

## STUDIES ON PROTEIN SYNTHESIS BY PROTOPLASTS OF *SACCHAROMYCES CARLSBERGENSIS*

### I. THE EFFECT OF RIBONUCLEASE ON PROTEIN SYNTHESIS

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#### SUMMARY

Ribonuclease was found to inhibit the protein synthesis in the naked yeast protoplast for nearly 100 %. Even small concentrations (5  $\mu\text{g/ml}$ ) were found inhibitory.

The cause of this inhibition can be attributed at least in part to a 90 % inhibition of the respiration. Amino acid uptake was found to be inhibited for 75 %, and glycolysis for 50 %.

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#### INTRODUCTION

Inhibition of protein synthesis by RNase has been reported in a number of cases<sup>1-4</sup>. For example, the amino acid incorporation into protein by the rabbit-liver pH 5 enzyme microsome system<sup>1</sup>, as well as protein synthesis by protoplasts of *B. megatherium* is inhibited<sup>2</sup>.

During the course of our studies on protein synthesis by yeast, we observed that protein synthesis by naked protoplasts is also strongly inhibited by RNase. In view of current theories according to which ribonucleic acid would be involved in protein synthesis<sup>5-7</sup>, we started studying the mechanism by which RNase interacts with our system.

As a measure of protein synthesis, we used both the incorporation of [<sup>14</sup>C]amino acids and the induced synthesis of the enzyme  $\alpha$ -glucosidase.

In this paper, some results of our work will be presented.

#### MATERIALS AND METHODS

The yeast was a strain of *Saccharomyces Carlsbergensis*. A culture medium was used, containing 2 g  $\text{KH}_2\text{PO}_4$ ; 6 g  $(\text{NH}_4)_2\text{SO}_4$ ; 0.25 g  $\text{MgSO}_4$ ; 0.25 g  $\text{CaCl}_2$ ; 5 g Bacto pepton (Difco Laboratories, Detroit, U.S.A.); 2.5 g yeast extract (Koninklijke Gist-en Spiritusfabrieken, Delft); 6 ml 60 % Na lactate and 40 g glucose/l. Yeast was grown overnight at  $\pm 27^\circ$  by inoculating 1 l medium with 0.4 ml of a 24-h old culture.

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Abbreviations: RNA, ribonucleic acid; s-RNA, ribonucleic acid from the soluble (100,000  $\times$  g) fraction of the cell; m-RNA, ribonucleic acid from the microsomal particulate fraction; RNase, ribonuclease; ATP, adenosine triphosphate; TCA, trichloroacetic acid; PNPG, *p*-nitrophenyl- $\alpha$ -D-glucoside.

RNase was a  $5 \times$  recrystallized product of Sigma Chemical Company (St. Louis, U.S.A.). The snail enzyme hemicellulase was prepared from the intestinal tract of *Helix Pomatia*.

The contents of the intestinal tract were isolated and centrifuged in the cold for 10 min at  $10,000 \times g$ . The supernatant was carefully dialysed against 20 volumes of water at  $2^\circ$ . The contents of the dialysis bag were centrifuged at  $10,000 \times g$  for 10 min and lyophilized. The dry powder was stable for at least half a year when stored in the refrigerator at  $-15^\circ$ . It contained the enzymes necessary for breaking down the yeast cell wall. Radio active amino acids were a product of Volk Radiochemical Co. (Chicago, U.S.A.).

PNPG was prepared according to AIZAWA<sup>8</sup>. The crystalline product melted at  $214^\circ$ . Maltose was a product of Kerfoot.

The yeast cells were collected from the culture medium by centrifugation and washed twice with distilled water. Protoplasts were prepared according to EDDY<sup>9</sup>. After being suspended in 10 ml 10 % mannitol containing 0.02 *M* citrate-phosphate buffer pH 5.7 and 50 mg snail enzyme, the cells were incubated at  $30^\circ$  with occasional gentle shaking. The disappearance of the cell walls was controlled with a phase contrast microscope. Usually all cells were converted into protoplasts after 1 h incubation with snail enzyme, as could be proved by osmotic lysis. After another washing by centrifugation with 10 % mannitol in 0.02 *M* citrate-phosphate buffer pH 5.7 the protoplasts were ready for use.

Radio active protein samples were prepared by precipitating and washing with cold and hot TCA, alcohol and ether according to SIEKEVITZ<sup>10</sup>. Radio activity was determined on aluminum platelets with a Philips electronic counter type PW 4032.

The activity of induced  $\alpha$ -glucosidase was determined according to HALVORSON AND ELLIAS<sup>11</sup>, using maltose as an inductor and the rate of splitting of the chromogenic substrate *p*-nitrophenyl- $\alpha$ -D-glucoside as a measure for  $\alpha$ -glucosidase activity. The assay mixture contained 2.7 ml 1/15 *M* Sørensen phosphate buffer pH 6.8, 0.1 ml 0.3 % PNPG solution and 0.1 ml 0.1 % reduced glutathion. 0.1 ml of the medium containing the enzyme to be tested was added: after 5 min incubation at  $30^\circ$ , the reaction was stopped by adding 0.5 ml 1 *M*  $\text{Na}_2\text{CO}_3$ . The extinction at 400  $\text{m}\mu$  was read in a Unicam spectrophotometer SP 600.

The protein contents were assayed according to LOWRY<sup>12</sup>. The enzymic activity was expressed as units of extinction per milligram protein/min.

Respiration and glycolysis were measured in a Warburg apparatus at  $30^\circ$ , using either air or nitrogen as the gas phase.

#### EXPERIMENTAL RESULTS

##### *Inhibition by RNase of the incorporation of [ $^{14}\text{C}$ ]amino acids by protoplasts of Sacch. Carlsbergensis*

When whole cells of *Saccharomyces Carlsbergensis* are pre-incubated with ribonuclease, protein synthesis, as determined by the incorporation of [ $^{14}\text{C}$ ]amino acids, proceeds unimpaired. However, the amino acid incorporation by naked protoplasts of the same organism is strongly inhibited after a similar treatment with RNase (Table I).

TABLE I

INCORPORATION OF [ $^{14}$ C]AMINO ACIDS BY RNase TREATED WHOLE CELLS AND PROTOPLASTS

The incubation medium contained 100 mg mannitol; 10 mg glucose; 50  $\mu$ moles K-Na-phosphate buffer pH 6.8; 0.075  $\mu$ mole [ $^{14}$ C]leucine containing 20,000 counts/min in a total volume of 1 ml.

After 15 min incubation at 30° the reaction was stopped by adding 1 ml 10 % TCA.

	+ 100 $\mu$ g RNase	— RNase
Whole cells 2 mg	2200 counts/mg/min	
Whole cells 2 mg		2000 counts/mg/min
Naked protoplasts 2 mg	10 counts/mg/min	
Naked protoplasts 2 mg		1200 counts/mg/min

TABLE II

EFFECT OF SMALL AMOUNTS OF RNase ON THE INCORPORATION OF AMINO ACIDS BY PROTOPLASTS

The experimental conditions were the same as described in Table I.

RNase concn. ( $\mu$ g/ml)	Counts/mg/min
100	2
50	5
25	16
10	18
5	39
0	527

Even small amounts of ribonuclease can inhibit protein synthesis by the protoplast (Table II).

Repeated washings of the protoplasts after a 30-min incubation with 100  $\mu$ g RNase/ml did not change the inhibiting effect. After three washings with 10 ml 10 % mannitol pH 5.8, RNase treated protoplasts did not incorporate appreciable amounts of radio activity in their protein.

#### *Inhibition by RNase of the induced synthesis of $\alpha$ -glucosidase by Sacch. Carlsbergensis*

Adaptive enzyme synthesis was equally inhibited by pre-incubation of the protoplasts with RNase (Fig. 1).

#### *Studies on the mechanism of RNase action on protein synthesis by protoplasts of Sacch. Carlsbergensis*

In a trial to analyse the effect of RNase on the protoplasmic synthesis of proteins, it could be shown that the protoplasts loses an appreciable amount of 260  $m\mu$  absorbing material (Fig. 2) during incubation with the enzyme.

It can be seen, that after a quick diffusion to the medium of about 15 % of the 260- $m\mu$  absorbing material originally present, the rate of diffusion becomes slower. Owing to lysis of the protoplasts after a prolonged incubation with RNase at 30°, it was not possible to determine the upper part of the curve any further. From these data it seems likely, that some ribonucleic acid compound inside the protoplast is broken down.

It is possible that RNase would exert some indirect action besides the direct action on the RNA of the cell. Therefore, measurements were performed of amino acid permeation, respiration and glycolysis.

After incubation with [ $^{14}\text{C}$ ]amino acids it was observed that the quantity of amino acid label present in the cold TCA extract of the protoplasts was at least 75 % less in RNase treated samples as compared with the untreated ones (Table III).

The results of Table III show that the uptake of the amino acid through the

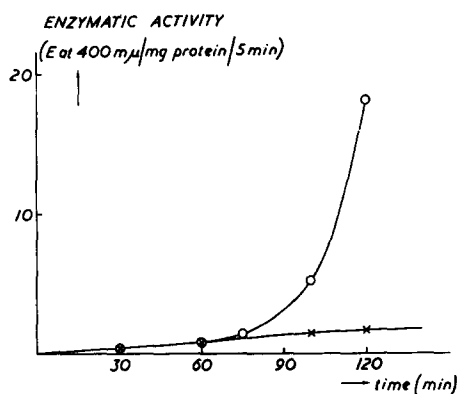


Fig. 1. Inhibition by RNase of the induced  $\alpha$ -glucosidase synthesis by protoplasts of *Sacch. Carlsbergensis*. The incubation medium contained 1 g mannitol, 0.16 mmole glucose, 0.9 mmole maltose, 100 mg casein hydrolysate, 500  $\mu$ moles citrate-phosphate buffer pH 4.7 and 20 mg protoplasmic protein in a total volume of 10 ml. Cells were incubated in 50-ml Erlenmeyer flasks with shaking at 30°. At appropriate times 2-ml samples were withdrawn, cooled to 0° and centrifuged. Cells were lysed by adding 1 ml distilled water and frozen. After thawing and centrifugation  $\alpha$ -glucosidase activity in the supernatant was determined as described under MATERIALS AND METHODS.

×—×, + 100  $\mu$ g RNase; ○—○, — RNase.

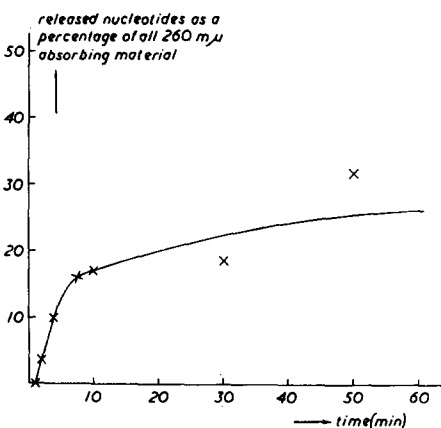


Fig. 2. The diffusion of 260- $\mu$  absorbing material from RNase treated protoplasts to the incubation medium. Protoplasts (3-4 mg/ml) were incubated with 100  $\mu$ g RNase/ml at 30° in 10 % mannitol pH 6.8. At appropriate times samples were removed, cooled till 0° and centrifuged. The 260- $\mu$  extinction was determined in the supernatant and corrected for the blank value, obtained from protoplasts without the addition of RNase.

TABLE III

THE EFFECT OF RNASE ON THE UPTAKE OF [ $^{14}\text{C}$ ]AMINO ACIDS  
BY PROTOPLASTS OF *Sacch. Carlsbergensis*

Protoplasts were incubated at 30° with 100  $\mu$ g RNase /ml; after 10 min, 0.075 mole [ $^{14}\text{C}$ ]leucine containing 20,000 counts/min were added to the medium. After 15 min the protoplasts were spun down at 2° and washed with 10 % mannitol containing 0.1  $\mu$ mole unlabelled leucine/ml. Protein was precipitated by adding 3 ml 5 % TCA to the sedimented protoplasts. The samples were respun and the protein was washed and counted as described previously. The cold TCA soluble extract was extracted with ether to remove the TCA and was counted after drying.

	+ 100 $\mu$ g RNase	— RNase	Zero time value
Cold TCA			
extract	170 counts/min/mg	624 counts/min/mg	4 counts/min/mg
Protein	80 counts/min/mg	4220 counts/min/mg	—

protoplasmic membrane is inhibited appreciably under conditions where RNase inhibited protein synthesis for nearly 100 %.

Furthermore glucose metabolism by the protoplasts is also considerably inhibited by RNase (Figs. 3, 4).

It can be concluded that under the experimental conditions RNase inhibits respiration of naked protoplasts of yeast for nearly 90 %, whereas glycolysis was inhibited for about 50 %.

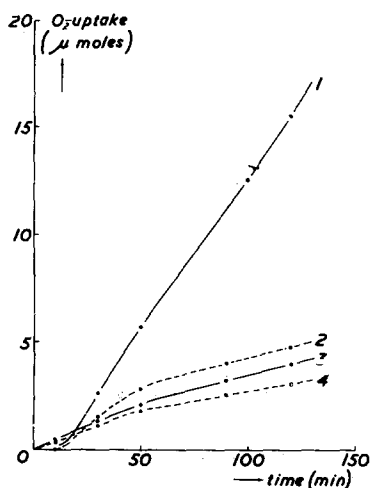


Fig. 3. The effect of RNase on glucose respiration by protoplasts of *Sacch. Carlsbergensis*. Protoplasts (5 mg protein/ml) were preincubated in Warburg vessels with 100  $\mu$ g RNase/ml in 1.7 ml 10 % mannitol containing 50  $\mu$ moles K-Na-phosphate buffer pH 6.8/ml. After 30 min at 30°, 0.2 ml 10 % mannitol containing 50  $\mu$ moles glucose/ml was added from the side arm and respiration was measured in air. 1, + glucose — RNase; 2, + glucose + RNase; 3, — endogenous — RNase; 4, endogenous + RNase.

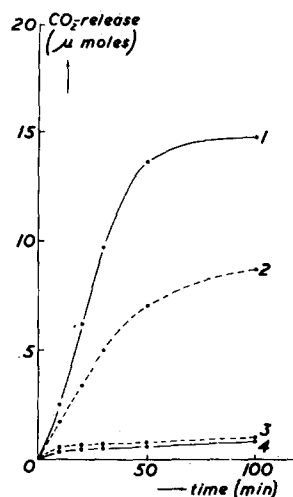


Fig. 4. The effect of RNase on glycolysis in protoplasts of *Sacch. Carlsbergensis*. Medium and circumstances as in Fig. 3. Measurements were done in a nitrogen atmosphere. 1, + glucose — RNase; 2, + glucose + RNase; 3, endogenous — RNase; 4, endogenous + RNase.

#### DISCUSSION

Recently, many data have been presented on the inhibition by RNase<sup>1-4</sup> of protein synthesis in various organisms and organs. It has been suggested that this inhibition should be attributed to the breakdown of RNA which is known to be involved in the synthesis of proteins.

In the present investigation it is shown that the action of RNase on the yeast protoplast inhibits protein synthesis in at least two ways.

1. By inhibition of energy yielding processes such as aerobic and anaerobic glucose dissimilation. The cause of this inhibition remains unclear. It is a wellknown fact that RNase can act as an uncoupler of oxidative phosphorylation in intact mitochondria<sup>13</sup>. Consequently, it interferes in the energy metabolism of the cell. It is unlikely that glucose permeation into the protoplast is inhibited to such an extent, that this would account for the total inhibition of respiration. Furthermore the endogeneous respiration is also slightly inhibited.

Yet it is likely, that an interference in the aerobic glucose dissimilation will result in an inhibition of protein synthesis simply by a lack of ATP, which is known to be necessary for amino acid activation<sup>14, 15</sup>.

2. By degrading part of the intracellular RNA. BRACHET *et al.*<sup>4</sup> reported a selective degradation of the s-RNA of onion root cells upon incubation with RNase. Experiments carried out with yeast protoplasts to elucidate whether also in this case a selective degradation of the s-RNA could be observed were inconclusive. The quick release of 260-m $\mu$  absorbing material to an amount of 15 to 20 % of the total contents of 260 m $\mu$  absorbing substances within the cell, followed by a much slower release is, however, suggestive for a selective degradation of an RNA fraction which is more easily accessible to RNase hydrolysis than the remaining RNA.

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