STUDIES ON PROTEIN SYNTHESIS BY PROTOPLASTS OF *SACCHAROMYCES CARLSBERGENSIS*

III. STUDIES ON THE SPECIFICITY AND THE MECHANISM OF THE ACTION OF RIBONUCLEASE ON PROTEIN SYNTHESIS

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SUMMARY

In this paper, the experimental results are presented of a continued study on the specificity and the mechanism of the inhibition by ribonuclease of protein synthesis in protoplasts of *Saccharomyces carlsbergensis*. By comparing the effects of native pancreatic ribonuclease with those of heat-denatured enzyme and protamine, it is shown that at least part of the observed inhibition of protein synthesis has to be attributed to the enzymic action of ribonuclease.

The enzyme was found to cause both a lysis of the protoplasts and an inhibition of protein synthesis. Mg^{2+} was found to abolish the lysing action of RNAase, whereas protein synthesis remained inhibited for 50–90 %. From the data it is concluded that RNAase inhibits protein synthesis primarily by its action on the yeast cell membrane.

INTRODUCTION

In two previous communications[1,2] the effect of pancreatic ribonuclease on protein synthesis by protoplasts of *Saccharomyces carlsbergensis* was described. Several polyanionic compounds were found to abolish the inhibiting action of ribonuclease on both the incorporation of ^{14}C-labelled amino a[illegible] into protein and on the induced synthesis of α-glucosidase.

Our first data[1] yielded indications that ribonuclease might interfere with the energy metabolism in the protoplast. However, continued investigations showed that this could not be the case and that the observed cessation of respiration and glycolysis was the result of a RNAase-caused lysis of the protoplasts.

In this paper the results are described of our continued studies on the specificity and the mechanism of the effect of ribonuclease on protein synthesis by yeast protoplasts.

MATERIALS AND METHODS

Protamine was a product of the British Drug House, Ltd.

Abbreviation: PMA, polymethacrylic acid; s-RNA, soluble RNA.

All other materials and methods were the same as described in our previous communications[1,2].

EXPERIMENTAL RESULTS

Studies on the specificity of the RNAase-effect

The effect of native ribonuclease on the incorporation of labelled amino acids was compared with the effect of heat-inactivated enzyme and of another basic protein protamine, in order to find out whether the observed inhibition of protein synthesis by RNAase[1,2] is due to the activity of the native enzyme.

That the effect of ribonuclease can be attributed to both a specific (enzymic) and an unspecific (structural) action is shown by our data as illustrated in Fig. 1. Although at higher concentrations of protamine and heat-inactivated ribonuclease an inhibition of amino acid incorporation can be observed, it is clear that at least part of the action of ribonuclease at low concentrations is highly specific and due to the enzymic properties of this protein.

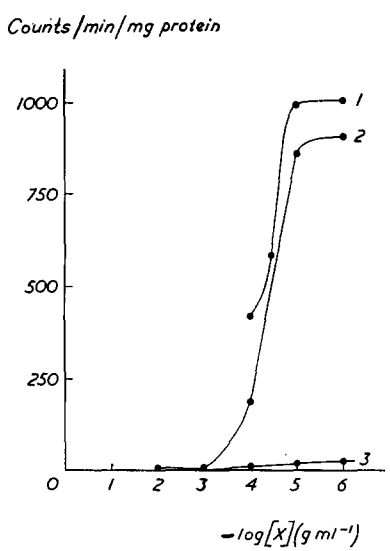

Fig. 1. The specificity of the action of ribonuclease on protein synthesis by yeast protoplasts. Experimental conditions as already described[1]. Ribonuclease was inactivated according to KUNITZ by heating a solution containing the enzyme for 10 min at 100° at pH 10. X = inhibitory compound added. Curve 1, heat-inactivated ribonuclease; Curve 2, protamine; Curve 3, ribonuclease.

Repeated washings of the protoplasts after 10 min of incubation with ribonuclease did not change the observed inhibiting effect. Furthermore, this effect was shown to be independent of the pH of the incubation medium between pH 4 and 7.

The mechanism of the inhibition by RNAase of protein synthesis

In an attempt to analyse the nature of the effect of ribonuclease on protein synthesis by yeast protoplasts a series of photographs was taken at 260 mμ with the aid of

an ultraviolet microscope*. These photographs showed a marked swelling of the protoplast after the addition of RNAase and, as could be seen in some cases, a swelling of the protoplasmic membrane (Plate I). Sooner or later lysis of the cells occurred, despite the fact that they were suspended in an isotonic medium. Heat inactivation,

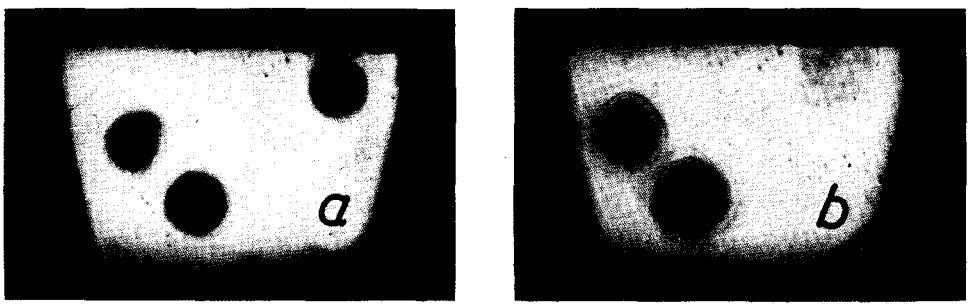

Plate I: Ultraviolet (260 mμ) microphoto's of yeast protoplasts: a, before; b, 10 min after the addition of ribonuclease.

which was shown to abolish the action of ribonuclease on protein synthesis was also effective in abolishing its lysing action. To exclude the possibility that the action of ribonuclease would be due to a contamination with lipid-or protein-splitting enzymes, lipase and trypsine were tested for their effect on yeast protoplasts. At pH 6.2 or 7.8 neither lipase, nor trypsine were found to cause a lysis of the protoplasts or to inhibit the incorporation of ^{14}C-labelled amino acids into the protein.

It was observed that the use of citrate buffer instead of phosphate buffer made the lysis proceed much faster at the same pH and ionic strength. Regarding the chelate-forming properties of the citrate ion, it was investigated whether di- and trivalent cations could inhibit the lysing action of ribonuclease on yeast protoplasts which were washed several times with 10 % mannitol containing 0.02 *M* Na-citrate–phosphate buffer pH 5.8.

Fig. 2 illustrates some determinations of the lysis of yeast protoplasts as a result of the action of ribonuclease in an isotonic medium. The absorbancy at 530 mμ of a protoplast suspension was chosen as a measure for the number of unlysed cells. Usually, total lysis of the protoplasts by osmotic shock resulted in a decrease of about 80 % in the 530 mμ absorbancy of the suspension. The data presented in Fig. 2 also show that the addition of very small amounts of Mg^{2+} results in a marked inhibition of the lysing effect of ribonuclease on yeast protoplasts.

Not only Mg^{2+} was found to abolish the lysing effect of ribonuclease on yeast protoplasts; 0.0005 *M* Al^{3+} and K^+ in concentrations of 0.5 *M* and more were also active. Higher Al^{3+} concentrations tended to cause a flocculation of the protoplasts, whereas K^+ in concentrations of 0.5 *M* and more was found to result in a marked inhibition of amino acid incorporation in protoplasts that were not treated with RNAase. Probably, the charge of these ions is responsible for preventing the lysis of the protoplasts by RNAase.

* The authors are very grateful to Professor Dr. M. T. JANSEN for taking the photographs.

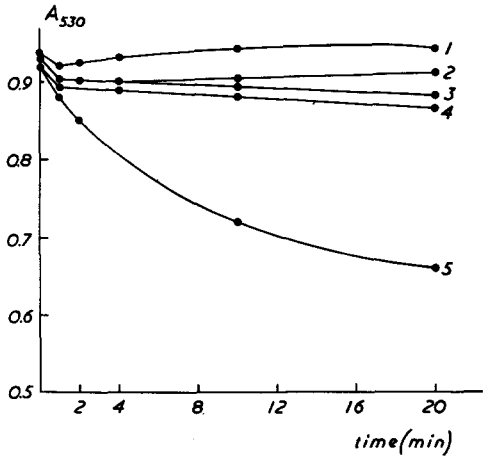

Fig. 2. Lysis of yeast protoplasts as a result of the action of ribonuclease. Protoplasts were incubated at 30° in a Unicam Ultraviolet Spectrophotometer U.P. 600. The medium contained 20 μmol K–Na-phosphate buffer pH 6.2 and 120 mg mannitol/ml and Mg^{2+} in the following concentrations: 1, 0.005 M; 2, 0.002 M; 3, 0.001 M; 4, 0.0005 M. Curve 5 represents the results of an experiment in which no magnesium was added. At zero time ribonuclease was added to a final concentration of 40 μg/ml.

To a varying and smaller extent the addition of Mg^{2+} also interferes with the inhibition by ribonuclease of the incorporation of ^{14}C-labelled amino acids into the yeast protoplasts (Fig. 3).

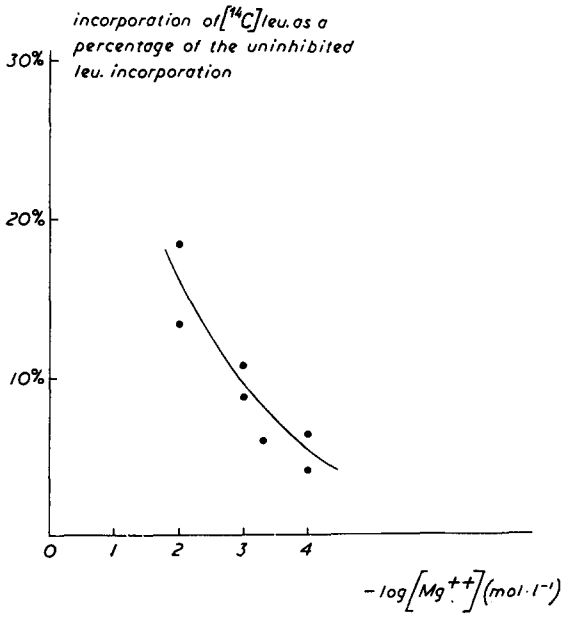

Fig. 3. Effect of Mg^{2+} on the inhibition by ribonuclease of the incorporation of ^{14}C-labelled amino acids. Experimental conditions as described previously[1]. Mg^{2+} was added in concentrations as indicated, before the addition of ribonuclease to a final concentration of 40 μg/ml.

The results from Fig. 3 show that the incorporation of amino acids remains inhibited to a great extent, despite the fact that no lysis of the protoplasts occurred. However, the results varied with different preparations and sometimes the inhibition by RNAase of the amino acid incorporation in the presence of added Mg^{2+} did not exceed 50 %.

At this point attention should be drawn to the fact that the observed inhibition of protein synthesis in protoplasts by RNAase cannot be attributed only to a lysis of the cells. Clearly, this follows from: (a) The observation of a marked (50–90 %) inhibition by RNAase of [^{14}C]amino acid incorporation in the presence of added Mg^{2+}, when no lysis occurs. (b) The observed complete reversal by PMA of RNAase inhibition of both amino acid incorporation and induced α-glucosidase synthesis[2]: this reversal cannot be obtained with lysed protoplasts as these are known not to be able to synthesize proteins[3].

When yeast protoplasts are incubated with ribonuclease, the 260 mμ absorbancy of the cold trichloroacetic acid extract increases, indicating the occurrence of nucleic acid degradation[1]. Immediately, the question rises whether this nucleic acid degradation involves a penetration of ribonuclease into the cell, or whether it occurs merely as a result of the lysis of some cells, after which the nucleic acid becomes accessible to the degrading action of ribonuclease. As a third possibility, a breakdown of RNA at or in the cell membrane should be considered.

In Figs. 4 and 5 the results are presented from determinations of both lysis and nucleic acid degradation when ribonuclease was allowed to act for 10 min on yeast protoplasts before Mg^{2+} was added to a final concentration of 0.005 *M*.

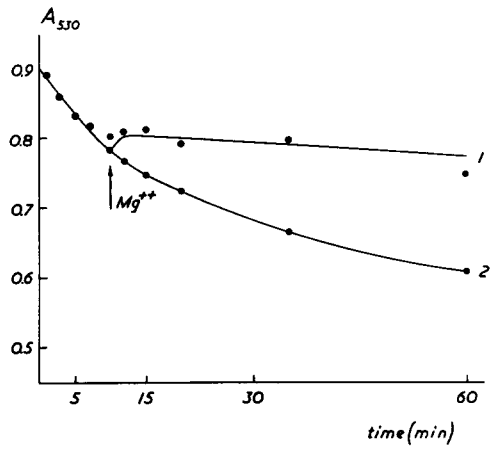

Fig. 4. Cessation of the lysis caused by ribonuclease after the addition of Mg^{2+}. Experimental conditions as described in the legend of Fig. 2 except that ribonuclease (40 μg/ml) was allowed to react for 10 min before Mg^{2+} was added to a final concentration of 0.005 *M* (Curve 1).

The results of Figs. 4 and 5 show that the addition of magnesium ions to the incubation medium results in an immediate cessation of both lysis and nucleic acid degradation. In our opinion, this proves that ribonuclease acts only on the cell membrane and not by penetrating into the yeast protoplast. The observed nucleic

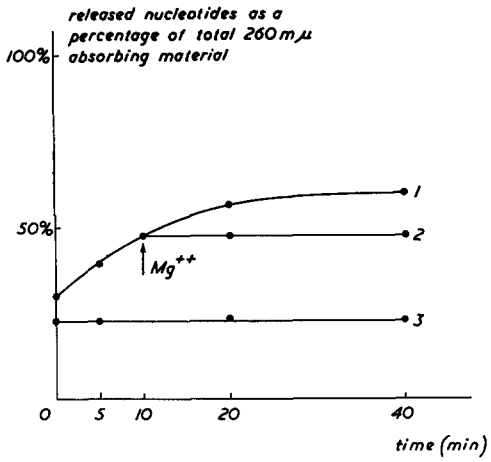

Fig. 5. Effect of Mg^{2+} on the release of cold trichloroacetic acid-soluble 260-mμ-absorbing material as a result of the action of ribonuclease on yeast protoplasts. Protoplasts (4 mg protein/ml) were incubated at 30° in a medium containing 120 mg mannitol and 20 μmol K–Na-phosphate buffer pH 6.2/ml. At zero time ribonuclease was added to a final concentration of 40 μg/ml. 1, without Mg^{2+}; 2, Mg^{2+} added after 10 min (final concentration 0.005 *M*); 3, Mg^{2+}, 0.005 *M*, added at zero time.

acid degradation then would occur either in or at the membrane only and/or after a lysis of the protoplasts by ribonuclease.

The effect of ribonuclease on the respiration and glycolysis of yeast protoplasts

In contradistinction to our former data[1], respiration and glycolysis were found not to be inhibited by ribonuclease, unless the cells were lysed. Lysis of the cells resulted in a complete inhibition of the glucose dissimilation. Table I shows the results of an experiment in which the effect of ribonuclease on protein synthesis was compared with the effect on glucose respiration.

TABLE I

EFFECT OF RIBONUCLEASE ON THE INCORPORATION OF [^{14}C]LEUCINE AS COMPARED WITH THE EFFECT ON RESPIRATION BY YEAST PROTOPLASTS

For respiration measurements, protoplasts (5 mg/ml) were preincubated in Warburg vessels for 10 min at 30° with ribonuclease (40 μg/ml) in a total volume of 1.7 ml containing 12 % mannitol, 20 μmoles K–Na-phosphate buffer pH 6.2 and 1 μmol $MgCl_2$/ml. Then 0.2 ml 12 % mannitol was added containing 10 μmoles/ml glucose and respiration was measured in air. [^{14}C]Leu incorporation was carried out for 30 min and determined as described previously.

	with RNAase	*without RNAase*
Respiration	6.0 μmoles O_2/h	6.6 μmoles O_2/h
Incorporation	138 counts/min/mg protein	832 counts/min/mg protein

The results of Table I show that ribonuclease does not affect the aerobic dissimilation of glucose, whereas leucine incorporation was inhibited for 85 %. Earlier data[1] yielded evidence that the respiration of the protoplasts was also inhibited by ribonuclease. A reinvestigation of this phenomenon revealed, however, that in the experi-

ments described before, probably lysis had occured during the prolonged incubation of the protoplasts with RNAase.

DISCUSSION

The results of our studies on the effect of pancreatic ribonuclease on protein synthesis in yeast protoplasts show that the enzyme causes both a strong inhibition of protein synthesis and a lysis of the protoplasts in the absence of bi- and/or trivalent cations. Both effects have to be attributed at least in part to the enzymic activity of RNAase. The unspecific inhibition of protein synthesis observed at higher concentrations of heat-denatured RNAase and protamine may be caused by the basic properties of these compounds.

As respiration was not affected and as RNAase did not uncouple oxidative phosphorylation[3] at the concentrations used (40 μg/ml or less), it seems probable that the enzyme does not inhibit protein synthesis by an interference with the energy production of the yeast protoplast. This finding is in accordance with that of other authors[4,5].

Since the hydrolytic action of RNAase on ribonucleic acid is the only known enzymic action of the enzyme, the observed lysis of the protoplasts and the inhibition of protein synthesis have to be attributed largely to this action. In an isotonic medium a lysis of the protoplast will occur only when the protoplasmic membrane is affected; therefore, RNAase may act primarily on this membrane.

Of course, the inhibition of protein synthesis by ribonuclease could also be the result of an enzymic hydrolysis of the cytoplasmic ribonucleic acid of the protoplast. Since intact ribosomes are rather resistant against ribonuclease degradation[3,6], this would mean that either the s-RNA, the nuclear RNA and/or some membraneous RNA are degraded. A hydrolysis of the s-RNA and/or the nuclear RNA would involve a penetration of ribonuclease into the yeast protoplast. Several authors[4] have claimed evidence for the occurrence of such a penetration in a variety of tissues and microorganisms. The following observations, however, have led to the assumption that ribonuclease does not penetrate into the yeast protoplast: The addition of polymethacrylic acid results in an immediate resumption of protein synthesis[2]; moreover, the addition of small amounts of Mg^{2+} causes an immediate cessation of the lysis of the protoplasts and of the accompanying ribonucleic acid degradation: these data do not suggest a penetration of the RNAase into the protoplast, for in that case one would expect a lagtime in the effects of both Mg^{2+} and polymethacrylic acid.

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REFERENCES

1 S. R. DE KLOET, R. K. A. VAN WERMESKERKEN AND V. V. KONINGSBERGER, *Biochim. Biophys. Acta*, 47 (1961) 138.
2 S. R. DE KLOET, R. K. A. VAN WERMESKERKEN AND V. V. KONINGSBERGER, *Biochim. Biophys. Acta*, 47 (1961) 144.
3 S. R. DE KLOET, unpublished.
4 J. BRACHET, *Biochemical Cytology*, Academic Press, New York, 1957, p. 264.
5 M. BELJANSKI, *Biochim. Biophys. Acta*, 15 (1954) 425.
6 H. T. SHIGEURA AND E. CHARGAFF, *Biochim. Biophys. Acta*, 37 (1960) 347.