Transport and Processing of Ribosomal RNA in Plant Cells after Treatment with Cycloheximide

Hans-Ullrich Gebauer, Ursula Seitz, and Ulrich Seitz

Institut für Biologie I, Universität Tübingen

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In freely suspended cells of parsley *(Petroselinum crispum)* protein synthesis is inhibited nearly totally by 5 μg/ml cycloheximide within 10 min. This very low dose has a slight effect on the rRNA synthesis too. During an incubation period of 60 min with [^{32}P]orthophosphate in the presence of the inhibitor (5 μg/ml) at least 65–70% mature rRNA are synthesized compared with the control. After 120 min the synthesis is progressively reduced to 60–65%, and after 240 min to 30–40%.

Cycloheximide causes a delay of the processing of the precursor RNA species leading to an accumulation of these components. In addition to the 2.3×10^6 daltons RNA, normally detectable in pulse experiments, two further precursor molecules do emerge which under normal circumstances apparently are shortlived. Their molecular weights are 2.0×10^6 and 0.9×10^6 daltons.

The pulse-chase technique and cell fractionation into nuclear and ribosomal parts enables us to demonstrate a rapid transfer of labelled 18S and 25S RNA during a 15 min chase treatment to the mature cytoplasmic ribosomes. Under these conditions no differential transport of the two components takes place.

The cells possess a pool of proteins. Therefore the formation of RNP-particles or ribosomes respectively and the transport from the nucleus to the cytoplasm are independent from protein synthesis for some time.

Introduction

It was becoming apparent, based on various arguments, that a relationship between synthesis and transport phenomena and protein synthesis does exist. Some of the arguments are: 1. The cleavage of the large precursor RNA depends on enzymatic activity. 2. The cells produce mature ribosomes only if there are ribosomal proteins in sufficient amounts. Besides these ribosomal proteins there are the true nucleolar proteins which are removed during the transport to the cytoplasm.

The dependence of the rRNA processing upon the protein synthesis has been described earlier in animal systems and in yeast [1, 2].

Inhibitors of the protein synthesis are good tools to solve these questions. An inhibitor is as good as its specifity. We have to minimize non-specific secondary effects by treatment with very low doses of the drug. Ellis and McDonald showed that cycloheximide has an influence on the energy balance and the ion turn-over [3]. In view of these data the cycloheximide concentration of 100 μg/ml applied by Udem and Warner appears to be too high [2].

Requests for reprints should be sent to Dr. Ursula Seitz, Institut für Biologie I der Universität, *D-7400 Tübingen*, Auf der Morgenstelle 1.

In this paper we intend to answer the following questions. 1. What is the time course of the cycloheximide inhibition and what are the concentrations necessary to inhibit the protein synthesis of parsley cells in suspension cultures? 2. Is the rRNA synthesis influenced by the antibiotic? If that is the case, at which stage of the processing? 3. Does the transport of the 25S and/or the 18S RNA depend on the protein synthesis?

In the context of the complex of questions posed in section 3, it is interesting to see further, if the transports of the 25S and the 18S RNA are regulated independently or whether they are based on the same mechanism. In several systems such a difference in the velocity of the transport has been observed [4–7]. In general the results of such experiments must provide clues in unravelling the regulatory mechanisms found in compartimented systems.

Results

The aim of the first series of experiments was to determine the minimal inhibitory dose of cycloheximide on the protein synthesis and to shorten the incubation time as much as possible. In order to solve these questions, the incorporation of [^{3}H]-leucine into the TCA-insoluble fraction was tested.

The parsley cells are very competent in leucine uptake. With 10 μCi/ml, 5 μCi/ml, 2.5 μCi/ml and 1 μCi/ml we got enough radioactivity into the cells. After an incubation period of 1 h and an isotope concentration of 5 μCi/ml (packed cell volume 0.09 cm^3/ml) about 20000 cpm/ml were incorporated. In all further experiments that [^{3}H]leucine concentration was applied.

Determination of the efficient cycloheximide concentrations. The following concentrations were tested: 1 μg, 5 μg, 10 μg, and 20 μg/ml. Within 1 h 20 μg/ml of the cycloheximide had an equal effect compared with the effect of 5 μg/ml.

The time course of the cycloheximide effect is shown in Fig. 1. Cells derived from the log-phase of a parsley suspension culture were incubated with [^{3}H]leucine in 2 identical samples (5 μCi/ml). To one sample cycloheximide was added and the TCA-insoluble activity was measured by liquid scintillation counting. From these curves it is evident that 10 min after addition of the drug a total inhibition takes place. No further radioactivity is incorporated.

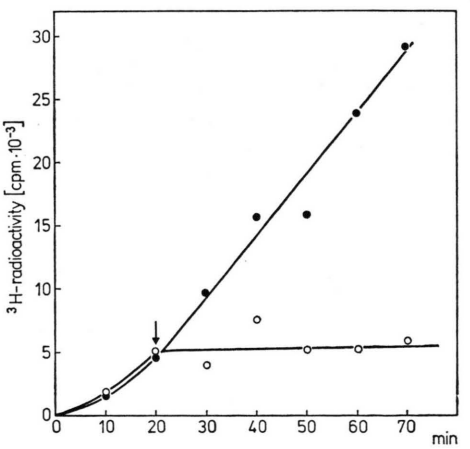
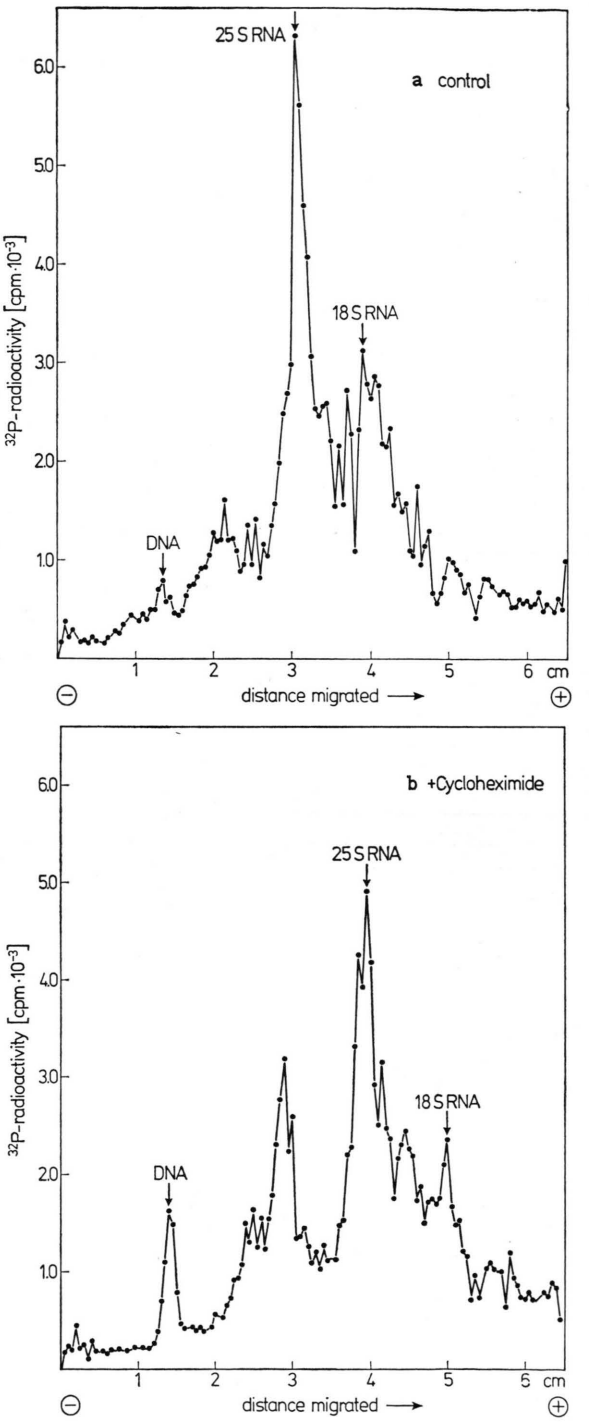


Fig. 1. Cycloheximide effect on the incorporation of [^{3}H]-leucine into the trichloroacetic acid-precipitable proteins of parsley cells grown in suspension culture. The arrow indicates the time of addition of the drug.

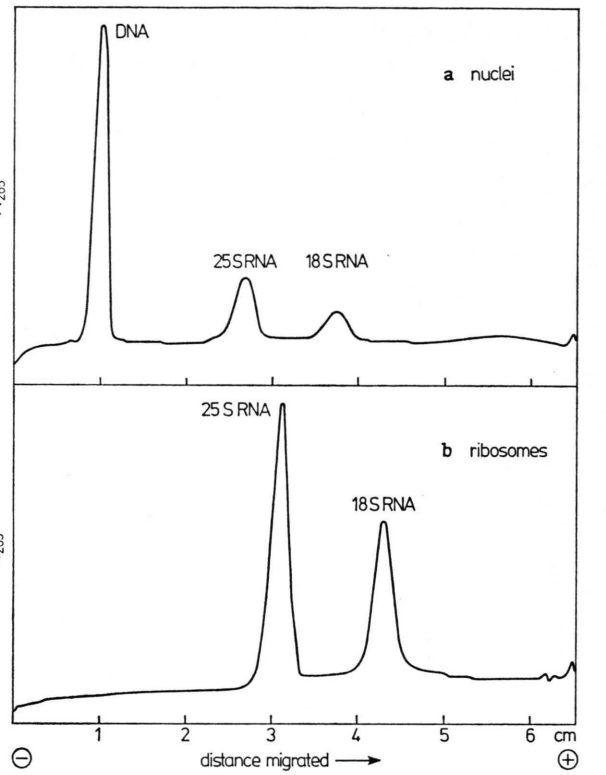
Cycloheximide effect on the rRNA synthesis

It was considered important to investigate the rate of rRNA synthesis in the presence of cycloheximide. Synthesis in this context means transcription and processing. This question had to be answered above all, because in the case of a total inhibition it would be impossible to study the transport from the nucleus to the cytoplasm in relation to the protein synthesis. The rRNA synthesis was measured as ^{32}P-



Figs 2 a and 2 b. Polyacrylamide (2.1%) gel electrophoresis of nucleic acids after a 120 min incubation with [^{32}P]orthophosphate. 9.8—10 μg nucleic acids were applied to 1 gel. The gels were chopped and the radioactivity was counted in a PPO/POPOP scintillation mixture. a. control; b. with 5 μg/ml cycloheximide during the incubation period. The peaks of the UV-scan are indicated by arrows.

radioactivity after incubation with [^{32}P]orthophosphate and fractionation of the nucleic acids in 2.1% polyacrylamide gels. The labelled compound had been offered for 60 min, 120 min and 240 min. During an incubation period of 60 min in the presence of the inhibitor 65 – 70% mature rRNA are synthesized compared with the control. A 120 min experiment is shown in Fig. 2. Fig. 2 a represents the control. The DNA, 25S RNA and 18S RNA contain the major part of the ^{32}P-label. The peak between DNA and 25S RNA represents the precursor RNA with a molecular weight of 2.3×10^6 daltons, which under the given conditions makes up only a little part compared with the mature rRNA. The electrophoretic pattern after cycloheximide treatment (Fig. 2 b) shows a reduction in rRNA synthesis. In the case of the 120 min incubation the label in the mature rRNA (25S and 18S RNA) is reduced to 60 – 65%. A 240 min incubation does not lead to higher activities in the mature RNA in relation to the 120 min values. Under those conditions only 30 – 40% of the mature rRNA are synthesized with regard to the control. A second result of the 120 min experiment is a very conspicuous accumulation of precursor RNA species. The characterization of these RNA molecules is described in the experiment shown in Fig. 6 (see also Table II).

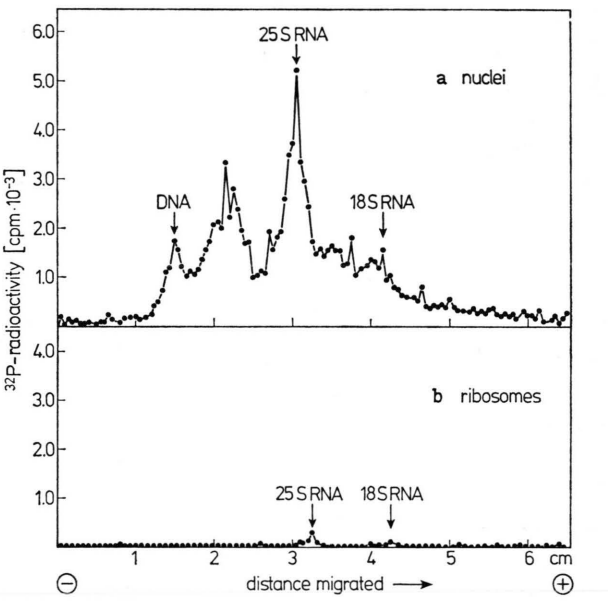


Figs 3 a and 3 b. Polyacrylamide (2.1%) gel electrophoresis of nucleic acids extracted from a. nuclei and b. ribosomes of parsley cells. The absorbance was recorded at 265 nm.

Pulse chase experiments and cell fractionation

As already mentioned at the beginning, the aim of our study was to analyse the transport from the nucleus to the cytoplasm after the disconnection of the protein synthesis by cycloheximide. The ribosomes had been prepared after the methods of Bielka and his co-workers [8]. The isolation procedure for the nuclei has to fulfill the following criteria: The method should be as fast as possible, and the intact organelles should be accumulated in considerable amount. The method of Tautvydas [9] is suited to our system. The criteria for the quality of the preparations were, besides the microscopic picture, the DNA – RNA ratio of approximately 3 : 1, determined by peak area measurements of the absorbance scan. In Figs 3 a and b the UV diagrams of the nucleic acids from nuclei and ribosomes are shown.

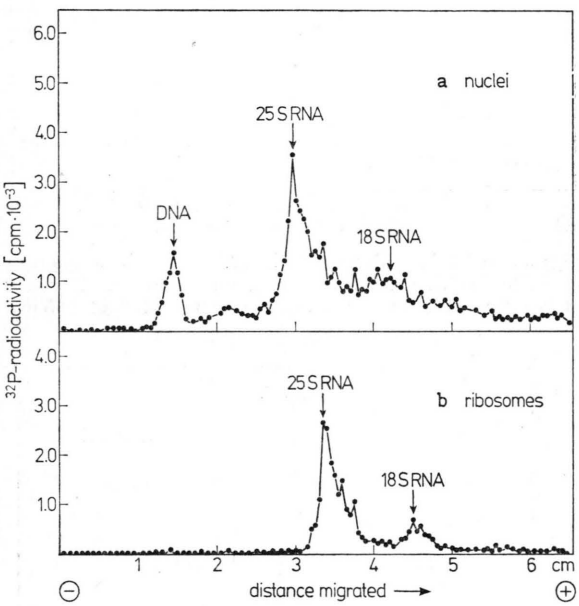
Pulse chase experiments. a. 30 min pulse, 15 min chase. The radioactivity diagram (Fig. 4 a) of the nuclear fraction shows 3 major components: The precursor RNA (2.3×10^6 daltons), the 25S RNA (1.3×10^6 daltons), and the 18S RNA (0.7×10^6



Figs 4 a and 4 b. Polyacrylamide (2.1%) gel electrophoresis of nucleic acids extracted from a. nuclei and b. ribosomes after a 30 min pulse with [^{32}P]orthophosphate followed by a 15 min chase incubation. Further conditions see Fig. 2.

daltons). Into the 25S component more label has been incorporated in proportion to the 18S RNA. This fact can be explained by the higher number of phosphate groups in the larger molecule. Compared with the ribosomal fraction (Fig. 4 b) it is obvious that after a 15 min chase treatment we caught just the right moment in which the newly synthesized material reaches the cytoplasm. The ratio between 18S RNA and 25S RNA is not shifted for the benefit of the 18S RNA. After these results we have to conclude that the transport of both has the same time constant.

b. 30 min pulse, 120 min chase. The results are presented in Fig. 5. The ribosomes now contain a high amount of label in the 25S and 18S RNA. The activity of the two mature RNA species of the nuclei



Figs 5 a and 5 b. Polyacrylamide (2.1%) gel electrophoresis of nucleic acids extracted from a. nuclei and b. ribosomes after a 30 min pulse with [^{32}P]orthophosphate followed by a 120 min chase incubation. Further conditions see Fig. 4.

is levelled down. In the gel region of the precursor RNA the label has disappeared nearly totally. In Table I the radioactivity values of the two experiments (15 min and 120 min chase) are summarized. The very close correspondence of the values obtained by addition of the counts and the homogeneous incorporation into the DNA bands are good signs for the standardization of our experiments. It can be taken from these data too that no transcriptional activity occurs during the chase treatment.

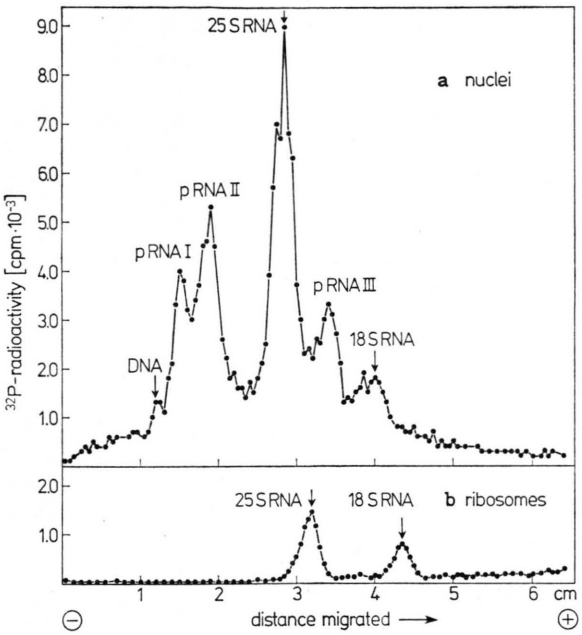
Table I. Radioactivity counted (cpm) in the peak fractions of a polyacrylamide gel electrophoresis (2.1%). Upper part: 30 min pulse, 15 min chase incubation. Lower part: 30 min pulse, 120 min chase.

	DNA	25S RNA	18S RNA
		30 min pulse, 15 min chase	
nuclei (1)	1800	5300	1400
ribosomes (2)		300	100
(1) and (2) added	1800	5600	1500
		30 min pulse, 120 min chase	
nuclei (1)	1600	3600	1000
ribosomes (2)		2600	700
(1) and (2) added	1600	6200	1700

Transport and protein synthesis

After the consideration of transport phenomena it was interesting to know something about the relationship of transport and protein synthesis. Since it is well known that the RNA cannot be transported as a naked molecule but only as a RNP particle [10] it is obvious that transport stops after the protein synthesis had been switched off. Alternatively it is possible that the nucleus contains a pool of transport proteins (ribosomal proteins) which enables the cell to continue that process. The following experiment (30 min pulse, followed by a 120 min chase under simultaneous treatment with cycloheximide) should give the answer. The chase period of 120 min had been selected for two reasons. 1. Within this period, as was evident from the experiment in Fig. 5, under normal conditions, the molecules are labelled markedly enough to allow an assessment of a reduced rate of incorporation, if this is indeed the case. 2. If the transport continues under cycloheximide treatment it would be possible to take from these experiments some information about the size of the protein pool.

The results shown in Fig. 6 clearly demonstrate that the precursor molecules (pRNA I, mol weight: 2.3×10^6 daltons and the pRNA II, mol weight: 2.0×10^6 daltons) are accumulated in the nucleus. In addition to that, between the two mature rRNA's a third one does appear (pRNA III). Based on the molecular weight which has been determined to be 0.9×10^6 daltons and its behaviour in chase experiments this component seems to be the direct precursor of the 18S RNA [11] (Table II). The pRNA II and the pRNA III are under normal conditions without cycloheximide shortlived, therefore they cannot be detected on the gel. These two additional pre-



Figs 6 a and 6 b. Polyacrylamide (2.1%) gel electrophoresis of nucleic acids extracted from a. nuclei and b. ribosomes after a 30 min pulse with [^{32}P]orthophosphate followed by a 120 min chase incubation under simultaneous treatment with 5 μg/ml cycloheximide. Further conditions see Fig. 4.

cursors do emerge in preparations of the total nucleic acids too (Fig. 2 b), but naturally in lower amounts than on gels with nucleic acids extracted from the nuclear fraction (Fig. 6 a).

Table II. Molecular weights of the ribosomal RNA components seperated on a 2.1% polyacrylamide gel (see Fig. 6 a) after a 30 min pulse with [^{32}P]orthophosphate followed by a 120 min chase incubation under simultaneous treatment with cycloheximide. Nucleic acids were extracted from the nuclei. The molecular weights were determined after the method described by Loening [18].

	mol. weights $\times 10^6$ daltons
p rRNA I	2.3
p rRNA II	2.0
25S RNA	1.3
p rRNA III	0.9
18S RNA	0.7

Discussion

1. Cycloheximide effect

In general, cycloheximide is considered to be a protein synthesis inhibitor in eucaryotes. But in addition, other processes, such as energy balance and ion uptake, are influenced by the drug too [3]. In our case, the inhibitory effect on the transcription observed by several authors is very important [1, 12, 13]. On the other hand, Pederson and Kumar [14] showed that transcription is only affected if protein synthesis is inhibited totally, whereas a slight inhibition only causes a reduction of the processing of the rRNA precursors. These facts have to be considered in the case of the experiments done by Willems and his co-workers [1] who applied very high doses of cycloheximide on the HeLa system.

We are interested especially in transport problems and the dependence on the protein supply. The optimum dose to inhibit the translation process in cells from parsley suspension cultures is 5 μg/ml. The amount of rRNA synthesis was determined for different incubation periods. After labelling the cells during 120 min and a simultaneous treatment with cycloheximide at least 60 – 65% are synthesized in relation to the control. It can be supposed that under these conditions secondary effects do not carry any weight. The reduction of the rRNA synthesis has to be considered as a result of regulatory processes. The protein synthesis however is not detectable under these circumstances.

2. Delay of the processing after inhibition of the protein synthesis

From plant and animal cells a series of examples are known: 35S, 27S and 20S RNA are accumulated in yeast [2] and a 32S RNA in *Phycomyces* [15]. Parsley cells amass 3 precursor molecules with the following molecular weights: 2.3×10^6, 2.0×10^6, and 0.9×10^6 daltons. Considering this effect, the inhibition with cycloheximide is a good tool to catch hold of shortlived RNA precursors and to characterize them.

3. The pool of ribosomal proteins and transport

The assembly of polynucleotides with proteins to RNP-particles is possible only if there are ribosomal proteins in sufficient quantities. One can imagine three possibilities for the inhibition of protein synthesis affecting this process. a. The transport is inhibited totally. b. In the case of a pool of ribosomal proteins the transport takes a more or less normal course. c. Different pools of protein exist which lead to a differential transport of the two ribosomal subunits. In our experiments, the labelled mature rRNA's appear in the ribosomal fraction in the same

ratio as measured in the control if cycloheximide is added during the chase treatment. That means a protein pool for the two ribosomal subunits exists, which makes the transport process independent from newly synthesized protein for some time. It follows from these results (and the one described earlier) that the drug has a weaker effect on the transport than on the processing provided low concentrations are employed.

Material and Methods

Cell growth conditions. The cells of *Petroselinum crispum* were propagated in suspension at 26 °C under aeration with sterile air as described by Seitz and Richter [16]. Growth of the cultures was measured by packed cell volume determinations.

Protein synthesis *in vivo*. Cells (packed cell volume 0.09 cm^3/ml) were preincubated in a leucine-free culture medium under sterile conditions. During the incubation time with L-[^{3}H]leucine (Amersham Buchler, Braunschweig) the cells were aerated with sterile air. Samples of 1 ml were taken from the suspension in 10 min intervals and sucked on nitrocellulose filters (SM 111, 3 μm, Sartorius, Göttingen). The protein synthesis was stopped by a TCA-treatment (5%). The free amino acids were removed by washing the filters thoroughly. After drying the filters at room temperature they were counted for ^{3}H-radioactivity in a PPO-POPOP-toluene cocktail. The cycloheximide was purchased from Boehringer (Mannheim).

^{32}P-labelling; pulse experiments. After a 12 h preincubation in a phosphate-free culture medium, 10 μCi/ml of [^{32}P]orthophosphate (carrier-free; Amersham Buchler, Braunschweig) were added. The incubation was terminated by a rapid cooling of the suspension on a salt-ice mixture.

Pulse chase experiments. At the end of the ^{32}P-pulse the cells were filtered, washed and resuspended in the normal culture medium with an excess of [^{31}P]phosphate and cultivated under the normal culture conditions. In the case of cycloheximide treatment the antibiotic (5 μg/ml) was added 10 min before the chase incubation starts.

Preparation of nuclei and ribosomes. 1. Nuclei. To avoid differences resulting from differences in the physiological state, the organelles were prepared from the same culture batch. The cell suspension was cooled down rapidly and centrifuged once more. The cells were disrupted in a potter homogenizer (Braun, Melsungen); 50 – 60 g fresh weight in an end volume of 120 ml; buffer: Tris-HCl-sucrose (0.05 M Tris, 0.002 M $MgSO_4$, 0.25 M sucrose, pH 7.5). The homogenate was filtered after the method of Tautvydas [9] through a series of nylon nets with various mesh widths (200 μm, 100 μm, 30 μm, 10 μm). After a 6800 × g centrifugation (10 min) the nuclei were gathered from the pellet. The supernatant contains the ribosomes. After washing (for buffer and centrifugation conditions see above) the nucleic acids were extracted from the nuclei according to the methods described previously [16, 7].

2. Ribosomes. The ribosomes were prepared according to Bielka *et al.* [8]. 1. Two centrifugations were made at 12000 × g for 10 min. 2. Supernatant was treated with Na-deoxycholate (0.5%, 15 min), followed by a centrifugation (see above). 3. The colourless pellet of a 90 min ultracentrifugation (Beckman rotor type 50 Ti, 39000 rpm) was suspended carefully in a Tris-HCl buffer (0.05 M Tris, 0.002 M $MgSO_4$, pH 7.5) and the nucleic acids were extracted by the phenol method.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out in 2.1% polyacrylamide gels [17]. The buffer system described by Loening [18] was used. In all cases 9.8 – 10 μg nucleic acids were applied to one gel. After ultraviolet scanning in a Polyfrac (Joyce Loebl, England) the gels were frozen in solid CO_2 and chopped in 0.5 mm slices. The radioactivity of dried gel slices was determined in a scintillation counter.

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