangements and the pores (Fig. 21).

The characteristics of the particles,—their having a size of about 100 A and a position at the cytoplasmic side of unit membranes, their circular arrangements (see Rebhun, 44, 45), and their probable involvement in protein synthesis, correspond to properties known to characterize the

FIGURE 17

The vesicular systems. The system of flat paired membranes, extending from the upper right to the lower left, is a reduced kind of endoplasmic reticulum. At the bottom, a vacuole in surface view, on the right, a cross-fractured nucleus. $\times 40,000$.

FIGURE 18

Endoplasmic reticulum connected to the nuclear envelope (upper center). $\times 40,000$.

FIGURE 19

Endoplasmic reticulum, fractured at a low angle, extending from the upper center to the lower right (compare with Fig. 3). The small pieces (one is marked by an arrow) show surface views. The background, on which these pieces are located, represents a surface view of the adjacent ground plasm. On this surface, circular areas can be recognized which are free of particles (ribosomes).

At the bottom and upper left, two mitochondria are visible in surface view. On the upper right, the surface of the ground plasm adjacent to a large vacuole is shown (compare with Fig. 3). On the lower left, the unit membrane (cytoplasmic membrane) can be seen forming an invagination. $\times 60,000$.

FIGURE 20

A large dumb-bell-shaped mitochondrion which is probably engaged in division. The upper left shows a surface view of the mitochondrial envelope, the lower right a cross-fracture which reveals the cristae mitochondriales. $\times 20,000$. The insert demonstrates the perforations of the mitochondrial envelope at higher magnification. $\times 75,000$.

FIGURE 21

A tangentially fractured piece of endoplasmic reticulum which is connected to the nuclear envelope (at the top). The reticular appearance is caused by the pores which perforate the membranes. $\times 60,000$.

FIGURE 22

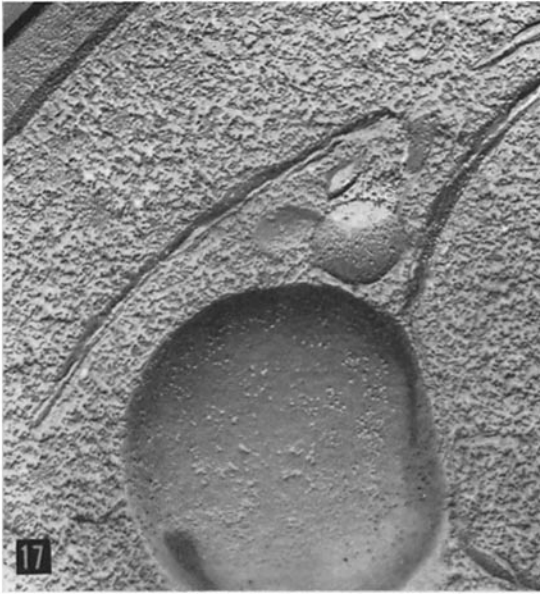
Surface view of a mitochondrion. $\times 40,000$.

FIGURE 23

Surface view of the cytoplasmic ground substance adjacent to a mitochondrion. $\times 40,000$.

FIGURES 22 AND 23

The arrows mark a piece of mitochondrial envelope which has been splintered out by the fracturing (Fig. 22) and left on the exposed surface of the cytoplasmic ground substance (Fig. 23).



ribosomes, *i.e.* Palade's small granules (5, 43). The identification of the particles as ribosomes however is based on circumstantial evidence and will have to be confirmed by direct methods demonstrating the RNA content. The striking fact that up to now electron microscopists have not been able to localize the ribosomes in the cells of baker's yeast may be explained on the basis of the properties of the fixatives used. Only permanganate, which does not preserve the ribosomal pattern, has revealed a clear image of the internal structures of *Saccharomyces*.

With respect to its width and content, the intracisternal space of the endoplasmic reticulum has the same properties as the perinuclear space demonstrated in the nuclear envelope (Figs. 17, 18). The network, visible in cross-fractured vacuoles (Fig. 25), is produced by small ice crystals, indicating the presence of dissolved substances. In cells originating from old cultures, the vacuoles may contain several dense globular bodies having a diameter of 0.1 to 1 micron (Fig. 25). Their occurrence in mature cultures, their size and their

position indicate that these bodies are the meta-chromatin granules described by Guilliermond (17). They are mainly composed of polymetaphosphate (60).

The description of vacuoles and endoplasmic reticulum has demonstrated the great differences between these organelles in regard to shape and content. But the similarity in the structure of the membranes of these organelles and of the nuclear envelope may indicate an ontogenetic relationship between all these membranes.

Mitochondria

In the last few years a number of investigators have reported electron microscopic evidence for the existence of mitochondria in yeast cells (2, 18, 21, 25-27, 29, 55, 57). All these authors agree in their description of the fine structure involved, which corresponds in principle to that of mitochondria of higher plant and animal cells. The identity of yeast mitochondria has been confirmed by the localization of characteristic enzymes in isolated organelles (56).

FIGURE 24

Surface view of a young vacuole, showing circularly arranged particles (ribosomes). $\times 50,000$.

FIGURE 25

Cross-fracture through an old vacuole containing dense globular bodies of "meta-chromatin," *i.e.* polymetaphosphate. $\times 25,000$.

FIGURE 26

The Golgi apparatus of *Saccharomyces*. The fracture plane has met tangentially the central pile of flattened sacs. $\times 60,000$.

FIGURE 27

A cross-fractured Golgi apparatus (left center) containing an expanded central vesicle. $\times 60,000$.

FIGURES 28 AND 29

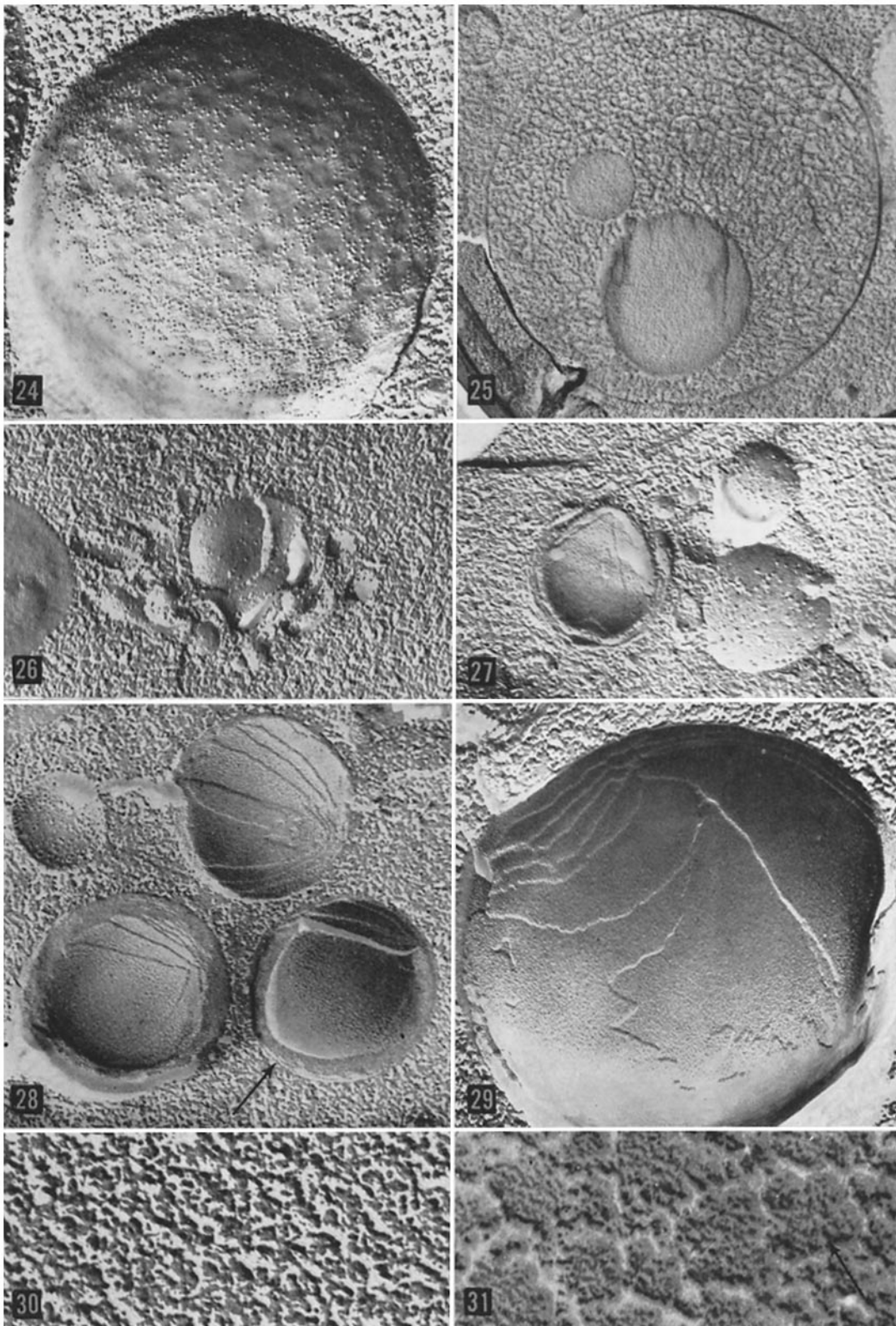
Lipid granules (storage granules) revealing a multilayered structure which indicates a concentric deposition of phosphatids. The arrow in Fig. 28 marks the unit membrane which surrounds a granule. $\times 80,000$.

FIGURE 30

The granular structure of the cytoplasmic ground substance of a young cell. $\times 150,000$.

FIGURE 31

The fine-granular structure of glycogen which is incorporated in the cytoplasmic ground substance of an older cell. The aggregation marked by the arrow is composed of four macromolecules of glycogen. $\times 150,000$.



In our specimens, the mitochondria have a structure similar to that described in the communications mentioned above. In growing cells, the mitochondria are usually about 1 to 2 microns long, 0.5 to 1 micron thick, and are shaped like a short sausage (Figs. 22, 23). Elongated dumb-bell-shaped organelles presumably are engaged in division (Fig. 20). In mature cells, the mitochondria are only 0.3 to 0.4 micron thick but may reach a length of 3 microns. In young and old cells they are situated close to the cytoplasmic membrane (Fig. 4). Cross-fractured mitochondria, which occur rather seldom, reveal the well known internal structure. The organelles are surrounded by two unit membranes, the inner membrane forming the few cristae which extend into the mitochondrial matrix (Fig. 20). In the mitochondrial envelope the membranes usually touch each other, while in the cristae they are separated by an obviously empty interlamellar space having a width of 50 to 100 A. (As a consequence of this structural peculiarity, the membranes of the cristae are much easier to recognize than those of the envelope.) The granular matrix seems to be denser and less affected by ice crystal formation than the cytoplasm.

In most cases the splintering procedure reveals a surface view of the mitochondria. The envelope shows a very uneven face which differs greatly from that of the limiting membranes of all the other organelles. Small pieces, 500 to 1000 A in diameter, may be splintered out of the envelope (arrow in Fig. 22) and adhere to the surface of the adjacent ground plasm (arrow in Fig. 23). Membranes that have been left more or less intact show many small perforations of irregular outline (Fig. 20). These perforations may reach a length of 1000 A, while their width probably does not exceed 100 A originally (insert in Fig. 20). Whether the perforations penetrate both membranes of the mitochondrial envelope cannot be determined by these observations, but the depth of these slits suggests that they do.

The similarity of mitochondrial structure revealed by frozen-etching to that seen in chemically fixed and embedded materials indicates that these organelles do not undergo as much morphological alteration during common preparative treatments as the nucleus and the vesicular systems. The relatively high resistance of the mitochondrial content to ice crystal formation suggests that the matrix contains many more hydrophilic sub-

stances, *i.e.* proteins, carbohydrates, and related compounds (33), than do the other organelles and the cytoplasmic ground substance. A comparable result has been shown by Rebhun (46) in frozen-substituted invertebrate oocytes. The mitochondrial matrix appears to be opaque, causing a "negative" staining of cristae. It demonstrates furthermore the high density of the original content which, as in our specimens, has had no chance to be washed out during fixation and dehydration.

The surface structure of the mitochondria leads to several considerations. (*a*) This type of surface, being characteristic of mitochondria, allows the identification of these organelles in surface views. (*b*) The described perforations may weaken the consistency of the membranes and most probably cause the brittleness of the envelope. (*c*) The size of the perforations could allow an exchange of macromolecular substances between the ground plasm and the mitochondrial matrix. If the perforations should be a common structure of mitochondria and if they also are present in erythroblasts of mammals, they would help to explain the uptake of ferritin (4). (*d*) These perforations may be related to the interruptions produced by a "reversal of mitochondrial membranes" detected in the hamster liver (9). (*e*) The invisibility of perforations and of any reversal in cross-fractured and cross-sectioned mitochondria of yeast cells may be caused by the small size and irregularity of these discontinuities.

Lipid Granules

The cells of frozen-etched baker's yeast generally contain a population of cytoplasmic granules clearly distinguishable from the organelles described above. They are characterized by a globular shape, a diameter of 1000 to about 7000 A, and a uniformly dense content. The most rigorous dehydration, attained by a slow, extracellular freezing (33), does not disfigure these bodies. In growing cultures usually 2 to 4 granules can be detected per cell, while specimens derived from mature cultures may be crowded with them. These granules generally have a central homogeneous core, 1000 to 2000 A in diameter surrounded by a multilayered shell up to 3000 A thick. This structure is revealed by oblique fractures accompanied by partial removal of the outer concentric layers (Figs. 28 and 29). A direct measurement of the thickness of the individual layers is hindered be-

cause of their invisibility in totally cross-fractured shells (see the lower granules in Fig. 28). The thickness of one layer however can be estimated from the height of the smallest steps on a partially splintered granule. A thickness of about 40 Å may be assumed, since the replicas, having an ultimate resolving power of 20 to 30 Å, are just able to reveal such steps. A triple layer, which is about 80 Å thick and which appears at the outer side of a cross-fractured shell, can be identified as a unit membrane surrounding the whole granule (arrow in Fig. 28).

Their insensitivity to dehydration indicates that these cytoplasmic granules do not contain either water or hydrophilic substances in significant quantities and are therefore most probably composed of lipids. The accumulation of granules in cells originating from mature cultures suggests that these granules are involved in storage. In fact, the only substance concentrated in distinct "droplets," which is known to be stored in the cytoplasm of yeasts, is fat. This population of granules therefore can be identified as the "granulations lipoidiques," described by Guilliermond (16), which have the same size, frequency, and chemical nature.

The fine structure of these "lipid granules" reveals a remarkable order. It suggests that the molecules are arranged in a characteristic lattice, which would correspond in size to the phosphatid lamellation shown by Stoeckenius (52). The considerable quantity of phosphatids present in the cells of *Saccharomyces cerevisiae* (28) exceeds the amount which could be localized in the membranes. It might be incorporated therefore in the shell of the lipid granules.

The occurrence of a limiting unit membrane, which has not been shown by the common techniques, indicates the lipid granule to be an organelle rather than simply the product of a separation of phases. The structure of the granule suggests that it grows by apposition, and the distinct task of storing lipids of these granules confirms this indication. Whether the lipids are only stored or are produced by the granules cannot be determined from these observations. The similarity of these "granulations" to the lipid granules of higher plant cells—the so called spherosomes (15, 42)—has been established by Guilliermond (17). A relationship may therefore exist between the lipid granules of yeast cells and the spherosomes. The surprising detection in the mouse kidney (54)

of a lipid granule having exactly the same size and structure as the storage granules of baker's yeast also suggests the presence of this organelle in animal cells.

Electron microscopists, applying common preparative methods to yeast cells, have not been able to reveal the structure of the lipid (storage) granules. Agar and Douglas (2), using osmium tetroxide, have shown clusters of dark stained bodies, while Vitols *et al.* (57) and others, preferring permanganate, have detected distinct empty bubbles similar in size to the granules described here. Both results can be explained by the common properties of the fixatives applied and their insufficiency in regard to the preservation and staining of pure lipid.

Golgi Apparatus

A further category of vesicles limited by unit membranes is also present in frozen-etched yeast cells. The different kinds of lamellar bodies, agglomerated at certain places in the cytoplasm, may be characterized as a central pile of about three flattened sacs surrounded by many very small bubbles (Fig. 26). In some cases only the groups of bubbles or the pile of sacs can be recognized. On the surface of the membranes there may be a few particles similar to the ribosomes in size. The smallest bubbles may show a dense, non-watery content. If visible at all, only one of these vesicular complexes may be seen in a cross-fractured cell from a growing culture. They have not been detected at all in starved specimens.

The structure of these complexes resembles in size and shape the Golgi apparatus found in meristematic plant cells (14, 36). The identity of these structures, suggested to be involved in the storage of lipids (10), may be confirmed by the presence of an expanded central vesicle, which obviously contains a substance similar to that of the lipid granules (Fig. 27). This blown-up vesicle, however, cannot be identified as the "central body" known to be a rather permanent constituent of certain Golgi apparatus. Groups of three lipid granules, as shown in Fig. 28, would indicate that all three sacs of a Golgi apparatus are able to differentiate in this direction. The fact that the disappearance of the Golgi vesicles during starvation coincides with an accumulation of lipid granules confirms the above indication.

The presence of the Golgi apparatus cannot be verified in each cell, probably because of its small

size and the very small number of units per cell (presumably 1 or 2). This may also explain the fact that up to now this organelle has not been identified in yeast cells. In anaerobically cultured cells of *Torulopsis utilis*, however, a "multimembrane system" appears which surrounds an "electron-transparent area" (27). This might be a hyperblastic kind of Golgi complex containing a lipid granule.

Cytoplasmic Ground Substance

The organelles described in the preceding sections are embedded in a matrix which may be called the "cytoplasmic ground substance" or "ground plasm." The similarity between this matrix and the nucleoplasm has been mentioned. Specimens frozen at different cooling rates have shown that both substances are most sensitive to freezing damage. Reticulate artifacts, which characterize poor freeze-fixation, have been demonstrated by several authors who have applied electron microscopy to frozen-dried objects (*e.g.*, 7, 37, 50). The granular structure of the ground plasm (Fig. 30) is revealed only in specimens frozen under the most favourable conditions (see section on Origin and Quality of Image). No trace of ice crystals which generally cause morphological alterations can be recognized. In this case, the yeast cells show a uniformly structured matrix containing granules 100 to 200 Å in size.

At the end of the growth phase, the cells of *Saccharomyces cerevisiae* are considerably less sensitive to freezing damage. The ground plasm shows an altered structure; *i.e.*, the appearance of a fine-granular material which predominates over the remaining constituents (Fig. 31). This material appears in lumps composed of globular units having a diameter of about 400 Å (arrow in Fig. 31). The fine granules within these units average 60 Å in size.

Since the texture of the ground plasm is similar in frozen-etched and in chemically fixed specimens it is apparent that chemical fixation by OsO₄ or KMnO₄ gives an adequate image of the natural structure. Thread-like and reticulate forms, *e.g.* the so called "cytonemata" which have been proposed as the elementary units of the cytoplasm (53), most probably are artifacts.

In growing and dividing cells of *Saccharomyces*, the ribosomes are known to be a prominent constituent of the cytoplasm. Koehler (26) has separated by analytical ultracentrifugation several

fractions ranging from 30 S to 120 S. The isolated particles show a diameter of 200 to about 400 Å. Neither Koehler nor any of the other electron microscopists who are investigating yeasts has been able to detect these rather large particles in cross-sectioned cells. The same difficulty arises in regard to cross-fractures of frozen-etched ground plasm. The globular constituents obviously do not exceed a size of 200 Å. This suggests that the ribosomes localized in their original position are much smaller than the isolated particles. Koehler (26) has described a further fraction of ribosomes, characterized by a sedimentation constant of about 10 S. The diameter of these particles can be calculated by an extrapolation from the data on the larger ribosomes. It could amount to about 120 Å. McCarthy and Aronson (30), investigating *Escherichia coli*, have detected RNA-containing fractions of 4 to 8 S. The calculated particle size would range from 70 to 100 Å. These values correspond to the size of the ribosomes (80 to 120 Å) which adhere to the cytoplasmic side of the unit membranes (see Sections on Nucleus and on Vacuoles and Endoplasmic Reticulum). This concordance confirms the actual presence of small ribosomes. The "big ribosomes," which are known to be composed of several subunits (24, 26, 30), therefore might be created artificially by an association of several smaller particles during the isolation.

The accumulation of a substance in the ground plasm of cells from mature cultures suggests that this substance is a stored product. Glycogen, known to accumulate at the end of the growth phase, is the only substance which really crowds the cytoplasm of *Saccharomyces*. Brüggemann and Drepper (8) report glycogen concentrations of 20 to 40 per cent of the dry-weight. If we assume that the glycogen is restricted to the ground substance, its concentration may be more than 50 per cent. This fact corresponds to the statement that the stored material predominates over the remaining constituents. The lumps of the fine-granular material therefore may be identified as aggregates of glycogen. A paper of Husemann and Ruska (23) reports the structure of glycogen having a molecular weight of about 1.5×10^6 . The iodine-stained and dried particles show a globular shape and a diameter of about 300 Å. In our specimens the globular units, having a diameter of 400 Å also might represent molecules of glycogen. The difference in size could be ex-

plained by a difference in molecular weight. Also, the isolated particles of Husemann and Ruska could have been reduced in size by partial water extraction. The fine granulation of the 400 A units suggests that the glycogen molecules are composed of 60 A subunits. The accumulation in the cytoplasm of very hydrophilic substances known to induce a remarkable resistance against ice crystal formation (33) helps to explain the increased resistance to freeze damage of yeast cells storing glycogen.

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