JOURNAL OF BACTERIOLOGY, Aug. 1974, p. 564-575
Vol. 119, No. 2

Effect of Polyoxin D on Chitin Synthesis and Septum Formation in *Saccharomyces cerevisiae*

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Received for publication 9 May 1974

The normal sequence of cell separation in *Saccharomyces cerevisiae* begins with the formation of a primary septum, presumably consisting of chitin, on which secondary septa are later deposited. In the presence of the antibiotic polyoxin D, a potent inhibitor of chitin synthetase, pairs of abnormal cells of two different types were observed by phase-contrast microscopy: the "exploded pair," consisting of two lysed cells from which the cytoplasm had been extruded at the cell junction, and the "refringent pair," consisting of two highly refractile cells joined by a thin bridge. Thus, in both cases the septal region appears to be affected. Observations with the electron microscope showed that the primary chitin septum was not formed in either of these cell types, and as a consequence secondary septa of varying thicknesses were laid down in an abnormal pattern. With [^{3}H]glucose as carbon source the incorporation of tritium into the chitin of abnormal cells was inhibited about 90%, whereas the labeling of mannan was normal and that of glucan somewhat reduced. The effective concentrations of polyoxin D (0.1 to 1 mg/ml) were much greater than those required to inhibit chitin synthesis in vitro. Dimethylsulfoxide and amphotericin B, both known to increase cell permeability, enhanced the action of the antibiotic.

It has been proposed (7) that chitin forms the primary septum in budding yeast, on the basis of the localization of this polysaccharide in the bud scar (5) and of its periodic synthesis during the cell cycle (6). To verify this hypothesis it was desirable to inhibit specifically the synthesis of chitin in vivo and to observe the consequences of the inhibition on the process of cell division. The polyoxins, a group of peptidylpyrimidine antibiotics (21), are powerful competitive inhibitors of chitin synthetases obtained from different organisms, including yeasts (13). In vivo effects have been observed on the cell wall of some fungi (3, 10), but yeast cells have been hitherto refractory to these antibiotics (12, 13). A possible explanation for these results was afforded by the finding (17) that peptides, usually present in growth media, inhibit polyoxin action. Recently, using a peptide-free synthetic medium and high concentrations of polyoxin D, we have observed striking effects of the antibiotic on cell division which are the subject of the present study.

MATERIALS AND METHODS

Culture conditions. *Saccharomyces cerevisiae* X2180 (ATCC 26109) was grown at 30 C in an incubator shaker in a minimal medium containing 0.7% yeast nitrogen base (Difco) and 2% glucose. The inoculum (80 ml) was started from an agar slant in the morning; approximately 8 h later, cells were transferred into 800 ml of medium in an amount estimated to produce, on the following morning, an absorbance of 0.2 at 660 nm in the Coleman Junior Spectrophotometer, using 1-cm diameter cells. The overall growth corresponded to about 12 cell divisions. Portions of this culture were diluted into fresh medium and used for the experiments. This procedure is critical, since cells which have undergone a larger number of generations become more resistant to polyoxin (see below).

Chemicals. Polyoxin D was a generous gift of H. Saito of the Kaken Chemical Company, Tokyo, Japan. D-[6-^{3}H]glucose, specific activity 8.45 mCi/μmol, and Aquasol were purchased from New England Nuclear. Renografin (Reno-M-76) was obtained from E. R. Squibb & Sons, Inc., New York, and polyvinyl pyrrolidone (PVP 360) was purchased from Sigma Chemical Co. Glucanase and chitinase were purified as described previously (5). Yeast mannan was prepared according to Cifonelli and Smith (8), and yeast glucan was prepared as already reported (5).

Observation of polyoxin effects by phase-contrast microscopy. Suspensions of 7 to 8 $\times$ 10^6 cells/ml were incubated in minimal medium at 37 C with shaking in the presence of a range of concentrations of polyoxin D. This temperature was chosen because the

effect of the antibiotic was more marked at 37 than at 30 C. At intervals over a period of four generations the absorbance at 660 nm was measured, and samples were monitored in the phase-contrast microscope under oil immersion. Several fields were scored for total and for abnormal cells, and the percentage of the latter was recorded. About 300 cells were counted each time.

Separation of normal and abnormal cells. For observations in the electron microscope and for studies of incorporation of radioactivity it was necessary to separate normal from abnormal cells. This was achieved by using a linear Renografin gradient of the type described by Hartwell (11). The limiting concentrations of Renografin were 25 and 40% (wt/vol), and the gradient contained 1% polyvinyl pyrrolidone (PVP 360). After centrifugation for 30 min at 12,000 rpm in the Sorvall HB-4 rotor, the normal cells were dispersed throughout the gradient, whereas the abnormal cells were found in the pellet.

Electron microscopy. Cells were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.3, for 1 h at room temperature, rinsed in the same buffer, pelleted, and enrobed in agar. Small blocks cut from the agar-enrobed pellet were post-fixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature. Dehydration in a graded series of ethanol solutions was followed by Epon embedding (14). The sections were stained with uranyl acetate and lead citrate.

Labeling of cells with tritiated glucose. The incubation mixture and conditions were the same as for the observation of polyoxin effects. A control culture and one containing 1 mg of polyoxin D per ml were processed simultaneously. Absorbance and percentage of abnormal cells were determined as above. To introduce the radioactive label at the time of maximal production of abnormal cells, growth was allowed to proceed in the presence of polyoxin D for about two generations; when the absorbance at 660 nm reached a value of 0.1 (about 3×10^7 cells/ml), 10 μliters of [^{3}H]glucose (2×10^7 counts/min) were added per ml of culture to both control and polyoxin vessels, and incubation was continued. After one additional division cycle, growth was stopped, and the percentage of abnormal cells was measured. The abnormal cells formed during the labeling period comprised 29% of the total number of abnormal cells present at the end of growth. This factor was used to correct for the "exploded" and "refringent" cells (see below) already present at the time of addition of radioactive glucose on the assumption that those abnormal cells would be unable to incorporate label.

Both cultures were centrifuged, the pellets were washed several times with water, and the polyoxin-treated cells were submitted to gradient fractionation as described above. The abnormal cells were recovered in the gradient pellet and washed again with water. Control and abnormal cells were counted in a hemocytometer.

Fractionation of cell wall polysaccharides. The general scheme of fractionation is shown in Fig. 14 (see Results). Carrier yeast cells were added before alkali extraction, and carrier mannan and glucan (5) were added before each of these polysaccharides was precipitated. The extraction of mannan and soluble glucan in 6% KOH was carried out for 90 min at 80 C. The precipitation of mannan from the extract with Fehling reagent was performed as described (1). Preparation of chitinase and glucanase and conditions for incubation with these enzymes have already been reported (5). After treatment with chitinase, the liberated diacetylchitobiose was chromatographed on Whatman no. 1 filter paper with pyridine-butanol-water (6:4:3, vol/vol/vol) as solvent.

Other methods. Radioactivity in water-soluble material was determined with Aquasol as the scintillation liquid. Insoluble samples were counted with a scintillation mixture containing a thixotrophic silica gel (13). Both determinations were performed with a Packard Tri-Carb spectrometer. Paper chromatography of polyoxin D was carried out using as solvent ethanol-1 M ammonium acetate (75:30) containing 1 mM ethylenediaminetetraacetic acid.

RESULTS

Observations by phase-contrast microscopy of abnormal cells induced by polyoxin D. Growth of yeasts in a minimal medium in the presence of polyoxin D (0.1 to 1 mg/ml) resulted in the appearance of two types of abnormal cells which were easily distinguishable under phase contrast (Fig. 1). One type, the

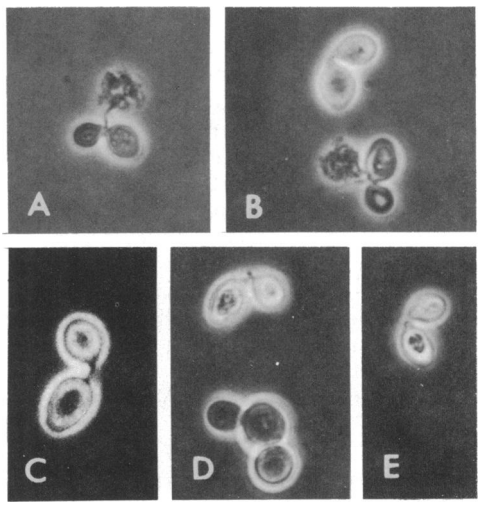


FIG. 1. *Appearance of abnormal cells from a polyoxin D-containing culture with phase optics. (A) An exploded pair in which the origin of the extruded material is clearly visible; (B) an exploded pair (below) and a refringent pair (above); (C) a refringent pair with an extremely long and thin junction. (D) A refringent pair (above), which presents some extruded cytoplasmic material at the connection between two cells (see black dot); normal cells are shown in the lower part of the picture for comparison. (E) A refringent pair has succeeded in completing division; observe the pointed ends of both cells. ×1,060.*

"exploded pair," consisted of a pair of cells which had lysed, invariably releasing intracellular material at the junction between mother cell and bud (Fig. 1A, B). The expulsion of cytoplasm, which sometimes could be observed under the microscope, occurred in less than 1 s and with explosive force, so that the material was sometimes found at a considerable distance from the cells (Fig. 1A).

The other type of abnormal cell, the "refractile pair," appeared as two cells, intensely luminous under phase contrast and often joined by a narrow bridge (see Fig. 1B, C, D). Both exploded and refractile pairs always consisted of a mother cell and a fully grown bud at the stage which usually precedes separation. However, a certain number of individual refractile cells were observed in bunches of several cells. In some cases, two refractile cells succeeded in separating without complete lysis, yielding single cells with pointed ends (Fig. 1E).

Electron microscopy. (i) Septum formation in normal yeast cells. For comparison with the polyoxin-treated cells, the sequence of septum formation in normal cells is shown in Fig. 2A-F. It may be seen that a very thin septum, the primary septum, grows centripetally from the somewhat thickened walls at the junction between mother cell and bud (Fig. 2A, B, C). After the primary septum is completed and the cytoplasmic membranes of both cells have separated, secondary septa are laid down from both sides (Fig. 2D, E). Finally, the cells are separated and a "bud scar" remains on the mother cell surface (Fig. 2F), whereas a much less conspicuous "birth scar" may be noticed on the daughter cell (not shown, see ref. 4). The electron-lucent areas, both in the lateral region and in the middle of the bud scar, correspond to the initial thickening of the cell wall and to the primary septum, respectively, and appear to consist of chitin (5, 7).

(ii) Abnormal cells. For observation with the electron microscope, the abnormal cells produced by polyoxin D were separated from their less dense normal counterparts by centrifugation in Renografin gradients (11). Electron microscopic images of these cells can easily be correlated with the two types of aberrant cells seen in the light microscope. In images corresponding to "refringent pairs" the cells are connected by an elongate "neck" of cytoplasm, with no evidence of septum initiation (Fig. 3). Images interpreted as a later stage in the budding process show the neck to be much narrower in the middle and lacking any discernible wall material in the central region (Fig. 4A). In other cases localized perforations of the wall occur in the neck region (Fig. 4B). In those pairs that successfully complete separation, cytoplasm protrudes in a pointed end and is covered by an unusually thin layer of wall material showing no evidence of a electron-lucent chitin layer (Fig. 5; cf. Fig. 1F). Frequently, a number of small (60 to 100 nm), membrane-bound vesicles were prominent in the cytoplasm of refractile cells. Similar structures were observed in normal cells of this strain, but they were both more numerous and more electron lucent in the polyoxin-treated cells. The vesicles were seen scattered singly or in groups of two to three throughout the cytoplasm but appeared to be concentrated in the septal region (Fig. 6). They were also seen concentrated in early buds (Fig. 7).

An exploded pair is shown in Fig. 8. The cytoplasm in these cells was completely disorganized and abnormal in appearance. In contrast to the refringent cells, the exploded cells presented a markedly thickened wall in the region of the presumptive septum (Fig. 9) and did not show the elongate neck of cytoplasm of refractile cell pairs. In the thickened layer of wall the fibers were longitudinally oriented, whereas in normal septum formation the predominant orientation appears to be transverse (cf. Fig. 2F and Fig. 9). The perforation of the wall through which the cytoplasm flowed out invariably occurred on the side of the daughter cell, but the channel connecting the two cells was not completely closed off by the abnormally thickened wall material (Fig. 10), so that the cytoplasm of the mother cell was also extruded.

The effect of different conditions on polyoxin D action. The percentage of abnormal cells observed depends on the concentration of polyoxin, although in a nonlinear fashion (Fig. 11). Except at the highest concentration used, aberrant cells did not appear until after about one generation time, and a maximum was reached after two to three generations; thereafter, a decline in the percentage of abnormal cells was observed (Fig. 11). (Although the percentage of abnormal cells decreased, the absolute number of aberrant cells did increase throughout growth.) These results suggested either that the cells were becoming more resistant to polyoxin with time or that the antibiotic was being inactivated. To verify the first of these alternatives, cells were allowed to grow through several passages during a total period of 72 h, after which their behavior in the presence of polyoxin D was examined in parallel with that of a 24-h culture obtained as described under

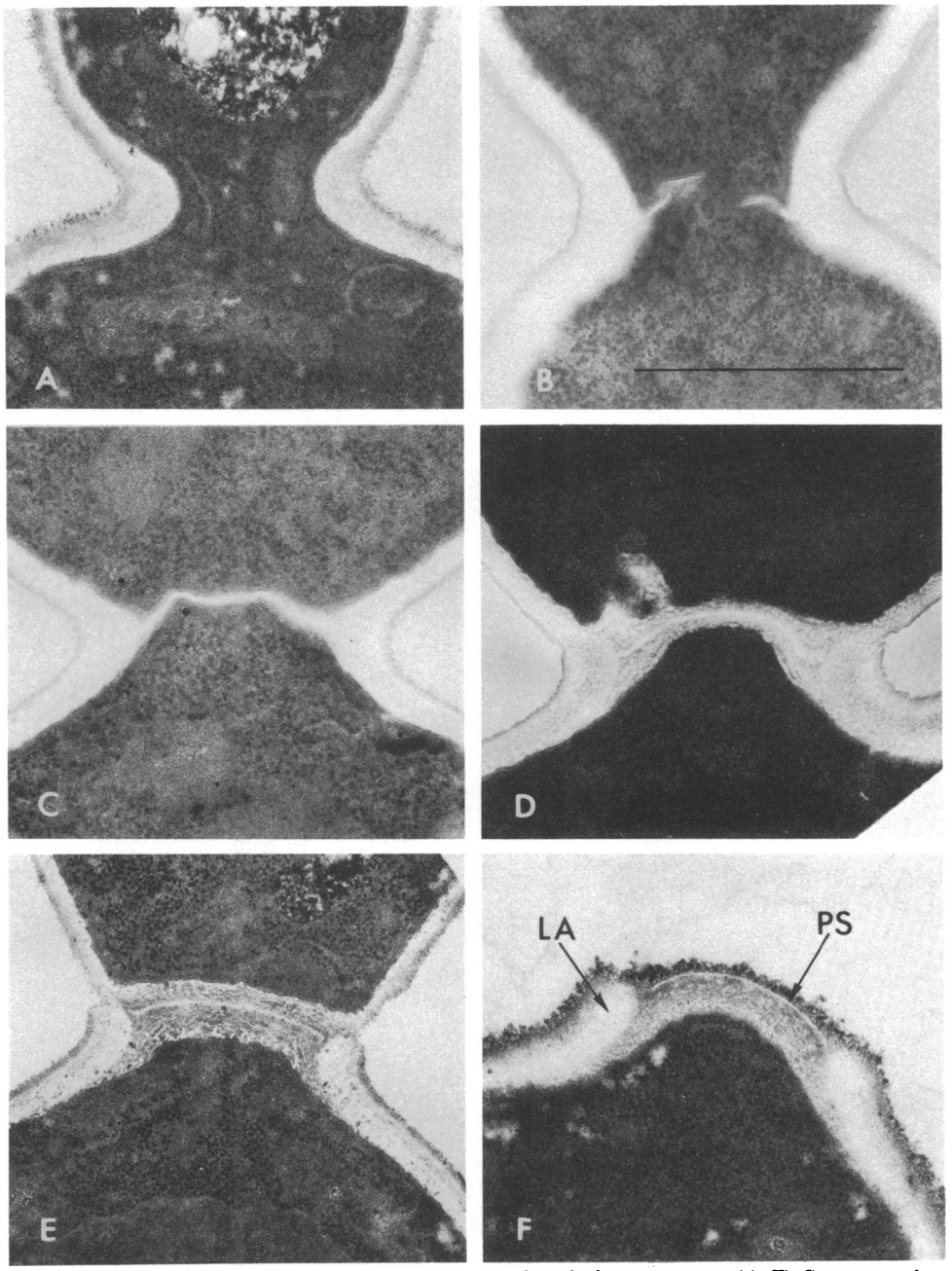
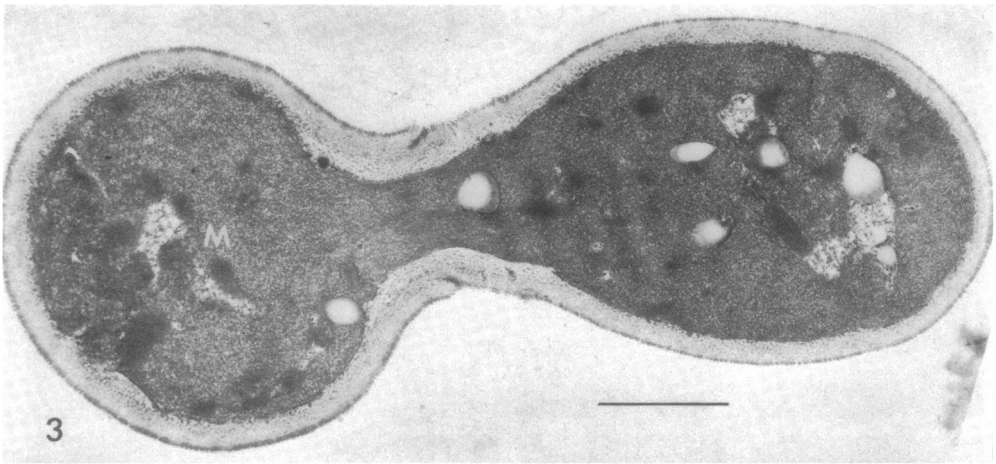
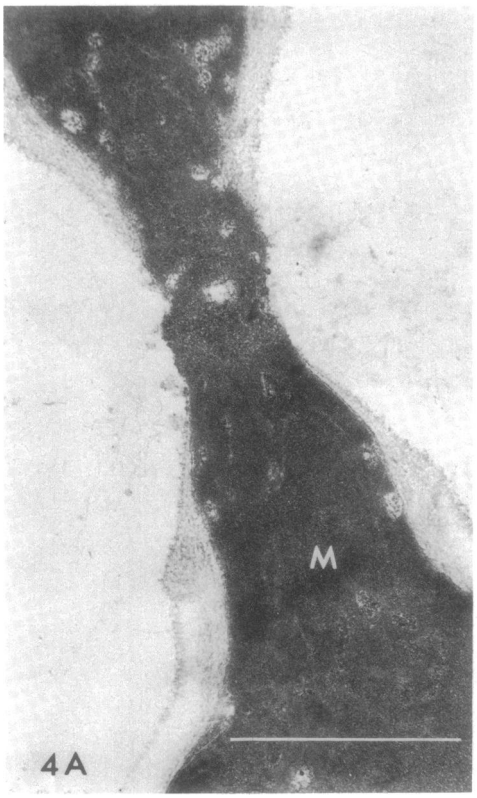


FIG. 2. *In all micrographs, M indicates mother cell and scale bars = 1 μm. (A–E) Sequence of septum formation in normal cells of strain X2180. (F) A bud scar after cell separation. The primary septum (PS) is still visible as a thin line parallel to the surface. Notice the clear lateral areas (LA) on both sides of the septum. ×35,000.*

Materials and Methods. The 72-h cells appeared to be more resistant to the antibiotic at a concentration of 0.2 mg/ml, whereas little difference was detected at a higher concentration (Fig. 12). On the other hand, chemical modification of the antibiotic by the cells does not seem to play a significant role. Polyoxin D, added to a growing culture, could be recovered





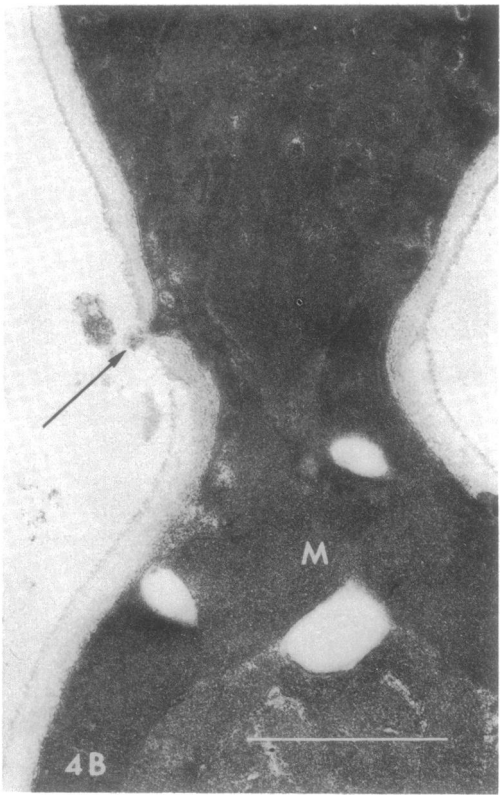


FIG. 3. *A refringent pair. Observe the long "neck" of the protoplasm, without appearance of a septum, despite the late stage in division.* ×*17,000.*

FIG. 4. *(A) A highly magnified view of the junction region of a refringent pair, which shows the complete absence of cell wall in the central portion.* ×*30,000. (B) Another cell junction, in which a small perforation with exit of cytoplasmic material can be seen (arrow).* ×*26,000.*

in good yield after a period equivalent to four generation times and, after purification by paper chromatography, was as effective in the production of abnormal cells as an unincubated control.

Cell doubling time was not markedly affected by the presence of polyoxin D in the culture medium (Fig. 11). The apparent decrease in growth rate evident after several divisions in high concentrations of the antibiotic can be

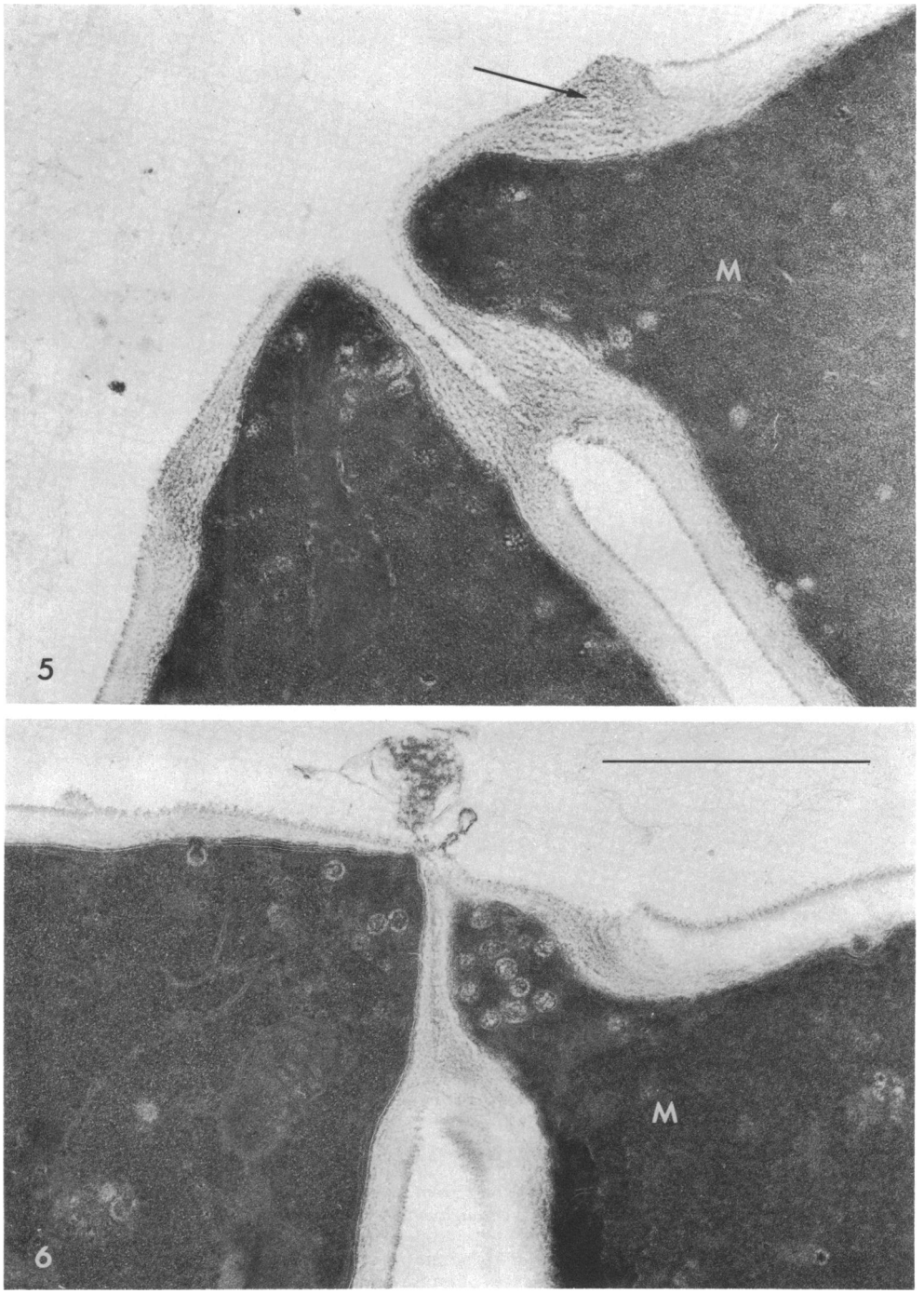


FIG. 5. *Detail of two cells from a refringent pair which have separated without apparent lysis. Notice the pointed end and the thin cell wall. Some thickening of the cell wall with parallel fibers at the base of the conical ends can be seen in the mother cell (arrow).* ×38,000.

FIG. 6. *Vesicles at the junction between two refringent cells from a polyoxin-containing culture.* ×38,000.

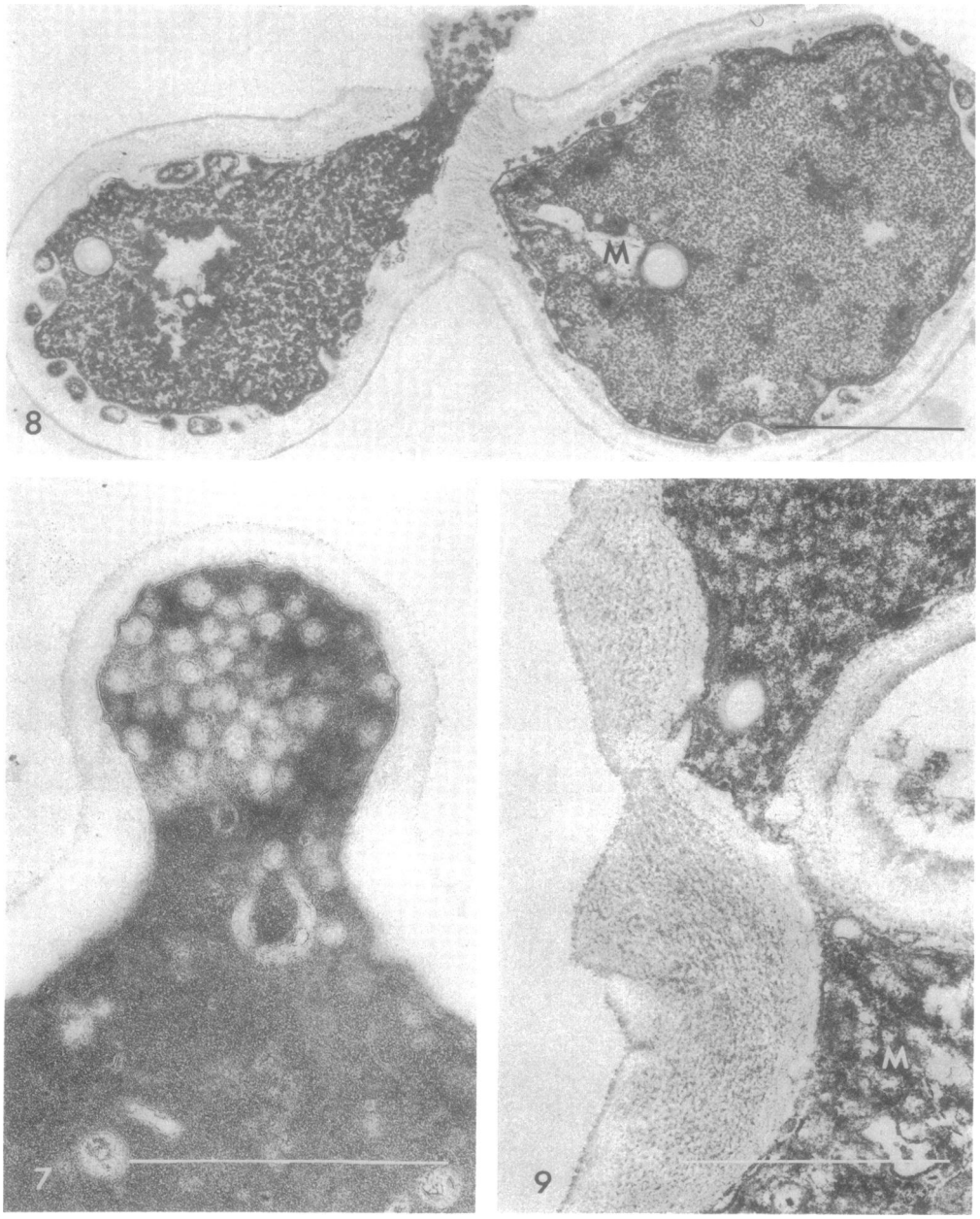


FIG. 7. *A concentration of small vesicles in a bud from a polyoxin-containing culture.* ×*43,000.*

FIG. 8. *An exploded pair. Notice the opening through which the cytoplasm has extruded and the abnormal septum.* ×*17,000.*

FIG. 9. *The septal region of an exploded pair at higher magnification showing fibers parallel to the connection between the two cells. The growth of the aberrant septum was quite asymmetric in this case.* ×*36,000.*

attributed to the presence of large numbers of abnormal cells that do not continue growth.

The concentration of polyoxin D required to produce visible effects on intact cells was much higher than that of the analogue, polyoxin A, which was inhibitory in vitro for the chitin synthetase of *Saccharomyces carlsbergensis* (13). This is due neither to a difference in strain

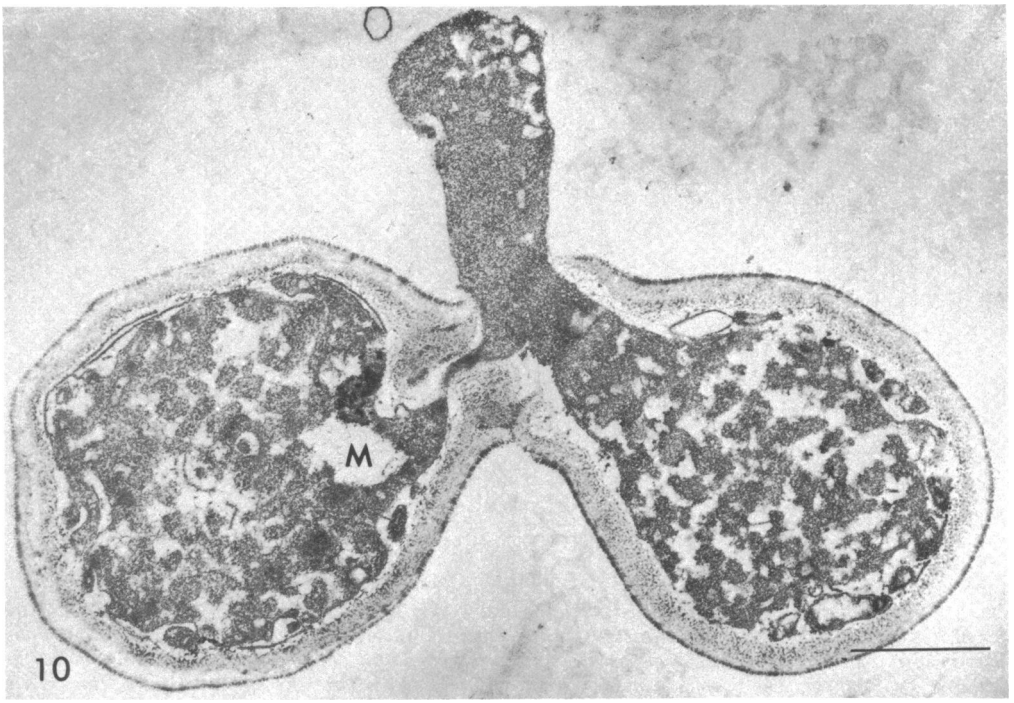


FIG. 10. *An exploded pair in which a communication between the two cells through the abnormal septum is visible.* ×*18,000.*

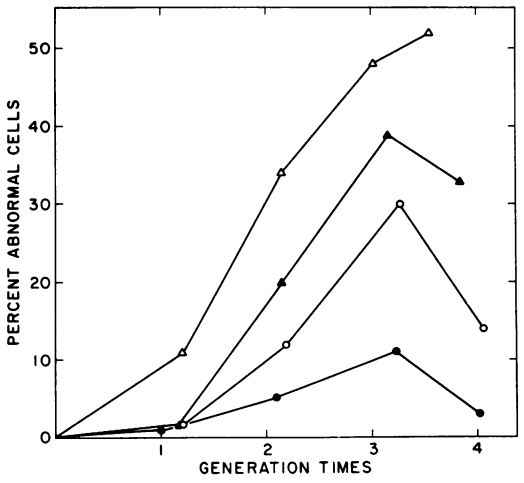


FIG. 11. *Effect of polyoxin D concentration on the production of abnormal cells. Cultures were monitored for increase in turbidity and percentage of abnormal cells at identical time intervals for all the curves. Lack of coincidence of points from the different curves with respect to the abscissa reflect differences in apparent growth rate. The concentrations of polyoxin D were 0.15 mg/ml (●), 0.3 mg/ml (○), 0.5 mg/ml (▲), and 1 mg/ml (△).*

nor in antibiotic. The K_i of polyoxin D, for chitin synthetase from *S. cerevisiae* X2180, was found to be 1×10^{-6} M, close to the value obtained for the enzyme from *S. carlsbergensis* with polyoxin A (13). It was suspected that the requirement for high levels of polyoxin might be due to poor permeability of the cells for the antibiotic. This supposition was borne out, in part, by the finding that the addition of dimethylsulfoxide to the medium enhanced the effect of polyoxin D (Fig. 13). The relative increase in abnormal cells caused by dimethylsulfoxide was greater at low concentrations of antibiotic. Amphotericin B (16) had a smaller but measurable effect. Cholate (18) could not be tried because the yeasts grew very poorly in minimal medium when this substance was present.

Finally, we confirmed the report of Mitani and Inoue (17) on the inhibition of polyoxin effects by peptides. When a peptone-containing growth medium (6) was used, polyoxin D at concentrations up to 1 mg/ml was completely ineffective.

Differential effect of polyoxin D on synthesis of cell wall polysaccharides. It was previously reported that polyoxin A inhibits chitin

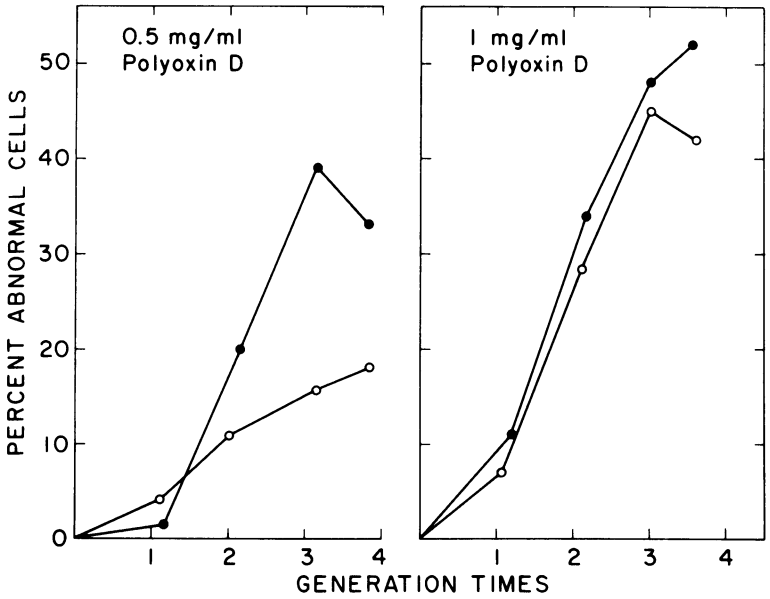


FIG. 12. *Effect of continued growth on resistance to polyoxin D. The conditions for growth were described under Materials and Methods. Two cultures from the same slant were maintained in the logarithmic phase of growth by carrying them through several passages of minimal medium. The total time of growth, previous to the addition of polyoxin D, was either 24 h (●) or 72 h (○). To eliminate the spent medium, the cells were centrifuged and washed with fresh minimal medium before being added to the polyoxin-containing medium.*

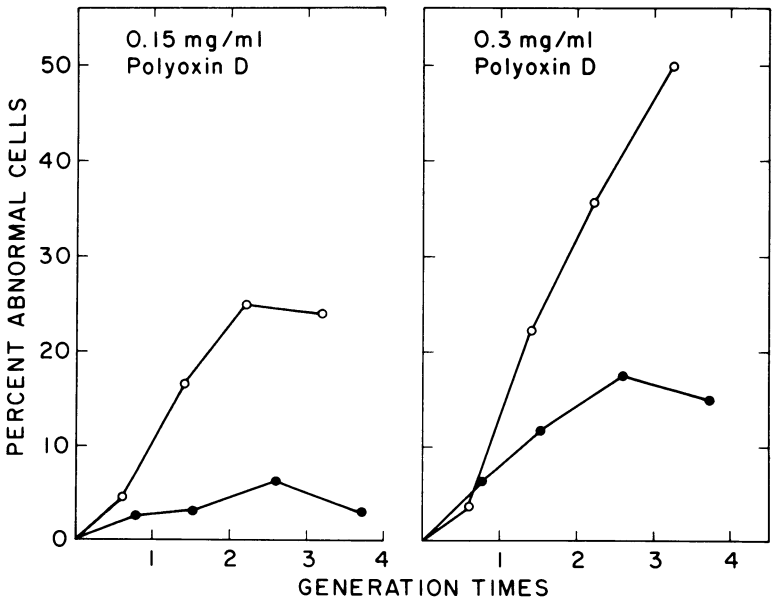


FIG. 13. *Effect of dimethylsulfoxide on the production of abnormal cells in the presence of polyoxin D. For the growth conditions, see Materials and Methods. Symbols: No dimethylsulfoxide, ●; 5% dimethylsulfoxide, ○. The presence of dimethylsulfoxide in the absence of antibiotic caused the appearance of a certain number of refringent cells, up to 14% of the total after four generations, usually not occurring in pairs. These cells were subtracted before plotting the results shown in the graph.*

synthetase, but is without effect on mannan and glycogen synthetases (13). It was important to ascertain whether the action of polyoxin D in vivo would also be directed specifically to chitin synthesis. For this purpose, [^{3}H]glucose was added to yeast cells growing in a medium with or without polyoxin D, and growth was allowed to continue for about one additional generation. The parallel cultures were harvested, and the yeasts from the polyoxin culture were fractionated with a Renografin gradient to isolate the aberrant cells. The incorporation of label in the different polysaccharide fractions of control and abnormal cells was determined according to the scheme of Fig. 14. The incorporation in the mannan of antibiotic-treated cells was about the same as in the control (Table 1), whereas that of the combined glucan fractions was 67% and that of chitin only 15% of the normal value. In another experiment, the results for mannan and glucan were again the same in both cases, and only 5% of the normal chitin labeling was found for the abnormal cells.

The proportion of radioactivity in the soluble glucan fraction is much higher for the abnormal than for the control cells (Table 1). The explanation probably lies in the fact that much more glucan is extracted by alkali from cell walls than from intact cells (2). Since many of the abnormal cells are partially lysed, they would resemble cell walls in this respect.

The fractionation scheme (Fig. 14) permits the measurement of mannan in two different fractions, i.e., one resulting from direct precipitation with Fehling solution and the other remaining after elimination of glucan with glu-

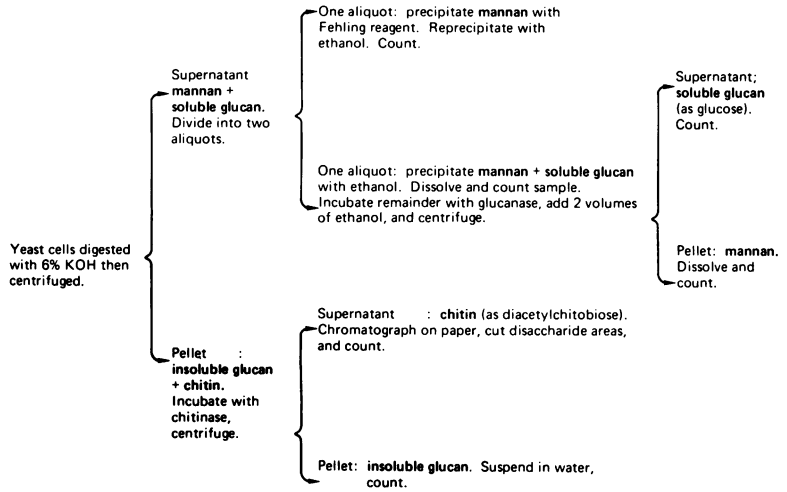


FIG. 14. *Scheme of fractionation of cell wall polysaccharides. For details see Materials and Methods.*

TABLE 1. *Effect of polyoxin D on the incorporation of radioactivity from [^{3}H]glucose into cell wall polysaccharides*[a]

Cells analyzed	Total cells	Mannan		Glucan				Chitin	
				KOH insoluble	KOH soluble	Total			
		Counts per min per cell $\times 10^4$	% Control	Counts per min per cell $\times 10^4$	Counts per min per cell $\times 10^4$	Counts per min per cell $\times 10^4$	% Control	Counts per min per cell $\times 10^5$	% Control
Control	6.7×10^7	5.2	100	5.5	0.9	6.4	100	2.1	100
Abnormal cells from polyoxin culture	2.04×10^7 [b]	4.95	95	2.25	2	4.25	67	0.3	14

[a] For experimental details see Materials and Methods and Fig. 12.

[b] Number of abnormal cells produced during growth in the presence of [^{3}H]glucose. The total number of cells was 7.04×10^7, and the correction was calculated as explained in Materials and Methods.

canase. Any glycogen present would be included in the latter mannan fraction, but not in the copper reagent precipitate. The results obtained with the Fehling-insoluble material are given in Table 1. Those from the other fraction were slightly lower, 4.9×10^{-4} counts per min per cell with normal cells and 4.5×10^{-4} counts per min per cell with polyoxin-treated cells. Thus, substantial amounts of glycogen do not appear to be present in this fraction.

DISCUSSION

The effects of polyoxin D are best discussed against the background of the normal process of septum formation in *S. cerevisiae* as represented schematically in Fig. 15. Cytokinesis starts with a thickening of the cell wall at the junction between mother cell and bud (Fig. 15A, B). The material which is accumulated has been reported to be more electron transparent than the rest of the cell wall (15). In a second stage a thin septum, which we call primary septum, grows inward until the channel between both cells is closed off (Fig. 15C, D). Later, secondary septa of similar appearance to the remainder of the cell wall are laid down from both sides onto the still visible primary septum (Fig. 2D, E, and Fig. 15E). Finally, the two cells separate, and a bud scar remains on the mother cell surface (Fig. 15F). According to this scheme, similar to that suggested by Shannon and Rothman for *Candida* (20), the primary septum would remain embedded in the bud scar. Indeed, this is the location at which we found a chitin disk (5) from which we conclude that the primary septum consists of chitin; this view is supported by a previous study on the timing of chitin synthesis in a synchronized yeast culture (6).

Most of the abnormal cells induced by polyoxin D are found either as "exploded pairs" or as "refringent pairs." In both cases the daughter cell is fully grown, i.e., the cell cycle is interrupted just prior to cell separation. There are several other indications that the septal region is the locus of antibiotic action: (i) in the exploded pairs, the cytoplasmic material is always extruded at the junction between the two cells: (ii) in the refringent pairs, the neck between mother cell and bud is unusually thin and elongated and often pierced; (iii) in no case has a primary septum been observed in these cells; and (iv) in electron micrographs the aberrant septa which are partially or totally formed show fibers running parallel, rather than transversal, to the channel between the two cells. We interpret these results to indicate that polyoxin D prevents the formation of the primary septum by inhibiting chitin synthesis, and that as a consequence the secondary septa lack the foundation on which they are usually laid down transversally. They are formed, if at all, along the lateral cell wall, thereby accounting for the longitudinally arranged fibers of Fig. 5, 8, and 9.

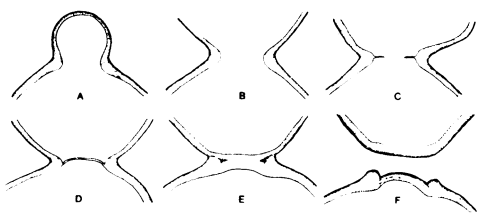


FIG. 15. *Schematic representation of septum formation in S. cerevisiae. White areas represent the chitin.*

It is not understood what factors determine whether a pair of cells will become refringent or will explode. The refringent cells are somewhat shrunken and heavier than the normal ones, as shown by their faster sedimentation during gradient centrifugation, and their cytoplasm appears to be poorly differentiated in the electron micrographs. It seems probable that these cells have lost some cytoplasm through holes too small to allow the organelles to escape. Refringent and exploded cells differ in the size of the aberrant secondary septa, which are much thicker in the exploded pairs. Conceivably, the difference between these aberrant patterns of cell division depends on the phase of the cell cycle at which a critical intracellular concentration of polyoxin is reached. However, secondary effects of the antibiotic at very high concentrations cannot be excluded.

The immediate cause for cell lysis is unknown. The orifices seen in the cell wall are presumably due to lytic action. It seems probable that in the normal process of cell division some breakdown of cell wall polysaccharide may be required for the separation of mother and daughter cell. Under the abnormal conditions resulting from the absence of primary septum, polysaccharide hydrolysis could occur in uncontrolled fashion, thereby causing irreparable damage to the cell wall. Perhaps the small vesicles frequently observed in the septal region of abnormal cells (Fig. 6) might have a role in the process. Similar vesicles have been described by other workers (9, 19) and reported to contain glucanase (9). Nevertheless, the higher number and visibility of these organelles in polyoxin-treated cells remains unexplained.

Despite the evidence that the action of polyoxin is exerted primarily at site of the septum, the need for concentrations of antibiotic higher by two orders of magnitude than those effective on chitin synthetase in vitro remained as a puzzling fact. Therefore, it was reassuring to find that in abnormal cells the incorporation of label into chitin was specifically inhibited, as compared with the other polysaccharides of the cell wall. The requirement for high concentrations of polyoxin could be due to several factors. The possibility that the antibiotic in the medium was destroyed has been eliminated. It is still conceivable that those molecules of polyoxin which penetrate the cells might be inactivated, either by binding or by chemical modification. An increase in the capacity for inactivation is one of the possible explanations for the augmented resistance of the cells to polyoxin after many generations of growth. The possibility has been ruled out that accumulated protein (peptides) in the medium of the older cultures might account for the difference in the apparently relative insensitivity of older cultures to polyoxin D. Another explanation is that permeability to the antibiotic plays a role both in the initial resistance and in its increase during growth. The facilitation of polyoxin action by amphotericin B and especially by dimethylsulfoxide supports this view, which is also in agreement with the delay in the appearance of abnormal cells, especially at low levels of polyoxin. Some delay was expected, perhaps about one-half the generation time, because the effect of polyoxin would be substantial only on cells which had not yet initiated their primary septum and would become evident only at the stage of cell separation.

ACKNOWLEDGMENTS

We are grateful to H. Saito of Kaken Chemical Company for a gift of polyoxin D and to G. Ashwell, W. B. Jakoby, and R. Ulane for useful criticism. Thanks are expressed to T. Olszewski for expert technical assistance.

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