Properties of Yeast Cell Ghosts Obtained by Ribonuclease Action

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ABSTRACT

Cell ghosts were obtained from Candida utilis and Saccharomyces cerevisiae by the action of some conformational isomers of pancreatic ribonuclease which passed through the cell wall and penetrated the cell membrane. In the interior, the enzyme caused extensive disorganization of the cellular structure as evidenced by the results of vital staining and electron microscopy. Ribonucleic acid was degraded to fragments that leaked out into the suspending medium. Other cytoplasmic constituents, including the amino acid pool, were released, but most of the cellular protein and deoxyribonucleic acid remained in the ghosts. The lesions in the cytoplasmic membrane were too small to be seen by conventional electron microscopy. The membrane clung to the cell wall even after obliteration of most of the intracellular structure.

Cells of Candida utilis and of Saccharomyces cerevisiae are susceptible to the action of pancreatic ribonuclease. Under suitable conditions, most of the ultraviolet-absorbing cellular components of the acid-extractable type are released into the suspending medium, and the cells lose their viability (13, 14).

A more detailed study of the substances released from the cells into the medium promised information on the effects of ribonuclease on the cytoplasmic membrane and on intracellular structures. Microscopic examination of the unstained ghosts revealed little change in their outline. However, electron microscopy and vital staining procedures have shown extensive cytological changes. This report describes in more detail the yeast cell ghosts obtained by the action of ribonuclease.

MATERIALS AND METHODS

Yeast culture. C. utilis (ATCC 9930) and S. cerevisiae (ATCC 7752) were cultivated as outlined in our earlier report (13), except for a decrease in the concentration of Mg++, Mn++, Zn++, and Ca++ to one-tenth of the values specified previously. After harvesting by centrifugation, the cells were washed twice with cold water; they proved suitable for experimentation for approximately 2 weeks if stored at 2 to 5°C. All weights specified in the experiments pertain to moist cells.

Treatment with ribonuclease. The washed cells were suspended in water to a final concentration of 2.0 mg/ml. Recrystallized pancreatic ribonuclease (Armour Pharmaceutical Co., Kankakee, Ill., Boehringer Mannheim Corp., New York, N.Y., or Nutritional Biochemicals Corp., Cleveland, Ohio) was used in a concentration of 0.1 or 0.2 mg/ml. As an alternative, chromatographically purified ribonuclease A or B (Sigma Chemical Co., St. Louis, Mo., preparation XII) could be used after heating their freshly prepared water solutions (1 mg/ml) at 70°C for 10 min as specified earlier (13). The enzyme preparations had to be free of salts, especially phosphates, and no buffer was used during incubation. The release into the medium of cellular constituents absorbing at 260 mμ served as a measure of enzyme action; after 30 to 45 min at 30°C, the maximal effect was reached. The cell residues (ghosts) were harvested by centrifugation, were washed, and were used for the experiments. In some instances, the enzyme treatment and washing of the cells was carried out in 1.0 M mannitol solution to minimize osmotic shock after the enzyme action on the cell membrane. Further details are given in connection with the experiments and in our earlier reports (13, 14).

Assay of deoxyribonucleic acid (DNA). The diphenylamine test of Dische (4) as detailed by Ashwell (1) was used. Thymus DNA (Calbiochem, Los Angeles, Calif.) served as reference standard; its concentration was determined by spectrophotometry. The procedure of Ogur and Rosen (10) was used for extraction of the yeast cells and ghosts.

Staining procedures. Methylene blue, neutral red, and acridine orange were used in 1:10,000 dilution in water or 1.0 M mannitol. The Hucker and Conn modification of the Gram stain was used as given in the Manual of Microbiological Methods of the Society of American Bacteriologists (16).

Electron microscopy. The cells were washed three times with distilled water, and the ghosts obtained

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after treatment with ribonuclease in 1.0 M mannitol were washed with water or mannitol, respectively. Pellets of the cells or ghosts obtained by centrifugation were fixed for 1 hr in 2% KMnO4, were washed with water, and were fixed for 2 hr in 2% osmic acid. After dehydration in a graded ethyl alcohol series (25 to 100%), the material was transferred to propylene oxide and then to an Epon 812 propylene oxide mixture for infiltration overnight prior to final embedding. Polymerization was for 8 hr at 37 C, 8 hr at 45 C, and 24 hr at 60 C. Sections were cut with a diamond knife, mounted on a Porter-Blum ultramicrotome, and floated on 200-mesh Cu grids. Additional staining was not required. The sections were observed with an RCA 3 G electron microscope equipped with a double condenser and cold stage.

RESULTS

Our earlier experiments on the leakage of cytoplasmic constituents from yeast cells after ribonuclease treatment (13, 14) have now been extended to the amino acid pool, intracellular proteins, and DNA.

The release of intracellular amino acids is exemplified by the following experiment: A quantity of 200 mg of cells of C. utilis in 100 ml of water was incubated with 40 mg of ribonuclease for 1 hr at 30 C. After centrifugation, the supernatant fluid showed an absorbancy of 0.61 at 260 m. thus indicating extensive action of the enzyme. The solution was evaporated and the residue taken up in 5.0 ml of 1.5 N perchloric acid.

For comparison, an extract of 200 mg of cells was prepared with 5.0 ml of 1.5 N perchloric acid. After 1 hr at 25 C, the debris was removed by centrifugation (15). Solid KHCO3 was added for removal of most of the perchloric acid and for adjustment to pH 5, which is suitable for the ninhydrin tests. After 1 hr in an ice bath, KClO3 was removed by centrifugation. A control experiment was carried out consisting of incubation of 200 mg of cells in water for 1 hr at 30 C, centrifugation, and concentration of the supernatant fluid to 5.0 ml. Equal amounts of each solution were spotted on Whatman no. 1 paper and were developed with 1-butanol-acetic acid-water (60:15:25, v/v). Spraying with ninhydrin revealed an almost identical pattern of numerous amino acids in the enzyme and perchloric acid extracts (Fig. 1), whereas the control experiment gave no ninhydrin-positive spots.

For quantitative evaluation of these extracts, ninhydrin tests of graded amounts of the appropriately diluted solutions were carried out according to the procedure of Yemm and Cocking (22) with leucine as the reference substance. In the supernatant fluid from the ribonuclease digest, a ninhydrin response equivalent to 26.6 μmoles of leucine was found for 100 mg of cells; in the perchloric acid extract, the leucine equivalent was 23.1 μmoles per 100 mg of cells. It is apparent, therefore, that ribonuclease under suitable conditions is as effective as perchloric acid for the release of ninhydrin-positive material.

Earlier analyses of the supernatant fluid after ribonuclease action on yeast cells had revealed the presence of significant amounts of protein (13). It could not be decided, however, whether this material had been released from the cells or was residual extraneous ribonuclease. This question was examined by labeling C. utilis with L-methionine-C14H2, glycine-2-C14, and uniformly labeled lysine-C14. The amino acids were incorporated into the culture medium in the quantities indicated in Table 1. The washed cells were exposed to ribonuclease or to 1.5 N perchloric acid, and the supernatant fluids were counted. The cell residues were washed and compared with intact cells by counting suspensions of each in the scintillation spectrometer. Concentrated supernatant fluid of the ribonuclease incubations was spotted on Whatman no. 1 paper in quantities corresponding to 1 and 2 mg of

![Fig. 1. Ascending paper chromatography of extracts obtained from Candida utilis by 1.5 N perchloric acid (PCA) and by pancreatic ribonuclease (RNASE). The quantities applied correspond to 1.6 mg of cells (wet weight). The numbers on the ordinate signify Rf values. Experimental details are listed in the text.](http://jb.asm.org/)
is apparent that *S. cerevisiae* and *C. utilis* lost only limited amounts of DNA. The action of ribonuclease did not facilitate the subsequent entry of deoxyribonuclease into the cell ghosts.

The analyses of the crude supernatant fluids after ribonuclease treatment indicate release of some DNA from the cells; these data, however, have to be interpreted with caution, because the diphenylamine test in presence of high concentrations of ribonucleic acid and amino acids is of low accuracy (1).

**Gram staining.** Ribonucleic acid (RNA) has played an important role in the interpretation of the gram-positive character of yeast cells (2, 3, 6). The removal of most of the ultraviolet-absorbing constituents from yeast cells by ribonuclease treatment suggested testing of the ghosts by the Gram-staining procedure. Table 3 shows results obtained with *S. cerevisiae* and *C. utilis* before and after treatment with ribonuclease. The results were the same when the exposure to the enzyme was extended to 6 or 24 hr. For comparison, ghosts obtained by extraction of the cells with 1.5 N perchloric acid for 1 hr at 25 C were included.

### Table 2. DNA in yeast cell ghostesa

<table>
<thead>
<tr>
<th>Material examined</th>
<th>Amt of DNA found in</th>
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<tbody>
<tr>
<td></td>
<td><em>Candida utilis</em></td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Intact cells</td>
<td>94</td>
</tr>
<tr>
<td>Ribonuclease ghosts</td>
<td>76</td>
</tr>
<tr>
<td>Ribonuclease ghosts, after treatment</td>
<td>73</td>
</tr>
<tr>
<td>of deoxyribonuclease</td>
<td></td>
</tr>
<tr>
<td>Supernatant fluid from</td>
<td>42</td>
</tr>
<tr>
<td>ribonuclease ghost preparation</td>
<td></td>
</tr>
<tr>
<td>Supernatant fluid from</td>
<td>6</td>
</tr>
<tr>
<td>deoxyribonuclease treatment</td>
<td></td>
</tr>
</tbody>
</table>

a In each experiment, a quantity of 100 mg of cells, or material derived therefrom, was examined. For preparation of ribonuclease ghosts, 2.0 mg of cells and 0.3 mg of ribonuclease per ml were used. The intact cells and ghosts were extracted with 1 N perchloric acid for 30 min at 80 C. The supernatant fluids obtained after exposure to ribonuclease were concentrated before the diphenylamine tests. For deoxyribonuclease treatment, the ghosts derived from 100 mg of cells were suspended in 1.0 ml of water and exposed to 1.0 mg of the enzyme for 1 hr at 30 C. After centrifugation, the sediment and the supernatant fluid were examined.

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yeast. The mobile components of the extracts were chromatographed with 1-butanol-acetic acid-water (60:15:25, v/v) or, in parallel experiments, with ethyl alcohol-acetic acid-water (65:2:33, v/v). The papers were surveyed by radioactivity scanning. Protein remains at the site of application in this procedure. No radioactivity which would indicate protein was detected at the spot of application.

The efficiency of counting suspensions of intact cells, cell ghosts, or cell debris after perchloric acid extraction (Table 1) was suboptimal. A comparison of the values shows, however, that much of the radioactivity was retained after ribonuclease or perchloric acid treatment of the cells. In no instances was the radioactivity in the ribonuclease ghosts lower than that in the cell residues after perchloric acid extraction; this would have indicated loss of protein from the ghosts.

An analysis of the ghosts for DNA was considered important, because the intracellular disarray caused by the action of ribonuclease may have led to autolysis of DNA and release of its fragments into the suspending medium. Cells, ghosts, and the supernatant fluid were examined, therefore, by the diphenylamine test (Table 2). It
in the examination. In our experience (15), these are the most lenient conditions under which the extraction of acid-soluble cellular constituents, including RNA and its fragments, is virtually complete. It appears from the data in Table 3 that removal of a high percentage of the cellular RNA has only moderate influence on the gram-positive character. The gram-negative staining of the cell residues after perchloric acid extraction shows that it is possible to remove the reactive material or to destroy the cellular sites that are responsible for the Gram staining.

Vital staining. Some vital stains that are employed frequently in yeast cytology were used to test the ghosts obtained by ribonuclease action. Methylene blue is used widely to detect dead cells by their immediate uptake of the stain (9). Details of the penetration of neutral red into yeast cells have been described by Guilliermond and Gautheret (5); the vacuole can be delineated particularly well although the stain is released again after some time. Acridine orange has been

<table>
<thead>
<tr>
<th>Organism examined</th>
<th>Untreated cells</th>
<th>Cell ghosts</th>
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<tbody>
<tr>
<td></td>
<td>Cell ghosts</td>
<td>After ribonuclease</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>&gt;95% gram-positive, &lt;5% gram-negative</td>
<td>&gt;90% gram-positive to intermediate, &lt;10% gram-negative</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>&gt;90% gram-positive, &lt;10% gram-negative</td>
<td>&gt;80% gram-positive to intermediate, &lt;20% gram-negative</td>
</tr>
</tbody>
</table>

* The concentrations were 2.0 mg of cells and 0.2 mg of ribonuclease (Armour) per ml of water. After 1 hr at 30 C, the enzymatic release of cellular constituents absorbing at 260 mµ was 83% for *S. cerevisiae* and 92% for *C. utilis*, compared with the extraction afforded by 1.5 N PCA (100%). The cell ghosts were washed twice with water before staining.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Untreated cells</th>
<th>After ribonuclease treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue</td>
<td>Almost colorless; &lt;5% dark blue dead cells.</td>
<td>Immediate uniform light blue; no intracellular detail visible.</td>
</tr>
<tr>
<td>Neutral red</td>
<td>Take stain rapidly; accumulate it in the vacuole.</td>
<td>Immediate diffuse light pink staining; nearly uniform internal distribution of stain. No intracellular detail visible.</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>Immediate light yellow staining; internal distribution varies with time; &lt;5% dark orange dead cells.</td>
<td>Immediate uniform light yellow staining, no change with time; no dark orange cells. No intracellular detail visible.</td>
</tr>
</tbody>
</table>

* The experimental conditions of the ribonuclease treatment were the same as those given in Table 3.
the stain solutions, mannitol was added to a final concentration 1.0 M. No significant difference from the observations listed in Table 4 was seen under these experimental conditions.

More specific staining techniques were not applied because concomitant electron microscopy showed that the cytological features of the cells were obliterated in the ghosts.

Electron microscopy. Figure 2 shows the results of electron microscopic examination of C. utilis and of ghosts obtained from it by the action of ribonuclease in water and in 1.0 M mannitol. The intracellular effects of the enzyme are obvious, particularly in Fig. 2C.

DISCUSSION

The present experiments indicate that lesions in the yeast cell membrane are inflicted by pancreatic ribonuclease. The alternative, transfer of ribonuclease into the cytoplasm by a process of
pinocytosis (7), seems excluded by the rapid loss of a variety of cellular constituents that would be retained by an intact membrane. The speed of action of ribonuclease excludes the possibility that the release of cellular constituents is merely a consequence of cell death and ensuing autolysis. The latter can be observed after killing yeast cells with ultraviolet light; however, under these conditions, it takes many hours to complete the autolysis of RNA and the discharge of ultraviolet-absorbing material into the surrounding medium (18).

In addition to RNA fragments and inorganic compounds (13), the release of the amino acid pool is demonstrated by the present experiments. The observation that intracellular protein and DNA are retained by the yeast cell indicates that the size of the lesions in the membrane or the pore size of the cell wall may set a limit for the dimensions of the molecules that can leak into the suspending medium.

The immediate penetration of vital stains into the cell ghosts was expected, but the intense staining with methylene blue that usually is considered to be characteristic of dead cells was not seen. Apparently, components essential for the staining have been removed from the cytoplasm. The low intensity of staining with acridine orange and neutral red is in agreement with this concept. On the other hand, the speed and type of staining permit one to distinguish with ease between ribonuclease ghosts and viable cells. Microscopy of ghosts treated with any of these stains reveals internal destruction whether the enzyme treatment has been carried out in water or in an isotonic milieu such as 1.0 M mannitol.

Changes in the staining properties of S. cerevisiae after exposure to ribonuclease have been noted already by Orskov (11). However, he came to the tentative conclusion that the morphological changes are not permanent. Competitive effects of ribonuclease on the binding capacity of yeast for ions and dyes have been investigated by Takada and Tokuno (20) and by Tokuno and Takada (21). Most of their observations were explained in terms of the ion-exchanging properties of the cell surface. It appears, however, that ribonuclease in these experiments must have penetrated also into the cytoplasm.

Our observations that the gram-positive or gram-intermediate character prevails after ribonuclease treatment of the yeast cells proves that the function of RNA, if any, in this process would have to be restricted to a small fraction, perhaps to the material firmly retained in the structure of the cell membrane.

Several features of the ghosts are revealed in more detail by electron microscopy. Pictures were taken after ribonuclease treatment of cell suspensions in water as well as in 1.0 M mannitol. In the latter, the effects of osmotic shock were greatly reduced. The structural changes in ghosts prepared under isotonic conditions must be attributed mainly to the intracellular action of ribonuclease. The collapse of the vacuole is the most consistent observation. Under other experimental conditions (19), this structure shows stability in 1.0 M mannitol solution for several hours, even in the isolated state. The instability in our present experiments, therefore, may be explained by the action of ribonuclease on the vacuolar membrane; its sensitivity appears to be similar to that of the cytoplasmic membrane. The damage to the nucleus and mitochondria in water is extensive. In 1.0 M mannitol (Fig. 2B), the nucleus appears to be undamaged, and the effect on the mitochondria is less severe than in water (Fig. 2C).

In water as well as in 1.0 M mannitol, the cell was not collapsed. This is in striking contrast to the collapse of the cells after ultraviolet damage (18). Some adherence or linkage of the membrane to the cell wall is indicated in the present experiments. The lesions produced by ribonuclease in the membrane are not visible. It is possible that the fissures are few and have eluded detection in serial sections of the cells. More probable, however, is the assumption that the pores are too small to be visualized by electron microscopy in our hands.

Many details of the action of pancreatic ribonuclease on the yeast cell membrane are still obscure. The enzyme is known to break internucleotide bonds adjoining pyrimidine bases (12). If the RNA is interlinked with other components of the membrane, the severance of the internucleotide bonds can only be the beginning of membrane lesions, because the dimensions of the structural fault created by this initial event would not be compatible with the size of the ribonuclease molecules that get into the cell, nor with the estimated thickness of the membrane. The secondary alterations initiated by ribonuclease are unknown.

The release of cytoplasmic constituents from yeast cells by ribonuclease occurs at biological pH and temperature without introduction of any reagent other than the enzyme. Thus, it is a very mild procedure which is selective, to some extent, in the size of the molecules removed from the cells. The retention of protein and DNA in the ghosts may prove to be of experimental interest.

ACKNOWLEDGMENT

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LITERATURE CITED


