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CHARACTERIZATION OF MESSENGER RNA IN PROTOPLASTS OF *SACCHAROMYCES CARLSBERGENSIS*

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SUMMARY

(1) The pulse-labeled RNA extracted from enriched polysomal fractions of *Saccharomyces carlsbergensis* has been characterized by sedimentation analysis, by the effect of actinomycin D on the observed sedimentation patterns, by its sensitivity to ribonuclease action and by determinations of its base composition. It is concluded that this pulse-labeled RNA shows many of the properties which in general are ascribed to messenger RNA.

(2) It is shown, that polysomes which are charged with nascent α-glucosidase can be precipitated rather specifically with the aid of a purified rabbit anti-α-glucosidase γ-globulin fraction and a purified anti-rabbit γ-globulin antiserum from the goat.

(3) The pulse-labeled RNA extracted from the precipitated polysomes charged with nascent α-glucosidase has a sucrose gradient sedimentation pattern which is quite distinct from that of the pulse-labeled RNA from the total polysomal fraction.

(4) From this pattern and from the patterns obtained in pulse-double-labeling experiments with maltose-induced and non-induced protoplasts, it is tentatively concluded that the messenger RNA coding for the inducible enzyme α-glucosidase has a sedimentation coefficient of about 28 S.

INTRODUCTION

The messenger RNA occurring in cells of various yeast strains has been characterized by a number of authors[1–3]. Protein synthesis by protoplasts of *S. carlsbergensis* and, in particular, the regulatory mechanism involved in inducible α-glucosidase synthesis by these protoplasts have been investigated by our group for a number of years[4]. Obviously, one aspect of such studies should concern the characterization of total messenger RNA and, in particular, of the RNA coding for the enzyme α-glucosidase.

In the first part of this paper, the results are presented of experiments which were performed to characterize the pulse-labeled RNA extracted from enriched polysomal preparations as messenger RNA.

Abbreviations: PNPG, *p*-nitrophenyl-α-D-glucoside; PPO, 2,5-diphenyloxazole; POPOP, 2-*p*-phenylene-bis(5-phenyloxazole).

A polysomal fraction was chosen as a favorable source to isolate messenger RNA from the protoplasts since:

(1) it was shown to be the most active fraction in subcellular protein synthesis[5], including that of inducible α-glucosidase[6],

(2) if whole protoplasts or isolated nuclei are chosen as the source, precursor ribosomal RNA and transfer RNA are also pulse-labeled[7,8],

(3) from the 100 000 × *g* supernatant fraction only transfer RNA can be isolated[8].

In the second part of this paper, a technique is described which may lead to a preliminary characterization of the messenger RNA coding for the inducible enzyme α-glucosidase.

MATERIALS AND METHODS

Materials

All chemicals used were analytical grade.

Bactopeptone was obtained from Difco Laboratories (Detroit, U.S.A.) and yeast extract was the product of Koninklijke Gist- en Spiritusfabrieken (Delft). For the conversion of yeast cells into protoplasts, an enzyme preparation was used from the intestinal tract of the snail *Helix pomatia*: it was obtained from L'Industrie Biologique Française (Genevilliers, France). Glucose and GSH were purchased from British Drug House Ltd. (Poole, Dorset, England), maltose from Fluka A.G. (Buchs, Switzerland). Casaminoacids were produced by Difco Laboratories (Detroit, Mich., U.S.A.). PNPG was prepared essentially according to AIZAWA[9]. Ribonuclease (2 × recrystallized) was the product of Nutritional Biochemical Corporation (Cleveland, Ohio, U.S.A.): 2′,3′-nucleoside monophosphates were purchased from Pabst Laboratories (Milwaukee, Wisc., U.S.A.). ^{14}C-labeled purines and pyrimidines were obtained from The Radiochemical Centre (Amersham, England) and ^{32}P-labeled sodium orthophosphate from Philips-Duphar (Petten, The Netherlands). PPO was purchased from Merck (Darmstadt, Germany) and POPOP from Nuclear Chicago Corporation (Des Plaines, Ill., U.S.A.). Sephadex G-200 was the product of Pharmacia (Uppsala, Sweden).

The actinomycin D was a generous gift of Merck, Sharp and Dohme (Rahway, N.J., U.S.A.).

Yeast culturing and the preparation of the protoplasts

Cells of *S. carlsbergensis* (strain 74, N.C.Y.C., England) were grown aerobically at 27° in a medium containing 40 g glucose, 5 g bactopeptone, 2.5 g yeast extract, 6 ml 60 % sodium lactate, 0.25 g $CaCl_2$, 0.25 g $MgSO_4 \cdot 7\ H_2O$, 2.0 g KH_2PO_4 and 6 g $(NH_4)_2SO_4$ per l (ref. 10). The cells were harvested during the later phase (after 15 h) of the logarithmic growth period and converted into protoplasts according to the method of EDDY AND WILLIAMSON[11]. Following their collection from the culture medium by centrifugation, the cells were washed twice with distilled water; they were incubated at 30° under continuous gentle shaking in a 12 % mannitol solution, containing 20 mmoles sodium citrate–phosphate buffer (pH 5.8) and 1–2 ampullae

of snail enzyme per l of yeast culture. The conversion of the cells into protoplasts was followed by observation with a phase contrast microscope of a small sample of the suspension diluted with water; usually, lysis was complete after 60–90 min. Subsequently, the protoplasts were centrifuged off and washed twice with 12 % mannitol. In a suspension in 12 % mannitol, the protoplasts could be stored at 2° for 2–3 days without losing their synthetic activities.

The induction of α-glucosidase synthesis

The induction of α-glucosidase synthesis was performed at 30° as has been described before[10], by incubating a suspension of protoplasts (0.5 mg protein/ml, $A_{500\ m\mu} = 1.5$) in a medium containing 3 mg glucose, 30 mg maltose, 10 mg casein-hydrolysate, 50 μmoles sodium–potassium phosphate buffer (pH 6.2) and 120 mg mannitol per ml. At appropriate times, samples were removed and lysed in ice-cold distilled water. After centrifugation of the lysate at 3000 × *g* and the subsequent removal of the debris, the α-glucosidase activity was determined as described before[10]; the samples were incubated at 30° in 2 ml containing 0.6 mg of the chromogenic substrate PNPG, 0.2 mg GSH and 200 μmoles sodium–potassium phosphate buffer (pH 6.8). The mixture was incubated for varying times, depending on the α-glucosidase activity being determined. Subsequently, after the addition of 1 ml 1 M Na_2CO_3, the absorbance at 400 mμ, due to the *p*-nitrophenol formed, was measured by means of a Unicam spectrophotometer SP 600, using a sample of PNPG without added enzyme as a blank. The specific α-glucosidase activity is defined as mμmoles of PNPG split per min per mg protein, the molar extinction coefficient of *p*-nitrophenol at pH 10 and at 400 mμ being $18 \cdot 10^3\ M^{-1}\ cm^{-1}$.

Fractionation procedures

Preparation of "enriched" polysomal fractions: It has been shown before[5] that the 100 000 × *g* sediment of the yeast protoplasts is active in subcellular protein synthesis; however, when subjected to ultracentrifugation through a linear (20–40 %) sucrose gradient the same fraction proved to consist for more than 90 % of free 80-S ribosomes (Fig. 1A).

As it was to be expected that most of the messenger RNA is associated with polysomes, an "enriched" polysomal fraction was isolated by ultracentrifugation through a layer of 40 % sucrose as follows:

Unless stated otherwise, all manipulations during fractionation were carried out at 4°. After collecting by centrifugation, the protoplasts were lysed in a 0.01 M sodium–potassium phosphate buffer (pH 6.0) containing 2 μmoles of $MgCl_2$ and 1 mg per ml of sodium bentonite. The lysate was centrifuged for 30 min at 13 000 × *g* in a PR International refrigerated centrifuge. The sediment, containing nuclei, mitochondria and cell debris was discarded. Subsequently, each 6 ml of the 13 000 × *g* supernatant was layered on 15 ml of a 40 % sucrose solution, prepared in 0.01 M sodium–potassium phosphate buffer (pH 6.0) being 0.002 M in $MgCl_2$ and centrifuged for 1 h at 100 000 × *g* in a 30 rotor of a Spinco model L ultracentrifuge. The sediment – the "enriched" polysomal fraction – was gently resuspended in 0.05 M Tris–HCl buffer (pH 7.4); in the immunological experiments, the polysomal suspension was centrifuged at 3000 × *g* during 15 min to remove any aggregated particles.

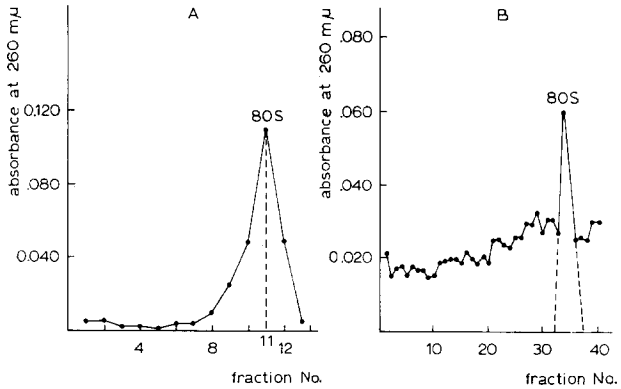


Fig. 1. A. Sucrose gradient ultracentrifugation of a ribosomal preparation (100 000 × *g* sediment) from protoplasts of *S. carlsbergensis*. Gradient ultracentrifugation (20–40 % sucrose in 0.01 M sodium–potassium phosphate buffer (pH 6.0) being 0.002 M in $MgCl_2$) was performed in a Spinco SW 39 rotor for 0.5 h at 39 000 rev./min. B. Sucrose gradient ultracentrifugation of the "enriched" polysomal fraction isolated as described in METHODS. Gradient ultracentrifugation (20–40 % sucrose in 0.01 M sodium–potassium phosphate buffer (pH 6.0) being 0.002 M in $MgCl_2$) was performed in a Spinco SW 25 rotor for 2.5 h at 25 000 rev./min.

From the pattern obtained after ultracentrifugation through a linear (20–40 %) sucrose gradient of the enriched polysomal fraction, it can be concluded that it consists for at least 50 % of polyribosomes (Fig. 1B). This conclusion was confirmed by the observation that the heavier material disappears and sediments with the 80-S peak after a treatment with ribonuclease.

Extraction of RNA

To the polysomal suspension sufficient of a solution, containing 5 % sodium dodecyl sulfate and 0.5 % naphthalene-1,5-disulfonic acid (sodium salt, adjusted to pH 7.4), was added to yield a final concentration of 0.5 % sodium dodecyl sulfate. Subsequently, the suspension was extracted with an equal volume of phenol reagent (90 % aqueous phenol containing 0.1 % 8-hydroxyquinoline) for 15 min at 4° by gentle shaking in a glass-stoppered flask. After centrifugation for 10 min at 3000 × *g*, the aqueous phase was separated; the phenol layer was re-extracted with one half its volume of 0.05 M Tris–HCl buffer (pH 7.4) and after centrifugation, the aqueous phase was pipetted off. To the combined aqueous phases sodium acetate was added to a final concentration of 2 % and, subsequently, cold (−20°) ethanol up to a concentration of 70 %. The RNA precipitated was collected by centrifugation and redissolved in 0.01 M sodium acetate buffer (pH 5.2) being 0.1 M in NaCl. Subsequently, it was reprecipitated with ethanol as described above and redissolved in an appropriate buffer, depending on the kind of experiment to be performed.

Examination of RNA by sucrose gradient centrifugation

0.4 ml of a solution containing approx. 0.4 mg of RNA dissolved in gradient buffer (0.01 M sodium–potassium phosphate (pH 6.8), 0.02 M KCl and 0.001 M EDTA)

was layered on top of 5 ml of a linear gradient of 5–20 % sucrose dissolved in the same buffer. The tubes were centrifuged in a Spinco SW 39 swinging-bucket rotor for 4 h at 40 000 rev./min. Subsequently, the bottom of the tubes was punctured and 10-drop fractions (about 0.2 ml each) were collected. The absorbance at 260 mμ of the fractions was read in a Unicam Spectrophotometer SP 600 after dilution with 2 ml water. The radioactivity of the fractions was determined routinely by precipitating the RNA with trichloroacetic acid, collecting the precipitates on millipore filters (0.45 μ) and washing of the precipitates on the filters with cold trichloroacetic acid and ethanol respectively.

Subsequently, the filters were dried and the radioactivity determined in a toluene–PPO–POPOP solution (1 l toluene containing 5 g PPO and 0.1 g POPOP) with the aid of a Nuclear Chicago Mark II liquid scintillation counter.

Determination of base ratios

The ^{32}P-labeled RNA was dried by lyophilization and subsequently hydrolyzed in 1 ml 0.33 M KOH during 18 h at 37°. The hydrolysate was adjusted to pH 4 with a 12 % $HClO_4$ solution at 4°. The $KClO_4$ precipitate was removed by centrifugation, the supernatant dried by lyophilization and the residue redissolved in 0.2 ml H_2O; subsequently, all four 2′,3′-ribonucleoside monophosphates were added as markers. A suitable aliquot was subjected to paper electrophoresis on Whatman No. 3 MM paper in 0.4 M sodium acetate buffer (pH 3.7) for 2 h at a constant current of 50 mA (about 50 V/cm). It appeared, that a good separation of all four nucleotides could be obtained after a single electrophoretic run; also, in most cases, the 2′- and the 3′-monophosphates of guanosine were nicely separated. After drying, the nucleotide spots on the paper were made visible with the aid of a Mineralight short wave (200–280 mμ) transilluminator (Ultraviolet Products, Inc., San Gabriel, California, U.S.A.), marked and cut out; the radioactivity of each nucleotide spot was determined in a Nuclear Chicago Mark II liquid scintillation counter.

Partial purification of inducible α-glucosidase

A 500-ml portion of yeast protoplasts was induced with maltose during 4 h at 30° as has been described above; it appeared, that after this period the rate of induced enzyme synthesis starts to decrease. After collection by centrifugation, the protoplasts were lysed in a 0.001 M potassium phosphate (pH 7.2) buffer solution. The lysate was centrifuged for 10 min at 13 000 × *g* in a 296 rotor of a PR International refrigerated centrifuge.

From the resulting 13 000 × *g* supernatant, α-glucosidase was partly purified by the procedure outlined in Fig. 2 and elaborated by Miss E. VAN HEE from our group[12]. As can be seen from Fig. 2, this procedure involves the removal of contaminating protein by isoelectric precipitation at pH 5.2, a fractionation with dilute (1.5 %) phenol, isoelectric precipitation at pH 4.8 and a further fractionation by Sephadex G-200 gel filtration.

As can be concluded from the data in Table I, the fractionation procedure leads to a 15-fold purification of α-glucosidase with respect to the 13 000 × *g* supernatant fraction.

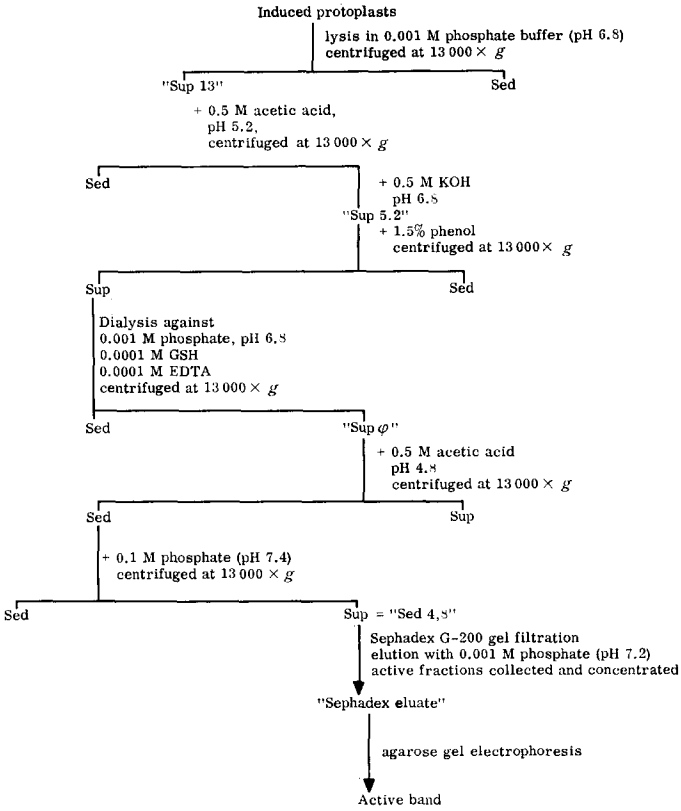


Fig. 2. Schematic outline of the procedure used for the partial purification of the inducible enzyme α-glucosidase from protoplasts of *S. carlsbergensis*.

From the preparation obtained after Sephadex G-200 gel filtration, 0.01-ml samples were subjected to agarose gel electrophoresis on a microscope slide (7.6 cm × 2.6 cm), carrying 1 % agarose gel in a 1/15 M potassium phosphate buffer solution (pH 7.4). Electrophoresis was performed according to WIEME AND RABAEY[13] for 2–3 h

TABLE I

YIELD AND SPECIFIC α-GLUCOSIDASE ACTIVITIES OF THE FRACTIONS OBTAINED DURING THE PARTIAL PURIFICATION OF THE ENZYME AS DESCRIBED IN THE TEXT AND OUTLINED IN FIG. 2.

U, unit of α-glucosidase activity, *i.e.* the amount of enzyme catalyzing the hydrolysis of one mμmole PNPG per min under the experimental conditions of the test (see METHODS).

Fraction	*Total protein (mg)*	*Specific activity (U/mg protein)*	*Total activity (U total)*	*Yield (% of total units in sup. 13)*
Sup. 13	2250	880	1 980 000	100
Sup. 5,2	750	1760	1 320 000	67
Sup. φ	288	3850	1 108 800	56
Sed. 4,8	120	6600	792 000	40
Sephadex eluate	24	13200	316 800	16

at a constant current of 30 mA. After drying, fixation and staining of one half of the agarose gel with amido black, usually 3–4 protein bands appeared; by cutting out 1-mm strips from the other half of the gel and a subsequent determination of α-glucosidase activity in the strips[6], it could be shown that all of this activity is present in the band marked with an arrow (Fig. 3A).

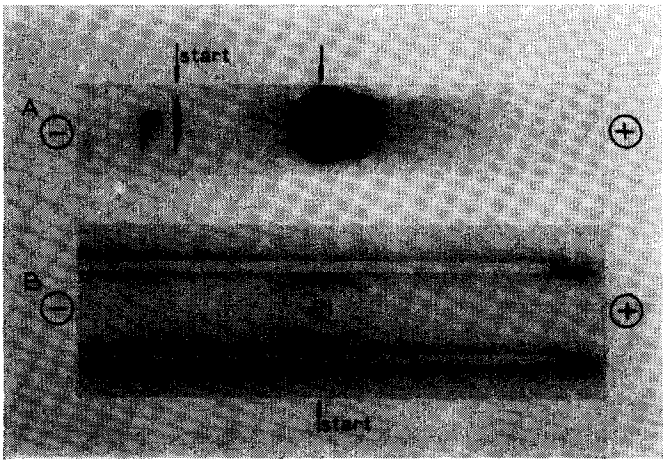


Fig. 3. A. Agarose gel micro-electrophoresis of a partly purified α-glucosidase preparation (Sephadex eluate, prepared as described in METHODS), in a 1/15 M potassium phosphate buffer (pH 7.4). The α-glucosidase activity was shown to be present in the band marked with an arrow. B. Agar-gel immunoelectrophoresis of a similarly purified α-glucosidase preparation in 0.05 M veronal buffer (pH 8.3). The rabbit anti-α-glucosidase antiserum used was obtained after immunization with a homogenate of the bands containing the enzyme as indicated in Fig. 3A. Experimental conditions and procedures as described in the text.

When electrophoresis was carried out during 60–90 min only, one more rather heavy amido black-positive band appeared, with a high mobility towards the anode; after prolonged electrophoresis (2–3 h, Fig. 3A) this band is no longer found. From the results obtained upon electrophoresis during 1–1.5 h, the purity of the α-glucosidase in the Sephadex eluates could be roughly estimated to be 10–20 %.

Eight of the bands containing the α-glucosidase activity were cut out and used as a homogenate to prepare the antiserum directed against purified α-glucosidase, from two rabbits.

Antisera

The purified γ-globulin fraction of the rabbit anti-α-glucosidase antiserum, the goat anti-rabbit γ-globulin antiserum and the purified γ-globulin fraction of this serum, were kindly prepared for us by Dr. R. E. P. A. BALLIEUX (Stichting Academisch Ziekenhuis, Utrecht, The Netherlands).

Antiserum against α-glucosidase was prepared by immunization of rabbits: this was done by subcutaneous administration of the homogenate of agarose gel with purified α-glucosidase in Freund Adjuvant Complete (Difco Laboratories, Detroit, Mich.). After six weeks, the rabbits were bled. The γ-globulin fractions were

obtained from this serum and from the goat anti-rabbit γ-globulin antiserum by means of the Rivanol–$(NH_4)_2SO_4$ procedure[14]; they were dialyzed against distilled water and lyophilized.

Immunodiffusion and immunoelectrophoresis

Immunodiffusion experiments were performed according to the method reported by MANSI[15]. Thin agar layers (about 1.5 mm) of 1 % agar in 0.01 M phosphate buffer (pH 7.0) were prepared by pipetting the hot agar solution on glass plates of 76 mm × 26 mm. After cooling, small holes were drilled in the agar and these were filled with the antigen and antibody solutions, respectively. The plates were kept in a water-saturated atmosphere for 24 h. During this period, precipitation lines consisting of the antigen–antibody complex are formed. After drying and fixation, the precipitated proteins were colored with amido-black.

For immunoelectrophoresis again the technique described by WIEME AND RABAEY[13] was used. Both agar and agarose gels were prepared as described above. Electrophoresis of solutions of partly purified α-glucosidase (Sephadex eluate) was carried out for 120 min at 25 mA in 0.05 M veronal buffer (pH 8.3) or in 1/15 M phosphate buffer (pH 7.4). The temperature was kept at about 13°. Subsequently, two small troughs were cut in the agar, parallel to the direction of electrophoresis; these were filled with the solution of purified anti-α-glucosidase rabbit γ-globulin fraction. Precipitation lines were allowed to form during 24 h in a water-saturated atmosphere; after washing, drying and fixation they were colored by means of amido black. A typical example of the results obtained upon immunoelectrophoresis of partly purified α-glucosidase (Sephadex eluate) in veronal buffer (pH 8.3) is presented in Fig. 3B. Clearly, only one precipitation line is formed with the antiserum from rabbits that were immunized with the bands in the agarose gel containing the enzymatic activity. It may be worth mentioning, that 4–5 precipitation lines were formed with antiserum from rabbits that were immunized with the total Sephadex eluate fraction. In all experiments reported in the second part of this paper, the purified γ-globuline fraction was used, forming one precipitation line upon immunoelectrophoresis with Sephadex eluate preparations.

RESULTS AND DISCUSSION

The experiments to be reported were designed for two purposes. The first was to show that most of the pulse-labeled RNA isolated from the enriched polysome fraction represents messenger RNA; four criteria for this which will be discussed are as follows: (a) the radioactivity patterns obtained after sucrose gradient ultracentrifugation, (b) the effect of actinomycin D on these patterns, (c) the extremely high sensitivity to the action of ribonuclease, (d) the base composition.

The second purpose of these experiments was to isolate a messenger RNA which might be specific for an inducible enzyme. By immunological techniques a fraction of the messenger RNA associated with polysomes presumably synthesizing α-glucosidase has been isolated and partially characterized.

Sedimentation analysis of [^{14}C]uracil pulse-labeled RNA

The molecular size of the pulse-labeled RNA from the "enriched" polysomal fractions was investigated by determining its distribution upon ultracentrifugation through a linear gradient of 5–20 % sucrose.

In Figs. 4A–D, the absorbance and radioactivity patterns are shown of the RNA extracted from polysomal fractions obtained from protoplasts that had been incubated with [^{14}C]uracil during varying lengths of time. In a large number of introductory experiments it was found, that the first (lower) fractions of the gradient contained little radioactivity, showing the same pattern as the absorbance. Therefore, in all further experiments the determination of radioactivity was started in Fraction 6. The distribution of total RNA through the gradient as determined by the absorbance at 260 mμ shows the two peaks (18 S and 28 S) which are characteristic for ribosomal RNA, as well as a small 4–5-S RNA peak.

The distribution of the radioactive RNA isolated after 3, 6 and 10 min pulse of [^{14}C]uracil clearly indicates its heterogeneity with respect to molecular weight, which is quite distinct from that of the bulk ribosomal RNA. As can be seen from Figs. 4A, B and C, the major part of the pulse-labeled RNA is distributed over the relatively high molecular weight region of 12–23 S.

When longer incubations with [^{14}C]uracil are performed, the radioactivity is

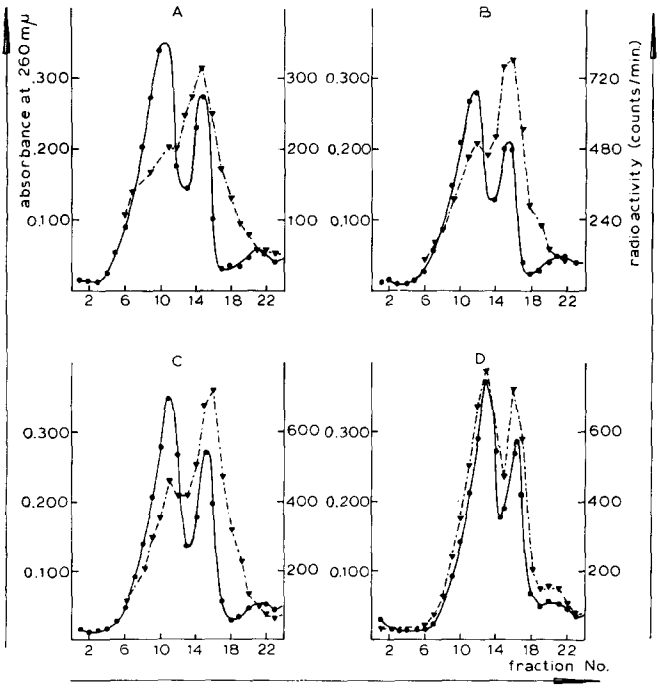


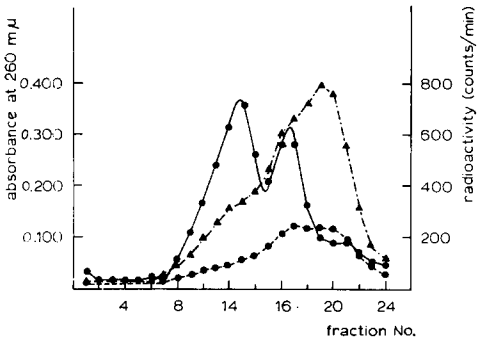
Fig. 4. Sucrose gradient (5–20 % sucrose) sedimentation patterns of the RNA extracted from the "enriched" polysomal fractions of maltose-induced protoplasts that had been labeled with [^{14}C]-uracil during (A) 3 min, (B) 6 min, (C) 10 min, and (D) 60 min. For experimental details see the text. ●–●, pattern of absorbance at 260 mμ; ▼ . – . ▼, radioactivity pattern.

soon found to enter the two ribosomal RNA peaks; thus, a change in the radioactivity pattern became visible after an incubation of more than 10 min with [^{14}C]uracil. After 60 min of incubation, the radioactivity pattern closely approaches that of the absorbance at 260 mμ (Fig. 4D).

Consequently, from the results presented in Figs. 4A, B and C, it can be concluded that the molecular weight distribution of the 3, 6 and 10 min pulse-labeled RNA is rather distinct from that of the bulk ribosomal RNA. Moreover, it is interesting to note the close similarity between the pulse-label RNA patterns illustrated in Fig. 4 and those obtained by FUKUHARA[16] with the pulse-labeled RNA extracted from a heavier (3000–24 000 $\times g$) particulate fraction of *S. cerevisiae* cells.

Effect of actinomycin D

Actinomycin D has been shown to be an effective and rather specific inhibitor of transcription and, consequently, of messenger RNA synthesis[17,18]. Therefore, an experiment was performed in which half of the protoplasts was used as a blank whereas the other half was incubated with 40 μg of actinomycin D per ml, 60 min before and during the incubation with [^{14}C]uracil. Preincubation and the rather high dose of actinomycin D are necessary as it appeared that the permeation of the antibiotic into the yeast protoplasts is rather slow*. After a 3-min pulse with [^{14}C]-uracil, the RNA was isolated from the polysomal fractions of untreated (blank) and actinomycin-treated protoplasts in the usual way, layered on a linear 5–20 % sucrose gradient and centrifuged.



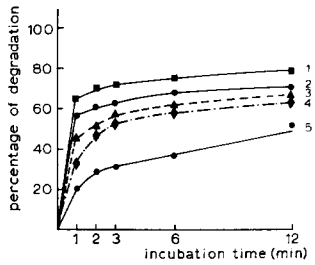


Fig. 5. Sucrose gradient (5–20 % sucrose) sedimentation patterns of [^{14}C]uracil pulse-labeled (3 min) RNA, extracted from the "enriched" polysomal fractions obtained from untreated (blank) and actinomycin-D-treated protoplasts. Experimental conditions and procedures as described in the text. ▲ . – . ▲, radioactivity pattern of RNA from blank protoplasts; ● - - - ●, radioactivity pattern of RNA from actinomycin-D-treated (40 μg/ml) protoplasts; ●–●, pattern of absorbance at 260 mμ.

Fig. 6. Degradation by ribonuclease of the labeled RNA present in the "enriched" polysomal fractions obtained from protoplasts that had been incubated with [^{14}C]uracil during varying lengths of time. Incubation with 10 μg/ml ribonuclease occurred at 30°. The breakdown of RNA was determined as the disappearance of cold trichloroacetic acid insoluble radioactive material. The protoplasts were incubated with [^{14}C]uracil during (1) 3 min, (2) 6 min, (3) 10 min, (4) 30 min and (5) 60 min.

* R. HARTLIEF, unpublished results.

From the radioactivity patterns as shown in Fig. 5 it can be seen, that the incubation of the protoplasts with actinomycin D causes the disappearance of about 75 % of the radioactive RNA in the region of 12–28 S. However, 25 % only of [^{14}C]uracil incorporation during 60 min into total RNA was found to be inhibited by the same concentration of actinomycin D as used in the experiment described above.

Therefore, the conclusion may be drawn that the rapidly labeled RNA from the polysomal fraction has a high turnover and that its synthesis is rather sensitive to the inhibitory action of actinomycin D.

Sensitivity to ribonuclease action

The data presented in Fig. 6 show the ribonuclease sensitivity of the radioactive RNA present in enriched polysomal fractions obtained from protoplasts that had been incubated with [^{14}C]uracil during varying lengths of time.

In the experiment, the 3-min pulse-labeled RNA shows the greatest, and the 60-min labeled RNA the smallest sensitivity to the action of the enzyme.

Moreover, in all cases the most rapid degradation of the RNA is observed during the first minute of incubation; during the following minutes, degradation proceeds much slower, indicating the occurrence of a very fast and a much slower degradative reaction, respectively.

From electron microscopic pictures it appears that the messenger RNA in a polysomal preparation has a rather open structure, explaining its high sensitivity to ribonuclease action[19–22]. On the other hand, the ribosomal RNA is somewhat protected by the ribosomal protein and consequently, much less sensitive to the action of the enzyme[22–25]. Thus, the most obvious explanation for the two-phase character of the rate of degradation as illustrated in Fig. 6 is that during the first minute mainly messenger RNA is broken down, whereas a small remainder of messenger RNA but mainly ribosomal RNA is degraded during the prolonged incubation times.

Base composition

Unfortunately, when the four ^{14}C-labeled bases were added as RNA precursors in experiments performed to determine the base ratios of the (pulse-)labeled RNA, two sources of considerable error were found to occur.

First, the rate of incorporation of any of the four bases into RNA proved to be strongly inhibited by the presence of the other three bases in the medium. This phenomenon, which also was noted during short incubations with radioactive precursor, may be due to an interference of one or more of the other bases with the permeation from the medium into the protoplasts of the one radioactive base to be incorporated. And secondly, even during the pulse-label experiments a significant exchange (of radioactivity) was found to occur between the purine and the pyrimidine bases respectively. Therefore, in our opinion, base ratios obtained from RNA labeled with the ^{14}C bases must be regarded as rather fortuitous.

Consequently, the base composition of a number of radioactive RNA preparations was determined after the labeling of the RNA with ^{32}P inorganic phosphate

in vivo. Alkaline hydrolysis of the RNA and the subsequent separation of the 2′, 3′-nucleotides were performed as described in METHODS.

TABLE II

BASE COMPOSITION OF ^{32}P-LABELED RNA, EXTRACTED FROM ENRICHED POLYSOMAL FRACTIONS AFTER THE INCUBATION OF THE PROTOPLASTS WITH ^{32}P-LABELED INORGANIC PHOSPHATE DURING VARYING LENGTHS OF TIME AT 30°

For experimental conditions and procedure, see METHODS.

	A	*U(T)*	*G*	*C*
Yeast DNA (ref. 26)	31.5	32.8	18.5	17.3
Yeast RNA (ref. 26)	25.4	26.0	28.6	20.2
RNA labeled during:				
3 min	31	32	16	21
6 min	28	31	18	22
60 min	28	29	21	21
150 min	25	28	22	22

From the data presented in Table II it can be concluded that the base ratios of the RNA preparations vary with the time of incubation of the protoplasts with [^{32}P]phosphate approaching, after 150 min, values which are more or less in agreement with the nucleotide composition of total yeast RNA. However, the RNA extracted from polysomal fractions of protoplasts that were labeled by a 3-min pulse, shows an equivalence of A and U and a composition which is rather similar to that of yeast DNA. Moreover, there is a rather good agreement between the data presented in Table II and those reported by FUKUHARA[16] on the base composition of the pulse-labeled RNA extracted from a still heavier particulate fraction of *S. cerevisiae* cells.

At first sight, however, a lack of agreement seems to exist between the data presented in Table II and those on the sedimentation analysis of RNA preparations as illustrated in Fig. 4. Thus, according to sedimentation analysis, the molecular size distribution of the pulse-labeled RNA rapidly changes towards that of the bulk ribosomal RNA: after a 60-min incubation of the protoplasts with [^{14}C]uracil the pattern of radioactive RNA shows a size distribution profile which coincides almost completely with the pattern of the bulk ribosomal RNA.

Nevertheless, according to the data shown in Table II, the chemical composition of the 60-min labeled RNA is still different from that of the 150-min labeled RNA and from that of the total RNA. Probably, this apparent lack of agreement is the result of a much lower permeability of the yeast protoplasts for phosphate than for the nucleic acid bases and/or of a relatively slow incorporation of the radioactive phosphate as a consequence of intracellular isotope dilution. Thus, in a number of experiments, the incorporation of [^{32}P]phosphate into RNA was found to be 100–200 times slower than that of the ^{14}C-labeled nucleic acid bases.

As the pulse-labeled (3 and 6 min) RNA appears to have a DNA-like base composition (Table II), it can be concluded that it consists mainly of messenger RNA. Thus, from the results presented sofar it appears that the rapidly labeled RNA extracted from the enriched polysomal fractions is clearly distinct from the bulk ribosomal RNA as it shows the following properties, which in general are ascribed to messenger RNA: (a) It has a heterogeneous molecular size distribution as shown

by the radioactivity pattern obtained after sucrose gradient ultracentrifugation. (b) This radioactivity pattern disappears for the greater part when the protoplasts are incubated with [^{14}C]uracil in the presence of actinomycin D. (c) It appears to be extremely sensitive to digestion with ribonuclease. (d) It has a DNA-like base composition.

Immune precipitation of polysomes charged with nascent α-glucosidase

One way to try to obtain a preliminary characterization of the messenger RNA coding for a particular protein would be to select the polysomes charged with nascent chains of that protein and subsequently to characterize the pulse-labeled RNA extracted from these polysomes, *e.g.* by sucrose gradient ultracentrifugation. Such an experimental approach could be successful, as a number of studies have made it possible to specifically identify nascent proteins associated with bacterial and mammalian ribosomes[27–32]. Thus, WARREN AND GOLDTHWAIT[29] have shown by immunological techniques that triose phosphate dehydrogenase is associated with a particular fraction of the total ribosomal population. Similar results have been obtained *e.g.* with albumin[31,33], β-galactosidase[32], catalase and glutamate dehydrogenase[33].

Thus, if it were possible to precipitate by an immunological method the family of ribosomes involved in the synthesis of α-glucosidase, one could demonstrate that the pulse-labeled RNA of this ribosomal fraction differs *e.g.* in its sedimentation pattern from that of the pulse-labeled RNA from the total polysomal population. If successful, such a method could facilitate the isolation of the messenger RNA coding for α-glucosidase or, more in general, for any one particular protein.

It appeared that it was not possible to precipitate any ribosomal material with the purified anti-α-glucosidase γ-globulin fraction from rabbits that were immunized with purified α-glucosidase. The introductory experiments were carried out with "enriched" polysomal fractions from maltose-induced (2 h) protoplasts, the RNA of which had been labeled with [^{14}C]uracil during the 2 h of incubation. In order to minimize the degradation of messenger RNA, a period of 1 h of incubation at 0° with varying concentrations of the purified rabbit antiserum was chosen. Neither during this short period, nor during prolonged times of incubation (up to 24 h) any formation of a radioactive precipitate was found to occur. This finding is in agreement *e.g.* with the data reported by SPIEGELMAN[32], indicating that *E. coli* (poly)ribosomes charged with nascent β-galactosidase bind anti-β-galactosidase antiserum without being precipitated by it. Following the suggestion by the same author, that anti-antiserum would cause a precipitation of the (poly)ribosome-nascent enzyme–antiserum complex, purified anti-rabbit γ-globulin antiserum from the goat was added in our following experiments.

First, the approximate optimal ratio of rabbit γ-globulin fraction and anti-rabbit γ-globulin antiserum concentrations was determined with an immunodiffusion test, using varying amounts of purified anti-serum and a constant amount of purified rabbit γ-globulin. The amount of anti-antiserum at which a sharp precipitation line was formed at an equal distance from the two holes, was found to be 4 mg of purified anti-antiserum per mg rabbit γ-globulin. The procedure used to determine the optimal conditions for the precipitation of ribosomes charged with nascent α-glucosidase was as follows:

The "enriched" polysomal fraction from induced protoplasts, the RNA of which was labeled (2 h) with [^{14}C]uracil, was incubated at 0° in the presence of purified rabbit anti-α-glucosidase γ-globulin fraction during 45 min. Subsequently, purified goat anti-rabbit γ-globulin antiserum was added to the mixture of polysomes and rabbit γ-globulin fraction. After a 5-min incubation, the mixture was at first diluted 10 times with distilled water, to minimize non-specific co-precipitation or occlusion of ribosomal material. The precipitate was collected by centrifugation for 15 min at 3000×*g* and subsequently assayed for radioactivity.

In preliminary experiments with total, *i.e.* unpurified, anti-rabbit γ-globulin antiserum from the goat it was observed that a considerable unspecific co-precipitation with the polysomes occurs. In order to remove any unspecific co-precipitate, the precipitates obtained were diluted and washed by centrifugation with varying NaCl concentrations. From the results presented in Fig. 7, it may be concluded that by diluting the precipitate 10 fold with a 0.9 % NaCl solution and subsequent washing by centrifugation not much non-specific precipitate is obtained.

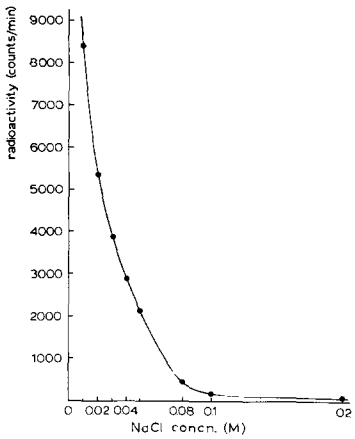


Fig. 7. The effect of washing with varying NaCl concentrations on the non-specific co-precipitation of polysomes from non-induced yeast protoplasts, caused by rabbit anti-α-glucosidase γ-globulin fraction and anti-rabbit γ-globulin antiserum from the goat.

To determine the optimal rabbit anti-α-glucosidase γ-globulin/polysome ratio, varying amounts of polysomal material containing [^{14}C]uracil-labeled (2 h) RNA were added to a constant amount (0.2 mg) of the purified rabbit antibody. The tubes were allowed to stand for 45 min at 0°. Subsequently, 0.8 mg purified anti-rabbit γ-globulin antiserum was added to each tube; after 5 min, the mixture was diluted 10 times with a 0.9 % NaCl solution. The precipitates were collected by centrifugation for 15 min at 3000×*g*, the radioactivity of the precipitates being determined according to the procedure described in METHODS.

In a series of experiments, maximal amounts varying from 4 to 10 % of the total radioactive label in the RNA of the polysomal fractions were found in the precipitate. The result of a rather illustrative experiment is presented in Table III, Column 3.

TABLE III

IMMUNE PRECIPITATION OF POLYSOMES FROM INDUCED AND NON-INDUCED PROTOPLASTS CAUSED BY PURIFIED RABBIT ANTI-α-GLUCOSIDASE γ-GLOBULIN FRACTION AND ANTI-RABBIT γ-GLOBULIN ANTISERUM FROM THE GOAT

Experimental conditions and procedures as described in the text.

mg polysomal protein	*mg rabbit anti-α-glucosidase γ-globulin fraction*	*[^{14}C]Uracil-labeled RNA precipitated as a percentage of the total radioactive polysomal RNA isolated from*	
		Induced protoplasts	*Non-induced protoplasts*
0.05	0.2	6.0	1.0
0.1	0.2	10.0	1.3
0.2	0.2	5.7	0.9
0.5	0.2	4.2	0.9

From such data as presented in Table III, it could be concluded that under the experimental conditions maximal precipitation occurs at an antibody/polysomal protein ratio of 2.

As a blank, in order to determine whether the precipitation of polysomal material was due to a specific antibody-antigen reaction, parallel experiments were performed with enriched polysomal fractions from non-induced protoplasts: for comparison, the results of such an experiment are shown in Table III, Column 4. In our opinion, two factors are responsible for the observed precipitation of some polysomal material from non-induced protoplasts; first, a little specific precipitation can be expected to occur as a consequence of the low but significant level of α-glucosidase activity which is always present in polysomal material from non-induced protoplasts. And secondly, despite the washing with 0.9 % NaCl solution some non-specific co-precipitation or occlusion of polysomal material by the purified goat anti-rabbit γ-globulin antiserum could still occur.

Nevertheless, the apparently specific precipitation of polysomes as suggested by the data in Table III could be due to the presence in the polysomal preparations of unspecifically adsorbed α-glucosidase. In order to exclude this possibility, another experiment was performed with an enriched polysomal preparation from [^{14}C]uracil-labeled non-induced protoplasts. Before ultracentrifugation through a 40 % sucrose layer, the labeled 13 000 × *g* supernatant fraction of these protoplasts was mixed with an equal amount of a 100 000 × *g* supernatant fraction from induced protoplasts, containing 880 units of α-glucosidase activity per mg protein. After reaction of the polysomal fraction thus obtained with the double antibody system, 1.5 % of the total radioactive label in the RNA was found in the precipitate (compare Table III, Column 4). This finding indicates that the observed precipitation of polysomes from induced protoplasts is not due to an unspecific adsorption of α-glucosidase to the ribosomal particles.

Consequently, the results as presented in Table III strongly suggest the occurrence of a specific immune precipitation of polysomes charged with nascent α-glucosidase. In order to obtain some additional and more direct evidence of such a specific precipitation, an attempt was made to prove that the α-glucosidase activity in the 3000 × *g* supernatant would decrease after an incubation of enriched polysomal fractions with rabbit anti-α-glucosidase γ-globulin fraction and goat anti-rabbit

γ-globulin antiserum under the optimal conditions as described above. It was found that the following facts made it difficult to obtain the accurate determinations wanted:

(1) The enriched polysomal fractions were not entirely free of soluble α-glucosidase: of course, this would not interfere very much during the experiments performed to characterize the pulse-labeled RNA extracted from the precipitated (poly)ribosomes (next section). However, in order to prove that ribosomes with nascent α-glucosidase are precipitated, it was necessary to wash the resuspended enriched polysomal fractions by another ultracentrifugation at 100 000 × *g* (compare ref. 35).

(2) It was observed, that the activity of the nascent enzyme is increased by 50 to 100 % during the incubation with the rabbit anti-α-glucosidase γ-globulin fraction. The same phenomenon was observed by SPIEGELMAN[32], when studying the interaction between ribosome-bound β-galactosidase and anti-β-galactosidase antiserum; possibly, this may be attributed to a change in the conformation of the nascent enzyme caused by the specific anti-enzyme antibody structure.

(3) Moreover, it was repeatedly found that the addition of rather large amounts of various proteins, *e.g.* of bovine serum albumin, in some unspecific way causes a small but significant increase in the activity of both the nascent and the purified enzyme[12,*].

As a consequence of these findings, suspensions were used of washed enriched polysomal fractions that had been preincubated during 2 h with 20 mg/ml of bovine serum albumin. With these suspensions, experiments have been performed as outlined in Table IV.

TABLE IV

IMMUNE PRECIPITATION OF RIBOSOME-BOUND NASCENT α-GLUCOSIDASE CAUSED BY PURIFIED RABBIT ANTI α-GLUCOSIDASE γ-GLOBULIN FRACTION AND ANTI-RABBIT γ-GLOBULIN ANTISERUM FROM THE GOAT

Experimental conditions and procedures as described below, and in the text.
Polysome concentration in all tubes, 1 mg protein per ml. The polysomes of tube No. 2 were preincubated during 2 h with 20 mg/ml bovine serum albumin (BSA). The polysomes of tube No. 3 were, after preincubation during 2 h with 20 mg/ml BSA, incubated during 45 min with 2 mg/ml of purified rabbit anti-α-glucosidase γ-globulin fraction (AB). The polysomes of tube No. 4 were treated in the same way as those of tube No. 3, and subsequently incubated during 5 min with 8 mg/ml of purified goat anti-rabbit γ-globulin antiserum (AAB). Tube No. 5, the same as tube No. 3, and a subsequent incubation during 5 min with 8 mg/ml BSA (blank for tube No. 4); the purified goat anti-antiserum has no effect on the enzymatic activity.

Tube No.	*Reactants*	*α-Glucosidase activity in the 3000 × g supernatant (mμmoles PNPG split per min, × 10²)*
1	Polysomes	0.616
2	Polysomes + BSA	0.682
3	Polysomes + BSA + AB	1.199
4	Polysomes + BSA + AB + AAB	0.836
5	Polysomes + BSA + AB + BSA	1.210

From the results of a number of such experiments, it was concluded that 25–30 % of the ribosome-bound α-glucosidase activity is precipitated (*i.e.* is spun down upon centrifugation at 3000 × *g*) after 45 min of incubation at 0° with the rabbit

* R. HARTLIEF, unpublished results.

anti-α-glucosidase γ-globulin fraction (ratio of polysomal protein: rabbit γ-globulin = 1:2) and a subsequent incubation during 5 min with purified goat anti-rabbit γ-globulin antiserum (ratio rabbit γ-globulin: goat γ-globulin = 1:4). In our further experiments (next section), it should be tried to prevent the breakdown of pulse-labeled RNA and, consequently, to make the immune precipitation procedure a short one rather than a quantitative one. Consequently, the experimental procedure for the precipitation of the (poly)ribosomes charged with nascent α-glucosidase was maintained as described above, the washing and preincubation with bovine serum albumin of the enriched polysomal fractions being left out.

Preliminary characterization of messenger RNA for α-glucosidase

After induction by incubation with maltose during 2 h (see METHODS), the protoplasts (100 ml, 0.5 mg protein/ml) were pulse-labeled for 3 min with [^{14}C]-uracil. The "enriched" polysomal fraction was isolated according to the procedure described under METHODS. Subsequently, the immune precipitation of the polysomal material charged with α-glucosidase was performed with the aid of the purified rabbit anti-α-glucosidase γ-globulin fraction and the purified anti-rabbit γ-globulin antiserum from the goat as described in the preceding section. The RNA extracted from the immune precipitate and from the 3000×g supernatant according to the

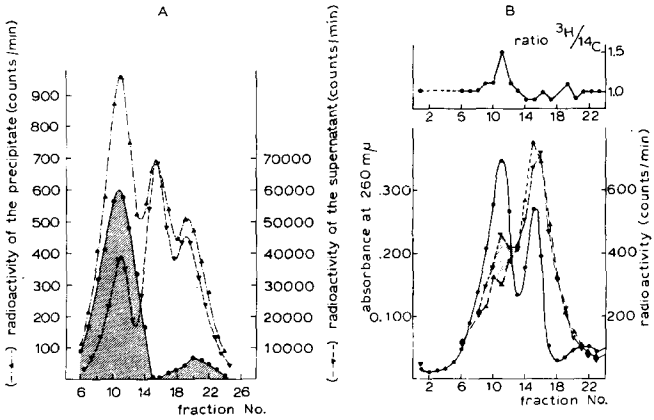


Fig. 8. A. Sucrose (5–20 %) gradient sedimentation patterns of the [^{14}C]uracil pulse-labeled (3 min) RNA extracted from the 3000×g precipitate and from the 3000×g supernatant obtained after the incubation of an enriched polysomal fraction with the rabbit anti-α-glucosidase γ-globulin fraction and the purified goat anti-rabbit γ-globulin antiserum. The main radioactive peak coincides with the peak in the absorbance at 260 mμ of the 28-S ribosomal RNA. ▲ . – . ▲, radioactivity pattern of the RNA extracted from the immune precipitate; ▼ - - - ▼, radioactivity pattern of the RNA extracted from the supernatant. The shaded area represents the radioactivity pattern of the RNA from the precipitate after correction for non-specific co-precipitation as described in the text. B. Sucrose (5–20 %) gradient sedimentation patterns of the pulse-labeled (10 min) RNA extracted from the enriched polysomal fractions from induced and from non-induced protoplasts. The maltose-induced protoplasts were incubated with [^{3}H]uracil, the non-induced protoplasts with [^{14}C]uracil. The protoplasts were incubated in the same medium, containing either 3 % maltose or 3 % glucose. ▼ - - - ▼, radioactivity pattern of the RNA extracted from induced protoplasts; ▲ - - - ▲, radioactivity pattern of the RNA extracted from non-induced protoplasts; ●–●, pattern of absorbance at 260 mμ. In the upper part, the ratio has been plotted of the radioactivity of [^{3}H]RNA and [^{14}C]RNA through the gradient. For further experimental details see the text.

procedure described under METHODS was subjected to ultracentrifugation through a linear sucrose gradient. The resulting radioactivity patterns obtained in such an experiment are shown in Fig. 8A.

Clearly, sedimentation analysis shows that the radioactivity pattern of the pulse-labeled RNA extracted from the precipitate is quite different from that of the 3-min pulse-labeled RNA fraction extracted from the total polysomal fraction and from the $3000 \times g$ supernatant obtained after the immune precipitation procedure (see Fig. 4A and Fig. 8A respectively).

Since a unique messenger RNA should have a unique molecular weight, the messenger RNA for α-glucosidase should show a narrow distribution through the gradient. In fact, the pulse-labeled RNA extracted from the precipitate clearly shows three distinguishable peaks with an approximate sedimentation coefficient of 28 S, 18 S and 8–10 S. However, most of the radioactivity in the smaller 18-S and 8–10-S peaks may be due to the unspecific co-precipitation or occlusion of polysomal material. After the application of a reasonable correction for the unspecific co-precipitation, *i.e.* after subtracting 1 % of the radioactivity of the supernatant RNA present in each gradient fraction from that of the corresponding fraction of the RNA from the precipitate (see the preceding section), only one symmetrical main peak is obtained with a sedimentation coefficient of about 28 S (Fig. 8). The very small amount of material with a lower rate of sedimentation may be due to some degradation of the pulse-labeled RNA during the incubation with antibody and anti-antibody.

From four experiments, all yielding similar results as that illustrated in Fig. 8A, it was tentatively concluded that the relatively high 28-S radioactivity peak might reflect the presence of messenger RNA for α-glucosidase. Encouraged by these results, we have tried to obtain some more experimental evidence in favor of this conclusion.

To this purpose, pulse-double-labeling experiments were carried out with induced and with non-induced protoplasts to look for a possible difference in the sucrose gradient sedimentation patterns of the RNA. Maltose-induced (90 min) protoplasts were incubated during 10 min with [^{3}H]uracil whereas non-induced protoplasts (same incubation medium, maltose replaced by glucose) of the same batch were simultaneously incubated with [^{14}C]uracil. An incubation time of 10 min for the labeling was chosen, as it was found that this leads to perfectly reproducible and significant results. A mixing of the two portions of protoplasts was performed immediately after labeling in order to assure that they were treated identically during the fractionation procedure and to eliminate differences which could arise during the extraction of the RNA.

The results of a representative experiment are shown in Fig. 8B. It can be seen, that the ratio of ^{3}H/^{14}C radioactivity remains relatively constant all through the gradient, except for a rather sharp increase in this ratio in the region of the 28-S ribosomal RNA peak. This finding proved to be quite reproducible; when – as a control – during incubation with or without the inducer maltose the labels were reversed, the same peak in the isotope ratio appears, but it is also reversed (*i.e.* the ratio of ^{14}C/^{3}H radioactivity increases sharply). This indicates that the peak in the isotope ratio is not an artifact caused by *e.g.* impurities in the radioactive precursor. Therefore, in a tentative way it is concluded that the messenger RNA coding for α-glucosidase indeed sediments with a coefficient of about 28 S.

The 28-S ribosomal RNA has a molecular weight of about $1.4 \cdot 10^6$ and it contains about 4700 nucleotides[34]. Such a RNA molecule could serve as a template for a protein with a molecular weight of about 160 000. Of course, our estimate is extremely rough, but it is clear that such a messenger RNA and, consequently, the messenger for α-glucosidase is probably large enough to be a polycistronic messenger.

Of course, it is realized that the preliminary data on the characterization of the messenger RNA for α-glucosidase, as presented in this paper, are still far from being complete. A further purification and characterization of this RNA fraction is needed in order to prove that it consists of or even contains messenger RNA for α-glucosidase. Obviously, one conclusive experiment would be to show that the synthesis of α-glucosidase occurs under the direction of this RNA in a heterologous cell-free system.

Nevertheless, it is inferred, that the use of specific antibody and anti-antibody preparations may help in the development of a more general procedure for isolating polysomes charged with one particular protein and, consequently, for the isolation and characterization of the messenger RNA coding for that protein.

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