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Publication Date

1961-06-20

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Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

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James K. Koehler

(Ph. D. Thesis)

June 20, 1961

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ABSTRACT

An electron microscope study of the internal organization of the baker's yeast (Saccharomyces cerevisiae) is presented with particular emphasis on the structural changes brought about by nitrogen starvation and x irradiation.

The general characteristics of normal growing yeast cells fixed with $KMnO_4$ are a dense granular cytoplasm devoid of most cytomembranes and mitochondria, and a well defined—usually homogeneous—nucleus located adjacent to the central vacuole. On the other hand, starving cells display a progressively less dense cytoplasmic matrix, resulting in enhancement of the visualization of membranous components.

The nuclear membrane is apparently a triple structure in growing cells, but has a "classical" double-membrane appearance in starved cells. Intranuclear objects believed to be chromosomes are described and their unusual staining properties discussed. A structure thought to correspond to the centriole apparently has an intravacuolar location that may be appropriate to the unusual mode of nuclear division in yeast.

Cells irradiated with 100,000 r of x rays undergo only slight changes during the first hours of postirradiation incubation, but after two days undergo drastic alterations suggestive of lysis. Unusual cell forms, referred to as ameboid, also appear during these late stages of radiation damage.

In order to obtain information on the fine structure of yeast at a higher stage of refinement, ribosomes were isolated from growing, starving, and irradiated cells; and subjected to parallel analysis with the ultracentrifuge and electron microscope.

Most striking is the discovery of a 65S ribosome present only in rapidly growing yeast cultures and absent from starving or stationary cells. This finding is discussed together with other recent data on the possible role of such a particle in protein synthesis. The sedimentation constants and relative percent abundances of the ribosomes vary in characteristic ways that are substantiated by size measurements made from electron microscope pictures of similar particles.

Radiation apparently has little immediate effect on the properties of the ribosomes studied in this work.

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I. INTRODUCTION

Perhaps no other organism has been so extensively investigated cytologically over such a long period of time with so little general agreement on findings as the yeast cell. This diverse spectrum of opinion is at least partially due to the very small size of this cell. Diploid S. cerevisiae measures 6 or 7 μ in diameter, and many of the organelles within the cell are of the order of magnitude of the resolving power of the ordinary light microscope. These dimensions, together with the thick cell wall, make the yeast cell an extremely difficult subject for cytological observations.

Diploid cells of the baker's yeast exist singly, or occasionally connected together in short budding chains. In logarithmic cultures the generation time is of the order of 1 to 2 hr. During this phase of growth a given cell can bud repeatedly, each bud producing a scar on the mother cell.

The yeast cell possesses a polysaccharide cell wall and a large central vacuole that is perhaps its most distinguishing feature. Indirect transport studies have for many years pointed to the existence of a plasma membrane in the yeast cell. Electron microscope observations have definitely shown that such a structure occurs, and that it is in intimate contact with the cell wall. The morphological nature of the nucleus and the genetic apparatus of yeast are still being actively investigated. Most workers, however, agree that the cell contains a discreet nucleus located adjacent to the vacuole, and that chromosomes are probably present within the nucleus. The characteristic mitotic apparatus of higher plant and animal cells such as the spindle, centrioles, and asters are not generally seen in the yeast cell.

The diploid cell can undergo meiosis under the proper cultural conditions and produce an ascus containing four haploid cells or spores. Free haploid cells of opposite mating type can combine sexually to form diploid cells, thus completing the life cycle. This study will concern itself only with nonsporulating diploid cells.

Electron microscopy has added somewhat to our knowledge of the internal structure of this cell during the past decade; however, these findings are also not in general agreement with one another nor exhaustive in their scope. It is the purpose of this work to amplify and extend knowledge of the cellular architecture of yeast, with particular emphasis on the variations in structure produced by changes in the physiology of the cell. It has been found that the subcellular particles known as ribosomes also vary in physical

properties with environmental changes of the cell; therefore, considerable information on isolated yeast ribosomes was also collected during the course of this work.

The yeast cell has been the object of intensive radiobiological research for many years; however, relatively little has been done regarding the morphological or fine-structure changes after irradiation. A rather definite objective in this research was, therefore, to study in some detail the structural changes which take place in cells and isolated ribosomes after irradiation.

II. LITERATURE SURVEY

Light Microscope Work

No attempt is made here to provide a complete synopsis of all morphological investigations on yeast; several good volumes exist to provide comprehensive information.[1,2,3] Reference is generally be made only to work that is particularly important or pertinent to the gist of this research.

Structural investigations on yeast date back to the time of Leeuwenhoek, who is known to have observed yeast cells in his original microscope about 1680.[2] Almost 200 years passed until the researches of Pasteur, who showed that fermentation does not take place without the presence of living yeast cells. Modern methodical research in yeast cytology goes back to the turn of the century and the extensive work of Emil C. Hansen and his collaborators at the Carlsberg Laboratorium.

Speculations on the internal structure of yeast have tended to fall into either one of two general schools. One view—originated by Wagner and Peniston,[4] and revived by Lindegran—holds that the vacuole plays a primary role in mitosis. An idea of the general philosophy of this school can be obtained from the following statement in a recent Lindegren publication:[5] "The spindle lies in the vacuole and mitosis occurring on the spindle spins out chromosomes which extend into the vacuole." According to Lindegren the nucleolus and volutin granules are also intravacuolar. On the other side of the controversy are the more conservative views voiced, for example, by Ganesan,[6] who showed that vacuoles are capable of fragmentation and are unstable. He reasoned that, "An entity which is so unstable and discontinuous and so sensitive to physiological conditions cannot possibly have any genetic role." Ganesan, who found no indication of a spindle, supports the view that mitosis is intranuclear, and that nuclear transport is strongly aided by conventional centrosomes.

The nucleus has probably received more attention than any other aspect of yeast cell structure. Guillermond[7] describes a small dense body situated at one side of the central vacuole that divides into two on budding, the daughter nucleus passing into the bud. Sinoto[8] and Subramanian[9] also support the view that the nucleus is a central, dense, Feulgen positive body that becomes dumbbell shaped and travels into the daughter cell during mitosis. DeLamater[10] claims to have observed functional centrioles carrying out this process of nuclear transfer. The pioneering uv microscope studies of Casperson included some work on yeast cells. Casperson and Brandt[11] showed a particularly

clear nucleus in starving cells, together with uv-absorbing granules which they believed to be volutin. Lindegren, as stated earlier, believes that the vacuole is central to the mitotic process. Richards[12] and Beams et al.[13] support an amitotic division in yeast on the rather questionable evidence of the lack of a colchincine effect on dividing cells. The confusion that exists regarding the interpretation of nuclear structures is summarized in Winge and Roberts' review,[2] in which, according to a scheme set up by Lindegren, it is seen that what Wagner and Peniston called the nucleolus, Guillermond called the cell nucleus, and Lindegren, the centrosome. Furthermore, what Wagner and Peniston called the nuclear vacuole, Janssens and Leblanc called the nucleus; and what Wagner and Peniston called the central volutin granule in the vacuole, Janssens, Leblanc, and Lindegren called the nucleolus.

The question of the nature and quantity of chromosomes in yeast is in a similarly unsettled state. Subramanian describes one large chromosome in haploid yeast.[14] Ganesan suggests that these bodies identified as chromosomes may be fixation artifacts.[15] Sinoto and Yuasa observed four chromosomes.[8] DeLamater found two pairs of chromosomes with Feulgen staining while, incidentally, rejecting Lindegren's centrosome and calling it the nucleus.[10] McClary et al. claim a diploid number of eight,[16] Leitz observed six,[17] and Levan says ten or more.[18] Recent extensive genetic analysis by Mortimer and Hawthorn points to the existence of at least ten linkage groups in the haploid cell.[19]

Most observers agree that mitochondria at least exist in yeast cells. Mudd found granules characteristic of mitochondria, by Janus green and tetrazolium staining methods.[20] Bautz and Marquardt, using Altmann's stain and the Nadi reaction, found about 13 mitochondria per cell and a smaller number in buds.[21] A rather vigorous controversy was initiated when Yotsuyanagi claimed to have observed mitochondria of greatly changing dimensions, depending on the nutrient and stage of growth of the yeast cells.[22] Williams, Lindegren, and Yuasa suggested that Yotsuyanagi's conclusions were due to artifacts resulting from crystal growth of the tetrazolium salts used in staining.[23] Yotsuyanagi again refuted Lindegren's attack in an appendum to the above paper.

Some general conclusions that have gained acceptance are that young cells are dense and devoid of structure, with relatively thick cell walls; as the cells grow older, the walls become thinner and the cells progressively fill with visible inclusions. The central vacuoles are thought to persist with little dependence of their structure on the nutritional state of the cell.

Electron Microscopy

Little could be done with the electron microscope in the investigation of yeast fine structure until the advent of thin sectioning several years ago. A heroic exception to this was the work of Bartholomew and Mitter, who used uv photolysis to spread the cells sufficiently thin for electron microscopy.[24,25] Only relatively gross structure could be observed; however, suggestive photomicrographs are displayed indicating nuclear progression into buds, and possible centriole structures. Since that time surprisingly little has been done with yeast electron microscopy, except by one or two groups that have been active in this area. Some of the reluctance to do electron microscopy with yeast can be traced to the almost complete failure of osmic acid to produce well-preserved specimens. The notable exception to this is the work of Agar and Douglas who found that good results could be obtained with osmic acid only if the cells were grown on Lindegren's presporulation medium before fixation.[26] In two earlier papers Agar and Douglas observed no structural detail in the cytoplasm of cells of various ages treated with a variety of fixatives.[27,28] These authors observed a single plasma membrane adjacent to the cell wall, and peripherally occurring mitochondria. They suggested that the cristae in these mitochondria are oriented parallel to their long axis although the micrographs are not definitely convincing concerning this structural point. Lipid granules are shown in the cytoplasm, and the vacuole takes a prominent position adjacent to, but not in contact with, the apparent nucleus. The nuclear membrane seemed to contain pores, although the double or single nature of the membrane could not be elucidated.

Hashimoto, Conti, and Naylor have published a series of papers[29,30,31] dealing with yeast electron microscopy, although the majority of these deal with ascospore structure, which is a specialized case and does not necessarily lend insight to the structure of vegetative cells. The gross features seen in their work are similar to those observed by Agar and Douglas,[26] although there are minor points of difference and the quality of the illustrative material leaves something to be desired. In their paper showing budding cells, nuclear bridges from mother to daughter cell are displayed, and the nuclear membrane was claimed to persist throughout nuclear division. The nuclei were very homogeneous and the view was put forth that no chromosomal structures existed in yeast, but that a diffuse dispersion of genetic material was present throughout the nucleus. In a very recent publication, Hashimoto et al.[32] have reversed their prior view towards the existence of chromosomes.

Yotsuyanagi has made one of the best studies to date, with the electron microscope, of yeast cell structure.[33,34] Displayed with particular clarity, especially in the earlier of his two works, are the nuclei and nuclear membranes, mitochondria, and other cytoplasmic inclusions. Prominent central vacuoles also appear in his stained material. In his second paper, Yotsuyanagi discusses the possibility of chromosome staining in yeast and illustrates with presumably chromosomal elements obtained in permanganate and uranyl acetate stained yeast.

A very recent series of studies by Mundkur utilizes freeze drying and electron microscopy, in conjunction with cytochemical techniques, in the elucidation of yeast fine structure.[35,36] The latter of these papers opens with the statement: "The homogeneously Feulgen positive, vesicular character

of the nucleus of Saccharomyces derives from a uniform dispersion of DNA; this condition persists throughout all stages of nuclear division... ." Mundkur rejects the findings based on permanganate fixation, on the basis that the vacuole is not preserved after such fixation, and therefore interpretation is impossible. It will later be shown that this is an incorrect assumption. Mundkur's electron micrographs reveal little structure within the yeast cell. The cytoplasm is composed of a fine granular material without membranes or mitochondria; and the nucleus, generally with no observable membrane, is homogeneous except for occasional granules of higher contrast.

The various questions raised here concerning the find strcture of yeast are more fully discussed in a subsequent section in the light of findings reported in this work.

Ribosome Fine Structure

A great deal of interest has been shown in the past few years in the small RNA-containing granules found in virtually all plant and animal cells. Much of the impetus for this research is no doubt due to the many indications that these particles are intimately involved in protein synthesis. Micro-organisms and yeast are a convenient source of these particles that have by convention been called ribosomes.

The first careful physicochemical study on yeast ribosomes was carried out by Chao and Schachman, who analyzed the stability of these particles to various solutions, by means of the analytical ultracentrifuge.[37] They found primarily 80S particles after grinding cells with 100-mesh carborundum. Also, Mg^{++} was found to be an important factor in the stability of the ribosomes. Tissieres and Watson found four recognizably different particles in log phase E. coli extracts.[38] All four classes seemed to have the same gross chemical composition: 40% protein and 60% RNA. The particles were characterized by analytical centrifugation as 32S, 51S, 70S and 100S. These workers also proposed that the 70S particle is composed of 51S and 32S particles, and that the 100S particle is a dimer of 70S particles.

The work of several groups suggests that there are variations in the physical and chemical properties of the ribosomes, dependent on the state of nutrition of the cell of origin. Bradfield found smaller and more numerous granules in young bacterial cultures.[39] The average diameter of the ribosomes increased from 20 mµ to 30 mµ throughout the growth cycle. Wolfe, working with yeast cells, found only 80S particles in old or starving cells, but several smaller peaks were observed in ultracentrifuge patterns of actively growing cells.[40] Apparently x irradiation did not alter the sedimentation pattern. Ashikawa, using the same system, made a more extensive study and showed that four new components appear during growth.[41] The ratios of the quantities of material in these peaks shift during the various phases of the growth cycle, and 80S particles are predominant at stationary phase. Wade and Morgan have made similar observations ultizing E. coli ribosomes; namely, that dividing E. coli has more RNA and two or three centrifuge peaks in addition to the 80S peak, whereas resting cells have less RNA and only low concentrations of the additional peaks.[42] Dagley and Sykes also confirmed these general results and suggested that the additional components might represent particles having key roles in enzyme production.[43]

Considerable work has been devoted to the elucidation of the fine structure of the ribosome. Much of this work has involved analytical ultra--centrifugation, such as the researches of Schachman and Tissieries discussed earlier. Perhaps the most useful approach to an understanding of ribosome fine structure has come from correlative electron microscopy and ultra-centrifugation. Two such projects have very recently been reported on E. coli ribosomes. In a rather brief paper, Hall and Slayter showed the 30S particles to be rod-like with an axial ratio of 2:1, the 50S particles to be spherical, the 70S particles to be composed of one 50S and one 30S particle, and the 100S particles to be dimers of the 70S particles.[44] These findings are in complete agreement with the earlier work of Tissieres and Watson[38] but with the added weight of electron microscope observations. A very extensive study by Huxley and Zubay revealed essentially the same results but with considerably more experimental evidence.[45] Negative staining with phosphotungstic acid was employed in the electron microscope to clearly show the separation between 50S and 30S particles in the 70S ribosome. It was also shown that under the proper in-vitro conditions 50S particles can dimerize to form 80S particles. By comparison of the stained ribosomes to similarly treated Turnip Yellow virus the authors concluded that the ribosomes, unlike the virus, were not covered with a protein coat.

Since agreement is not yet complete concerning the properties of normal ribosomes, it is not surprising that very little work has been carried out on the study of radiation damage to these organelles. Billen and Volkin observed changes in the ultracentrifuge patterns of irradiated E. coli ribosomes, but only after prolonged postirradiation incubation.[46] They interpreted these changes as a breakdown of the 40S component into smaller units. Pollard and Kennedy have reported that P^{32} uptake is smaller after irradiation, and that it decreases progressively with dose.[47] The suggestion is made that this is a reflection of damage to a relatively large and sensitive unit, presumably the ribosome.

III. EXPERIMENTAL PROCEDURES

Electron Microscopy of Cells

Diploid S. cerevisiae with the laboratory-strain designation X841 were routinely grown in liquid medium containing 1% yeast extract, 1% dextrose, and 1% peptone (abbreviated YEPD). A starvation medium was also extensively used in the research; it contained 0.078% succinic acid and 0.051% potassium hydroxide, resulting in a pH of approximately 5.0. This solution deprives the cells of a nitrogen source and hence acts as a starvation medium. All cultures were incubated in a shaker at 30°C.

Many fixatives were tried on the yeast cells, including osmic acid, permanganate, uranyl ion, ferric ion, mercury salts, lead hydroxide, and dichromate. Potassium permanganate gave the best results in terms of good preservation and contract enhancement. The integrity of the cells was apparently maintained in that cytoplasmic membranes, mitochondria, and the vacuole retained their structures, whereas with uranyl nitrate cells were produced that had no visible vacuoles and an agglomerated cytoplasm. Mitochondria were not observable with the uranyl nitrate treatment, although densely staining particulate bodies in the nucleus and cytoplasm indicated that there may be preferential uptake by certain cellular materials, possibly nucleic acids. Lead hydroxide prepared according to the manner of Watson[48] and applied to permanganate stained sections increased contrast, especially of membranous regions. The above considerations—together with the work of Luft,[49] and more recently Mollenhauer[50]—suggested that potassium permanganate should be used as the routine fixative for yeast. Various vehicles for the permanganate were tried, including Veronal buffer, phosphate buffers, and distilled water, but no apparent differences in the quality of the fixed material were observed. This finding is in agreement with the work of Mollenhauer,[50] who showed that buffered permanganate solutions are not superior to distilled water solutions, and in some instances are even detrimental to good fixation. Accordingly, a 1.5% solution of $KMnO_4$, —at pH 8.0, in distilled water—was used for most of the study reported here.

Cells were removed from their growth or starvation media and washed briefly in water, after which they were fixed in permanganate for one-half hour at room temperature. The cells were then centrifuged and resuspended in a series of alcohols (50%, 75%, and 95%) at half-hour intervals, and finally treated with two changes of absolute alcohol. Acetone dehydration was attempted but gave rather poor results and was not routinely used. The cells were placed in 20% methyl-80% butyl methacrylate overnight before they were placed in an oven at approximately 50°C. This prolonged treatment with methacrylate monomer seemed to improve the embedding quality and reduced the explosion of cells. Thin sections were cut with a Servall Porter-Blum ultramicrotome equipped with a diamond knife, and then were mounted on formvar-coated stainless steel or copper grids. All specimens were examined in an RCA EMU-2 electron microscope equipped with a Canalco electrostatic compensator for the objective lens.

Preparation and Manipulation of the Ribosomes

Yeast cells of strain X841 were grown in mass culture and harvested at various times during growth and starvation. A small aliquot of these cells was usually taken for electron microscopy just prior to the isolation of the ribosomes. The conditions of culture were arranged so that four or five grams of wet cells was obtained from each sample. Isolation proceeded by the methods of Wolfe[40] and Ashikawa.[41] The cells were hand ground in a mortar, together with about five times their weight of 100-mesh carborundum. This procedure was carried out in an ice bath for about ten minutes. Extraction followed with several volumes of Chao and Schachman's buffer[37] ($0.00125M$ $KH_2PO_4-K_2HPO_4$ 3:7, $0.001M$ $MgCl_2$ and $0.01M$ KCl). Preliminary centrifugation at 20,000 g in an International Refrigerated Centrifuge—equipped with a high speed head—sedimented cell debris, cell walls, nuclei, and mitochondria, and left the bulk of the ribosomes in the supernatent. Further sedimentation in a Spinco Model L ultracentrifuge at 120,000 g (40,000 rpm) for 1 hr pelleted the ribosomes. The slightly orange translucent pellets were carefully redissolved in buffered solution, and aliquots were taken for electron microscopy and the Spinco Model E analytical ultracentrifuge. Both of these operations were completed within 24 hr of the ribosome isolation. The preparations were stored in buffer at $4°C$ at all times during which they were not in use.

The ribosomes were prepared for electron microscopy in several different ways. Generally they were diluted to a proper concentration with buffer and placed on prepared microscope grids, the excess liquid being drawn off by touching the edge of the grid with absorbent tissue. The air-dried specimens were then shadowed with nichrome at an angle of about 3:1. Some samples were fixed in permanganate and other fixatives, dehydrated, and embedded in methacrylate analogously to the whole cells. Freeze drying of the ribosomes was also attempted but the morphology of the particles was not significantly different from air-dried samples, so the more complicated procedure was not routinely used.

Irradiation

A 50-kv x-ray machine operating at 25 ma and without filtration was used in irradiating the cells and isolated ribosomes. During the irradiation a dose rate of 240 r/sec was delivered to cells and particles placed in Petri dishes. A few experiments utilized the heavy-ion irradiation of the Heavy-Ion Linear Accelerator. Concentrated cell suspensions were placed on millipore filters over dampened pads, then irradiated in air.

Several analytical procedures were followed in analyzing the ribosomes. The ultracentrifuge runs provided Schlieren patterns from which Svedberg sedimentation rates were calculated for the various observed peaks. The areas under the peaks were measured by counting squares on graph paper tracings. These determinations give a measure of the type and relative quantity of ribosomes in a particular preparation. Electron micrographs at an enlargement of 150,000 X were used to measure the physical proportions of the ribosomes. Two measurements were made on the particles—the diameter was measured perpendicular to the shadow length, and the shadow length itself was measured in order to determine the relative heights of the particles.

In the case of greatly asymmetrical particles that represent a small pro-
portion of the total, the greater dimension was used. A Bausch and Lomb
7X magnifier with a scale calibrated to 0.1 mm was used in making these
measurements. These data have been plotted in several representations
and are fully treated in the section on results.

IV. EXPERIMENTAL RESULTS

Normal Growing Cells: General Structure

After a few hours in growth medium, yeast cells commence the
logarithmic phase of growth during which the doubling time is approximately
two hours. Samples of cells taken at this time reveal large numbers of
organisms in various stages of the budding process. Figure 1a shows what
is presumed to be a rather early stage, in which the nucleus has apparently
not yet begun the mitotic movement toward the daughter cell. Note the
thickening of the cell wall at the constriction between mother and daughter
cells. Some 1880-A polystyrene reference particles can be seen in this
and many of the other figures dealing with sectioned yeast cells. The RNAase
and DNAase extractions performed on similar sections cleared of metha-
crylate indicate that the body described above is the DNA-containing organelle
of the yeast cell; it will be referred to as the nucleus. From the many buds
observed, it is believed that the movement of daughter nucleus into daughter
cell does not begin until an appreciable fraction of the new bud has been formed.
The mother cell contains prominent vacuoles and occasional mitochondria.
Figure 1b shows two cells in which nuclei have taken their places in the
respective mother-daughter pairs. Occasionally one can find cells in which
nuclear bridges connecting mother and daughter nuclei are still intact. Such
a configuration can be seen in Fig. 1c. Sometimes these nuclear transfers
appear to be very complex morphologically. Figure 1d illustrates a very
tortuous nuclear bridge and Fig. 2a, a somewhat atypical lobulated nucleus
in the process of transfer.

S. cerevisiae generally exists in the vegetative phase as single cells;
that is, the daughter cells separate from their mothers after budding. Some-
times, however, separation does not take place and short budding chains of
cells are produced. Figure 2b illustrates such a configuration, in which
nuclear transfer is seen to be taking place in the uppermost cells. Figure 2c
shows a budding cell in a later stage. The nuclei and central vacuoles have
taken their appropriate places, and cytokinesis is proceeding to completion.
During the budding or growth stage relatively little structure can be observed
in the cytoplasm. The predominant feature is the dense particulate matrix,
probably representing the ribosomes of the cell. Occasional mitochondria
and cytomembranes, both of which seem to occur primarily on the cell
periphery, can be seen in budding cells. Figure 2d illustrates these points.
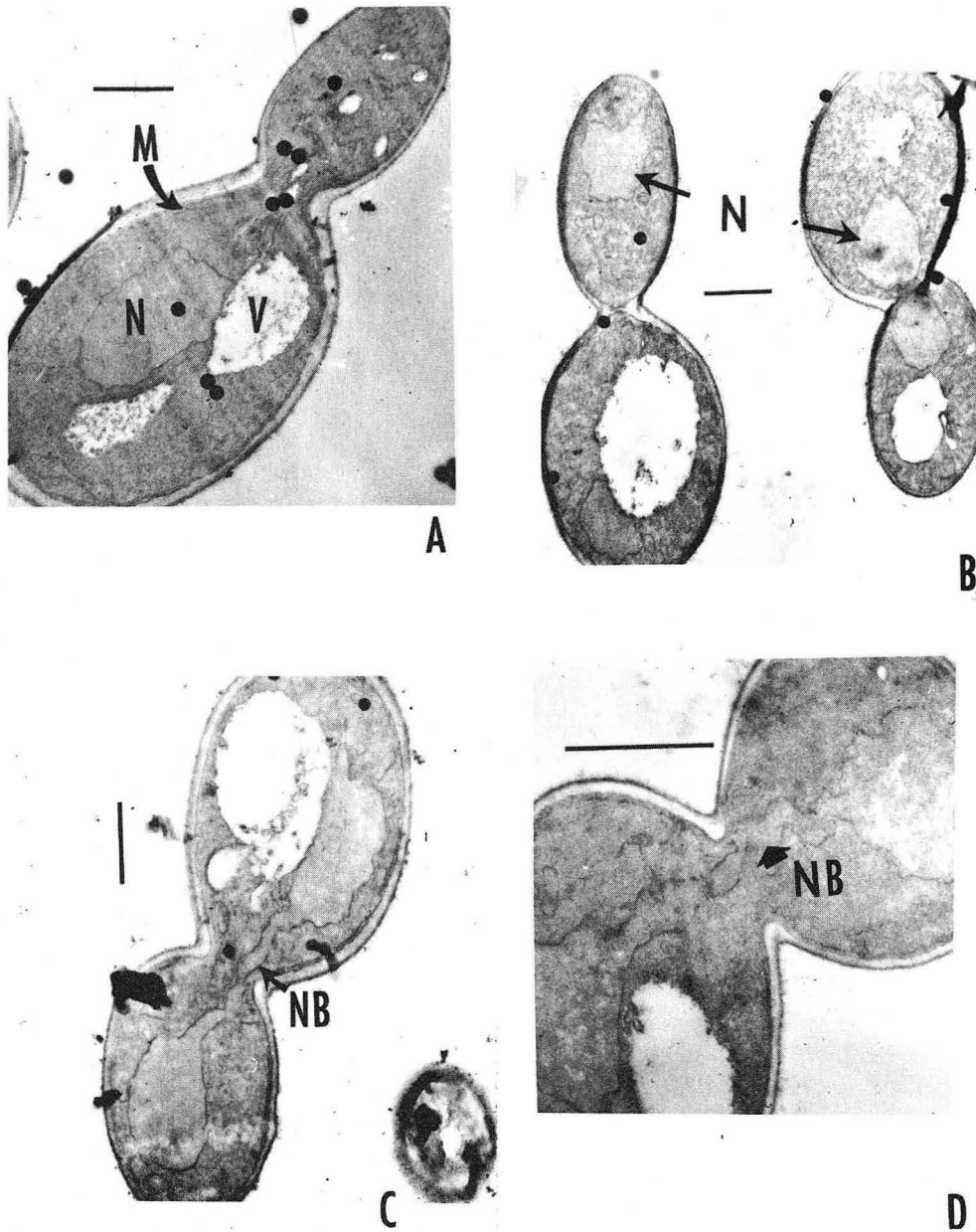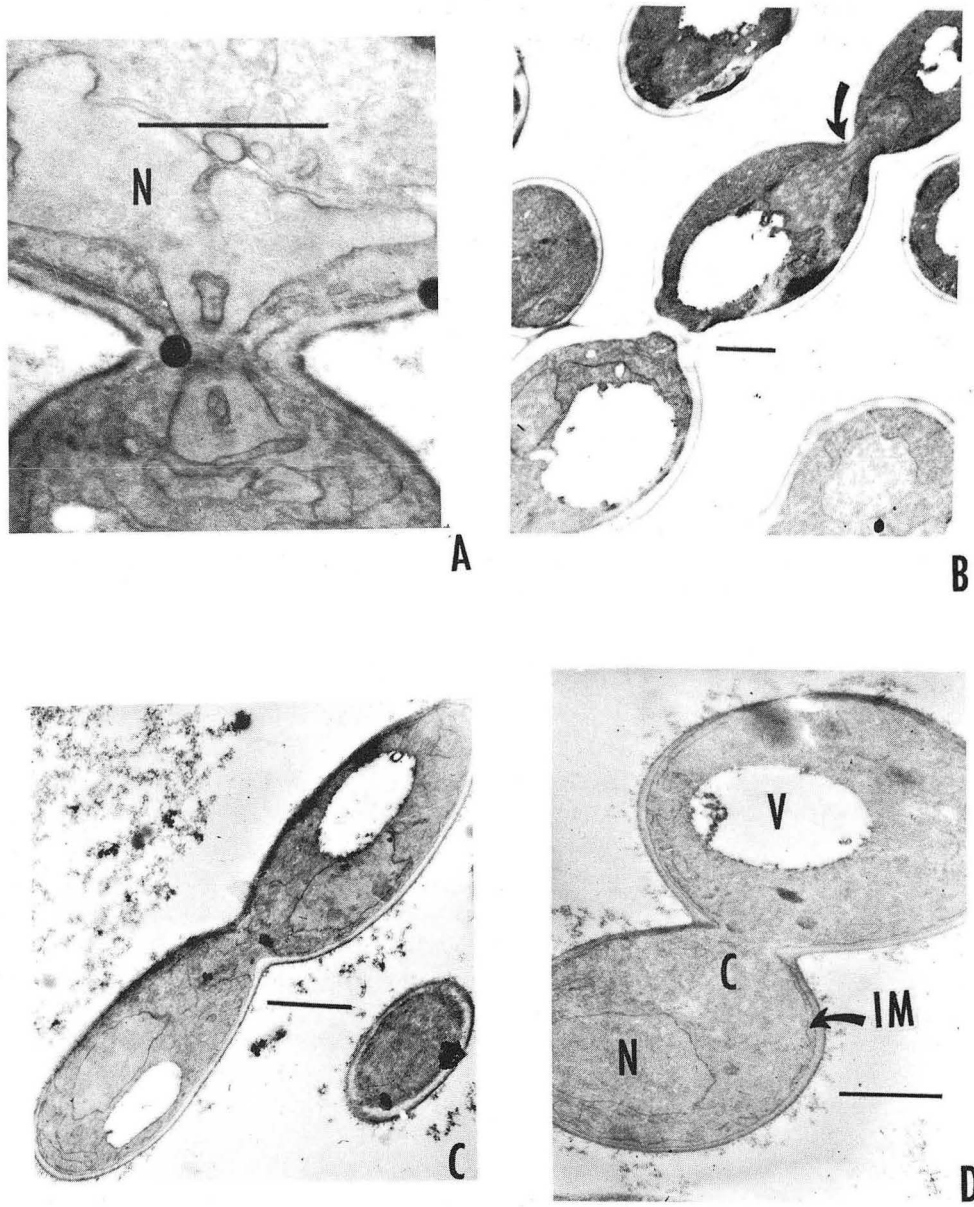
Fig. 1a. Budding yeast cell after 4 hr in nutrient medium.
   b.   Budding cells after 6 hr in nutrient medium.
   c.   Budding cell after 6 hr in nutrient medium.
   d.   Budding cell after 6 hr in nutrient medium.
   (See page 75 for table of abbreviations used on figures.)

ZN-2844

Fig. 2a. Budding cell after 2-1/2 hr in starvation medium.
   b. Budding chain of cells after 4 hr in growth medium.
   c. Budding cell after 5 hr in growth medium.
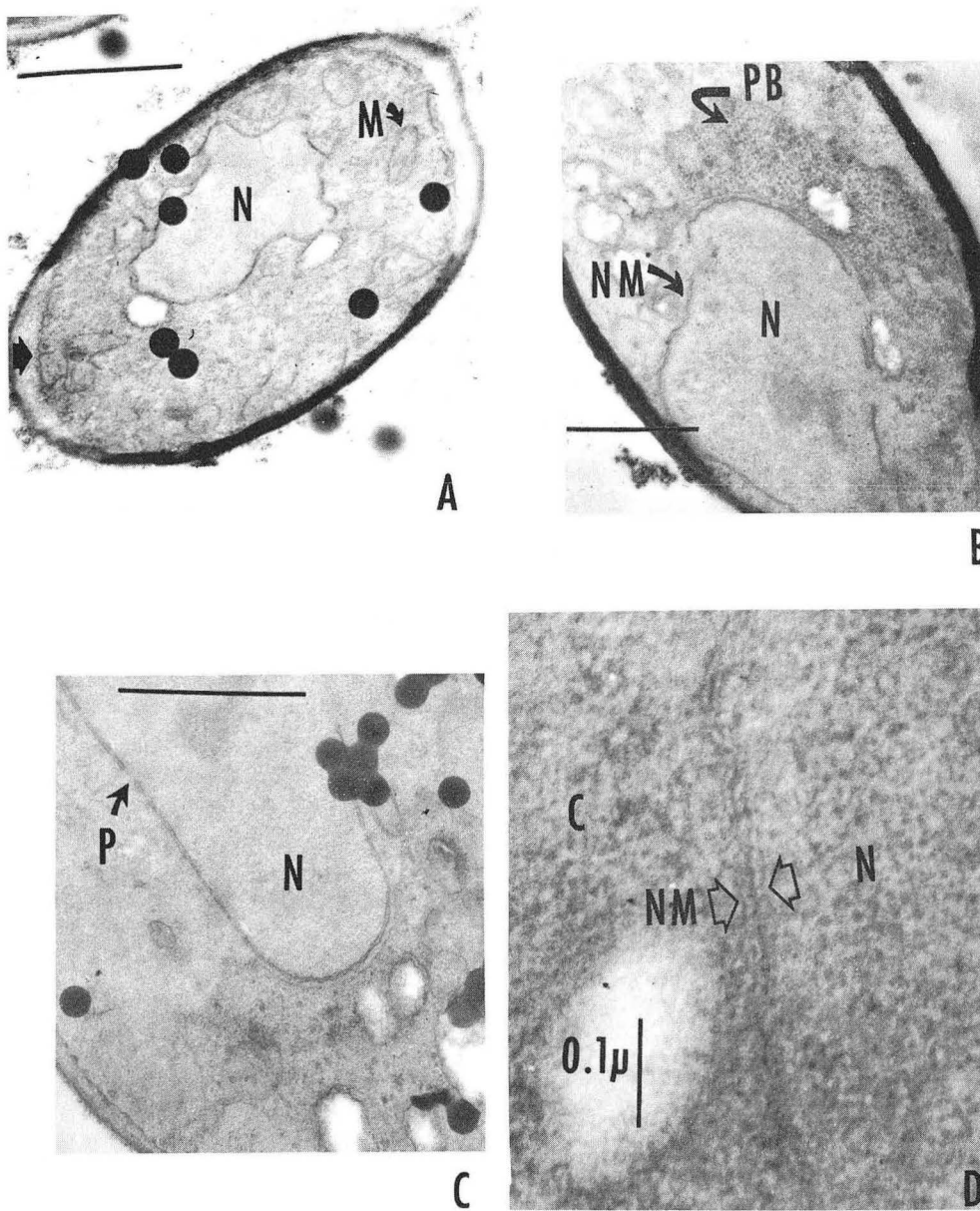   d. Budding cell after 6 hr in growth medium.

Normal Starved Cells: General Structure

The process of nitrogen starvation seems to have a considerable morphological effect on yeast cells. It should be pointed out that cells kept on a starvation medium indefinitely will eventually die; however, such extended starvation was not carried out in this work, and cells showed essentially 100% viability over the range studied. In general one finds a gradual reduction in cytoplasmic density concommitant with starvation. This reduction brings with it the desirable effect of enhancing the contrast of membranous systems within the cell. Figure 3a shows a cell starved for 4 1/2 hr. Considerable structure can now be seen, including mitochondria containing relatively few cristae and some membranes of the endoplasmic reticulum. Note the highly invaginated plasma membrane in certain area (see arrow). During later stages of starvation the large central vacuole is generally not seen; instead, a number of small condensed vacuoles is seen, often containing considerable amounts of material. Quite regularly a dense particulate component with particle sizes of the order of 500 A appears in these starved cells. These particles are sometimes located in an area suggestive of the position occupied by the central vacuole in growing cells. Figures 3b and 3c showing cells starved for 4 and 22 hr, respectively, illustrate these observations. Note the clarity with which the nuclear membranes stand out as contrasted with those of growing cells.

Nuclear Structure

The fine structure of the nucleus and nuclear membrane of yeast seems to be highly dependent on the physiological state of the cell. During nutrition the nuclear envelope appears to have a rather complex fibrillar appearance. Sometimes this can be resolved into a membranous system of three or more components, as shown in Fig. 3d. Starvation brings about a dramatic change in nuclear membrane structure. Figure 3c shows a cell starved for 22 hr that has the typical double-membrane structure of higher plant and animal nuclear envelopes. Note also the numerous pores in the nuclear membrane, which can be seen at higher magnification in Fig. 4a. There are apparently no diaphragm-like membranes within the pores, as has been suggested for higher plant materials by Alfzelius.[51] Nuclei are sometimes seen, particularly in starving cells, which display bleb-like outpocketings of the nuclear membrane. Such structure is shown in Fig. 4b, in which a whole series of such blebs is forming at the nuclear surface.

The nucleoplasm of the majority of cells is fairly homogeneous, although a small fraction of the cells show nuclear structures presumed to be chromosomes. Under the conditions of fixation employed here, these structures are generally less dense than the surrounding nucleoplasm. Examples of such bodies can be seen in Figs. 4c and 4d. Occasionally, dark intranuclear masses are also visualized in yeast cells which have approximately the same configurations as the electron-transparent structures. Figures 5a and 5b display such dark objects, which are configurationally suggestive of chromosomes. The identity of these structures in the functional sense has not been established in this work; however, the term chromosome will be applied to these objects as an operational definition. Very rarely one can find nuclei which have both light and dark chromosome-like structures. A nucleus of this type is shown in adjacent sections in Figs. 5c and 5d. The significance of these structures is discussed in a subsequent section.
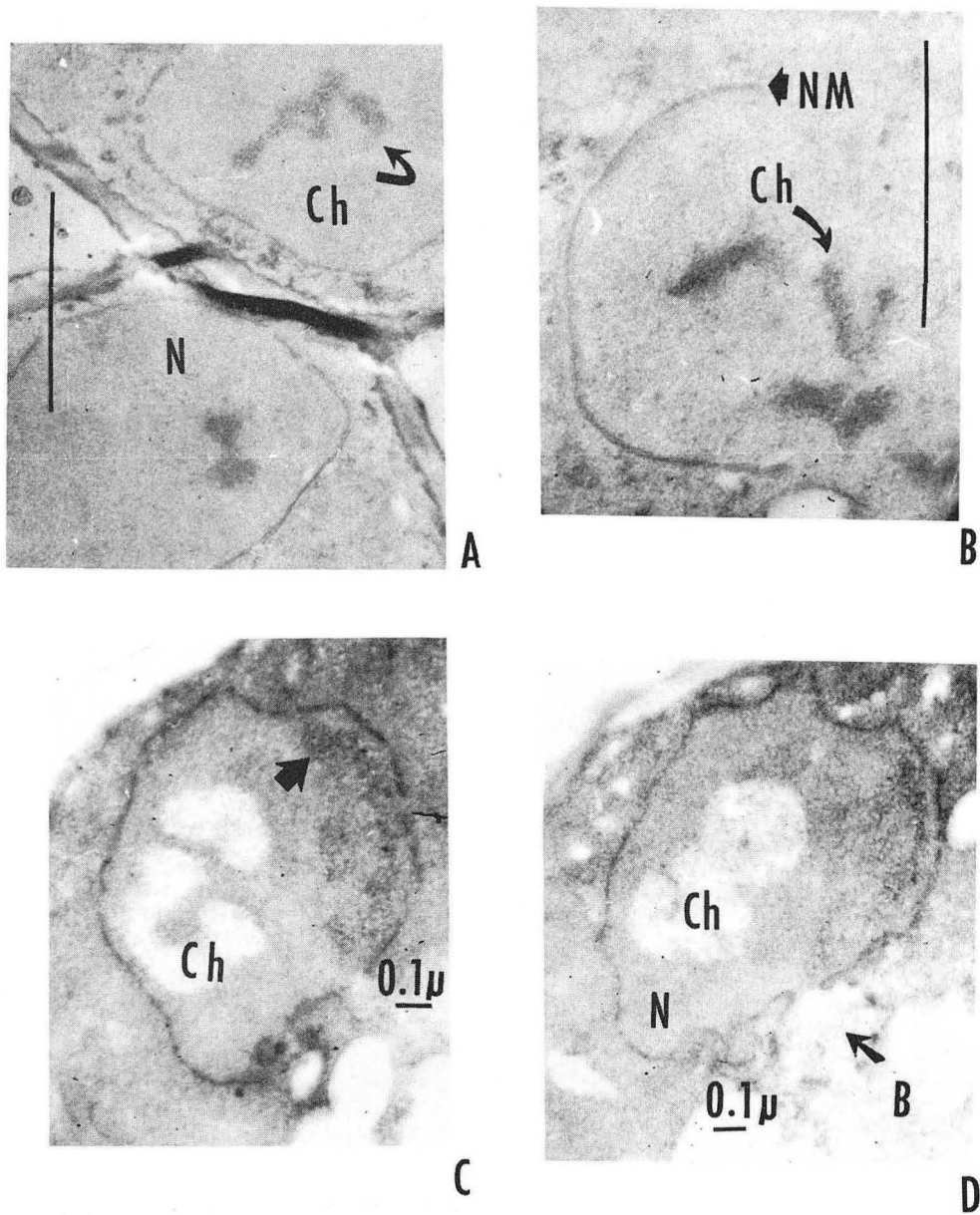
Fig. 3a. Yeast cell after 4-1/2 hr in starvation medium.
      b. Yeast cell after 4 hr in starvation medium.
      c. Yeast cell after 22 hr in starvation medium.
      d. Nuclear membrane of a cell after 4 hr in growth medium.
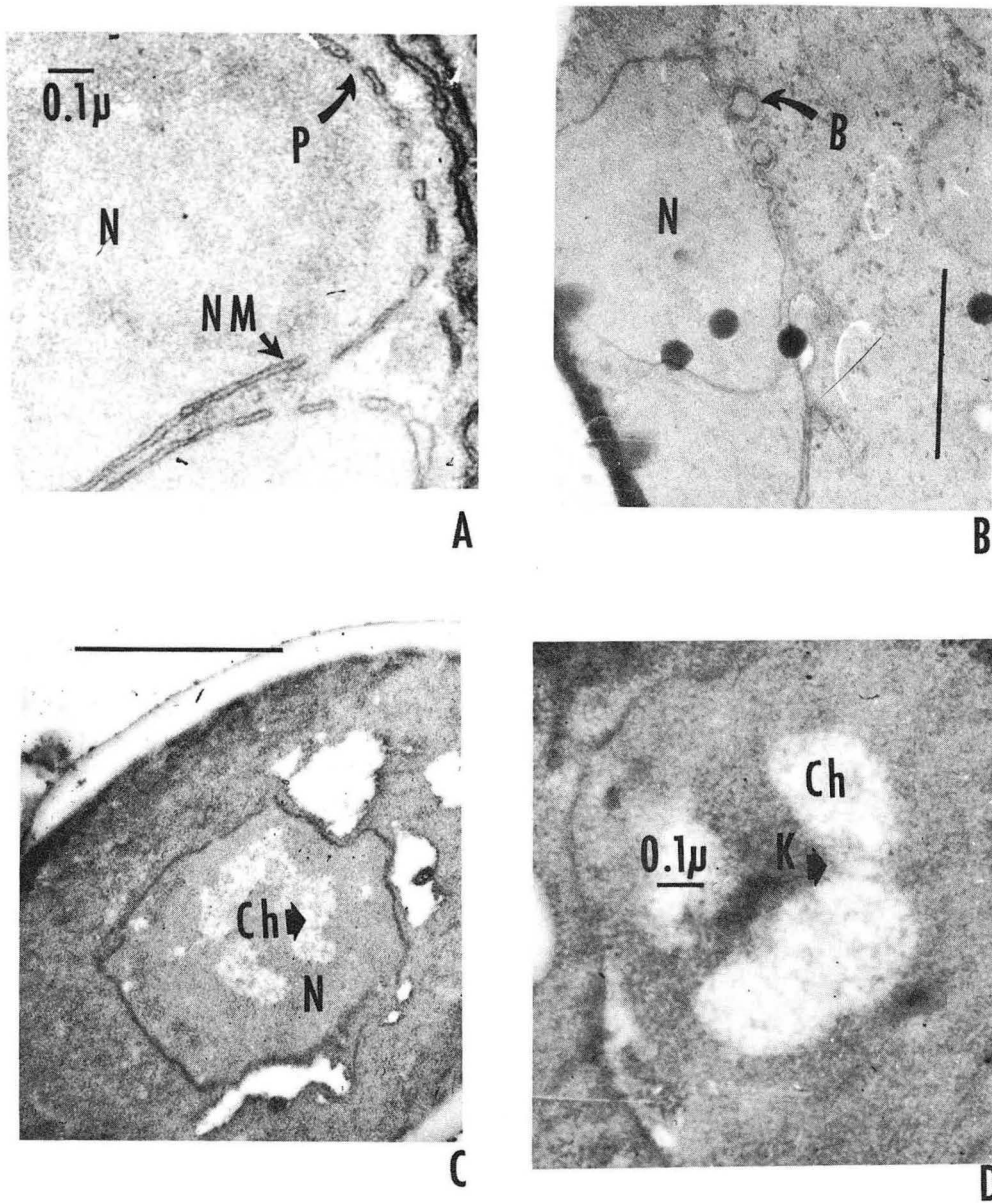
Fig. 4a. Nuclear membrane with pores in a cell 24 hr in growth
          medium after receiving 10^5r of x rays.
       b. Nuclear blebs in a cell after 24 hr in starvation medium.
       c. Light chromosomes in a cell after 22 hr in growth medium.
       d. Light chromosomes in a cell after 42 hr in growth medium.

ZN-2850

Fig. 5a. Dark chromosomes in a cell after 22 hr in starvation medium.
    b. Dark chromosomes in a cell after 22 hr in starvation
    medium.
    c. Light and dark bodies in a cell after 42 hr in growth
    medium.
    d. Light and dark bodies in a cell after 42 hr in growth
    medium.

Owing to the importance of the centriole in mitosis and its close re-
lationship to the nucleus, it is not out of place to make a few statements here
concerning the possible existence of centrioles in the yeast cell. The nucleus
in yeast is generally in very close proximity to the central vacuole. Indeed,
it is not unusual for a cytoplasmic space of only 100 or 200 A to separate the
nuclear membrane from the vacuolar membrane. Quite often one or two dense
ringlike objects 0.2 to 0.3 μ in diameter can be seen in close association with
these membranes. Figures 6a and 6b show these objects, which are discussed
later in relation to centriole structure.

Mitochondria

The question of the existence of mitochondria in yeast has been settled
to the satisfaction of most investigators, but the problem of their fine structure
and its variation during the various physiological stages of growth is still
open for discussion. Figures 6c, 6d, and 7 show the considerable spectrum
of mitochondrial structures that can be found in starved and irradiated yeast
cells. Note particularly the apparent difference between mitochondria found
in starved and irradiated cells and mitochondria in normal growing cells
shown earlier. Starved mitochondria seem to be better differentiated from
surrounding cytoplasm than are their nutriated counterparts. As stated
before, this is probably due to a general diminution of cytoplasmic matrix
density rather than a specific change in mitochondrial density. Note that
there are relatively few cristae per mitochondrial profile, and that they are
variably arranged with respect to the long axis of the mitochondrion, although
in a given mitochondrion the cristae run primarily transversely or longitudinally.
Occasional small circular profiles are seen within the mitochondria which
suggest that the cristae are at least partially of the tubular type. Some
mitochondria show a very "open" or loose architecture, even to the point of
having cristae-like extensions into the cytoplasm, as in Fig. 7a.

Endoplasmic Reticulum

Membranous systems do exist in the yeast cell, although it is somewhat
difficult to determine the "smoothness" or "roughness" of these membranes
because of the overpowering background ribosome granularity. Sometimes in
purified ribosome samples one does come across apparently membranous
segments with particulate attachments, as is shown in a subsequent section
(Fig. 17).

A particularly constant feature of yeast fine structure is the presence
of a discontinuous double membrane extending around the periphery of the
cells in very close proximity to the plasma membrane. This membrane is
sometimes in close association with mitochondria, as shown in Fig. 8a.
Occasional membrane connections to the nuclear envelope are also seen, as
in Fig. 8b. The endoplasmic reticulum may play an important role in yeast
cell physiology but it no doubt represents a rather minor component in terms
of cytoplasmic quantity.
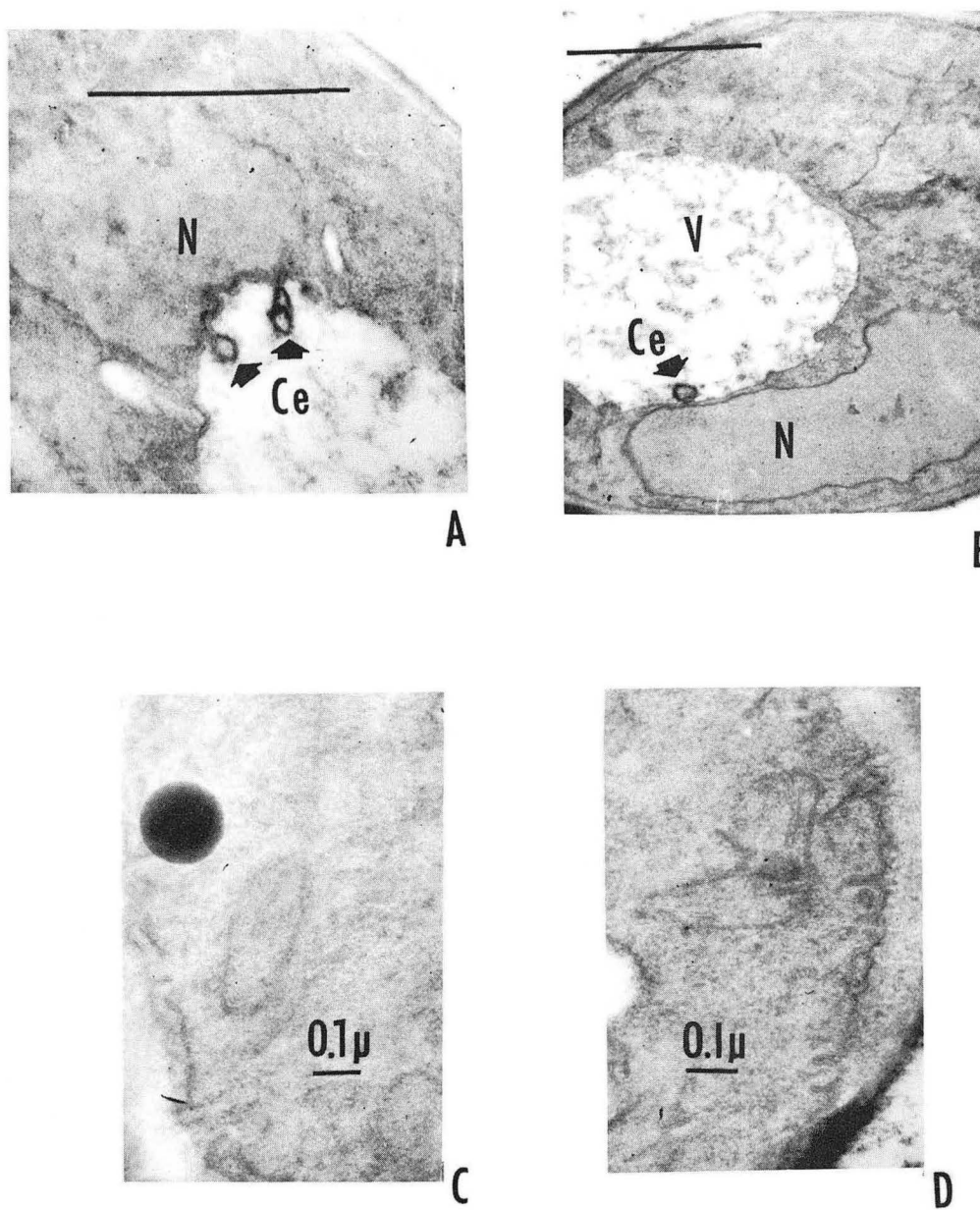
Fig. 6a. Centriole-like structures in a cell after 2 hr in growth
          medium.
      b. Centriole-like structures in a cell after 7 hr in growth
          medium.
      c. Mitochondrion in yeast cell after 5 hr in starvation
          medium.
      d. Mitochondrion in yeast cell after 5 hr in starvation
          medium.
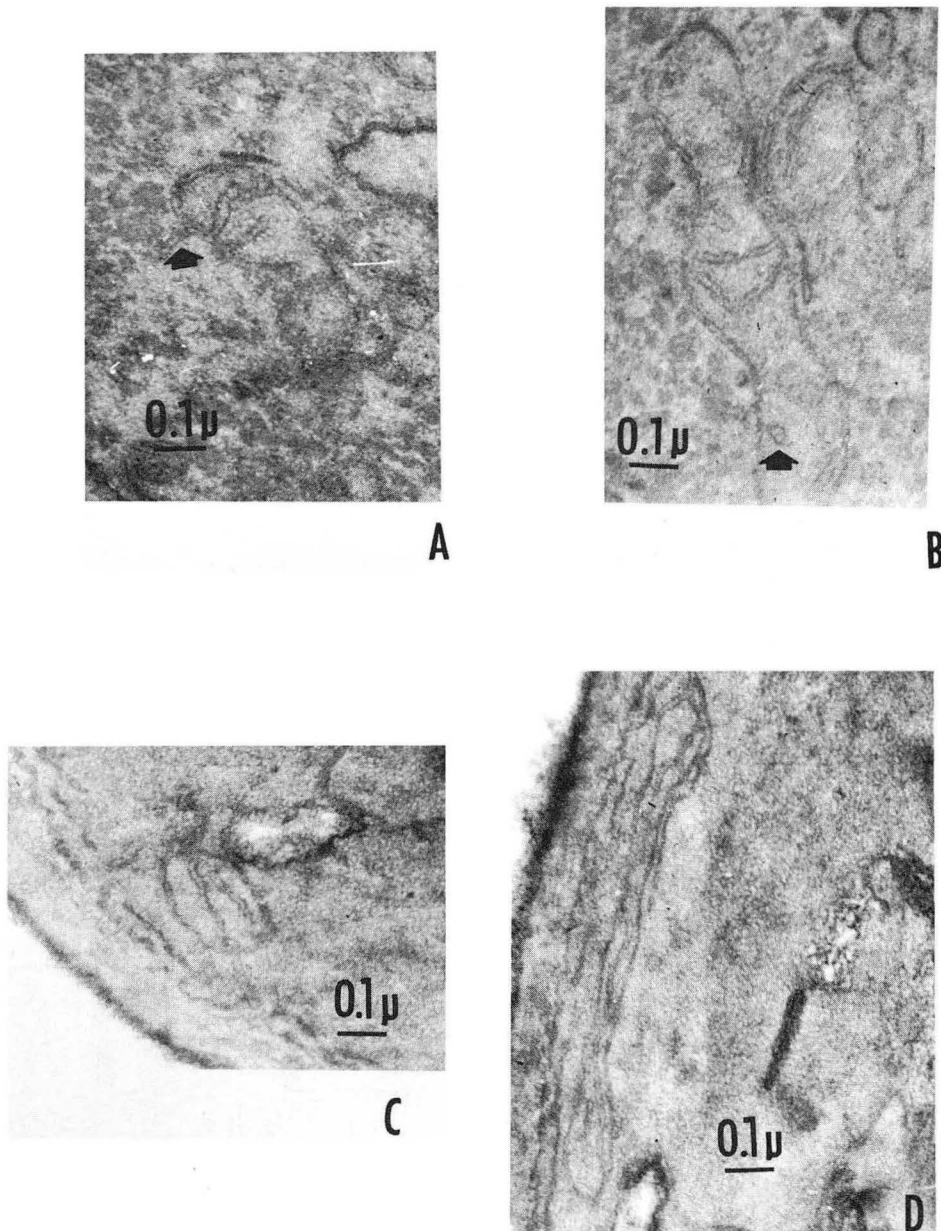
Fig. 7a.   Mitochondrion in a cell 1/2 hr in growth medium
          after receiving 10^5r of x rays.
      b.   Mitochondrion in a cell 1/2 hr in growth medium
          after receiving 10^5r of x rays.
      c.   Mitochondrion in a cell 3 hr in growth medium
          after receiving 10^5 rads of Ne particles.
      d.   Mitochondrion in a cell 3 hr in growth medium
          after receiving 10^5 rads of Ne particles.
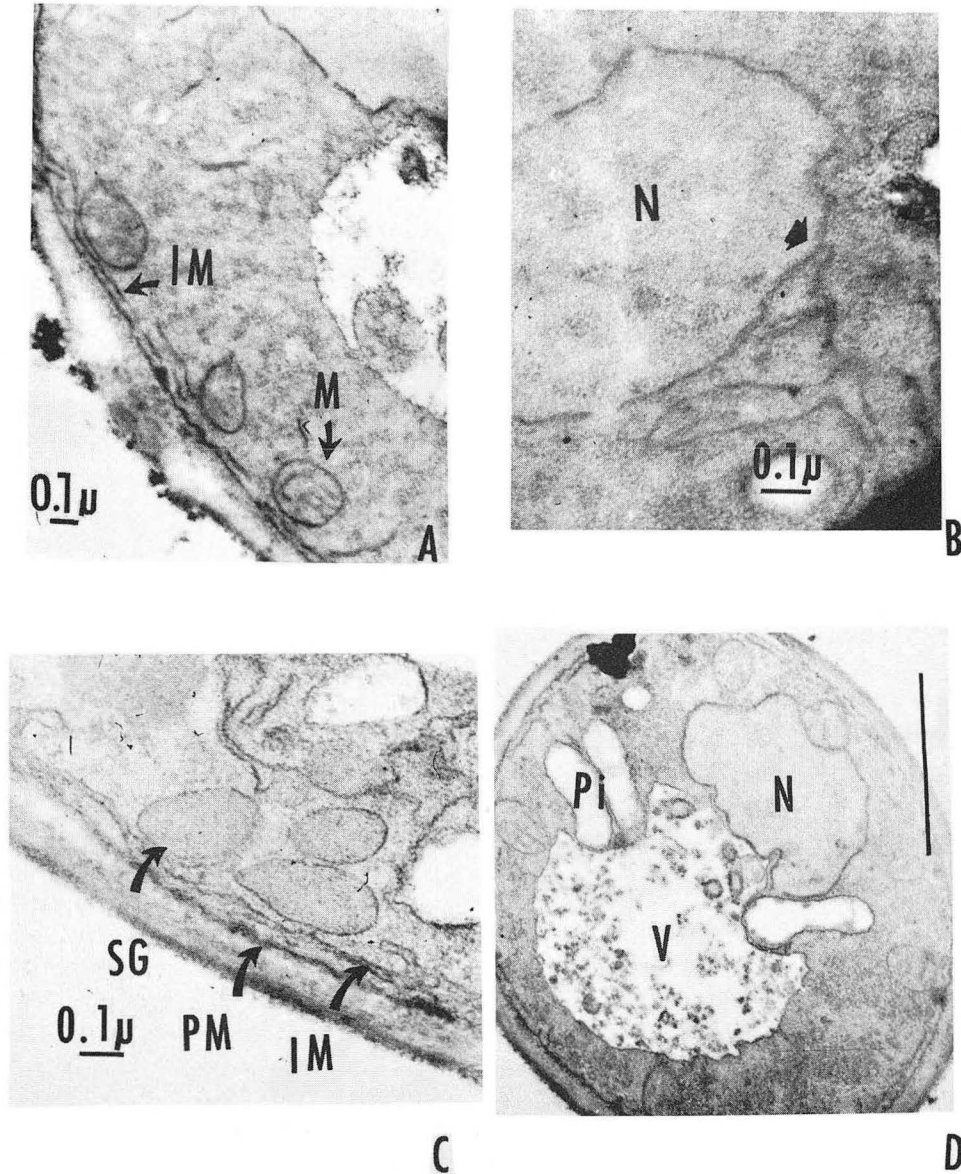
ZN-2848

Fig. 8a. Mitochondria and internal membrane in a cell after
          3 hr in growth medium and after receiving 10^5r of x rays.
          b. Nuclear-cytomembrane connection.
          c. Storage granules in a cell after 24 hr in growth
          medium and after receiving 10^5r of x rays.
          d.   Unusual vacuole configurations in a cell after
          48 hr in growth medium and after receiving 10^5r
          of x rays.

Other Cytoplasmic Structures

Quite often in older well-nourished yeast cells one finds round, or oval, rather dense fibrillar sacks surrounded by a single membrane. These structures are generally a few tenths of a micron in diameter and similar in appearance to so-called storage granules found in other plant and animal cells. Figure 8c shows these structures, which are presumed to be storage or glycogen granules.

One other point of interest regarding cytoplasmic structures is the area of the vacuolar membrane or tonoplast. In growing cells the vacuole is generally smoothly oval or round, with relatively little intravacuolar material; but in starved or irradiated cells the vacuole appears fragmented or condensed, and is sometimes full of granular material. For example, compare Fig. 2c with Fig. 3b. In addition, vesicles are sometimes observed in the vacuolar membrane that are suggestive of a pinocytotic process within the vacuole. This is particularly true in the case of starved or irradiated cells, as shown in Fig. 8d. Of course, extreme caution must be exercised in the interpretation of vacuolar changes because this structure is known to be very labile and sensitive to the production of artifacts.

Irradiated Cells

The results of radiation damage to yeast cells can be classified broadly into two parts: early effects that may or may not be quite subtle with respect to fine structure changes, and late effects that are usually rather disastrous and produce considerable morphological alteration. Cells placed in succinate starvation medium after irradiation seem to undergo little, if any, radiation-induced change. It seems to be necessary to provide yeast with nutrient before such changes become visible.

Figures 9a and 9b illustrate the most common gross abnormality encountered in post irradiation budding cells. One of the two cells of the budding pair, usually the daughter, is greatly elongated, whereas the other cell has the normal round or oval shape. Usually only one of these doublet cells possesses a nucleus. Such configurations seem to persist with little change for long periods of time. Occasionally budding cells are encountered that appear to have a drastically altered nuclear morphology. Figure 9c illustrates a cell in which extensive nuclear fragmentation or degeneration has taken place. Figure 9d shows a budding cell that might be interpreted as undergoing an abortment of nuclear migration.

A fairly constant feature of the earlier stages of radiation damage in yeast is an apparent thickening of the nuclear membrane to two or three times its normal dimension. Figure 10a illustrates this effect. Signs of degenerative membrane changes can sometimes be seen during earlier stages of post-irradiation incubation. Figures 10b and 10c show such areas in the cytoplasm which may be interpreted as degenerating mitochondria or other membranous organelles. Note also, however, that normal mitochondria are also present in these sections. The central vacuoles of these irradiated cells are seldom found to be the relatively clear large structures of their unirradiated counterparts. Rather, they are generally fragmented or irregularly shaped, and often filled with inclusions—as shown in Fig. 10b, for example.
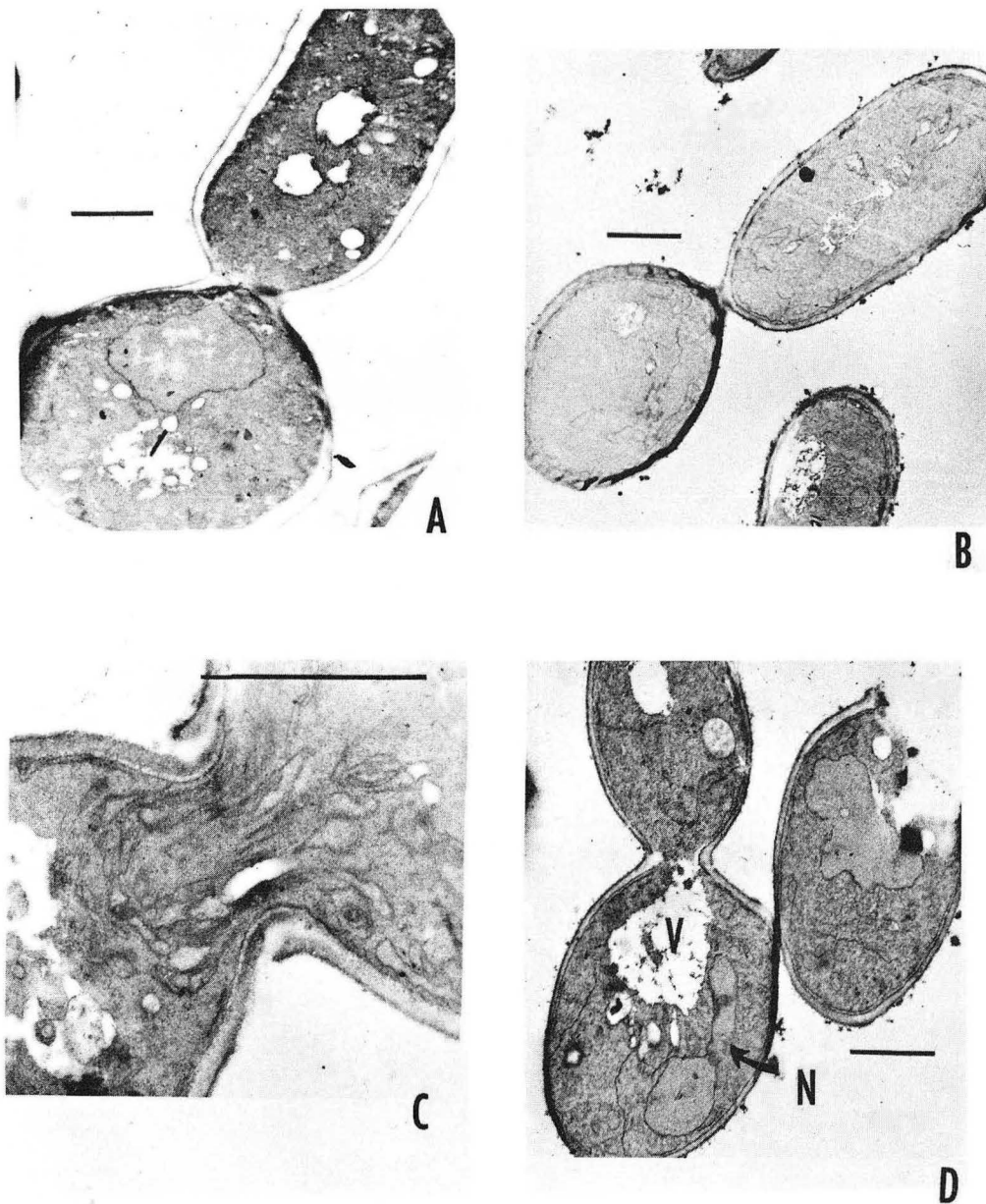
ZN-2852

Fig. 9a. Radiation-induced doublet cells after 24 hr in growth
          medium and after receiving 10^5r of x rays.
       b. Radiation-induced doublet cells after 3 hr in growth
          medium and after receiving 10^4 rads of Ne particles.
       c. Anomalous budding formation in a cell after 1/2 hr
          in growth medium and after receiving 10^5r of x rays.
       d. Budding cell after 7 hr in growth medium and after
          receiving 10^4 rads of Ne particles.
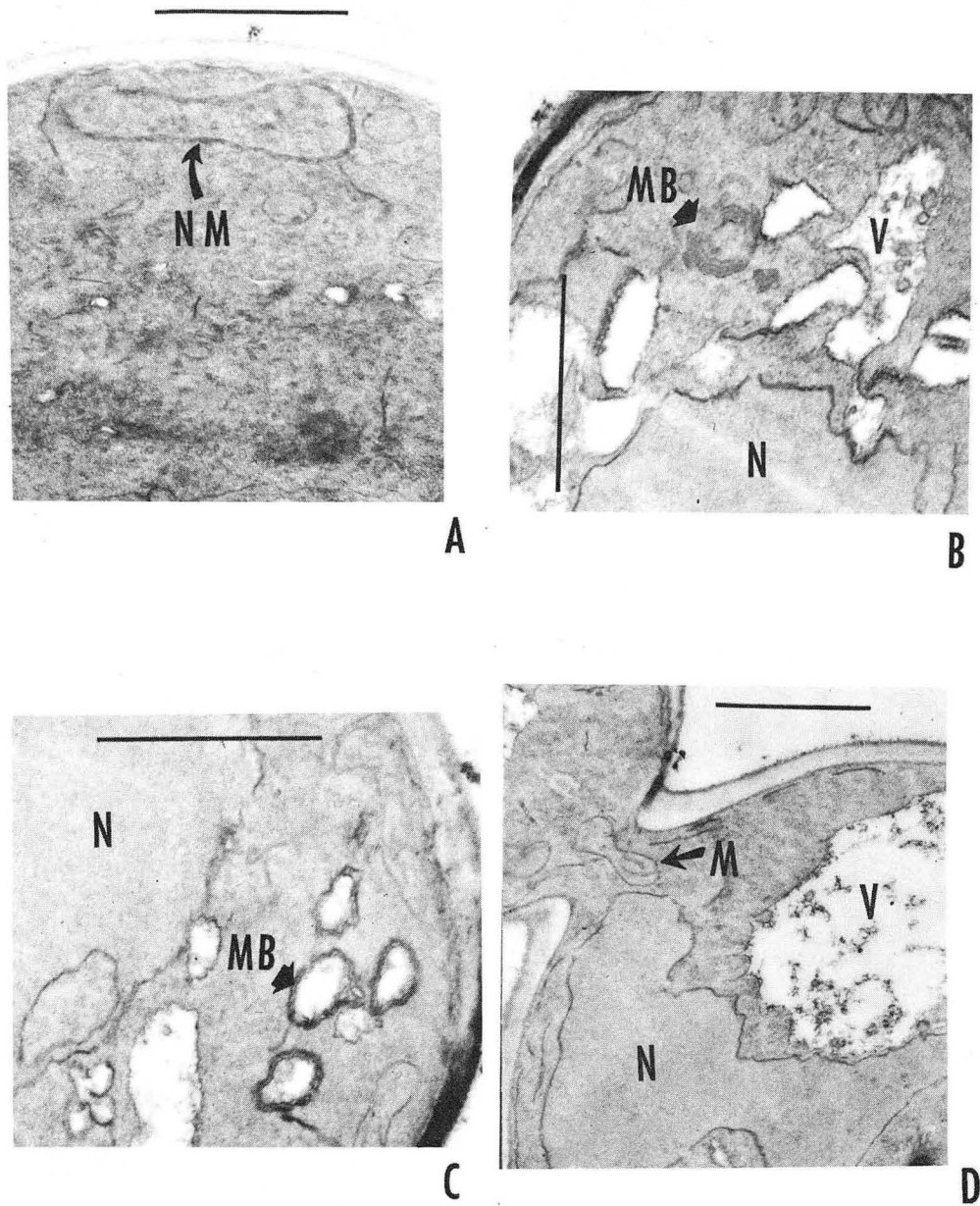
Fig. 10a. Abnormal nuclear membrane in a cell after 7 hr in
            growth medium and after receiving 10^5 rads of Ne particles.
       b. Membranous body in a cell after 1/2 hr in growth medium
            and after receiving 10^5 rads of Ne particles.
       c. Membranous body in a cell after 1/2 hr in growth medium
            and after receiving 10^5 rads of Ne particles.
       d. Budding cell after 7 hr in growth medium and after
            receiving 10^4 rads of Ne particles.

The over-all appearance of cells in the early stages of postirradiation damage is not unlike that of starved cells. The cytoplasm is generally rather clear, and membranous components stand out very well. Figures 10d and 8a illustrate this effect. Note the mitochondrion in Fig. 10d at the bud attachment point, and the membrane system in close association with the plasma membrane, as mentioned earlier in connection with normal cells. In Fig. 11a the nucleus is highly distored, and portions of the vacuole appear to have been filled with a particulate material. Notice the close association of the mitochondrion, marked M, with the membrane system discussed above. For the sake of brevity this membrane, or membranes, will be termed the "internal membrane." Occasionally this internal membrane can be seen to be continuous with the plasma membrane, as shown in Fig. 11b.

Unusual nuclear morphology that cannot be designated as degenerative in nature but, rather, merely abnormal is sometimes seen in irradiated cells. Whereas some irradiated nuclei are obviously atrophic with congealed and vacuolated nucleoplasms, others display apparently normal internal and membrane structures, but differ from the normal nuclei in size, degree of lobulation, and possibly number. Figure 11c illustrates an unusually large nucleus, or nuclei, in a cell 24 hr after irradiation. The degree of lobulation in irradiated nuclei is much greater than in normal cells, so it cannot be ascertained without extensive serial sectioning whether such a profile in reality corresponds to two lobes of the same nucleus or to two independent nuclei. Note the extremely clear nuclear pores and the internal membrane, which in this case runs continuously around the periphery of the cell and can be seen to be "smooth" and double. Figure 11d shows a cell to which similar comments apply as far as nuclear morphology is concerned, and which contains rather nice examples of yeast mitochondria. Note the concentric ring structure between the two nuclear portions. Such circular double-membrane profiles are quite commonly seen in irradiated cells. Their size and double-membrane internal structure are suggestive of mitochondria, but their functional identity with mitochondria has not been determined.

More drastic effects on cell morphology are noticeable if samples are allowed to incubate for two days or longer after irradiation. Figures 12a and 12b illustrate the amorphous appearance that some of the cells exhibit at this time. The dark particulate bodies seen in the vacuoles of these cells are often encountered in cells several days post irradiation. Figure 12a also shows a rather unusual nuclear membrane lobe projecting into the central vacuole. Note that mitochondria or membranous portions of the cytoplasm are absent or unobservable in these sections. Beyond 2 days postirradiation, many areas in thin sections are encountered that contain drastically altered cells. Figure 12c shows—at low magnification—a field of cells, most of which have obviously undergone lysis. The nuclei are sometimes still observable, and a dense coagulated cytoplasmic residue remains; but the great majority of these cells are no doubt dead in all respects. Figure 12d shows, at higher magnification, a cell that has undergone a process very suggestive of explosion.

Lysis does not seem to be the only end point that postirradion yeasts reach in the later stages of radiation damage. Cells are often encountered that have a relatively normal cytoplasmic appearance, but whose external morphology is more ameboid than yeast-like. Figure 13a illustrates—at low magnification—some of these ameboid forms, together with lysed cells discussed
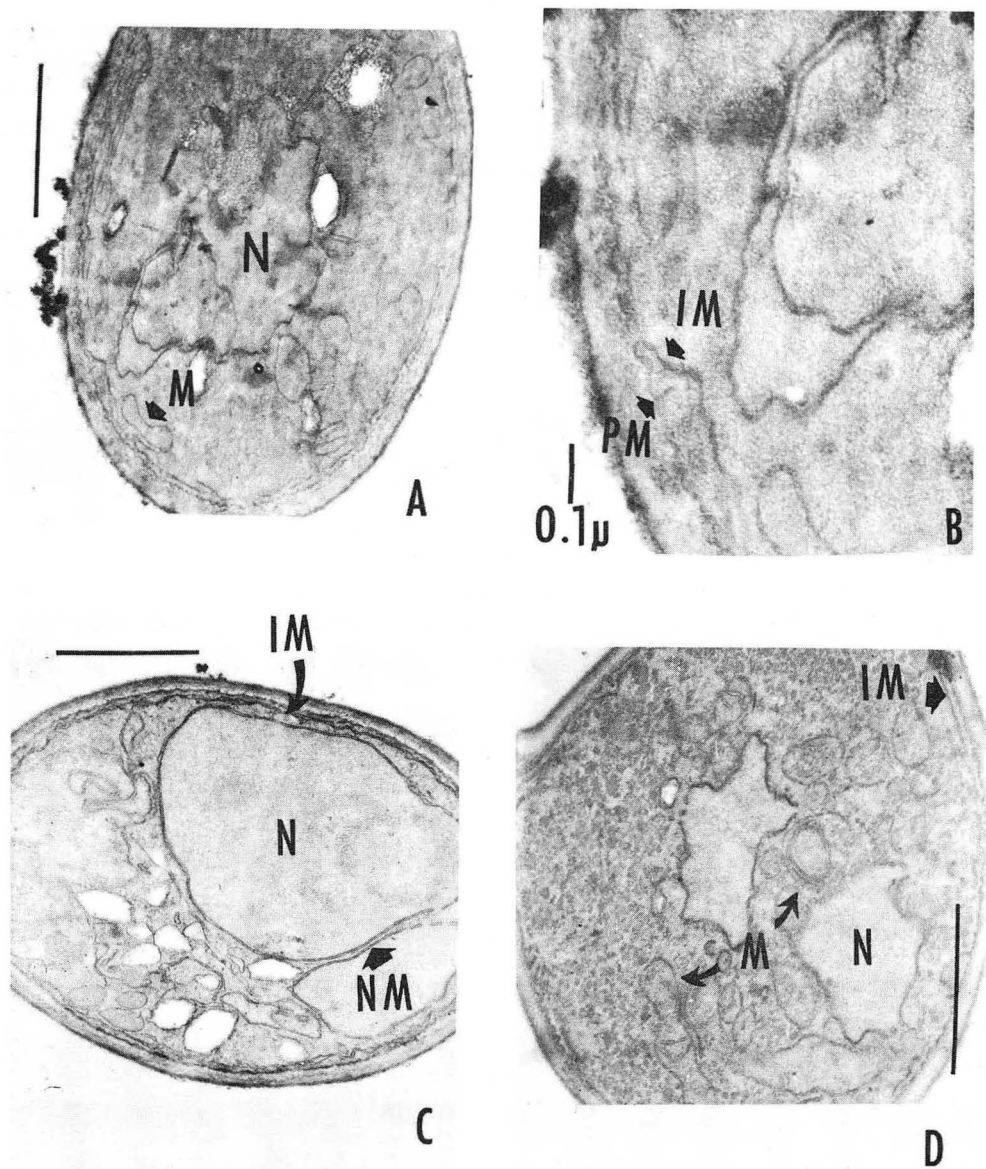
Fig. 11a.   Yeast cell after 3 hr in nutrient medium and
            after receiving 10^4 rads of Ne particles.
        b.   Plasma—internal-membrane connection in a cell
            after 3 hr in nutrient medium and after receiving 10^4
            rads of Ne particles.
        c.   Yeast cell after 24 hr in nutrient medium and after
            receiving 10^5r x rays.
        d.   Yeast cell after 1/2 hr in nutrient medium and after
            receiving 10^5r of x rays.
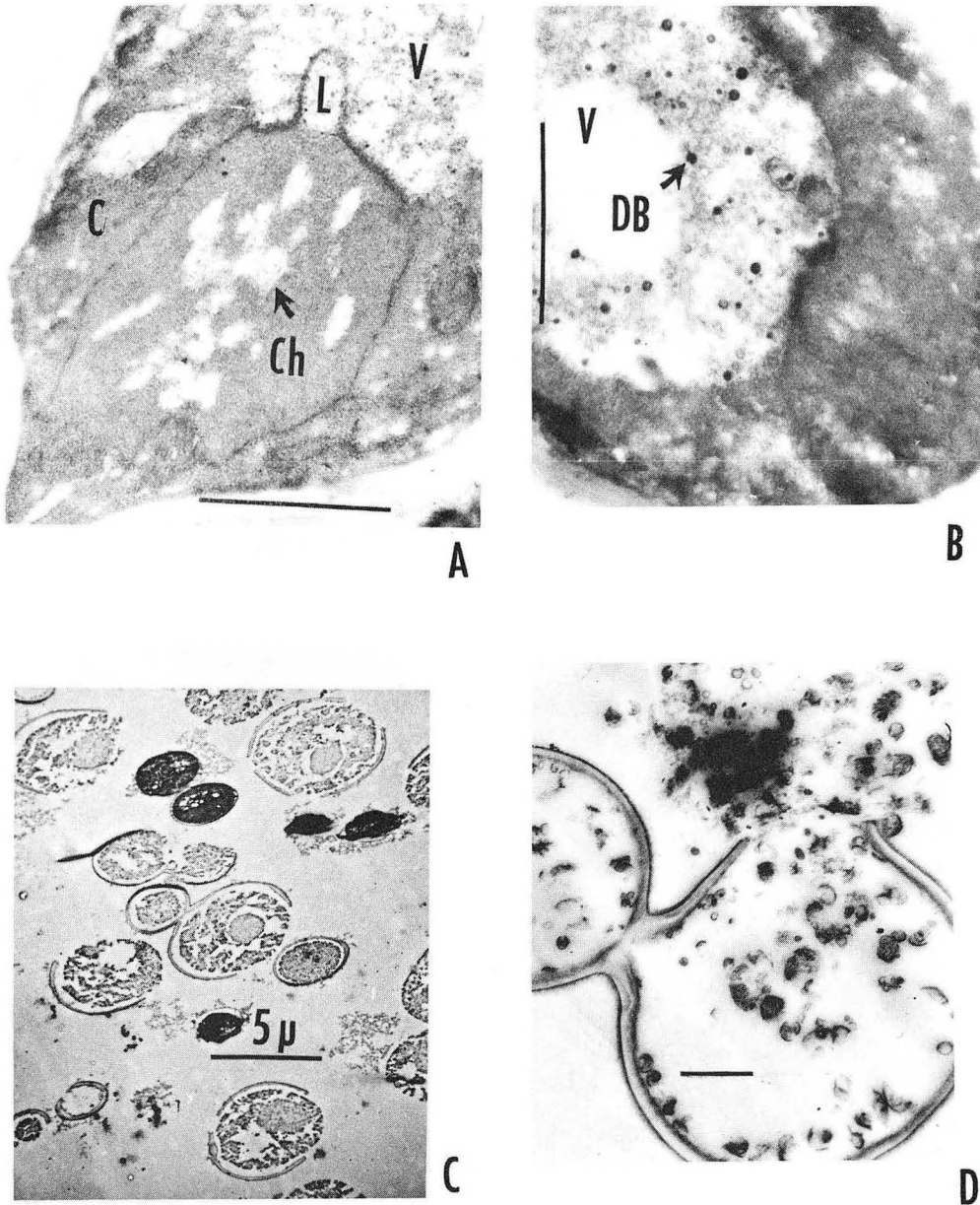
ZN-2855

ZN-2854

Fig. 12a. Degenerative cell after 42 hr in growth medium and
              after receiving 10^5r of x rays.
        b. Degenerative cell after 42 hr in growth medium and
              after receiving 10^5r of x rays.
        c. Lysed cells after 48 hr in growth medium and after
              receiving 10^5r of x rays.
        d. Lysed cell after 30 hr in growth medium and after
              receiving 10^5r of x rays.
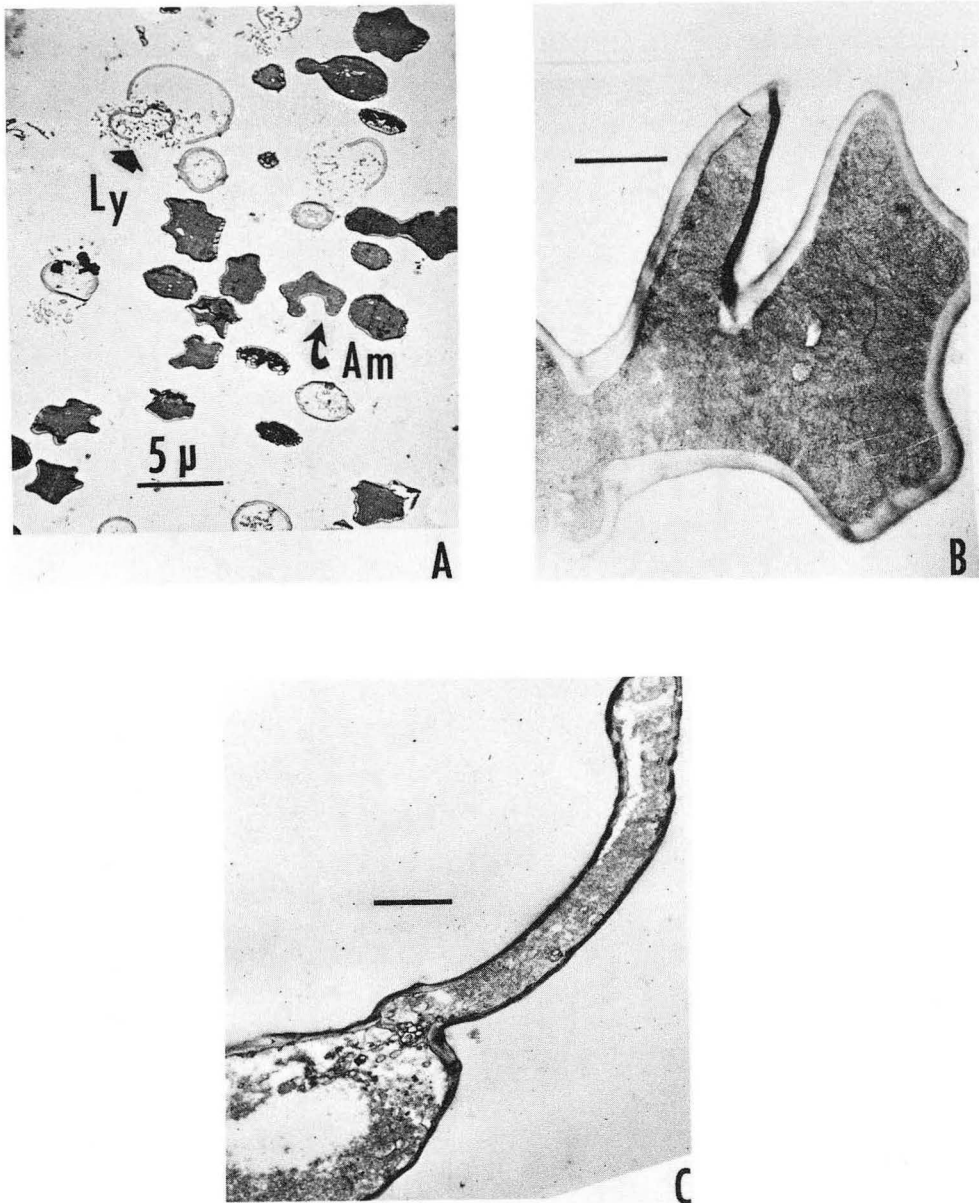
ZN-2856

Fig. 13a. Lysed and ameboid cells after 96 hr in growth medium
and after receiving $10^5 r$ of x rays.
b. Ameboid cell after 96 hr in growth medium and after
$10^5 r$ of x rays.
c. Ameboid cell after 96 hr in growth medium and after
$10^5 r$ of x rays.

above.   Upon closer examination, some of these deformed cells appear to
have a dense healthy cytoplasm, not dissimilar to cells in logarithmic growth
phase, such as in Fig. 13b. More commonly, they have a highly modified
cytoplasm containing many bizarre bodies; and few, if any, of the normal
components generally associated with the cytoplasm, as in Fig. 13c. Of
great interest is that few of these later type cells seem to possess a nucleus.

Ribosomes

It was decided to carry the fine-structure investigations of yeast to a
somewhat greater stage of refinement, by isolating the ribosome complement
of the cells and determining their structure and character by parallel electron
microscopy and ultracentrifugation. Ribosomes were isolated from yeast
cells after various times in growth or starvation media, by the usual centrifuge
methods described in Section III.

Sandritter et al. have shown that characteristic changes in the uv ab-
sorption spectrum occur after RNAase-heat treatment of liver ribosomes.[52]
Several preparations suspended in Chao and Schachman's buffer were examined
in a Beckman uv spectrophotometer to determine if we were indeed dealing
with the RNA-containing ribosomes. Figure 14 shows such a spectrum de-
termination, with the absorption at 260 mμ typical of nucleic acid, and the
same preparation after heat and ribonuclease treatment. The results are
essentially identical to those of Sandritter et al.,[52] but are somewhat puzzling,
as it would be expected that a 260 mμ peak due to free nucleotides would occur
in the treated material. Upon standing at room temperature for one to two
hours the treated solutions exhibited flocculant white precipitates.

Various preparative methods were employed to visualize the ribosomes
in the electron microscope. Figure 15a shows thin-sectioned ribosomes
after KMnO$_4$ fixation. Figure 15b illustrates the increased contrast possible
after a brief overstaining with Pb(OH)$_2$ according to the procedure of Watson.[48]
Relatively little structural detail can be obtained from such preparations, so
they were not routinely used in the analytical aspects of the research. Figure
16a shows whole ribosomes treated with uranyl nitrate, and Fig. 16b shows
the familiar metal-shadowed particles after a freeze drying procedure similar
to that of Williams[53] was carried out. Note the dimeric particle marked with
an arrow and the occurrence of a cleft in some of the other particles. This
cleft is felt to be on artifact since it is observed only on the shadow side of
the particles. More probably this represents the region of association of two
subunits that is obscured by metal and is visible only in the area of relative
shadow. The shadowing technique was employed routinely in obtaining micro-
graphs of ribosomes for measurement and size comparison.

The ribosome preparations viewed in the electron microscope generally
appear to consist of randomly dispersed particles of various sizes. Sometimes,
however, particles are also seen that are apparently strung together. From
the described work on sectioned cells it is known that yeasts contain a small
but definite amount of endoplasmic reticulum. Figure 17 illustrates the type
of configuration that could be explained as originating from this source.
Figure 17 (insert) shows such an area at greater magnification; the membranous
element can be seen to be closely associated with the particles. Note also
that in some instances the particles appear to be attached only to one side of
the membrane (see arrow). It must also be mentioned that these apparent
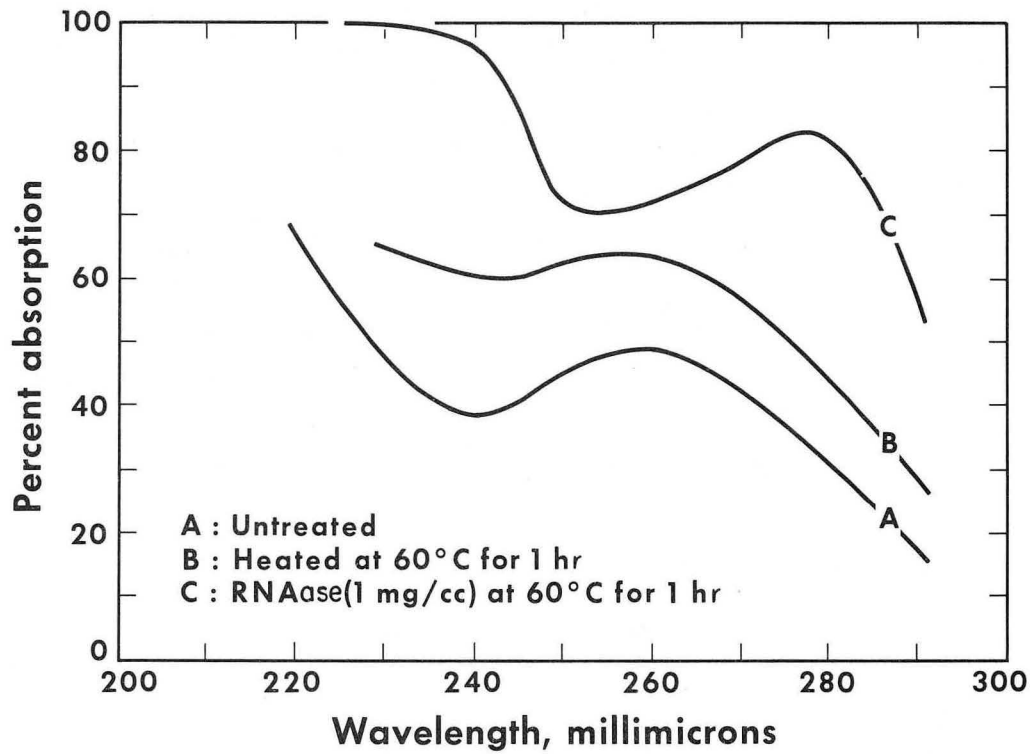
Fig. 14.   Absorption spectrum of isolated yeast ribosomes in
         Chao and Schachman's buffer at pH 6.5.
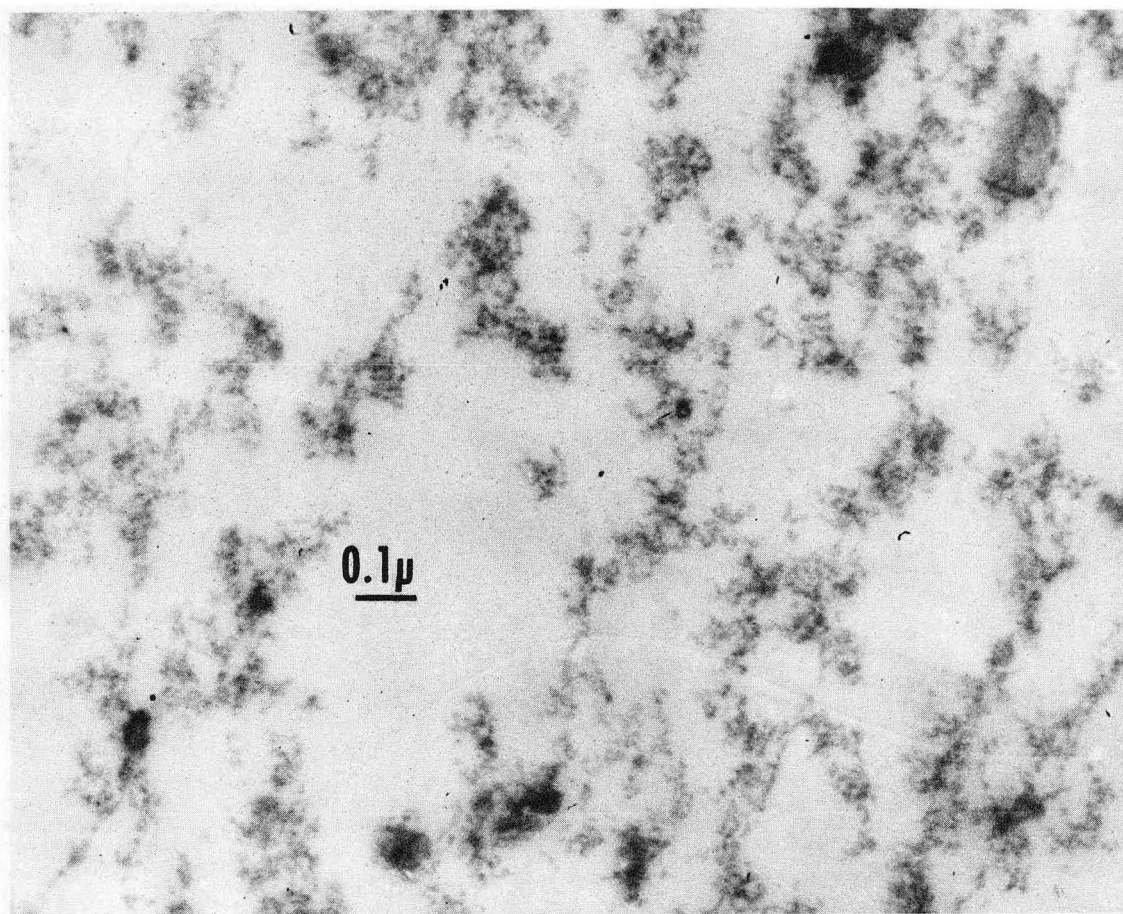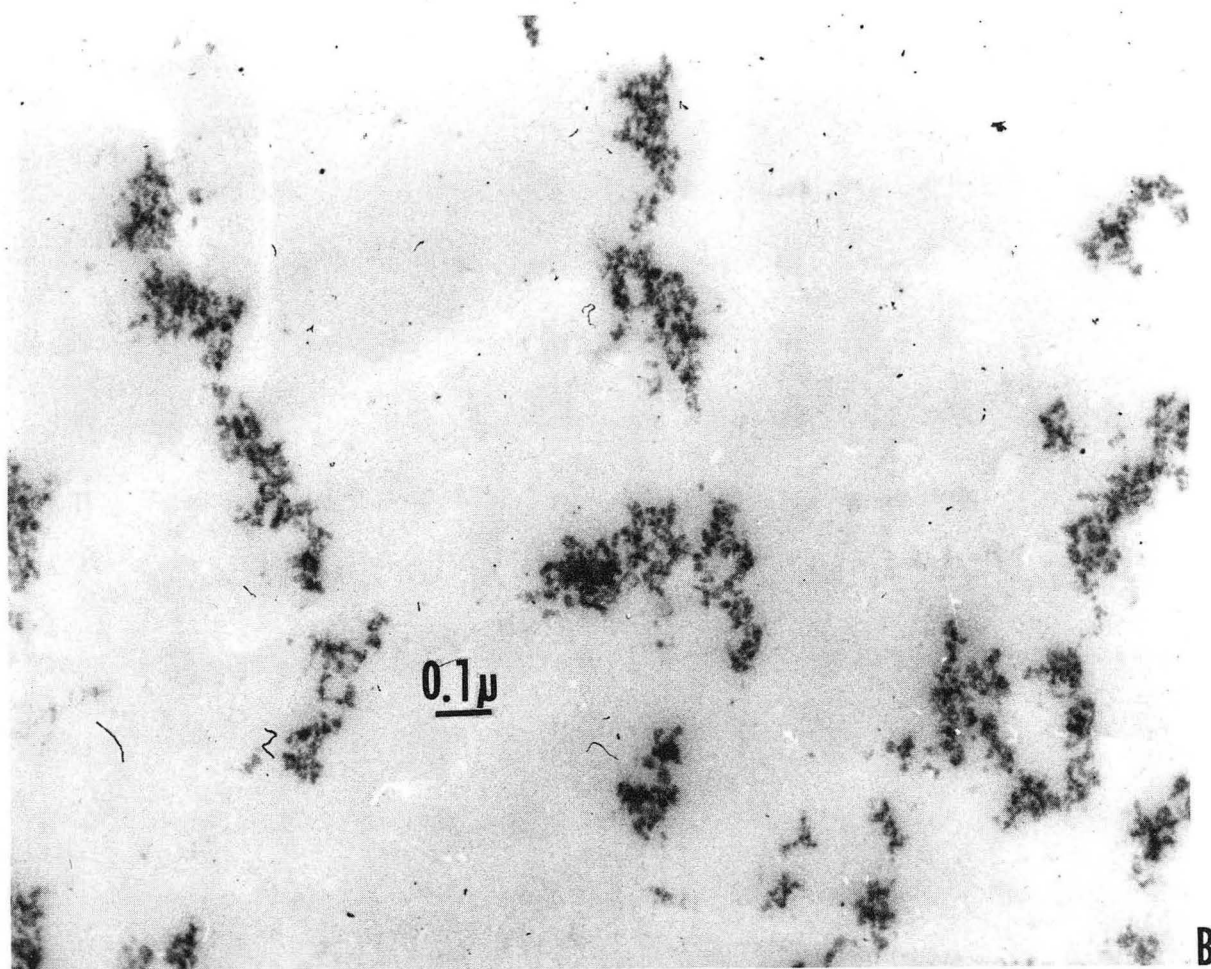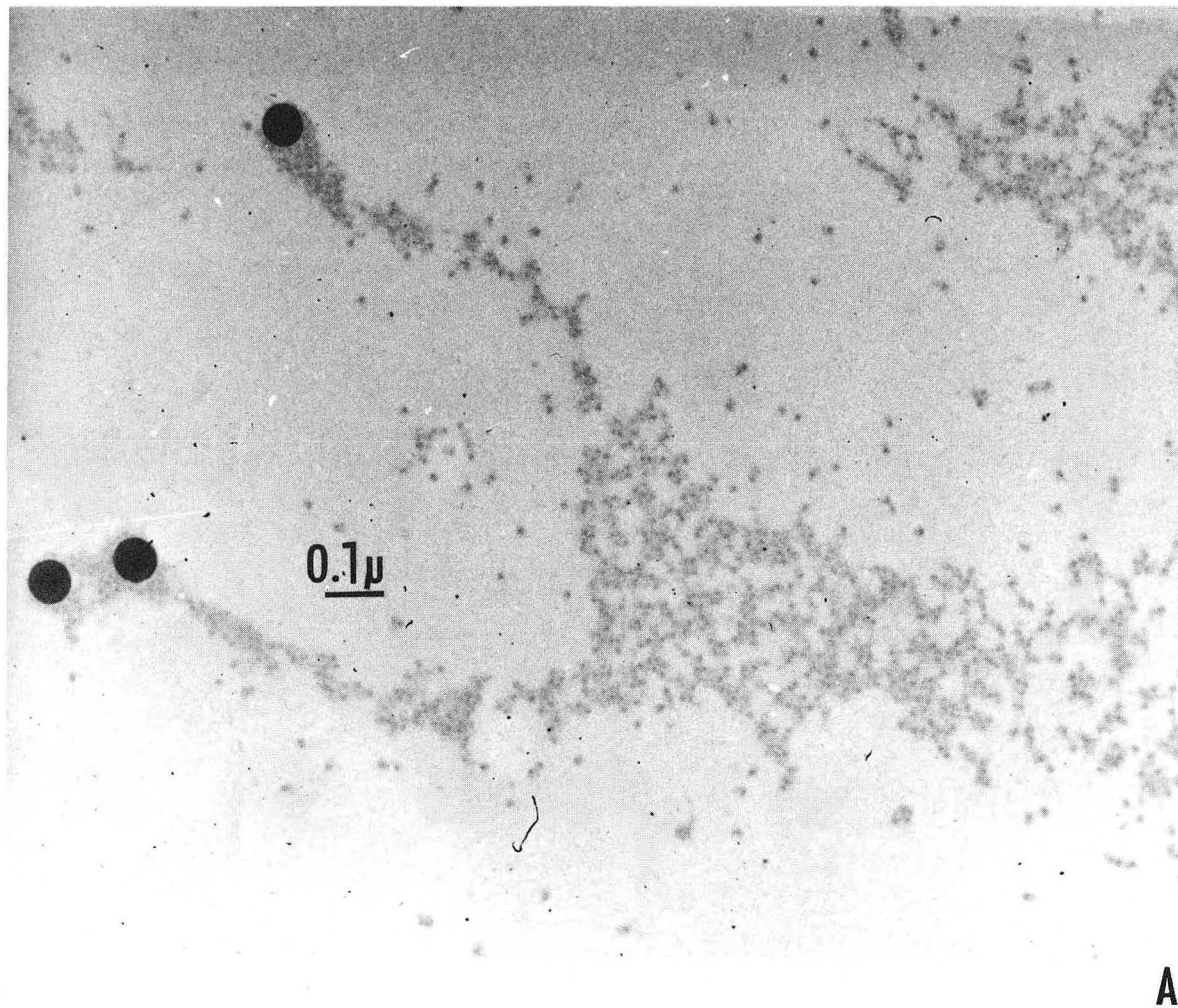
ZN-2857

Fig. 15a. Isolated yeast ribosomes, permanganate fixed
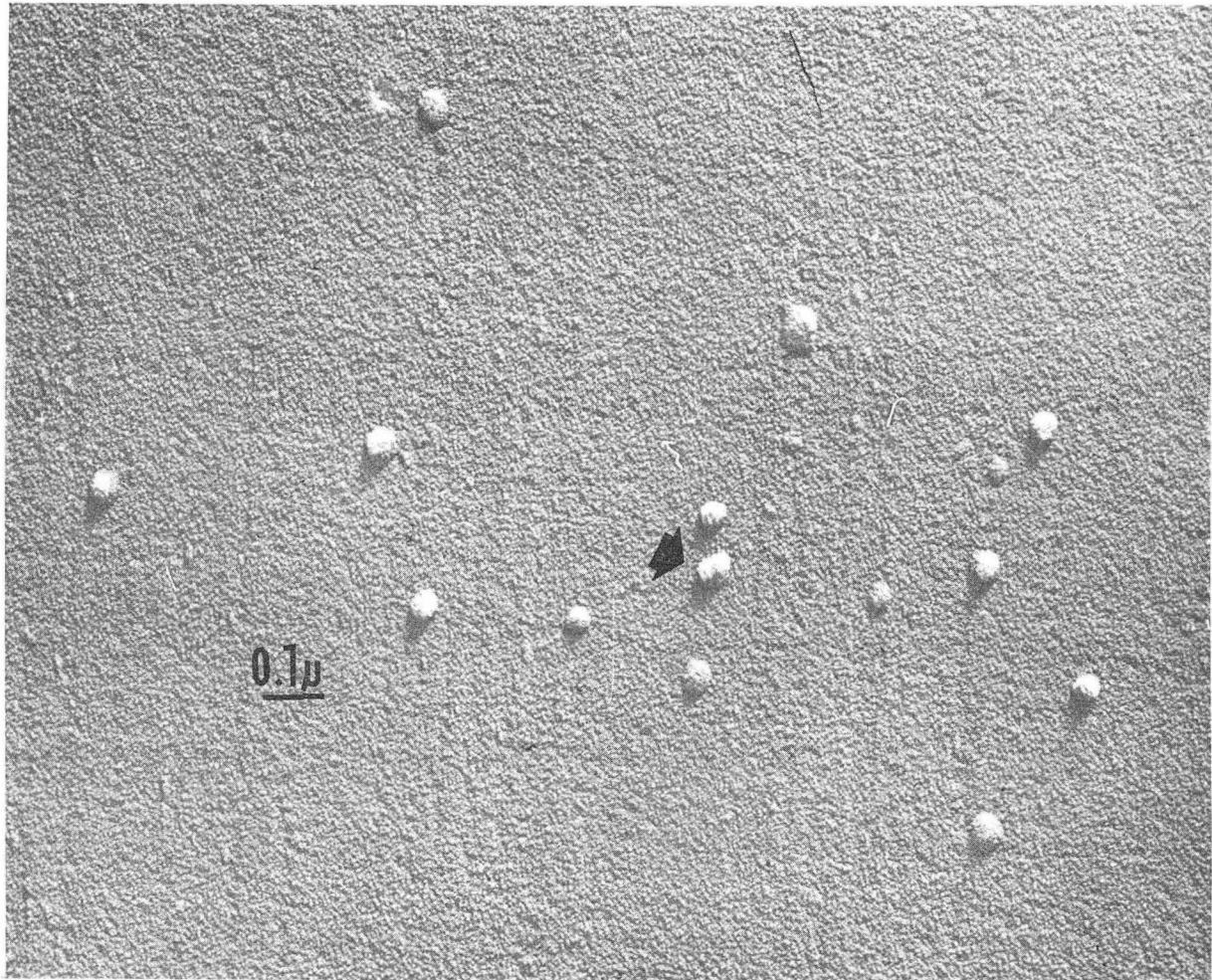and thin sectioned for electron microscopy.

Fig. 15b. Isolated yeast ribosomes, permanganate fixed,
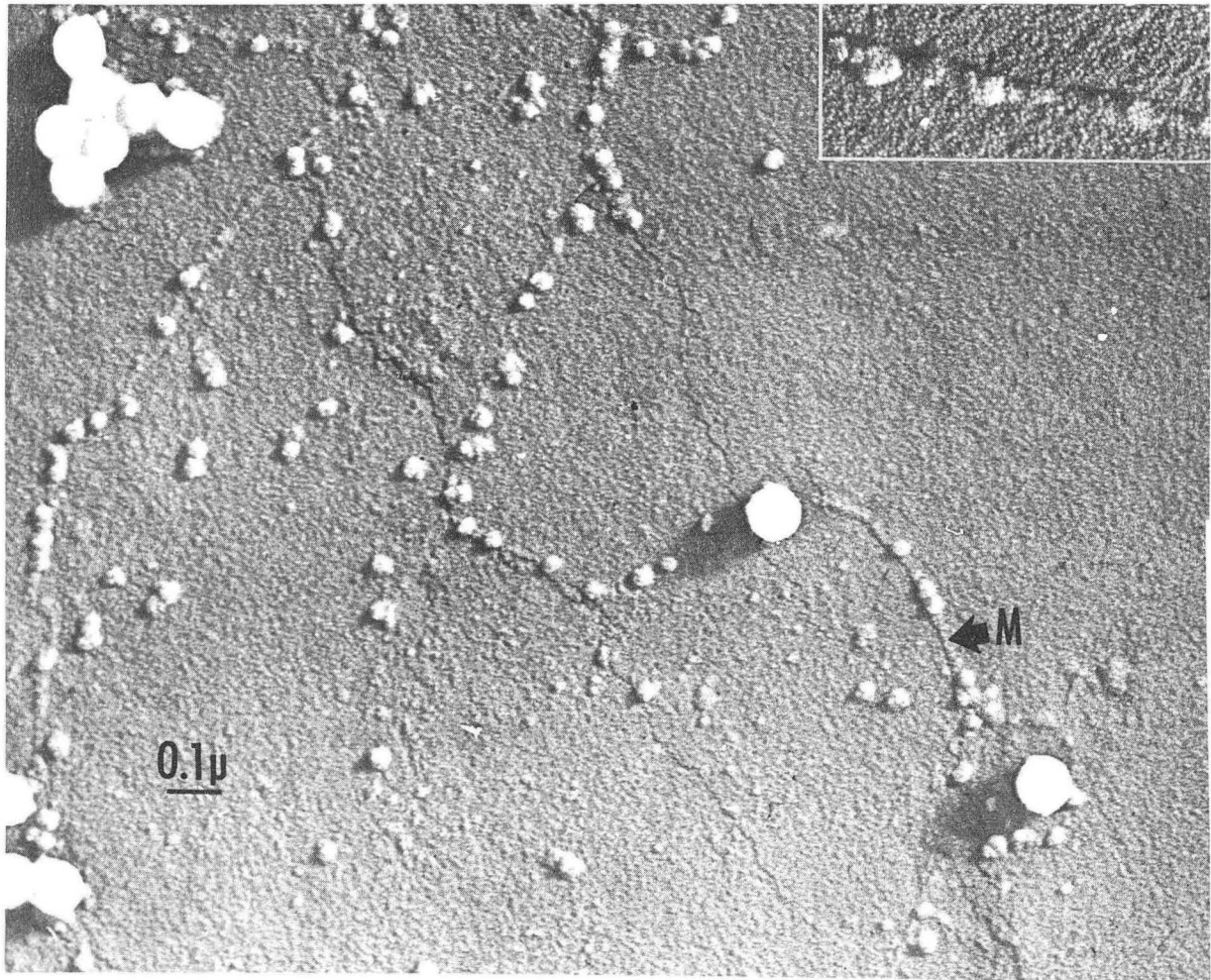   thin sectioned, and overstained with lead hydroxide.

ZN-2860

Fig. 16a.   Whole isolated yeast ribosomes fixed with uranyl
            nitrate.

ZN-2859

Fig. 16b. Isolated yeast ribosomes frozen dried and shadowed
          with nichrome.

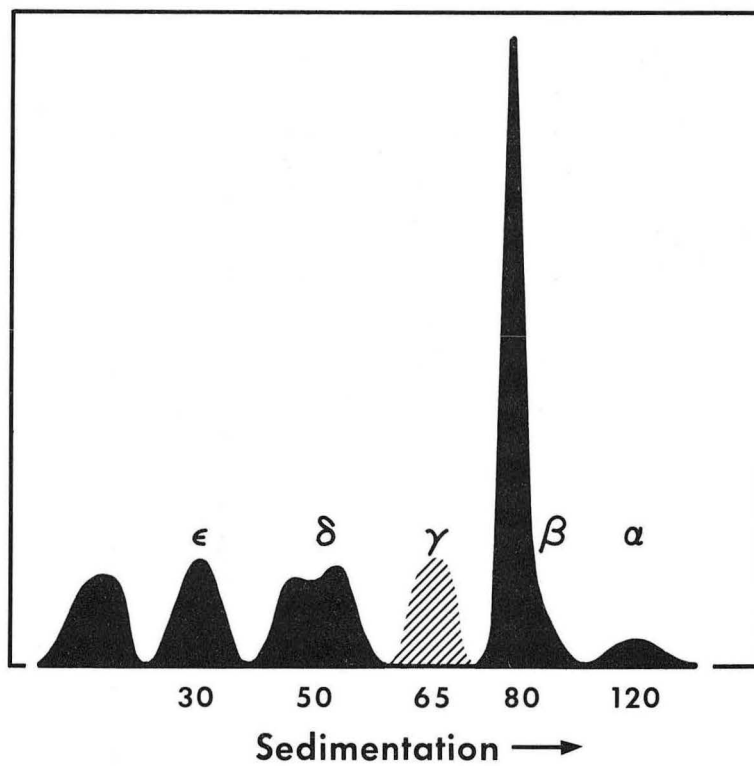ZN-2861

Fig. 17. Isolated yeast ribosomes air dried and shadowed with
          nichrome.

membranes may only be artifacts produced during the drying process by
surface-tension forces acting on a small molecular component in the solution.

Figure 18 shows a schematic representation of the analytical ultracentri-
fuge pattern of yeast ribosomes in Chao and Schachman's buffer at pH 6.5.
The alpha peak is generally more pronounced in starving cells, whereas the
gamma peak and the double nature of the delta peak are present only in growing
cells. The slowest moving peak (not labeled in the figure) is probably RNA
or RN protein of low molecular weight, as the supernatant of the ribosome-
pelleting centrifugation step contains large quantities of this material having
a sedimentation rate of approximately 10S. It exhibits the characteristic
260-mμ absorption peak, but may also contain protein or lipoprotein molecules.
Because the preparative sedimentation of this component was far from complete,
it was viewed as a contaminant and will not enter the discussion. Figure 19
typifies the ribosome sedimentation patterns obtained from physiologically
differing cells. The top pattern represents ribosomes from cells after 72 hr
in succinate starvation medium. The bottom pattern shows a ribosome prep-
aration from cells incubated for 4 hr in growth medium at pH 6.5. The ribo-
somes were always centrifuged in Chao and Schachman' buffer, and the
Schlieren pictures were taken 2 min apart after the running speed of 52,600
rpm has been reached. Note the complete absence of the gamma or 65S peak
in the starved ribosome preparation.

Figures 20 through 38 detail the information obtained from the various
samples of yeast ribosomes. Histograms that represent raw data, because
no correction was applied for the thickness of shadowing metal, were prepared
from enlarged micrographs.   These measurements were made in order to
obtain information on the variation of ribosome structure with cell physiology,
independent of the sedimentation data. Such a check is useful even though the
hydrodynamic method has the greater inherent sensitivity, being proportional
to the mass rather than diameter of the particles as determined by electron
microscopy. Visual comparison of the histograms is difficult since they are
quite similar in distribution. It will, however, be useful to draw attention to
several points of interest. Consider the distribution of particles from cells
isolated after 48 hr in succinate (Fig. 21). Those particles about 200 A in
size probably represent the 30S particles, whereas those between 400 and
500 A no doubt represent the 120S particles. Notice that the distribution is
fairly sharp around 300 A, representing the 80S particles. Figure 31 shows
a similar distribution for ribosomes isolated from 8-hr growing cells. The
distribution is roughly the same but with an added spike at 230 A, presumably
representing the new 65S component.

Figure 39 shows a χ^2 plot for the various histograms of ribosome sizes,
in order to obtain a more quantitative comparison of the various physiological
states. It can be seen that ribosomes obtained during logarithmic growth vary
considerably in distribution from those in resting or starved conditions. Again,
this is not doubt due to the appearance of the 65S particles during growth phase.

Figure 40 gives the mean ribosome size, determined from histograms
described above. There is a decided drop in mean size at the onset of growth.
This would indicate either a general shift of the entire population or the
diminution of one or more of the larger size classes with respect to the smaller
classes. Further analysis of the hydrodynamic data will probably indicate that
the latter mechanism is responsible for the effect. The open circles represent
irradiated samples and vary in no interpretable manner from the controls.

Fig. 18. Schematic representation of isolated yeast ribosome
centrifuge pattern as determined in Chao and Schachman's
buffer at pH 6.5 and concentration of about 1 g/100 ml.
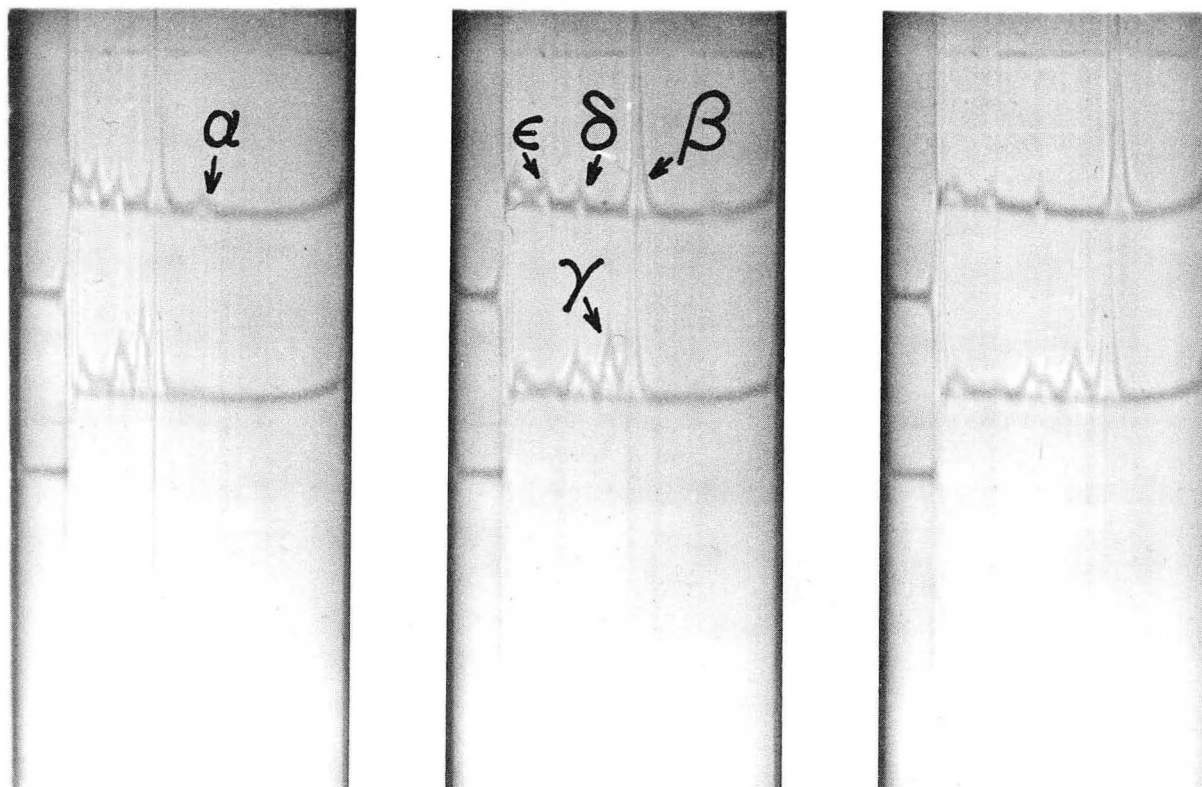
ZN-2862

Fig. 19. Schlieren patterns of typical yeast ribosome isolates
in Chao and Schachman's buffer at pH 6.5 and a concentration
of about 1 g/100 ml. Top: From cells 72 hr in starvation
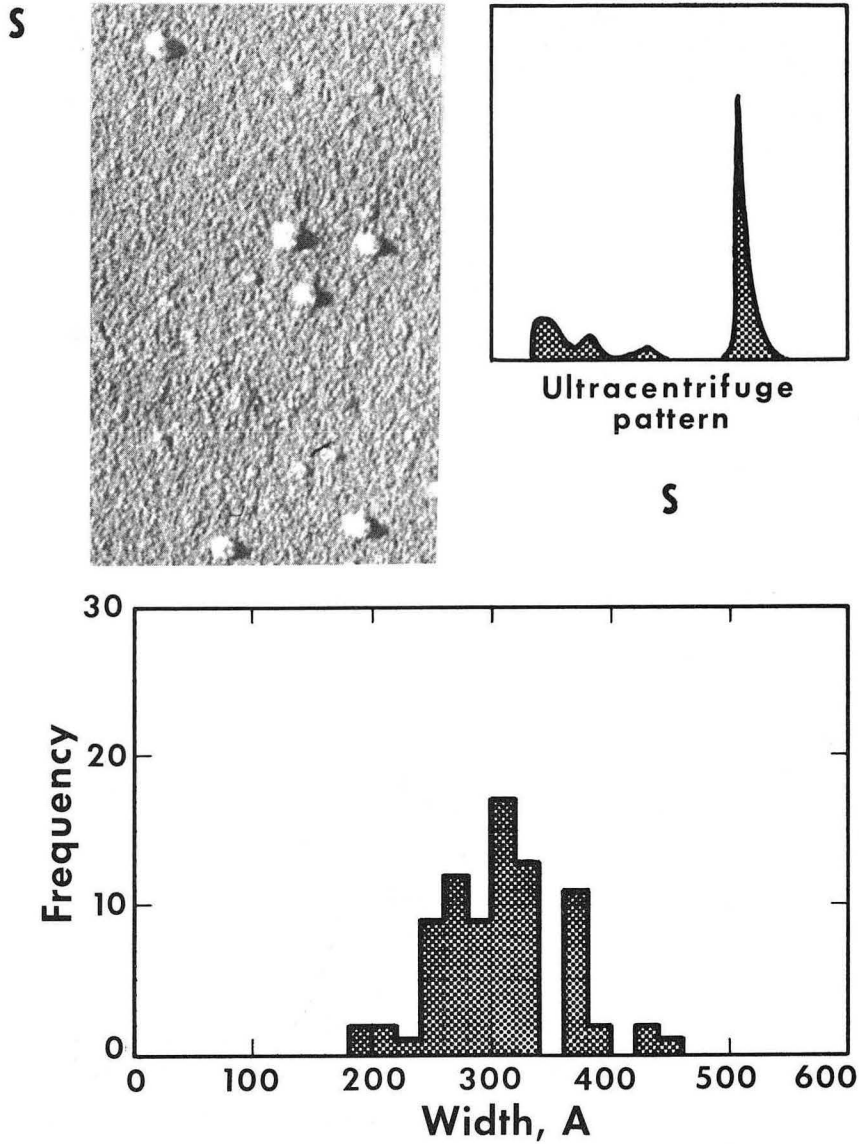medium. Bottom: From cells 4 hr in growth medium.
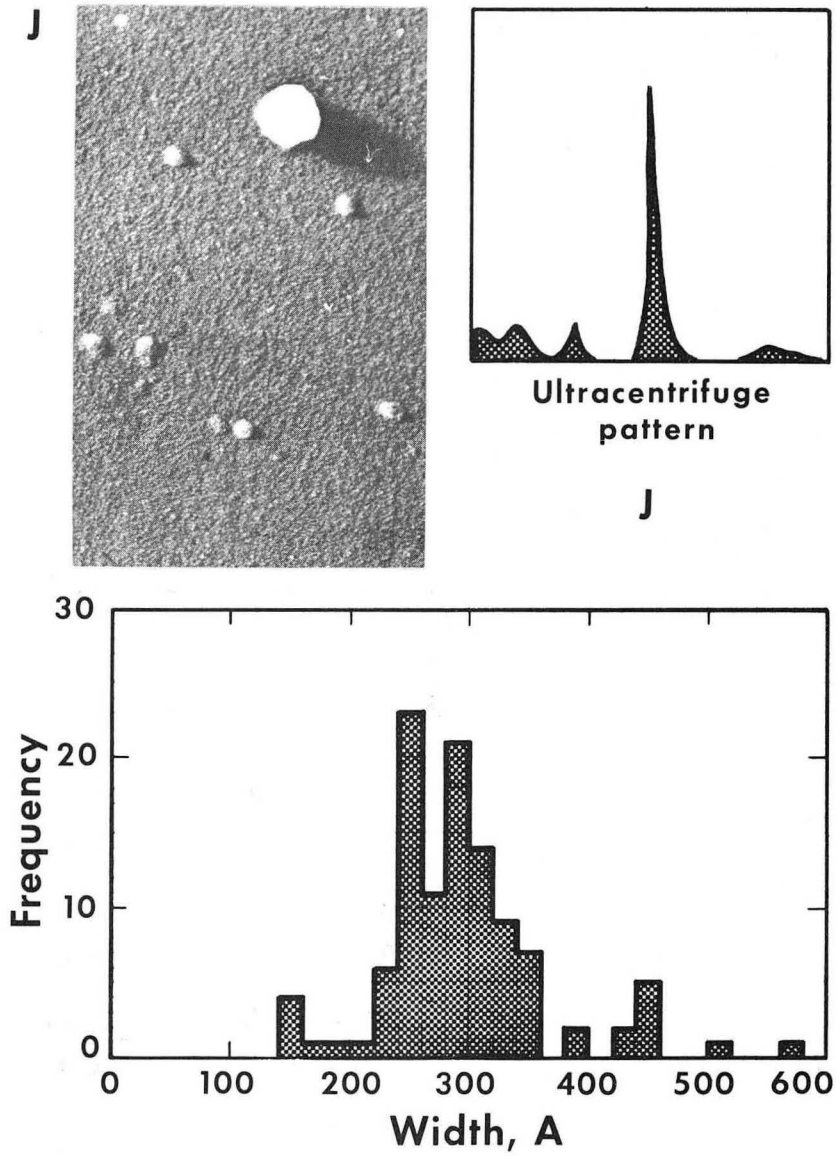
Fig. 20. Electron micrograph, ultracentrifuge pattern, and
          histogram of ribosome size distribution for yeast cells
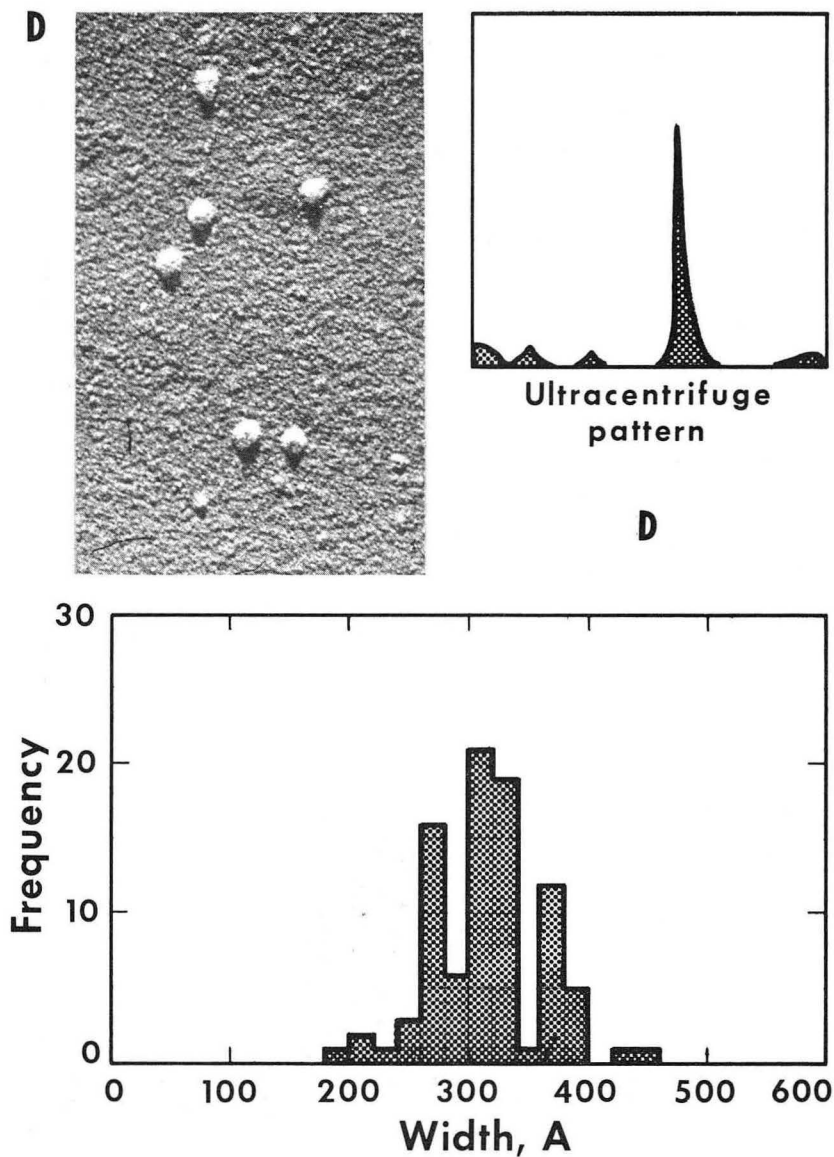   after 72 hr in starvation medium.
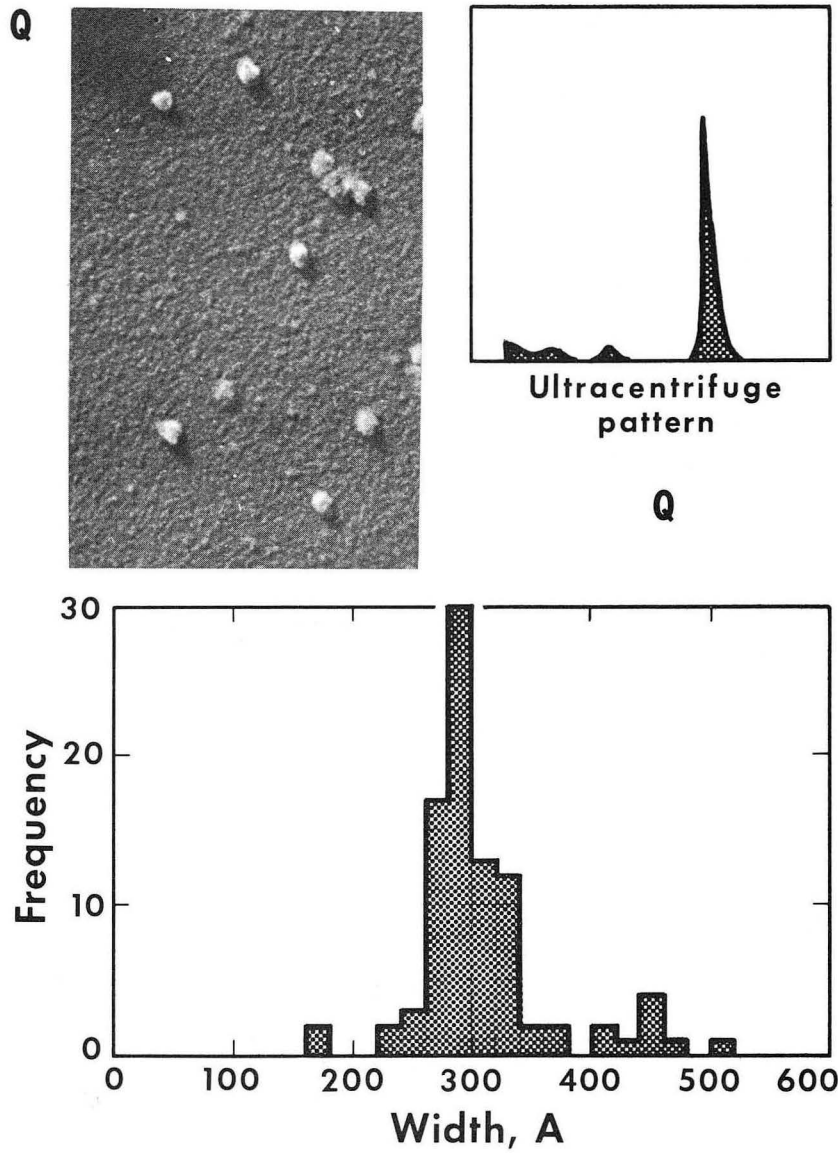
Fig. 21. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
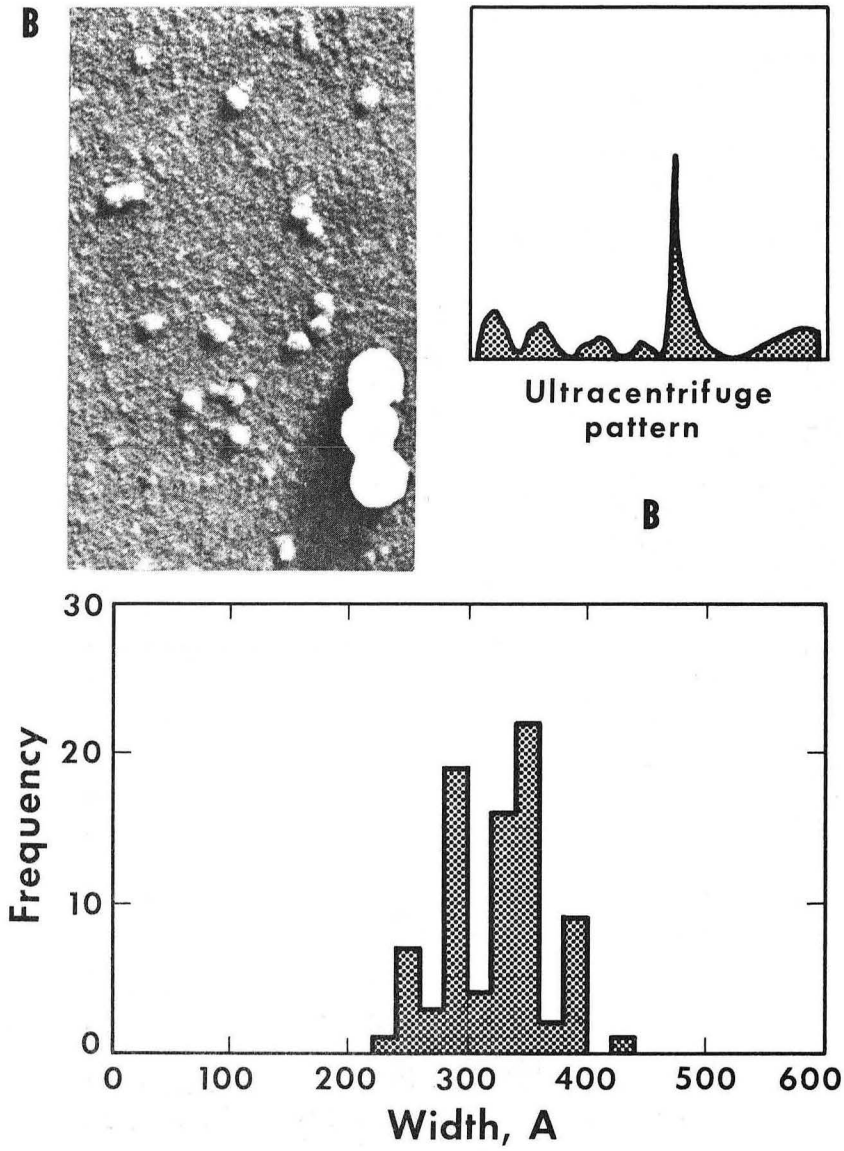after 48 hr in starvation medium.

MU-23741

Fig. 22. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
after 22-1/2 hr in starvation medium.

Fig. 23. Electron micrograph, ultracentrifuge pattern, and
          histogram of ribosome size distribution for yeast cells
          after 71-1/2 hr in starvation medium.

Fig. 24. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
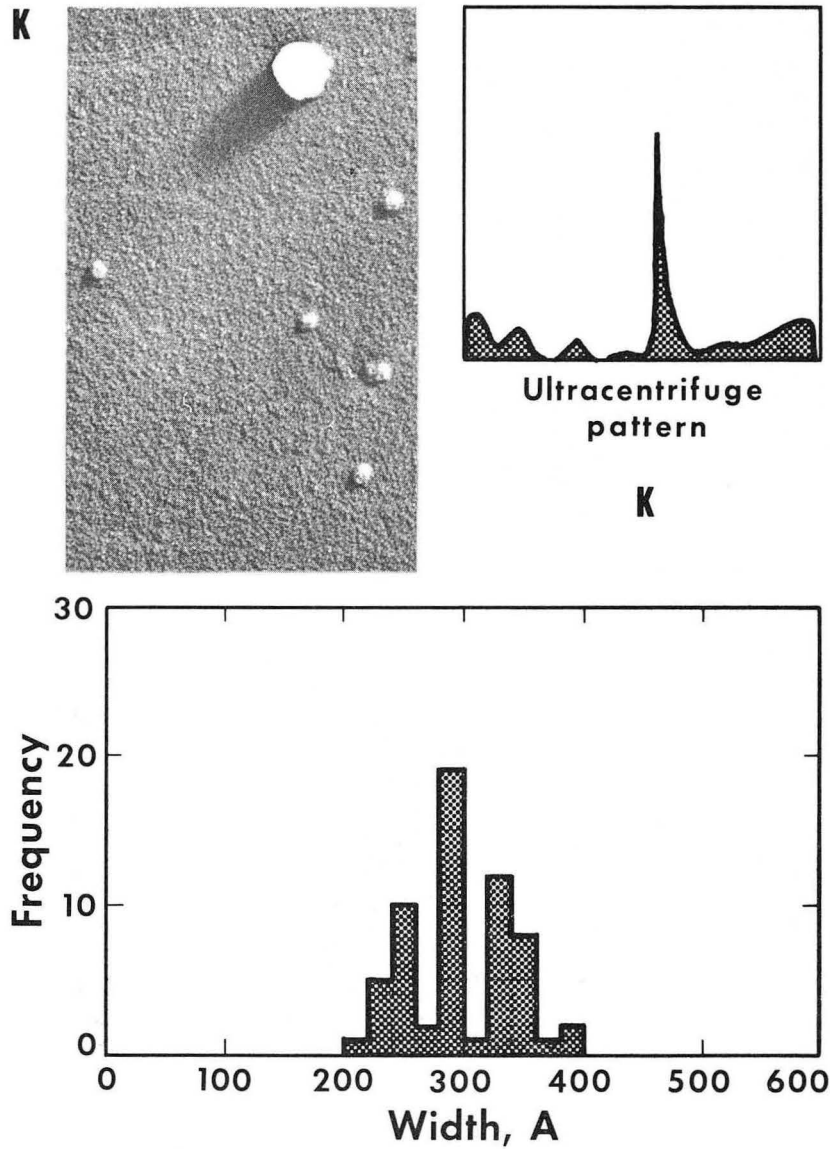after 1 hr in starvation medium.

MU-23746

Fig. 25. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
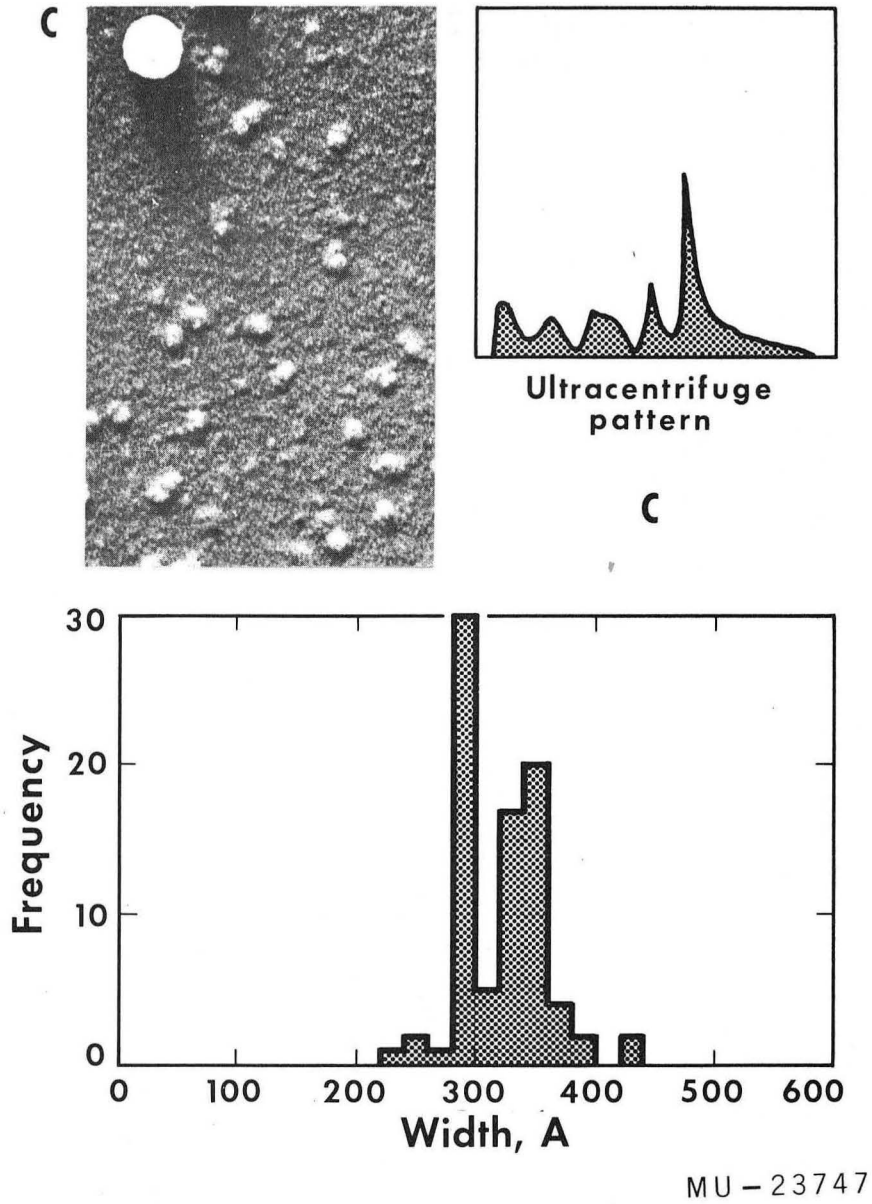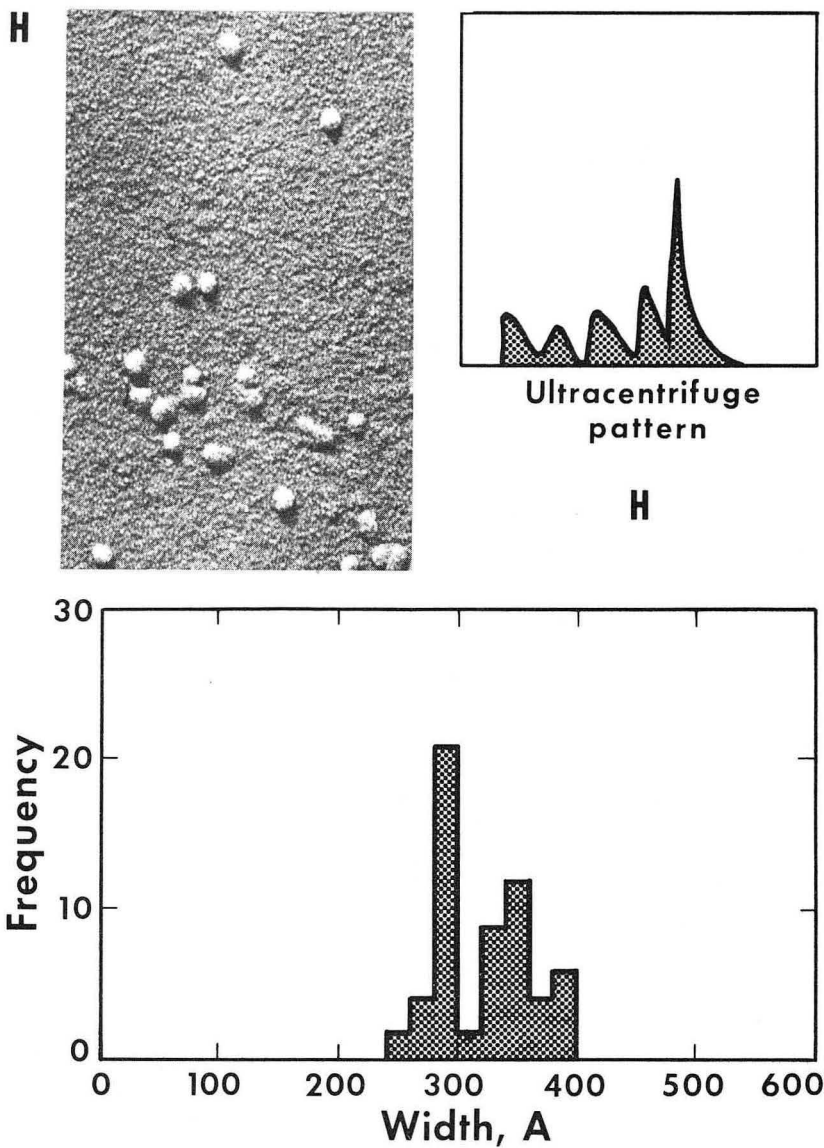after 1 hr in starvation medium and 10^5r x rays.

MU—23745

Fig. 26. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
after 1 hr in growth medium.

MU – 23748

Fig. 27. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
after 1 hr in growth medium and 10^5r x rays.
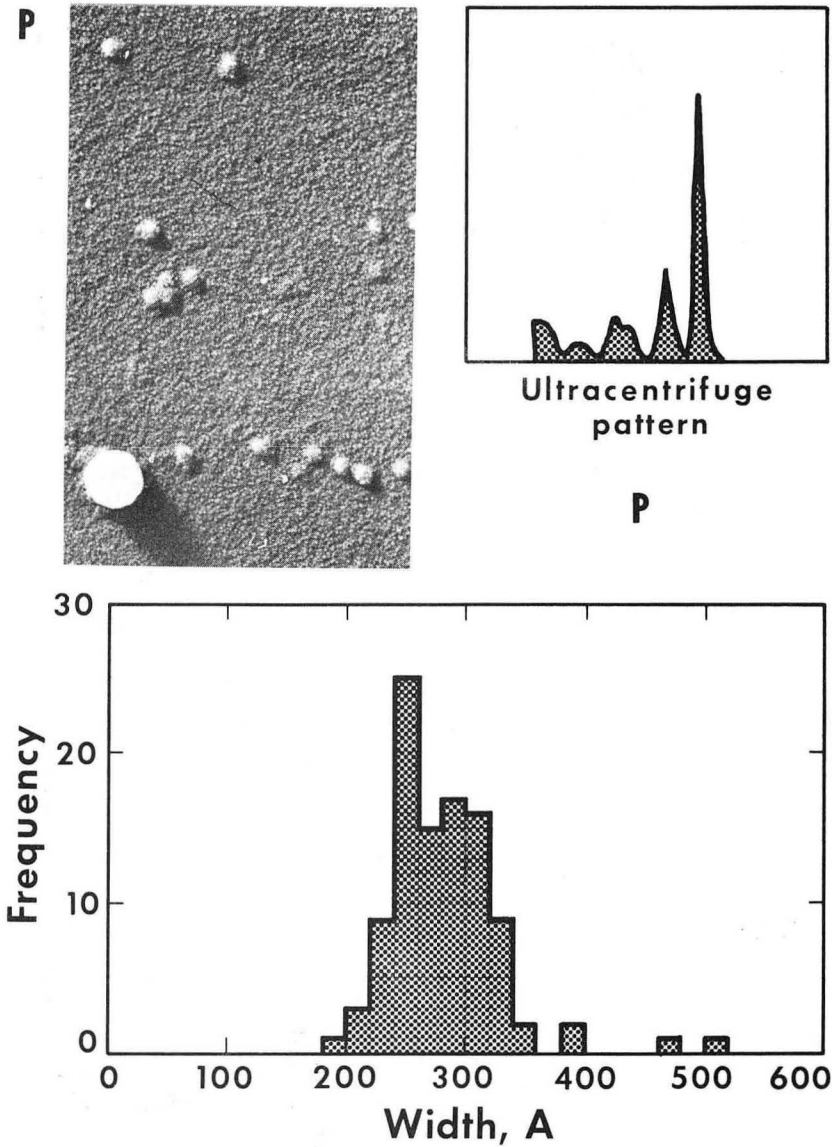
Fig. 28. Electron micrograph, ultracentrifuge pattern, and
          histogram of ribosome size distribution for yeast cells
          after 4 hr in growth medium.

Fig. 29. Electron micrograph, ultracentrifuge pattern, and
          histogram of ribosome size distribution for yeast
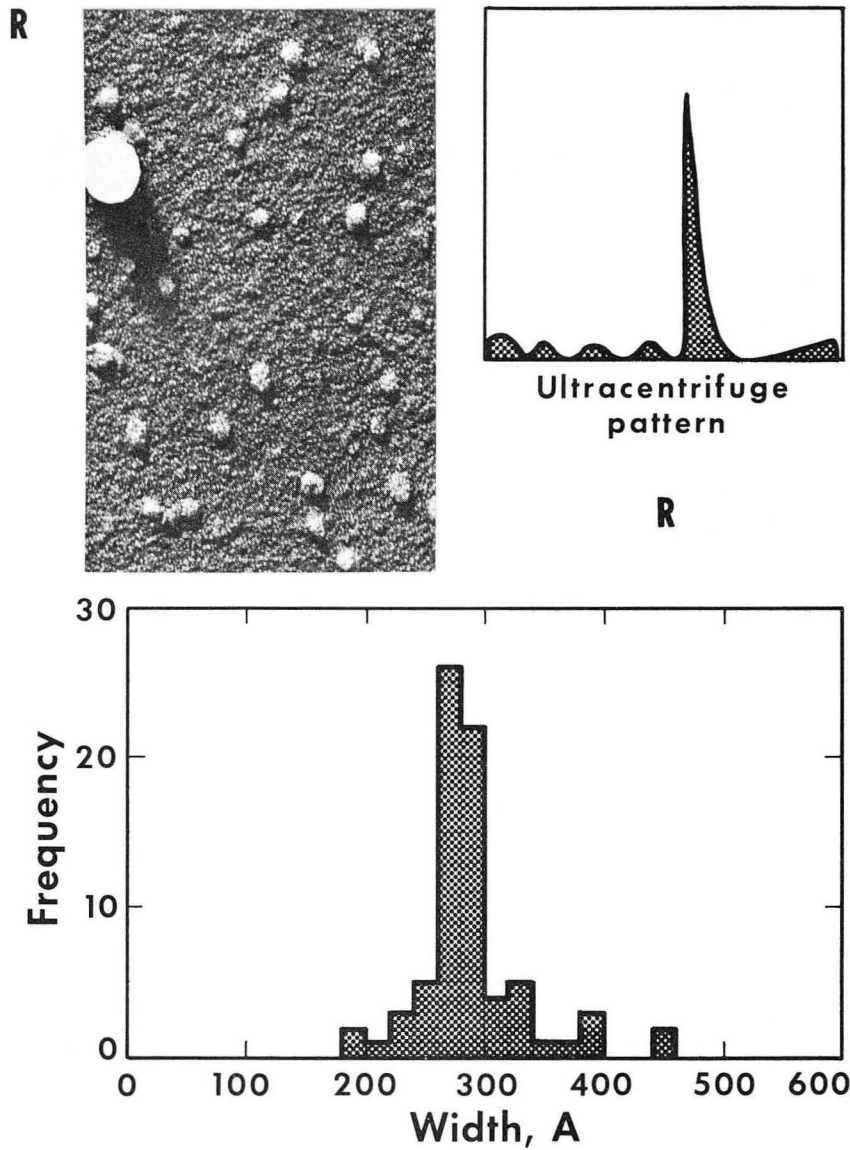   cells after 4 hr in growth medium.

T

Ultracentrifuge
pattern

T

MU – 23752

Fig. 30.   Electron micrograph, ultracentrifuge pattern, and
        histogram of ribosome size distribution for yeast cells
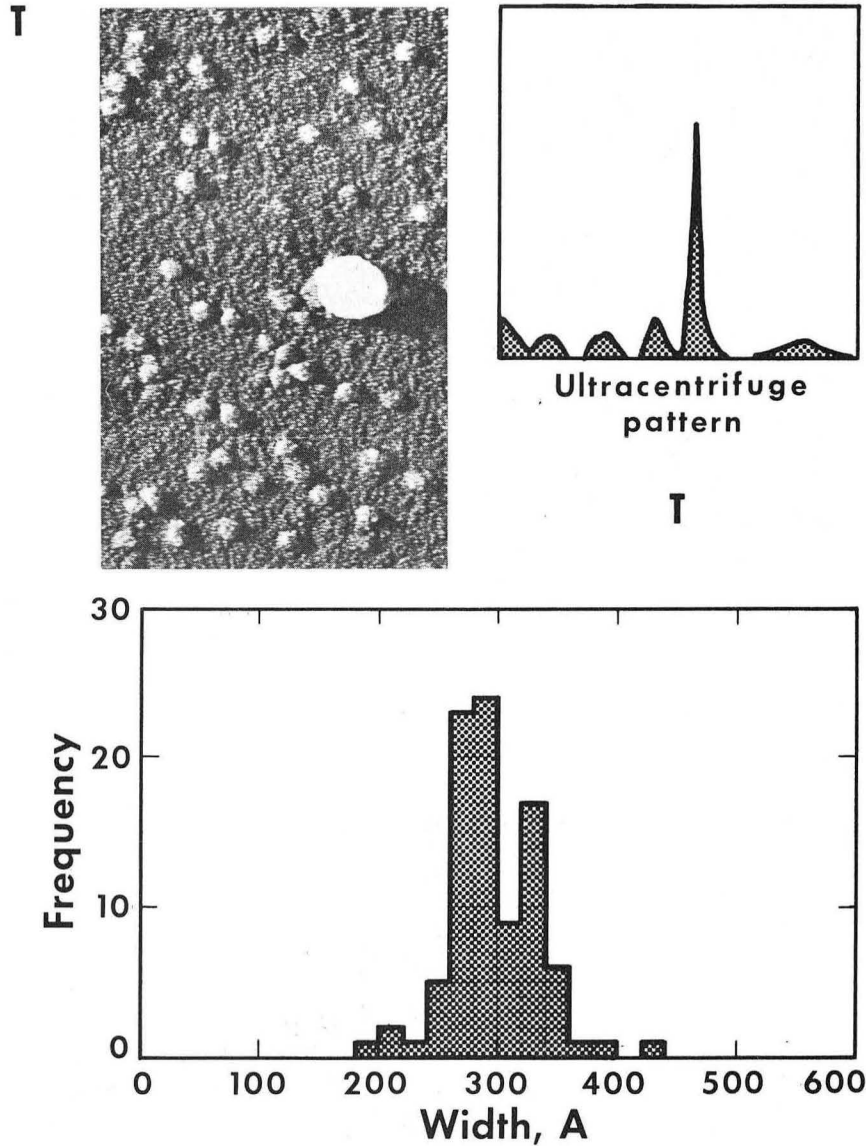   after 4 hr in growth medium and 10^5r x rays.

Fig. 31. Electron micrograph, ultracentrifuge pattern, and
          histogram of ribosome size distribution for yeast cells
          after 8 hr in growth medium.

MU — 23751

Fig. 32. Electron micrograph, ultracentrifuge pattern, and
          histogram of ribosome size distribution for yeast cells
          after 19 hr in growth medium.

Fig. 33. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
after 26 hr in growth medium.

Fig. 34. Electron micrograph, ultracentrifuge pattern, and
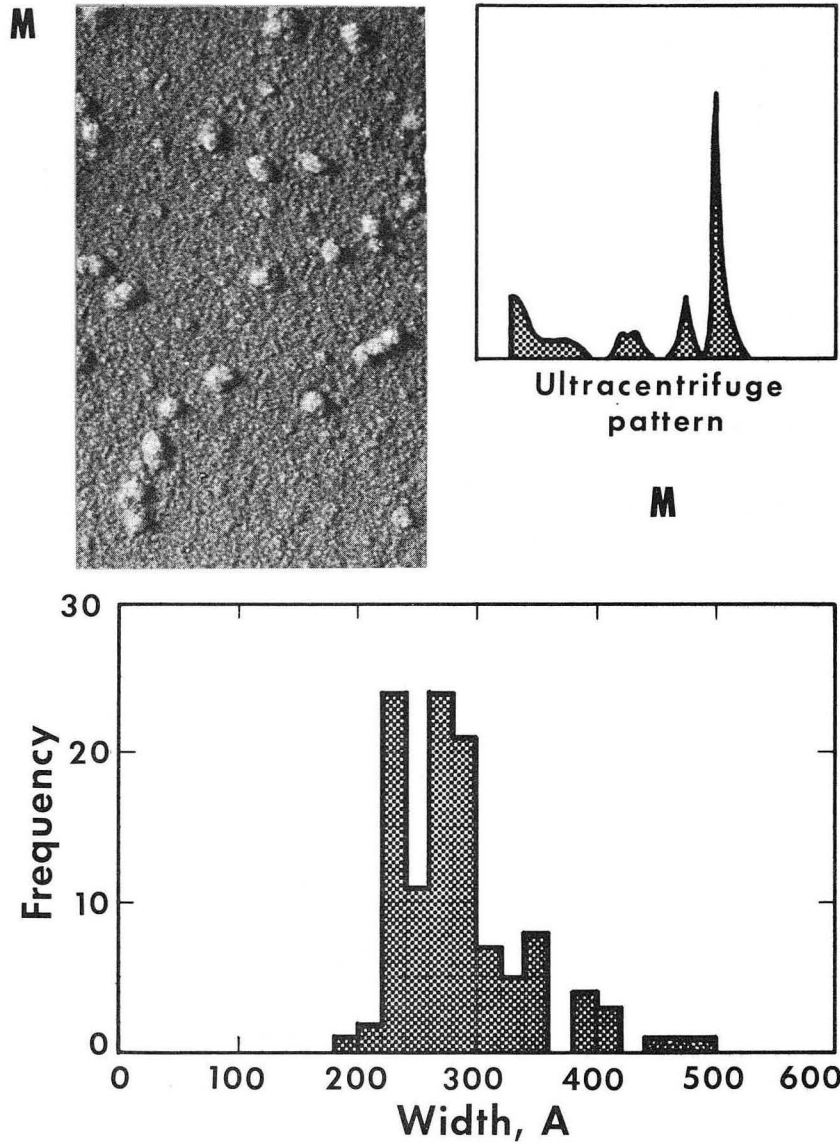histogram of ribosome size distribution for yeast cells
after 26 hr in growth medium and 10^5r x rays.
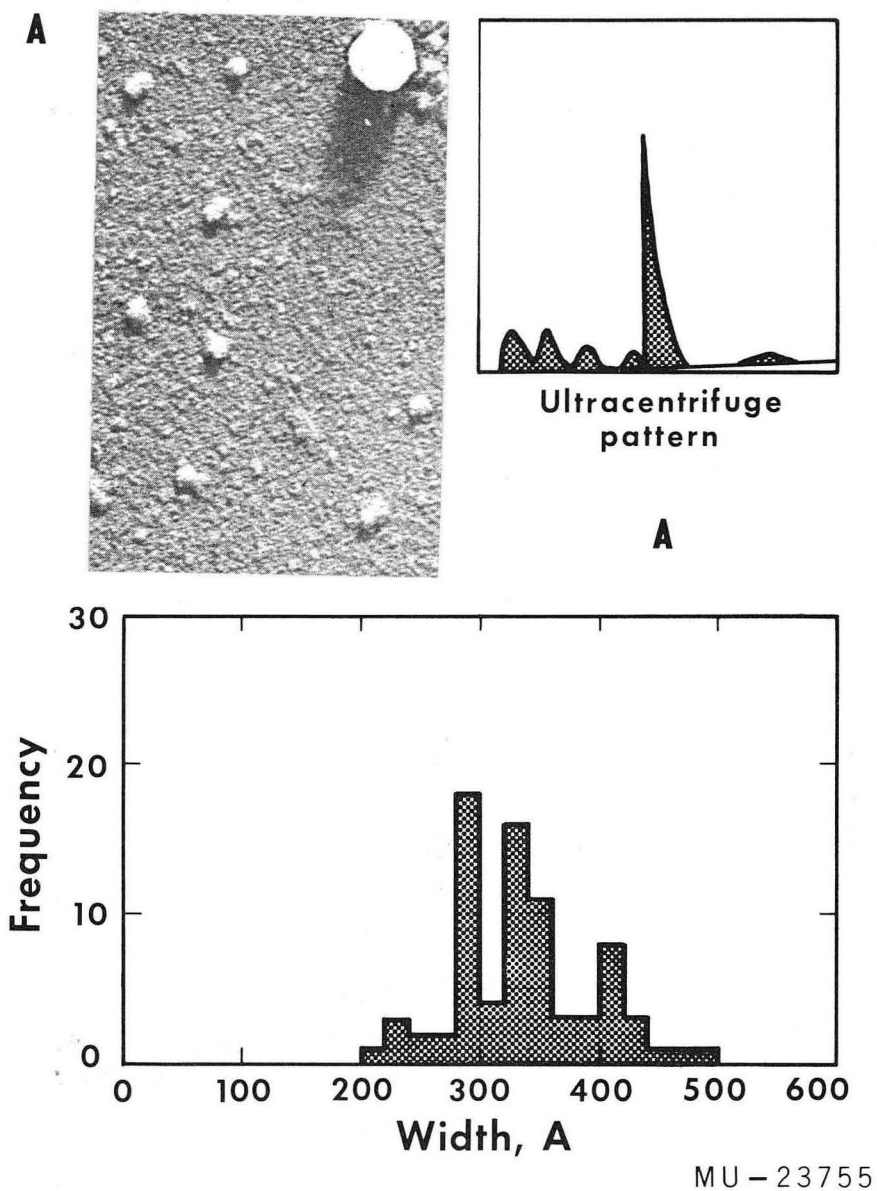
Fig. 35. Electron micrograph, ultracentrifuge pattern, and
          histogram of ribosome size distribution for yeast cells
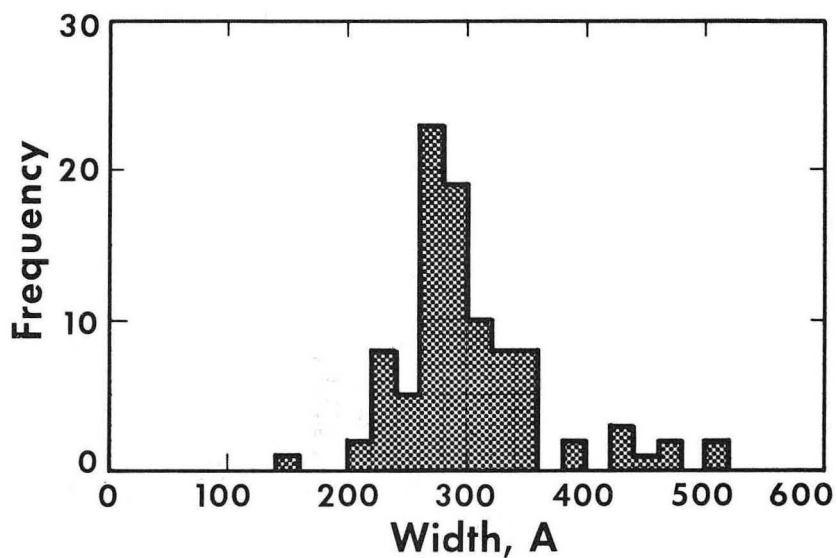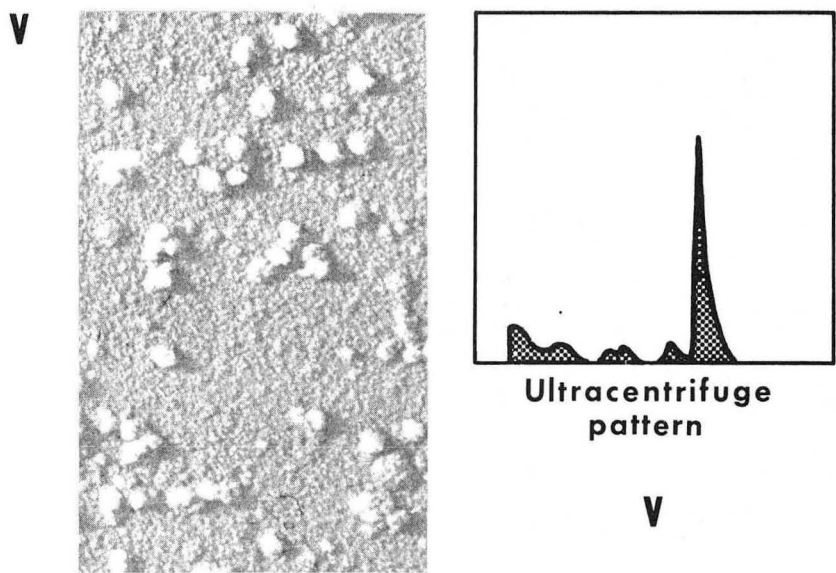   after 48 hr in growth medium.

Fig. 36. Electron micrograph, ultracentrifuge pattern, and
histogram of ribosome size distribution for yeast cells
after 48 hr in growth medium and 10^5r of x rays.

MU – 23758

Fig. 37. Electron micrograph, ultracentrifuge pattern, and histogram of ribosome size distribution for yeast cells after 48 hr in growth medium and 2×10^5r of x rays.
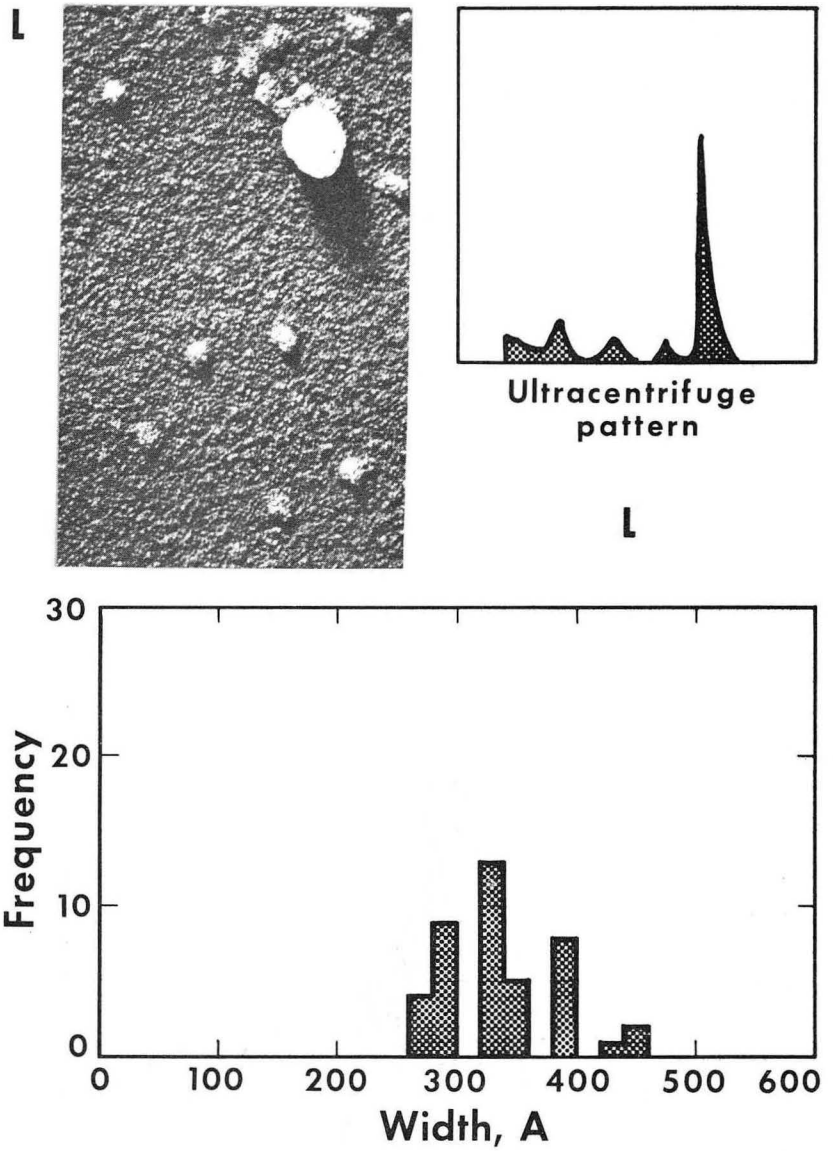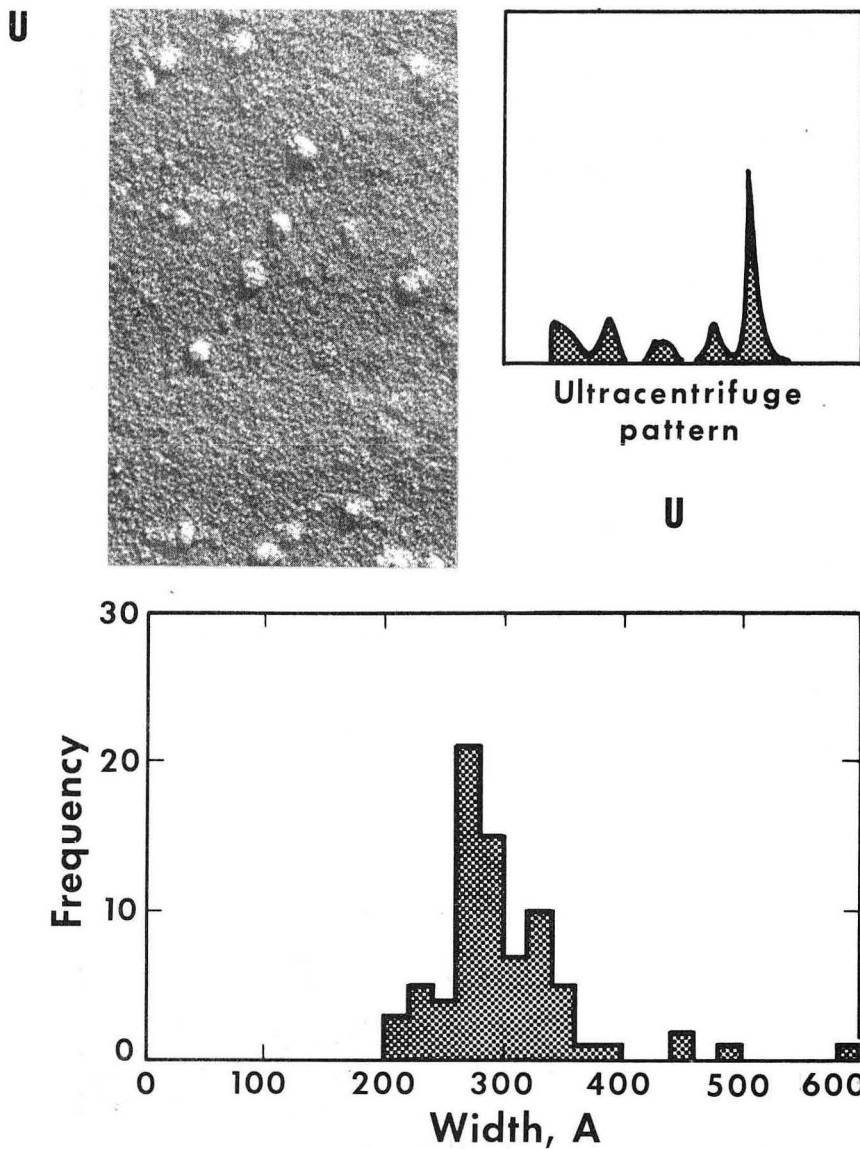
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Fig. 38. Electron micrograph, ultracentrifuge pattern, and histogram of ribosome size distribution for yeast cells after 48 hr in growth medium and $3x10^5r$ of x rays.
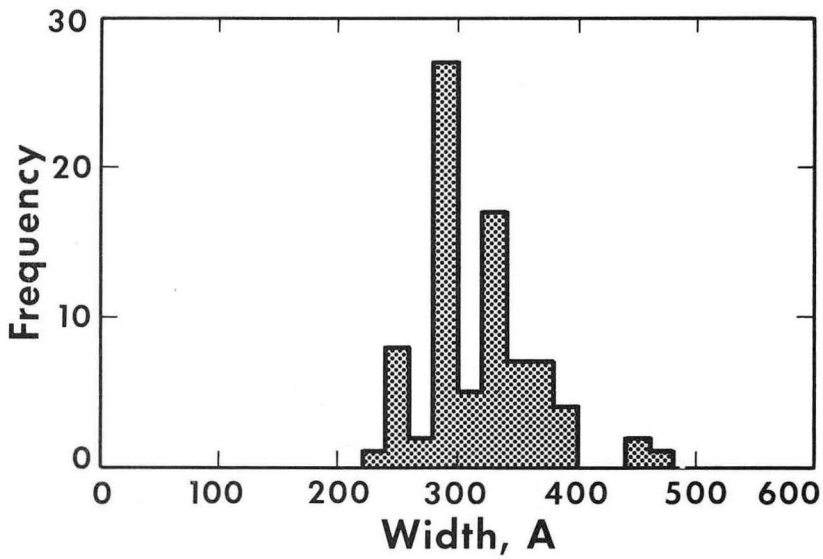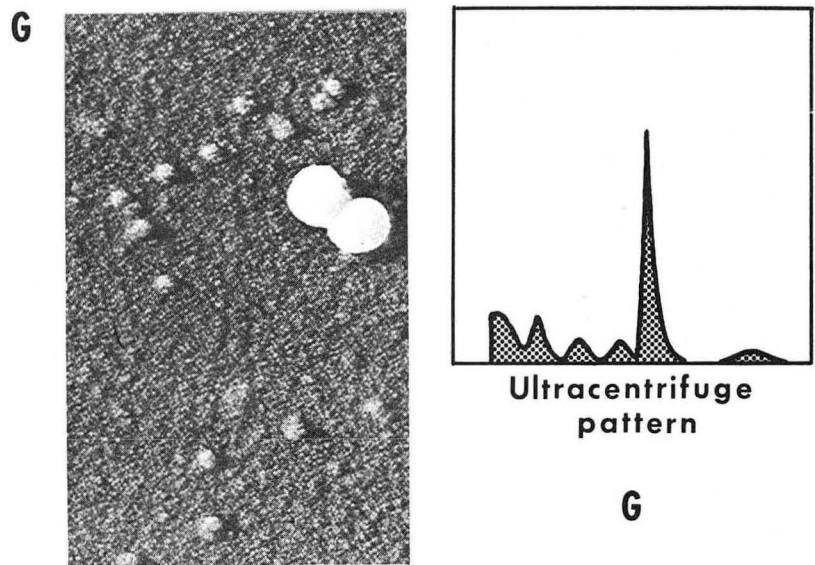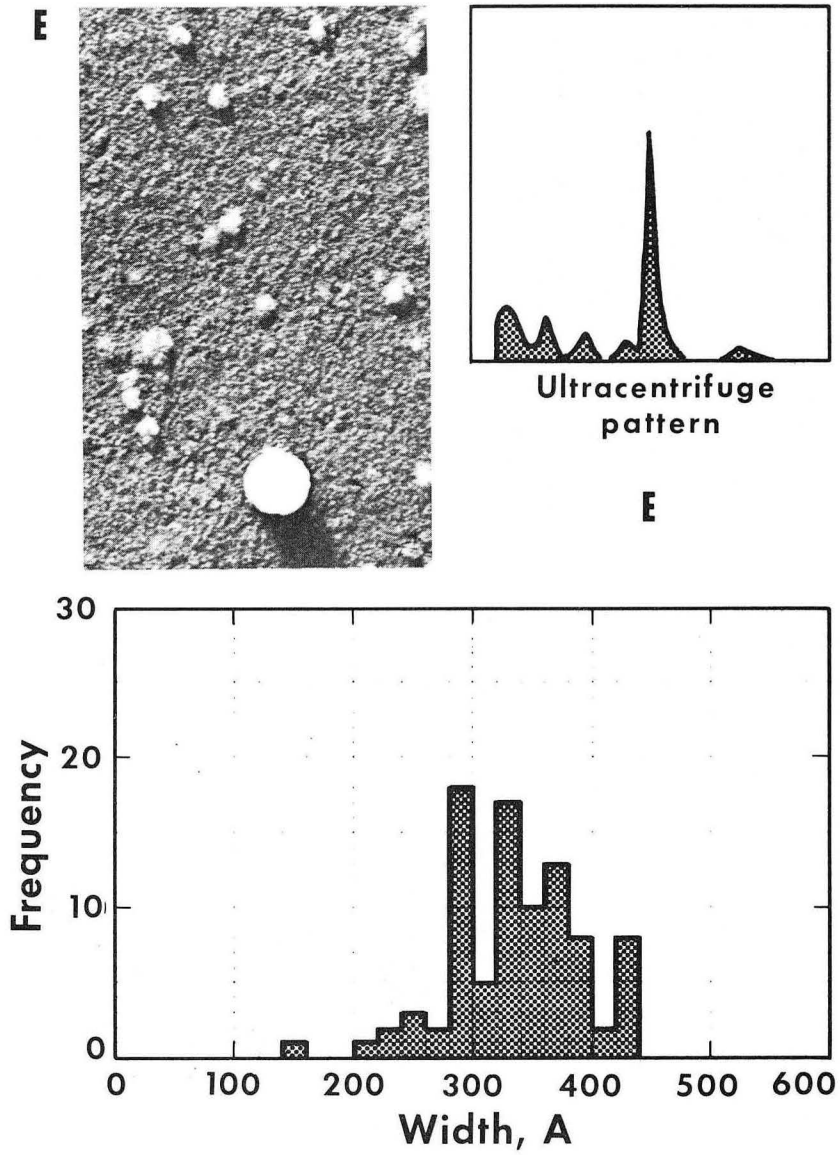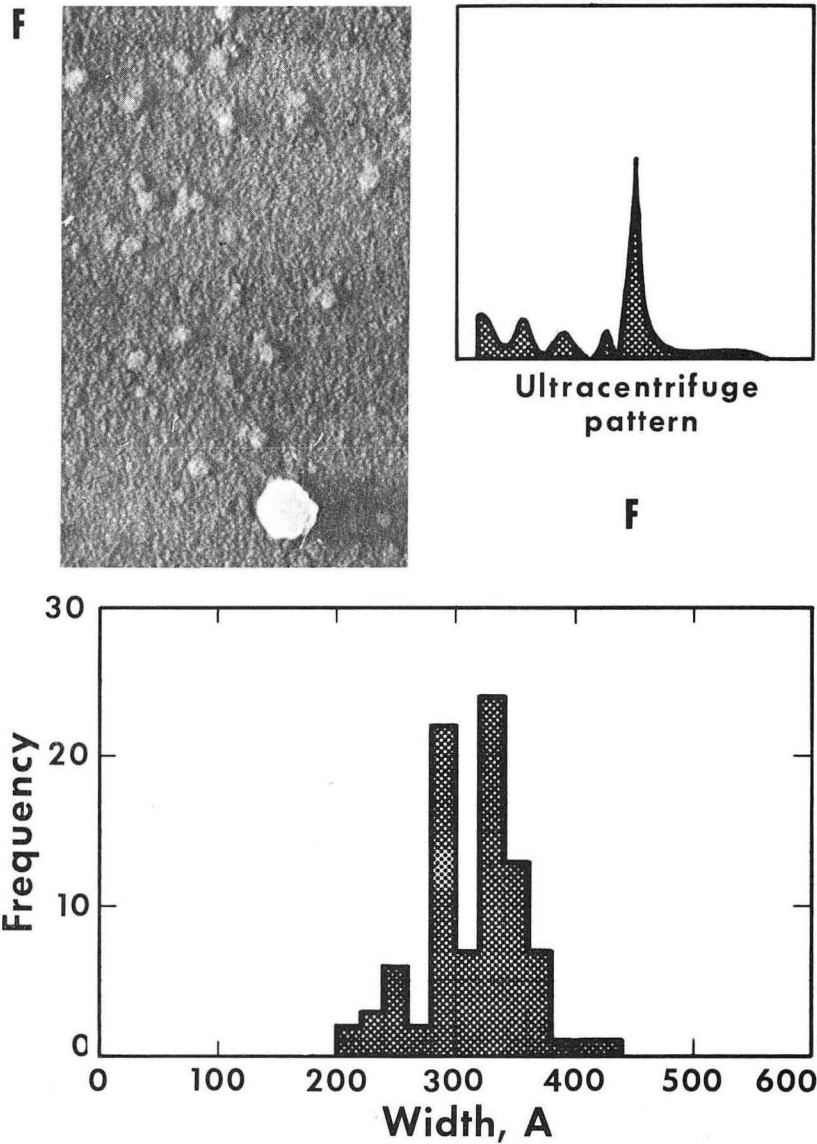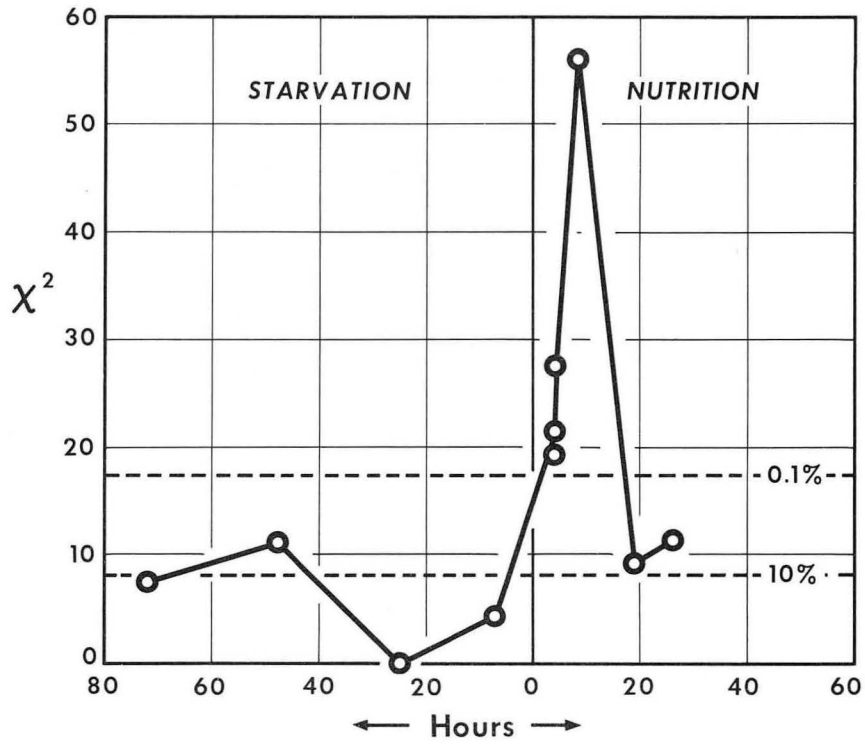
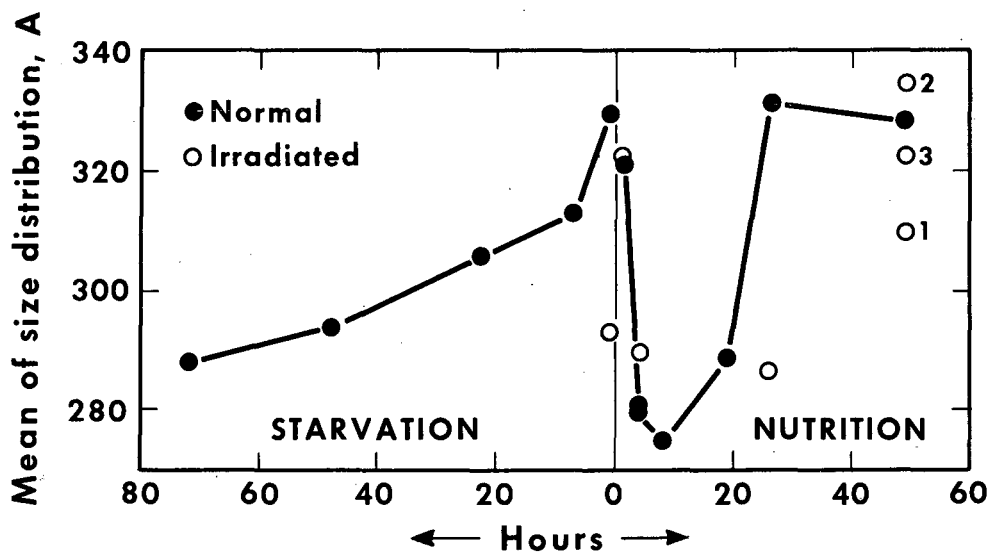Fig. 39. χ^2 plot showing the variation between histograms of size distribution of isolated yeast ribosomes.

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Fig. 40.   Mean of size distribution of isolated yeast ribosomes
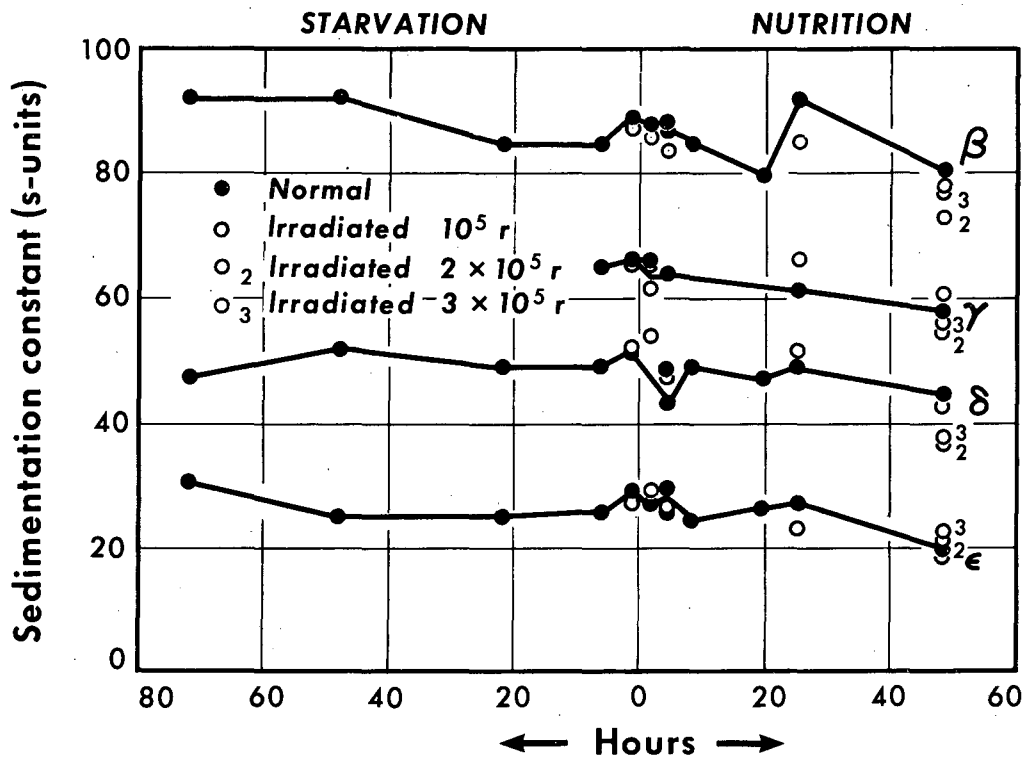   as a function of physiological state.

Sedimentation constants and relative percent abundances were calculated for the various components, and it has been attempted to relate that parameters to the physiological state of the cell from which the ribosomes were isolated. Figure 41 gives the sedimentation-constant data for the ribosomes. The various ribosome preparations ranged in total concentrations from about 0.5 to 1.5 g per 100 ml, as determined by oven-drying overnight and weighing. An approximate correction of the sedimentation rate to zero concentration was made by plotting the separate component concentrations against sedimentation rate and drawing the best straight line to zero. The assumption of linearity is made somewhat more reasonable by Chao and Schachman's[37] finding of very precise linearity for the concentration dependence of the 80S component in E. coli. The average concentration dependence correction was found to increase the observed sedimentation rate by about 10%. The standard correction of the sedimentation rate to the viscosity of water at 20°C was also applied, as the centrifugal determinations were carried out at 26°C. This correction decreased the observed data by about 10% and tended to counteract the concentration correction. All references to S constant will refer to these corrected data, except where rounded constants are used merely as a convenient nomenclature for the various components.

Although there are apparent fluctuations of S with the nutritional state of the cells, almost all the data can be fitted by straight lines having a slight negative slope ranging from 0.03 to 0.10 S/hr. This would suggest that ribosomes isolated from starving cells are larger or denser than their counterparts isolated from cells suspended in nutrient.

The open circles represent ribosome preparations irradiated with 100,000 r of x rays; they seem to be little affected in terms of sedimentation behavior.

Figure 42 shows the relative percent of the various components as a function of cell nutrition. Note that the 80S particles are always the majority component; however, during starvation these ribosomes represent 85 to 90% of the total, whereas during growth they average about 70% of the total. This finding is consistent with the conclusion drawn from the mean size data (Fig. 40) concerning the drop in mean ribosome size during logarithmic growth. Concomitant with this decrease in 80S particles is the appearance of the gamma or 65S ribosome. During early growth phase these particles represent about 20% of the total and decay to just a few percent within 20 hr in growth medium. The other components also change throughout growth and starvation but not so dramatically.

The search for a fairly immediate radiation effect on these particles was not very fruitful either in a change of sedimentation constant or in relative abundance. Figure 43 shows a typical experiment in which doses of 1×10^5, 2×10^5, and 3×10^5 r were delivered to freshly isolated aliquots of ribosomes. The irradiated particles were analyzed immediately in the ultracentrifuge and it can be seen that they exhibit essentially control values. A qualitative effect was found at very high doses—a broadening of the 80 and 120S peaks. This effect is shown in Figs. 25 and 38, and possibly represents a degradation of the largest particles into smaller constituents.

Fig. 41.   Variation of sedimentation constant of the yeast ribosome
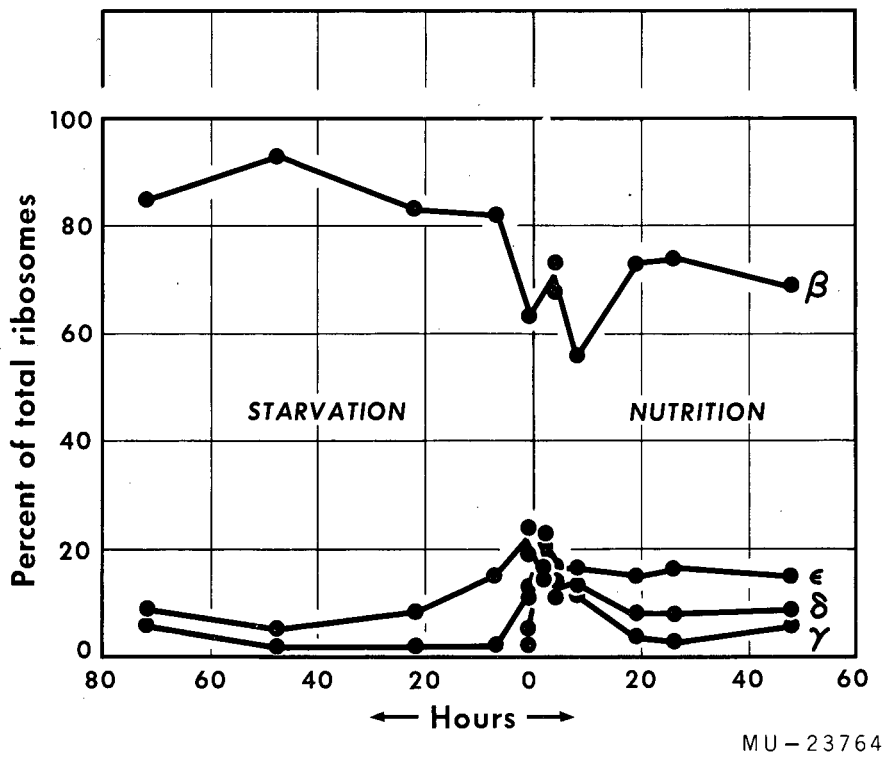   size classes with physiological state.

Fig. 42. Relative precentage of the various ribosome size classes as a function of physiological state.

V. DISCUSSION AND CONCLUSIONS

Normal Yeast Fine Structure: Growing Cells

Cells observed throughout the growth cycle have a dense particulate cytoplasm. A single plasma membrane is present adjacent to the cell wall and sometimes shows shallow invaginations into the cytoplasm. Often a double membrane closely associated with the plasma membrane can also be observed. Structure similar to the internal membrane has been noted in bacteria by Glauert et al.[54] They interpret this structure as part of a multiplamellar plasma membrane. This interpretation cannot be unreservedly applied to the yeast cell, as the internal membrane is not always present and usually exhibits discontinuous and variable structure dependent on the physiological state of the cell. It is possible that the internal membrane is derived from the plasma membrane since occasional connections have been observed, and that it performs a synthetic or other physiological function in the cell. It is not unusual for the internal membrane to be intimately associated with mitochondria that are usually found at the cell periphery. Association of mitochondria with endoplasmic reticulum has been observed in a number of investigations; for example, in the pseudobranchial cells of teleosts by Copeland and Dalton.[55] In cases in which the internal membrane could be carefully visualized, it appeared to have a smooth character without ribosomal particle attachments.

Yeast seems to possess very little endoplasmic reticulum in the growing state, or for that matter, in any of the physiological states investigated. Some membranes exist in the cytoplasm that probably fall into this category, and occasionally membrane attachments to the nuclear membrane and mitochondria are observed.

Mitochondria are sometimes discernible in growing cells but tend to be ill defined and difficult to distinguish from the cytoplasmic matrix, because of their rather loose structure and the fact that they have few cristae. This may help to explain the results of Bautz and Marquardt,[21] who in a quantitative light microscope study found fewer mitochondria in budding cells. The view of Agar and Douglas[26] that the cristae of yeast mitochondria are oriented parallel to the long axis has not been substantiated by this research. Yotsuyanagi's[22] light microscope work indicating a great dependence of mitochondrial form on cell nutrition seems to be borne out to the extent that growing cell mitochondria are generally small and poorly developed while those in starving cells are large and possess the usual mitochondrial form. Mundkur has been unable to demonstrate the existence of mitochondria in frozen dried preparations of yeast, viewed with the electron microscope.[35, 36]

The central vacuole of yeast seems to have a greatly changing appearance in cells of different ages and nutritional states. The vacuoles of budding cells are generally large oval structures with a single delineating membrane, and are relatively free of intravacuolar material. These observations are contrary to the suggestion of Mundkur, who considers $KMnO_4$ a poor fixative for yeast, as he contends that it does not preserve the central vacuole.
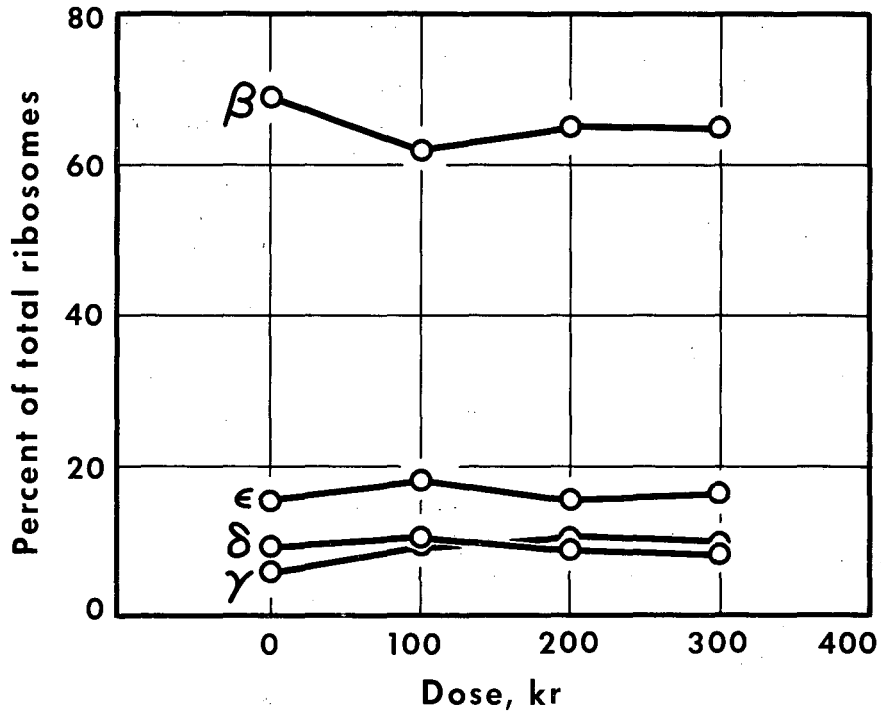
Fig. 43.   Relative percentage of the various ribosome size classes
           as a function of x ray dose.

Adjacent to the vacuolar membrane lies a rounded, sometimes gently
lobulated body considered to be the cell nucleus. As mentioned earlier,
there are no apparent evidences of nucleoli in these nuclei, although on a very
few occasions dark-staining intranuclear objects are seen that might, at least
on a morphological basis, fit this category. The nuclear membrane under
growth conditions has been found to be a complex structure composed of approxi-
mately three filamentous membranes. Evidence has been given earlier which
suggests that the nuclear membrane is produced by the coalescence of two
double membranes to produce a triple membrane, in which the middle com-
ponent is twice as thick as the outer components. Pores are occasionally
seen in this membrane but not as frequently as in nongrowing cells. Hashimoto
et al. claim to have found a double nuclear membrane in ascospore nuclei;[29]
however, their evidence is not very convincing in this respect. Agar and
Douglas also support the double nuclear membrane structure for yeast.[26]
Mundkur was unable to demonstrate a nuclear membrane in his frozen dried
material and, furthermore, suggested that $KMnO_4$-stained material showed
no density differences between nucleus and cytoplasm[35,36]—an obviously
erroneous statement in view of the many examples of such differences illustrated
earlier. The conclusion of Agar and Douglas[26] that the nuclear membrane is
retained throughout mitosis has been generally substantiated by this work; how-
ever, it should be pointed out that occasional cells have been observed in which
substantial portions of the nuclear membrane are missing, and hence a brief
mitotic phase in which the membrane has dissolved cannot be discounted.

Objects believed to be chromosomes occur within the nuclei of cells
both in budding and in resting stages. Configurations similar to those observed
in the anaphase movement of higher cell chromosomes are seen especially in
early budding cells. They appear to be analogous to other chromosomes in-
cluding the possession of a kinetochore, but they are toward the small extreme
on a scale of chromosome sizes. Approximations of their dimensions give a
thickness of 0.2 to 0.3μ and a length of about 0.5 to 1.0 μ. As used routinely
in this work, $KMnO_4$ generally produces chromosome images which appear
less dense than the surrounding nucleoplasm. Porter and Machado have ob-
served similar electron-transparent structures, known to correspond to
chromosomes, in onion root tip cells.[56] They found that the electron density
of the chromosomal material was related to the duration of permanganate
fixation. Short exposures produced electron-transparent chromosomes, and
prolonged fixation yielded dense chromosomes. This explanation is not entirely
satisfactory in yeast chromosome staining, because sometimes dense chromo-
somes are observed in yeast even though fixation times are identical with those
for specimens yielding transparent chromosomes. Of particular interest is
the occurrence of light and dark chromosome-like objects within the same
yeast nucleus. Recent work by Kaufmann et al. on heterochromatic regions
in drosophila chromosomes indicates that these regions appear as very dark-
staining in the electron microscope.[57] It is suggested that the dark areas
seen also in certain yeast nuclei might represent heterochromatin. The
possibility that some or all of the dark-staining material may represent the
nucleolus cannot be excluded, but the general morphology of these structures
is much more suggestive of chromosomes. Only Yotsuyanagi[34] and, more
recently, Hashimoto et al.[32] have been willing to acknowledge that intra-
nuclear bodies in their material might represent chromosomes. The study
reported here completely supports this hypothesis with very suggestive
illustrations.

Although centrioles in yeast have been described by DeLamater[10] and Mundkur[58] with light microscopy, and a rather tenuous description has been given by Bartholomew and Mittwer[24] utilizing electron microscopy of whole photolyzed cells, no such structure has yet been described in thin sections of yeast cells. Unfortunately, it has not been possible to determine the ultra-fine structure of the proposed yeast centrioles in this work to establish their exact homology with the mannalian centrioles investigated by deHarven and Bernhard.[59] However, the general structure, location, and behavior of these organelles are very suggestive of centrioles, and there is no reason to expect an exact similarity of organelle structure in such widely differing cellular types. The atypical mode of nuclear division in yeast may very well require a greatly modified centriole, in structure as well as function, compared with that of higher cells. The location of the proposed centrioles, within, or connected to, the vacuolar membrane could evoke considerable speculation on the role played by this structure in the mechanism of mitosis, but it would be pre-mature to add more unknown to an already unsubstantiated hypothesis. It might be mentioned, however, that this centriole locus is the only evidence even remotely connecting the vacuole to the mitotic process, and this may help to explain some of the peculiar controversies among earlier light micros-copists concerning the relative role of the vacuole or nucleus in cell division.

Normal Yeast Fine Structure: Starving Cells

The most general and constant morphological effect of nitrogen starva-tion of yeast is a gradual reduction of cytoplasmic density and granularity. This observation is in agreement with the classical uv studies by Caspersson and Brandt.[11] They showed that growing yeast cells exhibit a strong diffuse nucleic acid absorption throughout the cytoplasm, whereas starving cells have generally less absorbing material but possess highly absorbing particles near the vacuole that they considered to be volutin. It has been mentioned earlier that granular areas are usually present in starved cells, and it may be that the ribosomes clump together forming larger units during starvation, thus giving the impression of a lowered over-all opacity in the electron microscope. Unfortunately, the work on the ribosome fraction isolated from such cells does not show a significant effect of this type, hence the question remains unsettled.

The lessening of cytoplasmic background opacity, as viewed in the electron microscope, produces the secondary effect of improving the contrast and, therefore, the visibility of membranous cell constituents such as mito-chondria. From qualitative observations, it is also believed that the number of well-defined mitochondria actually increases in starving or stationary cells.

The effect of starvation on the ultrastructure of the nuclear membrane is also quite striking. Whereas growing cells for the most part exhibit the complex, apparently triple, nuclear membrane discussed earlier, starved cells usually possess a clearly "classical" double membraned nuclear envelope. Many pores, containing no diaphragm-like internal structures, are also present in these membranes. Both membrane and pore structure are very similar to those of onion root tip cells investigated by Marinos.[60] The cause of this dramatic difference between starved and proliferating cells is not known.

Starvation often produces a fragmentation or condensation of the central vacuole. Particular material often fills the vacuolar space, and it is possible that these events are the result of a physiological response to the starved environment. This observation is in agreement with the work of Brandt, who showed a similar change of vacuole structure, utilizing living yeast cells and the uv microscope.[61] It must, however, be reiterated that the vacuole is prone to artifactual displays and that its structural alterations should be treated with caution.

Fine Structure of Irradiation Cells

The first (in time) postirradiation response noted in the electron microscope is the production of cell doublets, one of which is generally deformed and elongated. Usually only one of these doublets possesses a nucleus. This finding is in complete agreement with the work of Mortimer, who described such doublets after x irradiation.[62] By Feulgen staining he observed only one nucleus in each of the doublet pairs. Mortimer attributes the effect to an inhibition of nuclear division, with the essentially nongenetic, cytoplasmic process of budding left unaffected. This may well be the case; however, electron microscope specimens have been observed that possess enlarged, and occasionally divided or partially divided, nuclei. This suggests that the principal radiation effect producing such doublet cells may be in the transport system that conveys the daughter nucleus into the bud. The strong possibility of centriole involvement in the transport mechanism, and observations on the radiation damage to this organelle described by Rustad[63] make it entirely possible to account for the production of singly nucleated doublets without invoking mitotic inhibition.

There have been no previous investigations on the ultrastructual alterations induced in yeast by ionizing radiation; indeed, only a few studies of this nature have been carried out with any cell type.

Nebel has studied mouse reproductive cells after irradiation, and found intranuclear structures, presumed to be meiotic chromosomes, to be more plainly visible than in unirradiated controls.[64] This effect has been qualitatively found in yeast cells, as it has been stated that irradiated cells are less dense and tend to look more like starved cells. Nebel ascribes this phenomenon to an autolytic process initiated by radiation. The recent work of Pollard and Dresden[65] on the radiation-induced release of RNAase from E. coli ribosomes, and the research of Roth and Eichel[66] on a similar effect in irradiated mammalian spleen might provide the required biochemical basis for Nebel's suggestion.

McQuade and Evans[67] investigated the effects of autolysis, ligation, and I^{131} irradiation on the thyroid with the electron microscope, and found similar degenerative changes for all three cell insults. These were a swelling of the endoplasmic reticulum, mitochondrial abnormalities, together with an effect on the nuclear envelope.[68] Because yeast does not possess a well-defined endoplasmic reticulum, no effect on this entity was noticed; however, mitochondrial and nuclear membrane changes were observed, and are in most respects similar

to the above investigations. Signs of mitochondrial degeneration, such as
very dense or heavily membraned forms were observed. Various aberrant
species, such as concentric ring structures, were seen in irradiated yeast;
these also may have been of mitochondrial origin. Nuclear membrane thick-
ening was also mentioned as one of the earlier effects. Here we are no doubt
dealing with secondary degenerative physiological effects that would be ex-
pected to cause similar morphological alterations in many different cell types
and with many toxic agents, including radiation. Tahmisian has observed, in
the electron microscope, profound radiation-induced changes in the develop-
ment and differentiation of grasshopper spermatids, including retardation of
nebenkern formation, production of supernumerary centrioles, flagellar
filaments, and acrosomes.[69,70] These results are interesting and in some
respects parallel the observations on yeast, although they represent a some-
what different category of radiation effect from the rather immediate structural
damage looked for in this work.

With respect to degenerative changes in vacuole structure and the
formation of pinocytosis vacuoles, it is interesting to note that the induction
of pinocytosis in amoeba by uv irradiation has been observed by Rinaldi.[71]
It is possible that in irradiated cells as well as in starved cells the vacuole
is called upon to play a physiological role that manifests itself in structural
changes. The dynamic nature of the vacuole has been described in detailed
light microscope work by Ganesan,[6] who found that the vacuole undergoes
fragmentation into smaller vesicles by the build-up of cytoplasmic filaments
throughout its volume.

Cell enlargement is a very general and universal reaction to radiation.
Brace has shown by indirect specific-gravity and protein-nitrogen measure-
ments that irradiated yeast cells increase in volume two to three times over
unirradiated controls, and concluded that this increase represented general
cytoplasmic growth rather than mere water uptake.[72] It is not surprising
that little or no enlargement of yeast cells were noticed in the thin sections
viewed with the electron microscope. The radius of these cells, approxi-
mated as spherical, would rise only as the cube root of the volume; hence,
even a threefold volume increase would result in a radius increase of only
about 1.4. In view of the heterogeneous size of a random population of cells,
this factor would not be recognized unless very extensive measurements
were made.

The bizarre ameboid forms observed in yeast several days postirradi-
ation might be explained by the collapse of giant water-impregnated cells
during the dehydration steps in preparing the cells for electron microscopy.
Just as likely, in view of Brace's work, is a radiation effect on the structural
integrity of the cell wall, causing its deformation.

Ribosome Studies

The earlier studies by Wolfe[40] and Ashikawa,[41] indicating that the
hydrodynamic properties of yeast ribosomes are dependent on the physiological
state of the cell of origin, have been generally supported by this research.
Both the sedimentation constants and relative quantity of the various components
change in characteristic ways throughout growth and starvation. Histograms
of ribosome size measured from electron microscope observations also change
in a similar fashion. The most striking difference between starved and growing
cells is the rapid appearance of a 65S ribosome component at the onset of
growth, whereas this particle is completely absent in starving yeast cells.
Peterman has very recently reported the occurrence of a 65S ribosome in a
mammalian cancer cell strain.[73]

Although the general consensus favors the theory that ribosomes
represent the synthetic site for cellular proteins, several dissenting claims
have recently been voiced. Osawa and Hotta, in turnover studies utilizing
radioactive amino acids, suggest that in growing yeast cells "the RNA particles
do not represent precursors for soluble proteins."[74] Similar views have been
expressed for the E. coli system by Roberts et al.,[75] although a very recent
report by McCarthy and Aronson,[76] working in Robert's group, suggests that
small RNA (4 to 8S) builds up 20, 30, and 50S ribosomes, which in turn
produce 70S particles acknowledged to be active in protein synthesis. Very
recent work of Tissieres et al. indicates that the incorporation of labeled
amino acids in E. coli is not uniform over the various ribosome size classes.[77]
A special kind of 70S ribosome called "active 70S" was shown to incorporate
15 to 40 times as much amino acids as 30 or 50S particles, and constitute only
10 percent of the total ribosome complement. It is possible that the 65S yeast
ribosome plays a similar role and might help to explain its rapid appearance
during the early growth stage.

Huxley and Zubay, working with E. coli ribosomes, have shown by the
use of positive uranyl and negative phosphotungstic staining techniques that
the 100S particles constitute two 70S particles in dimeric form.[45] The 70S
units in turn contain two subunits, probably representing 50 and 30S particles.
It was further shown that 50S units could combine under the proper conditions
to form 80S particles. It is possible that the predominant 80S units of yeast
are of this structure, and that the 65S units are actually the 70S units of
Huxley and Zubay, the difference being of the order of magnitude of the errors
involved in the ultracentrifugal determination. That there may be significant
difference between E. coli and yeast ribosomes is substantiated by the ob-
servation of a 120S component in yeast, rather than 100S. This may represent
the dimerization of 80S rather than 70S particles. Hall and Slayter,[44] using
the shadowing technique, have reached conclusions similar to those of Huxley
and Zubay on the structure of E. coli ribosomes. Figure 44 gives the com-
parative structures and approximate sedimentation rates for E. coli and
yeast ribosomes, as they are presently believed to exist. It must be stated
that no definitive evidence has been obtained favoring the existence of the
quadrupole 50S particle representing the 120S ribosome.

E. COLI

S constant Structure

100

70

50

30

YEAST

S constant Structure

120

80

65

50

30
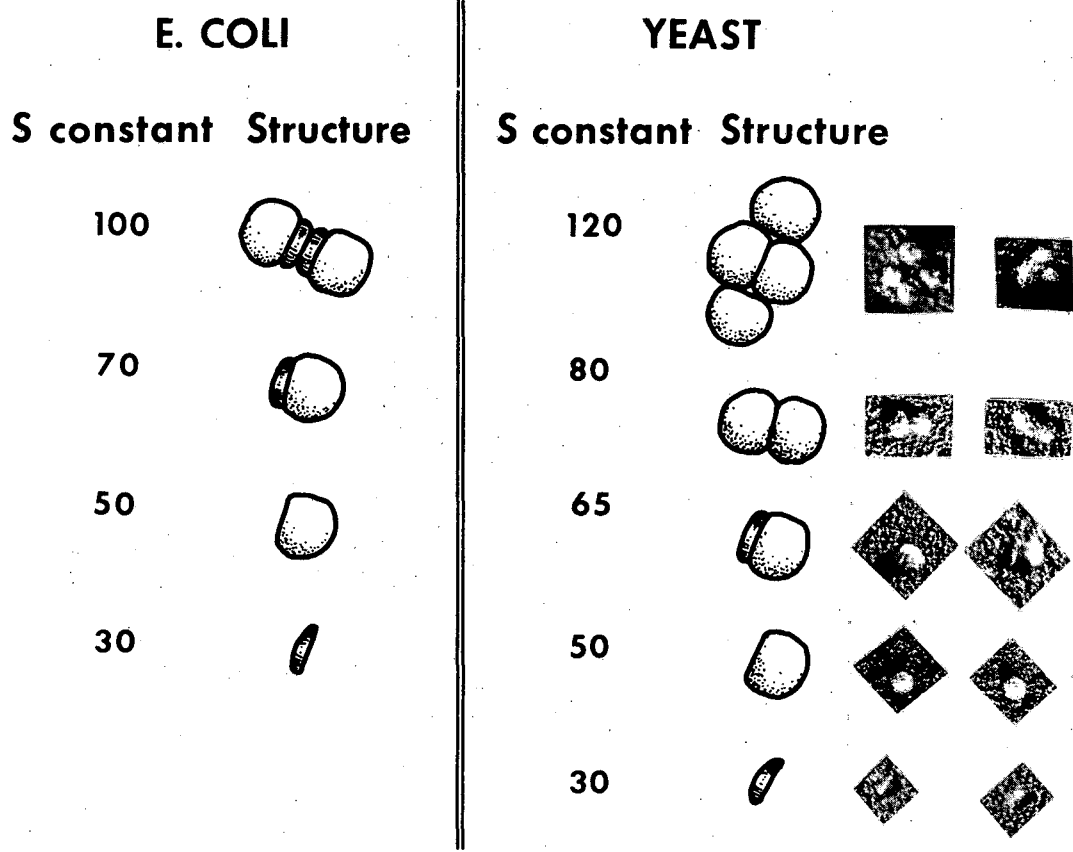
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Fig. 44. Hypothetical structures of E. coli and yeast ribosomes
adapted from this and other recent work showing also electron
micrographs of typical representative particles.

Radiation Experiments on Ribosomes

In view of the report of Pollard[47] that phosphate incorporation into ribosomes of E. coli is drastically reduced after irradiation, it was felt that a structural alteration in the yeast ribosomes might be found as a fairly immediate postirradiation effect. Only the slightest indication of such an effect was found, however, in the broadening of 80 and 120S peaks after heavy doses. Billen and Volkin found rather similar results with E. coli after 99% inactivating doses, and noted a decrease in ribosome components only after prolonged incubation of the cells in growth medium.[46] Billen in later work showed that doses of 60,000 r to E. coli caused the loss of nucleic acid fragments, the effect being inhibited by the absence of an exogenous metabolite.[78] This observation, together with the possibility of radiation-induced intracellular RNAase release,[65] may help explain the decrease in electron density noted in yeast soon after irradiation. It must be concluded that radiation has little or no effect on the structural parameters investigated, and that biochemical changes which have been noted apparently take place on a rather subtle molecular level.

SUMMARY

Cells of the baker's yeast (S. cerevisiae) have been studied in the electron microscope with the intention of elucidating the fine structure of growing, starving, and irradiated cells. The more important observations and conclusions of this study are:

1. The cytoplasm of growing cells is dense and quite uniform, with few membranous regions or mitochondria. Starvation decreases this cytoplasmic density and enhances the visualization of cytomembranes and mitochondria.

2. The nucleus of growing cells often contains objects believed to be chromosomes, and is generally surrounded by a complex membrane containing occasional pores.

3. A body residing in the vacuole adjacent to the nucleus has been described as a possible candidate for the yeast centriole. No structure corresponding to a mitotic spindle has been observed, and during budding the daughter nuclear allotment is apparently conveyed to the bud by a process involving the intact mother-daughter nucleus.

4. Nitrogen starvation decreases the cytoplasmic density and appears to affect the structure of the nuclear membrane. The central vacuole is altered from the clear oval form in growing cells to a fragmented structure containing granular inclusions.

5. Radiation induces structural changes of a subtle nature on the nuclear membrane, mitochondria, and central vacuole that in over-all perspective resemble the alterations obtained by starving cells. Late effects include cell lysis or explosion, and the production of ameboid cell forms.

In order to obtain more insight into the architecture of yeast ribosomes and their possible roles in the structural alterations induced by starvation and irradiation, a series of experiments was conducted on isolated ribosomes

from a variety of yeast nutritional states. These ribosomes were examined with the electron microscope and analytical ultracentrifuge. The primary results of these investigations are:

1. There are five yeast ribosome size classes distinguishable with the analytical ultracentrifuge. These groups are designated as 120S, 80S, 65S, 50S, and 30S. They vary in relative abundance, and slightly in apparent sedimentation rate, with the physiological state of the cell.

2. The 65S component appears only during the early growth phase of yeast cells and is not present in starved cultures. The appearance of these particles is reflected in histograms of ribosome sizes determined at various times during growth and starvation. The hypothesis is advanced that these particles possibly correspond to the "active" protein synthesizer of the ribosome spectrum.

3. The structure of these various particles conforms to recent results by other workers on E. coli, and suggests that the 120S particles are dimers of two 80S particles that in turn are composed of two 50S particles. The 65S particle possibly conforms to the 70S particle of E. coli, and is composed of a 50S and a 30S particle.

4. Even at very large doses, x irradiation does not appreciably effect the sedimentation constant or the relative abundance of the various ribosome size classes. A qualitative change in the Schlieren pattern of heavily irradiated ribosomes was noted, but it must be concluded that the immediate effect of radiation on the ribosome parameters studied was not severe.

ACKNOWLEDGMENTS

ABBREVIATIONS

Am - Ameboid Cell

B   - Bleb

C   - Cytoplasm

Ce - Centriole

Ch - Chromosome

DB - Dense Body

IM - Internal Membrane

K   - Kinetochore

L   - Loop

Ly - Lysed Cell

M   - Mitochondrion

Me - Membrane

MB - Membranous Body

N   - Nucleus

NB - Nuclear Bridge

P   - Pore

PB - Particulate Body

Pi - Pinocytotic Vacuole

PM - Plasma Membrane

SG - Storage Granule

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