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THE IONIC REQUIREMENT FOR ISOLATION OF STABLE POLYRIBOSOMES FROM CEREAL PLANTS AND ITS RELATION TO SULFHYDRYL PROTECTION

BY

MERLIN DENNIS BREEN

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Chemistry, South Dakota
State University

1969

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THE IONIC REQUIREMENT FOR ISOLATION OF STABLE POLYRIBOSOMES FROM CEREAL PLANTS AND ITS RELATION TO SULFHYDRYL PROTECTION

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Head, Chemistry Department Date

ACKNOWLEDGMENTS

The author wishes to express his gratitude and appreciation of Dr. D. G. Kenefick, Associate Professor in Agronomy and Station Biochemistry at South Dakota State University, for his guidance, patience, assistance and encouragement throughout the duration of the study. Sincere appreciation is also extended to other staff members, especially to Professor Eugene Whitehead for reviewing the manuscript. Appreciation is also extended to Teresa DeBoise for her technical assistance.

Special thanks is also extended to my wife, Janet, for her patience and understanding during the course of the study.

MDB

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INTRODUCTION AND REVIEW OF LITERATURE

Early attempts to isolate polyribosomes from barley tissue by the procedure of Lin and Key (14) were unsuccessful. When analyzed by sucrose gradient centrifugation such preparations showed a particle distribution predominately in the monomer region of the gradient similar to data of Srivastava and Arglebe (19). Similar difficulties were also encountered with certain animal tissues (7,4,11). They were resolved by the addition of higher levels of KCl to the extraction medium than used initially by Wettstein et al. (27).

The beneficial effects of monovalent ions can be explained in various ways. Petermann and Pavlovec (16) suggested that high salt levels prevented the binding of extraneous proteins, basic proteins in particular, to the ribosomal particles. Earl and Korner (6) demonstrated that amino acid incorporation into proteins was inhibited when polyribosomes were extracted from muscle tissue under low ionic conditions. Particles isolated in this manner were shown to have an usually high protein to nucleic acid ratio. Earl and Morgan (7) have more recently provided verification of these earlier results. Heywood et al. (11) have shown that high ionic strength was necessary to prevent co-precipitation of polyribosomes with myosin, a result similar to that reported by Earl and Morgan (7) for preparations from cardiac muscle.

Hayes *et al.* (8) have shown that 0.60 M NaCl favored the binding of ribosomal subunits to the polyribonucleotides, poly C, poly U and poly G, and this was attributed to a favorable environment for base pairing. These workers were also able to demonstrate a non-enzymatic binding of ribosomal RNA (rRNA) with natural occurring messenger RNA (mRNA) under these ionic conditions, again attributable to the interaction of complementary bases (9). These results suggested that if rRNA-mRNA formation was favored by high ionic conditions, similar associations occurring in the cell may also be stabilized by high ionic strength during extraction.

Still another explanation for polyribosome stability in high ionic strength is provided by the data of Wilson (28) in which conditions for maximal extraction of ribonuclease (RNase) from corn seedlings were investigated. He found that the inclusion of 0.50 M KCl was essential for the removal of this basic protein from microsomes. The results are reminiscent of the basic protein data presented by Petermann and Pavlovec (16) discussed above. Successful preparation of polyribosomes from tissue depends upon preventing degrading effects of RNase. The following is a report of preparative conditions essential for the extraction of stable polyribosomes from barley tissue. Preliminary data are also presented for corn, rye and wheat.

MATERIALS AND METHODS

Plant Material: Forty winter barley seeds (*Hordeum vulgare* 'Dicktoo') were placed on a double layer of blotter paper in 4-3/8" square plastic boxes and watered with 25 ml of 1 x 10^{-4} M $CaCl_2$. These boxes were covered and placed in a 10 x 21" plastic tray which was covered with a sheet of 4 mil black plastic. A constant 100% relative humidity was maintained during germination by the addition of 100 ml of water to this tray. The tray was placed in a 25° growth chamber and the seedlings were germinated for 4 days, at which time the shoots were about 40-50 mm in length. Two 40-seed lots provided adequate plant material (3 gm) for the extraction of polyribosomes for one treatment. Winter wheat (*Triticum aestivum* 'Scout 66') and winter rye (*Secale cereale* 'Bonel') were germinated in a similar manner, except 50 seeds were added per box. Corn (*Zea mays* 'SD 5 x M 14') was germinated with 25 seeds added per box. The seed was treated either by soaking for 6 minutes in a solution of Ceresan M (1.4 g/liter), rinsing 3 times in water and immediately air drying, or by dusting with Ceresan M.

Solutions: The extraction medium used for barley (except where noted otherwise) was composed of 0.25 M sucrose, 0.05 M Tris-HCl pH 8.0, 0.40 M KCl, 0.020 M $MgCl_2$ and 0.005 M mercaptoethanol. The gradient buffer was 0.20 M KCl, 0.05 M Tris-HCl pH 7.8 and 0.010 M $MgCl_2$. The 10 and 35% (w/v) sucrose solutions used for the continuous

sucrose gradient were made from a 60% sucrose solution, purified by the method of Braake[1]. The concentrated sucrose solution was diluted to 10 and 35% using a refractometer to determine final concentrations. Approximate molar concentrations of the salts used for the gradient buffer were added in crystalline form to these solutions. The pH was then adjusted to 7.8 by the dropwise addition of 4 N HCl. All chemicals were reagent grade and were dissolved in twice glass-distilled water.

Ribonuclease A (type I-A, bovine pancreas, 5 x crystallized), deoxycholate (DOC) and cycloheximide were obtained from Sigma Chemical Co.; dithiothreitol (DTT) was purchased from Calbiochem and ferritin (horse, 5 x crystallized) from Mann Research Laboratories.

Extraction of Polyribosomes: The procedure for the preparation of polyribosomes is outlined in figure 1. Three grams of 4-day shoots were excised, placed in an aluminum foil envelope and immediately covered with powdered dry ice. After a 10 to 15 minute freeze period, the shoots were pulverized in an iced-in mortar. The powdered tissue was transferred to a test tube and warmed to 0°. All subsequent operations were performed at 0° unless otherwise noted. Eight ml of extraction medium were added to each tissue sample, which was then suspended by 30 vertical strokes of a loose-fitting teflon pestle.

1) The ISCO manual, 4700 Superior, Lincoln, Neb. 68504.

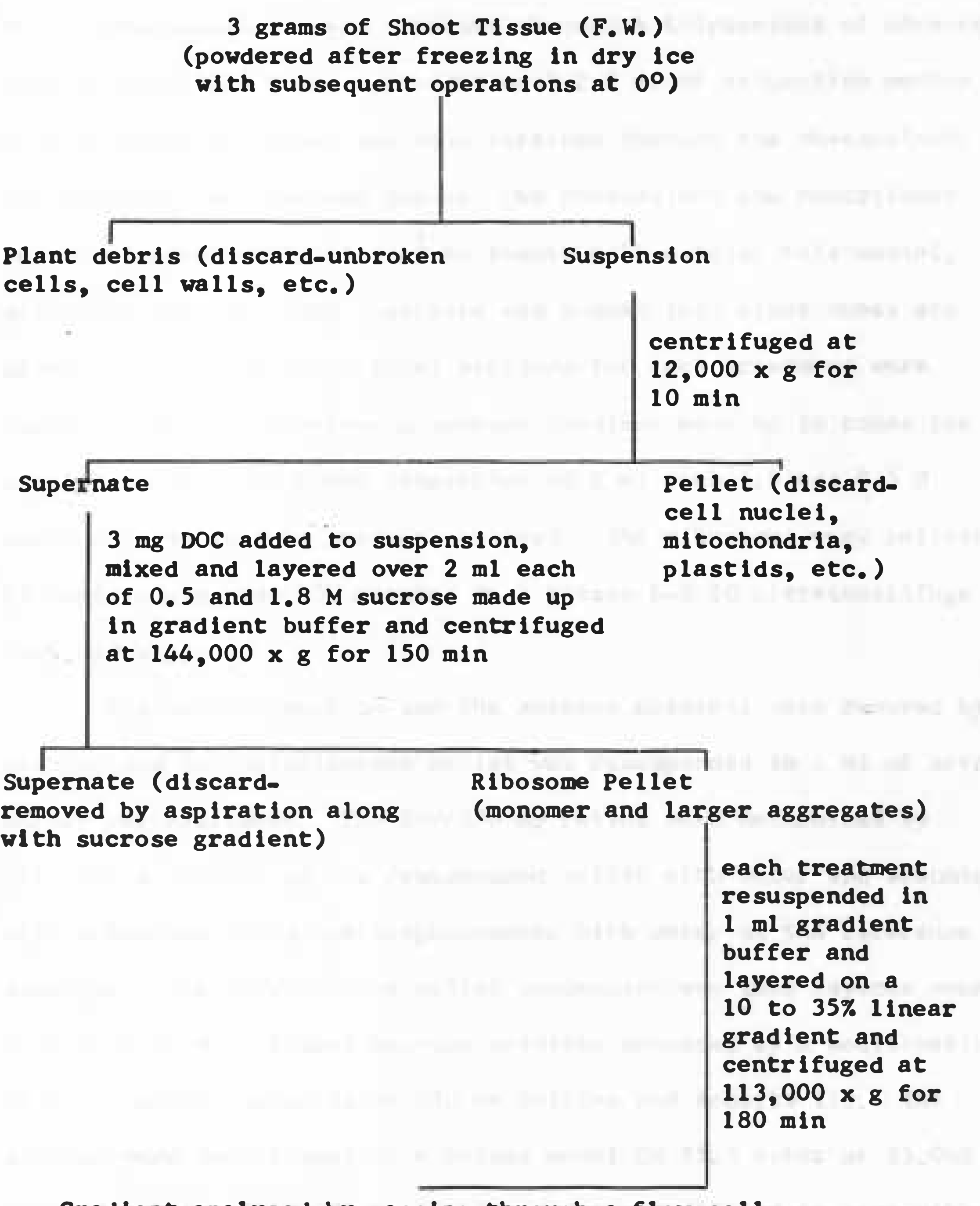

Fig. 1. Procedure for preparation of polyribosomes.

The suspension was strained through 4 thicknesses of cheesecloth into a centrifuge tube. An additional 2.8 ml of extraction medium, used to rinse the tube, was also strained through the cheesecloth. The filtrate was squeezed through the cheesecloth and centrifuged for 10 minutes at 12,000 x g[1] to remove cell debris, cell nuclei, mitochondria, etc. The supernate was poured into clean tubes and mixed with 3 mg of DOC. Equal portions for each treatment were layered over a discontinuous sucrose gradient made up in tubes for the Spinco model 40 rotor consisting of 2 ml each 1.8 and 0.5 M sucrose (dissolved in gradient buffer). The ribosomes were pelleted by centrifuging for 150 minutes in a Spinco L-2 50 ultracentrifuge (144,000 x g).

The soluble portion and the sucrose gradient were removed by suction and the polyribosome pellet was resuspended in 1 ml of gradient buffer per treatment. The 260/280 mμ ratios were determined by diluting a portion of the resuspended pellet with water and scanning with a Beckman DK-2A spectrophotometer with water as the reference solution. The polyribosome pellet suspension was then layered over a 10 to 35% (w/v) linear sucrose gradient prepared by a modification of the gradient maker described by Britten and Roberts (1). The samples were centrifuged in a Spinco model SW 25.3 rotor at 25,000 rpm for 180 minutes (113,000 x g). The rotor was allowed to stop without the use of the brake.

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1) Forces for rotors are stated at maximum rotor radius.

Analysis of Gradient: The gradients were analyzed using a teflon density gradient flow cell which incorporated an adaptor for the centrifuge tube. The flow cell was mounted in an ISCO dual beam optical unit. The optical density was monitored at 254 mμ with an ISCO UA-2 ultraviolet analyzer in conjunction with a Honeywell recorder. Direct tracings from this strip chart recorder were used for the results presented here. Areas under the curves were determined by use of a planimeter. Peaks not clearly separated were estimated by dividing the peak areas with a vertical line from the lowest point to the baseline.

RESULTS AND DISCUSSION

Polyribosomes are aggregates of ribosomes held together by messenger RNA (mRNA) which has the function, in living systems, of transcribing the genetic message from the nucleus during the growth process. Under careful extraction conditions polyribosomes can be isolated from tissue by differential centrifugation. The physical properties of the particles obtained in this investigation were similar to those reported by other workers (12,14,28).

For purposes of this report the polyribosome fraction was defined as the UV absorbing extract from plant tissue which sedimented below the monomer in the sucrose density gradients used. The monomer was located in the gradient by comparing it with ferritin (fig. 2), which has a sedimentation constant of 65S, and by degrading the polyribosomes to monomers with RNase (fig. 2). This polyribosome fraction has been further characterized by its requirement for Mg^{++} during extraction. Figure 3 shows a stepwise increase in heavy particles with increasing amounts of Mg^{++} in the extraction medium. The dependence of tissue upon adequate levels of oxygen for aerobic metabolism (14,20) has also been shown to be a factor in polyribosome stability. More recently the effect of the respiratory inhibitors, azide and dinitrophenol, has been shown to influence the maintenance of a normal distribution of particles in the gradient (13).

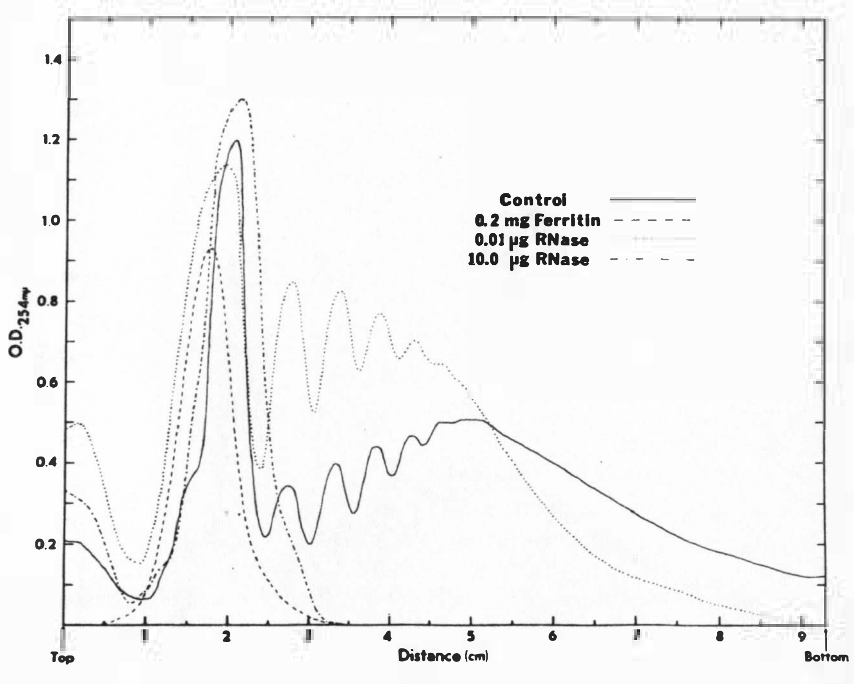

Fig. 2. A comparison of the normal stable polyribosome profile with profiles of ferritin (0.2 mg), of resuspended polyribosome pellet incubated for 10 minutes with 0.01 µg of RNase, and of supernate incubated for 20 minutes with 10 µg of RNase (one-half of resulting preparation was layered). The profiles were developed in 10 to 35% (w/v) linear sucrose gradient by centrifuging at 113,000 x g for 180 minutes. The sucrose solution contained 0.20 M KCl, 0.01 M $MgCl_2$ and 0.05 M Tris-HCl pH 7.8. The monomer (first peak on the left) was identified by comparison with the ferritin peak (sedimentation constant of 65S) and RNase treatment of the polyribosomes; the change in profile reflected a shift of UV absorbing material from the denser region toward the monomer with the more severe RNase treatment. The most positive slope of a line connecting the OD maxima of the peaks following the monomer was used to judge favorable extraction conditions.

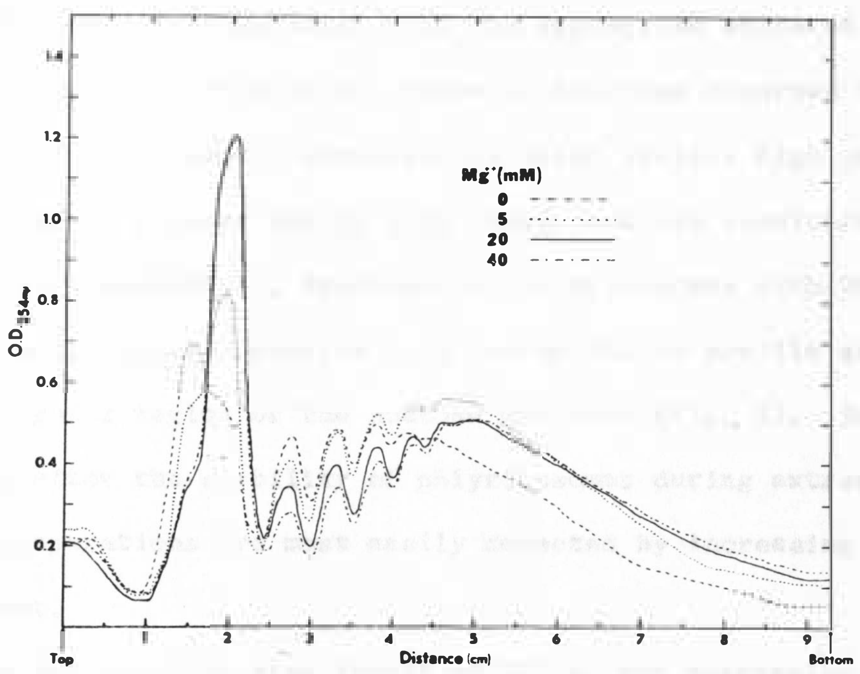

Fig. 3. A comparison of the influence of varying levels of Mg^{++} in the extraction medium on the stability of polyribosomes from 4-day barley shoots, when prepared in the presence of 0.40 M KCl, 0.05 M tris-HCl pH 8.0, 0.25 M sucrose and .005 M mercaptoethanol. The profiles were developed on a sucrose gradient as described in fig. 2. The 0 value reflects a breakdown of monomers to subunits, but the Mg^{++} level present in the tissue apparently was sufficient to maintain most of the polyribosome stability. A stepwise increase in the amount of denser particles is seen with increasing Mg^{++} levels.

The data that follow demonstrate several ways by which the particle distribution in the gradients can be altered. Maximum protein synthesis is associated with the aggregated state of ribosomes, whereas little or no synthesis has been observed for the monomers (27). Presumedly preparations which yield a high percentage of polyribosomes reflect the in situ status and are considered stable preparations. Conversely, treatment of polyribosomes with RNase prior to centrifugation results in a sedimentation profile skewed into the lighter region of the sucrose gradient (fig. 2). Several conditions alter the stability of polyribosomes during extraction and unstable preparations are most easily detected by increasing levels of the dimer.

The effect of varying levels of KCl in the extraction medium used to isolate polyribosomes from barley shoots tissue is shown in fig. 4. Omission of KCl during the extraction essentially eliminated particles sedimenting below the monomer in the gradient. With increasing levels of KCl a progressive increase in UV absorbing material was observed below the 2 cm distance in the tube. A comparison between 0.40 and 0.60 M KCl showed essentially no difference in the denser polyribosome region of the gradient. A depression in the amount of monomer was observed by the addition of 0.60 M KCl compared with other concentrations of the salt. Centrifugation conditions used were not adequate to resolve what appeared to be a shift from the monomer to possibly ribosomal subunits with this KCl level. The total yield of polyribosomal material was also depressed by this highest salt level.

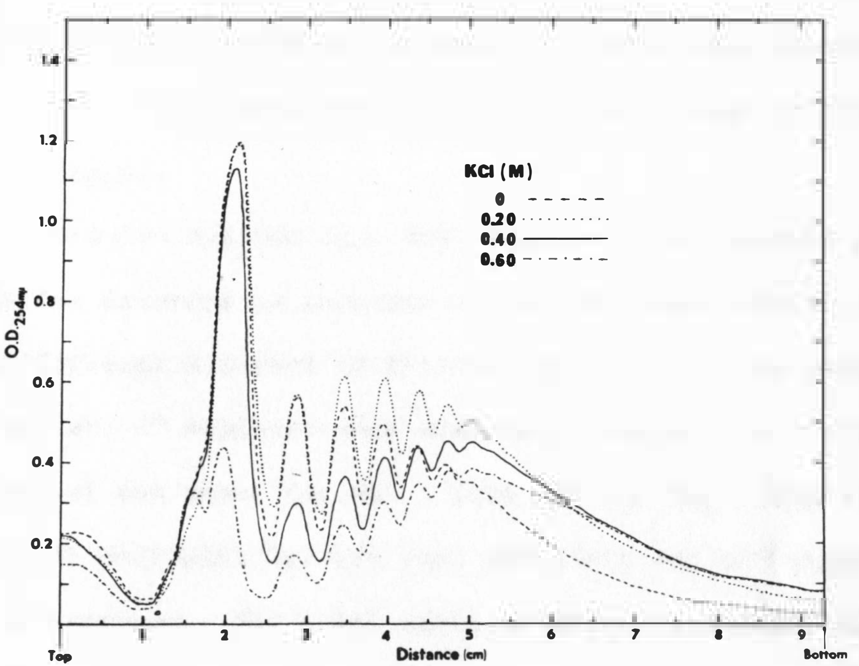

Fig. 4. A comparison of varying levels of KCl in the extraction medium (pH 7.8) on the yield and qualitative characteristics of polyribosomes from 3 grams of 4-day barley shoots. The Mg^{++} level was 0.02 M; other conditions were the same as in fig. 3.

Polyribosomes were also extracted in the presence of various monovalent ions. The chloride salts of Li^+, Na^+, K^+ and NH_4^+ at the 0.4 M concentration were used to extract barley tissue (pH 7.8), while similar salts at the 0.2 M level (pH 8.2) were used to obtain polyribosomes from rye (table I). As will be established later these two species required different extraction conditions; therefore, direct comparisons between the two sets of data shown in table I may not be meaningful.

The results did not show any appreciable difference in the sedimentation patterns in response to varying atomic radii in contrast to the differences reported by Phillips _et al._ (18) for bacteria. In barley, Na^+ and K^+ extracts were comparable except for a slightly lower level of the dimer for Na^+. Both Li^+ and NH_4^+ depressed the proportion of particles heavier than the dimer and Li^+ significantly reduced the monomer. The total yield of barley ribosomal material was higher for the cations of smaller atomic radii. With rye the effect of these salts was less pronounced. The principle differences were a lower percentage of dimer for Li^+ and K^+ and a reduction in total yield in the presence of K^+.

In addition to the stabilizing influence of higher KCl concentrations, pH was also shown to be important. In fact, the effects of KCl and pH were shown to be interrelated (fig. 5 and 6). The results indicated that higher KCl concentrations were less important as the pH of the extraction medium was increased. Using

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Table I. The Influence of Varying Atomic Radii on the Recovery and Stability of Polyribosomes from Barley and Rye Tissue

Relative yield of ribosomes and aggregates[1]		Area per cent[2]		
		Monomer	Dimer	>Dimer
	Barley[3]			
Li^+		29.3	4.3	66.4
Na^+		25.3	3.9	70.8
K^+		25.2	4.3	70.5
NH^+		31.6	3.9	64.4
	Rye[3]			
Li^+		25.2	5.6	69.2
Na^+		23.5	6.9	69.6
K^+		25.4	5.2	69.4
NH^+		25.4	6.6	68.0

1 Yield of this fraction was determined by calculating with a planimeter the total area under the curve from traces obtained by measuring absorbance at 254 mμ of 10 to 35% (w/v) linear sucrose gradients, which had been layered with plant extracts prior to centrifugation. Extracts represent 3 g fresh weight of 4-day shoot tissue.

2 Particle distribution was evaluated with a planimeter on traces from centrifuged samples.

3 Extraction conditions are described in Materials and Methods and Fig. 1 and were the same for barley and rye except the former was extracted with buffer containing 0.40 M KCl at pH 7.8, while rye polyribosomes were prepared with 0.20 M KCl at pH 8.2.

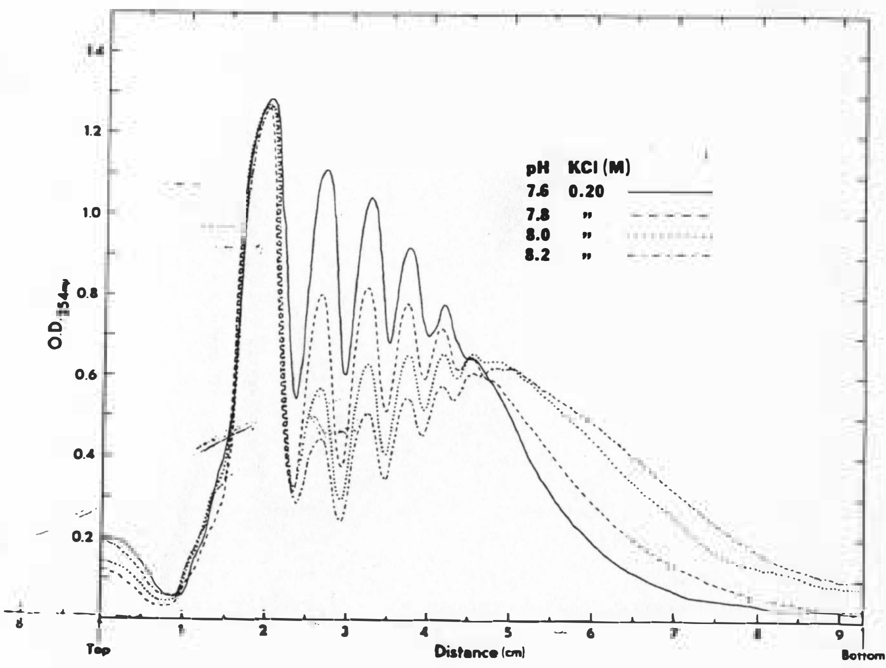

Fig. 5. The influence of pH of the extraction medium on stability of polyribosomes from 4-day barley shoots prepared in the presence of 0.20 M KCl; other conditions were the same as described in fig. 3. Following tissue extraction the pH dropped about 0.2 unit for each treatment, as measured in the 144,000 x g supernate fraction. At this KCl concentration the pH 8.2 extraction provided the best polyribosome preparation, when measured by the criterion outlined in fig. 2.

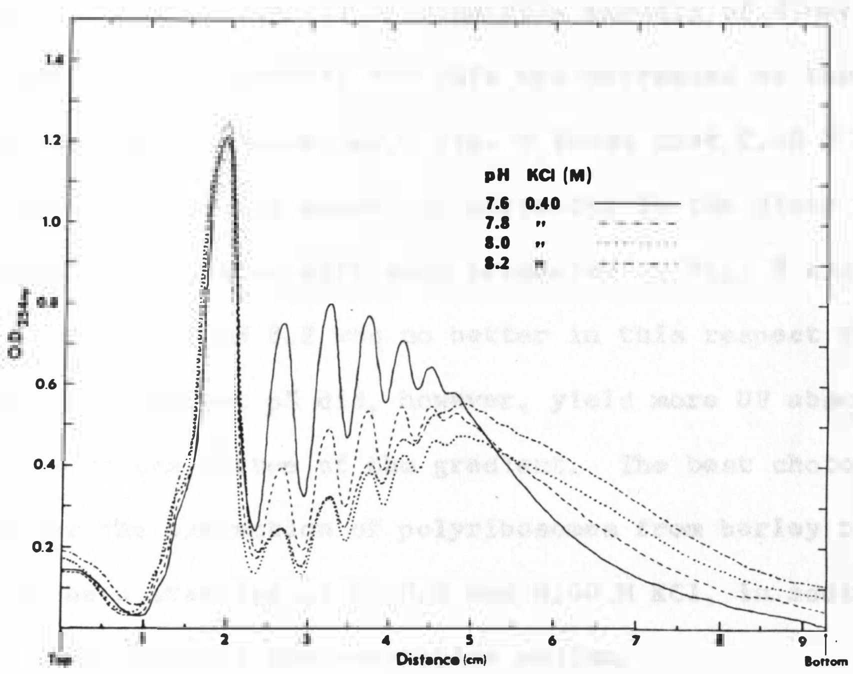

Fig. 6. The influence of pH of the extraction medium on stability of polyribosomes from 4-day barley shoots prepared in the presence of 0.40 M KCl; other conditions were the same as described in fig. 5. At this higher salt concentration, the higher pH values for the extraction medium were not as critical for obtaining stable polyribosome preparations. Again, when compared with the data in fig. 5, a drop of about 0.2 pH unit occurred during the extraction procedure.

0.05 M Tris buffer, the pH dropped about 0.2 unit from the original value during extraction of the plant tissue.

At 0.20 M KCl (fig. 5) considerable amounts of dimer were found in the barley extracts, but this was depressed as the pH of the extraction medium was increased. Fig. 6 shows that 0.40 M KCl effectively depressed the amount of particles in the dimer region of the gradient, as compared with data presented in Fig. 5 and that an extraction medium of pH 8.2 was no better in this respect than one of pH of 8.0. The highest pH did, however, yield more UV absorbing material toward the bottom of the gradient. The best choice of these variables for the extraction of polyribosomes from barley tissue appeared to be a starting pH of 8.0 and 0.40 M KCl, in addition to the other components of the extraction medium.

The unique KCl requirement for polyribosome stability in this tissue raised some questions as to similar requirements for other monocot species. Density gradient centrifugation was used on extracts from barley, rye, wheat and corn, each of which were extracted in medium containing either 0, 0.20, 0.40 or 0.60 M KCl (pH 7.8).

The results (table II) were evaluated on the basis of 1) the relative amounts of rRNA distributed in the monomer, dimer and the greater than dimer regions of the gradient as indicated by area per cents, 2) the total recovery of rRNA aggregates from 3 gm of tissue for each species, and 3) the 260/280 mμ ratio, indicating the extent to which ionic strength influenced the binding of protein to the particles.

Table II. The Quantitative and Qualitative Characteristics of Polyribosomes from Four Plant Species as Influenced by KCl Concentration during Extraction

KCl (M)	Relative yield of ribosomes and aggregates[1]	Area per cent[1]			260/280[2]
		Monomer	Dimer	>Dimer	
		Barley[3]			
0.0		35.1	10.4	54.4	1.86
0.2		27.6	7.7	64.7	1.89
0.4		28.1	5.4	66.5	1.88
0.6		17.6	4.6	77.8	1.78
		Rye[3]			
0.0		13.7	6.4	79.9	1.84
0.2		11.8	5.3	82.8	1.84
0.4		11.5	4.4	84.1	1.81
0.6		5.3	2.9	91.8	1.80
		Wheat[3]			
0.0		16.2	7.2	76.6	1.86
0.2		14.7	7.7	77.6	1.86
0.4		16.9	4.7	78.4	1.86
0.6		12.1	4.7	83.2	1.82
		Corn[3]			
0.0		28.4	15.7	55.9	1.68
0.2		18.1	10.5	71.4	1.71
0.4		20.4	5.8	73.7	1.93
0.6		15.1	4.1	80.8	1.57

[1] Calculated as described in table I.

[2] 260/280 ratios were calculated from wavelength scan plots on the total ribosome extract.

[3] 3 g of 4-day shoot tissue was extracted for each KCl variable. Initial pH of the extraction medium was 7.8 which dropped to about 7.6 after cell disruption.

As the salt concentration of the extraction medium was changed from 0.0 to 0.60 M, the amount of monomer and dimer decreased while particles larger than dimers increased. In the absence of KCl it was observed that 1) preparations from barley showed the highest level of monomer, followed (in order of decreasing amounts) by corn, wheat and rye, 2) preparations from corn showed the highest level of dimers, followed by barley, wheat and rye, and 3) preparations from barley showed the lowest level of particles greater in size than dimers, followed (in order of increasing amounts) by corn, wheat and rye. Based upon the observed changes in extractions made without KCl, the order of stability of polyribosomes was rye   wheat corn   barley.

The area per cents for monomers in 0.60 M KCl extracts showed the same species order as in the absence of the salt, with that of barley being the highest. As before, the species lined up in inverse order when the area per cents were compared for particles larger than dimers. The data suggested that the KCl requirement for polyribosome stability was related to plant species and that the need of a monovalent cation during extraction was greatest for barley tissue.

At the highest salt level the monomer was depressed in all species. Two apparent explanations existed for this observed depression. As shown in fig. 3, this high level of KCl apparently replaced some of the Mg^{++} required to bind the ribosomal subunits together, which resulted in the dispersion of the monomer (24,29).

A second possibility was that in this high ionic environment, free monomers aggregated with polyribosomes, as suggested by Petermann and Pavlovec (17). Table II also shows a quantitative difference among species in the amount of ribosomal material extracted from 3 gm of tissue. Regardless of KCl concentration, it appeared that barley yielded the lowest amount followed by corn and wheat or rye. It is not known if this observation reflects the actual number of polyribosomes per cell, the number bound to membranes and, therefore, discarded with the cell debris or the number of cells per unit fresh weight of tissue.

The 260/280 mμ values varied with respect to KCl levels within species and among species. No consistency was observed in these values to aid in establishing the optimum KCl concentration for extraction of polyribosomes. A fact which might be explained by differences in base composition of these species. Hayes _et al_. (8) have demonstrated that rRNA will bind non-enzymatically in high ionic conditions to poly U, poly C and poly G, but not to poly A. Different ratios of bases would be expected among different species, resulting in different levels of adenine bases. This could account for the different KCl requirements in the extraction of ribosomal material from barley, rye, wheat and corn.

At the two extreme KCl levels studied, the 260/280 mμ ratios decreased for all species observed except for rye (table II). The possible significance of this information in terms of the binding of extraneous protein (6,15) will be discussed further below. The reduction in yield of total aggregated and nonaggregated rRNA for

all species at 0.60 M KCl, the general loss of monomers and associated increase in ribosomal subunits, and the marked drop in the 260/280 mμ ratios all helped rule out this highest concentration of KCl as being desirable for the preparation of polyribosomes. Thus, an evaluation of only the area per cent of the fraction larger than dimers was not an adequate measure of polyribosome extraction conditions.

Two possible ways of explaining the beneficial effect of KCl on polyribosomes during extraction are: 1) that the high ionic environment reduced the binding of endogenous RNase, thus stabilizing polyribosomes by separation from the enzyme with eventual complete removal by centrifugation; 2) that the high salt concentration actually inhibited endogenous RNase, preventing its attack on the vulnerable mRNA. It seemed possible to distinguish between these two alternative explanations for the stabilizing influence of KCl. The approach used to ascertain the function of KCl was to incubate the cell extract at 25° for 20 minutes 1) before removal of cell debris, 2) before removal of the mitochondrial fraction and the addition of DOC and 3) after removal of the mitochondrial fraction. A similar incubation was made of the resuspended polyribosome pellet.

Removal of the cell debris, the 12,000 x g or the 144,000 x g fraction prior to the 25° incubation had no differential influence on the polyribosomes nor did the preparations show any indication of instability following such treatment (fig. 7). It appeared logical

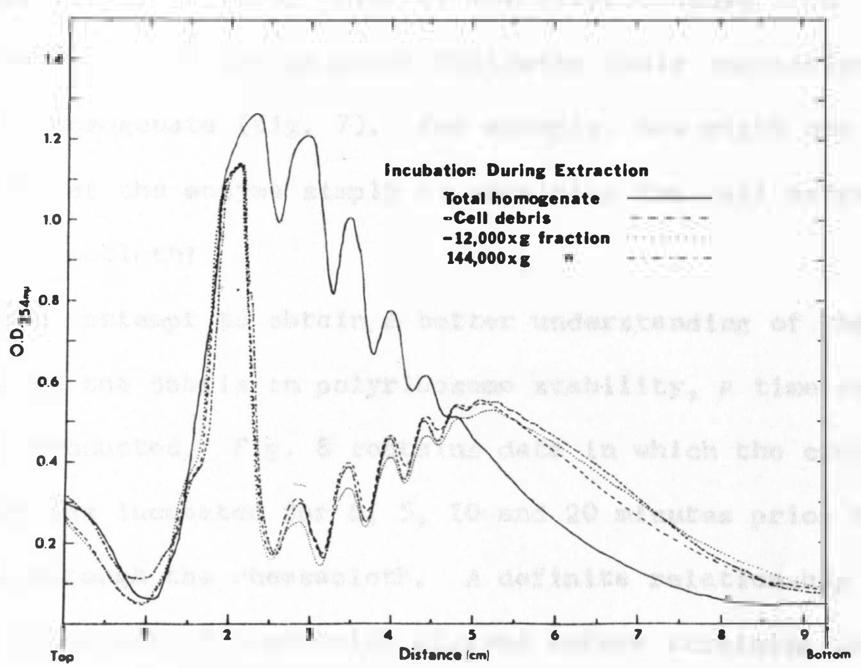

Fig. 7. The influence of incubation at 25° for 20 minutes on polyribosome stability during sequential stages of their extraction from 3 gm of 4-day barley tissue. The extraction medium contained 0.40 M KCl adjusted to pH 8.0. All other preparative conditions were the same as described in fig. 3. Incubation in the total homogenate had a pronounced effect of shifting ribosomal material into the lighter region of the gradient. Incubation after removal of cell debris or subsequent stages in preparation resulted in stable preparations, indicating inhibition or elimination of RNase by the procedure followed.

to conclude that KCl had actually inhibited the endogenous RNase, since only at the final step of the preparation were the polyribosomes removed from the cytoplasmic proteins. It was difficult, however, to develop any kind of rationale, based upon RNase activity, which would explain the striking shift of the polyribosomes into the less-dense region of the gradient following their incubation in the total cell homogenate (fig. 7). For example, how might one explain the removal of the enzyme simply by straining the cell extract through cheesecloth?

In an attempt to obtain a better understanding of the influence of the debris on polyribosome stability, a time-course study was conducted. Fig. 8 contains data in which the crude homogenate was incubated for 0, 5, 10 and 20 minutes prior to straining through the cheesecloth. A definite relationship between the length of time which elapsed before straining off the cell debris and the degree of polyribosome shift into the lighter region of the gradient was observed. The results at this point showed that the degrading effect of incubation could be controlled by removing the debris and that the preparations were stable for the remaining six hours required to complete the polyribosome extraction and analysis.

Consideration was next given to other chemical systems that might explain a loss in structure of polyribosomes in the presence of the cell debris. The possibility of an unfavorable oxidation-reduction environment was examined. Initially the contribution of

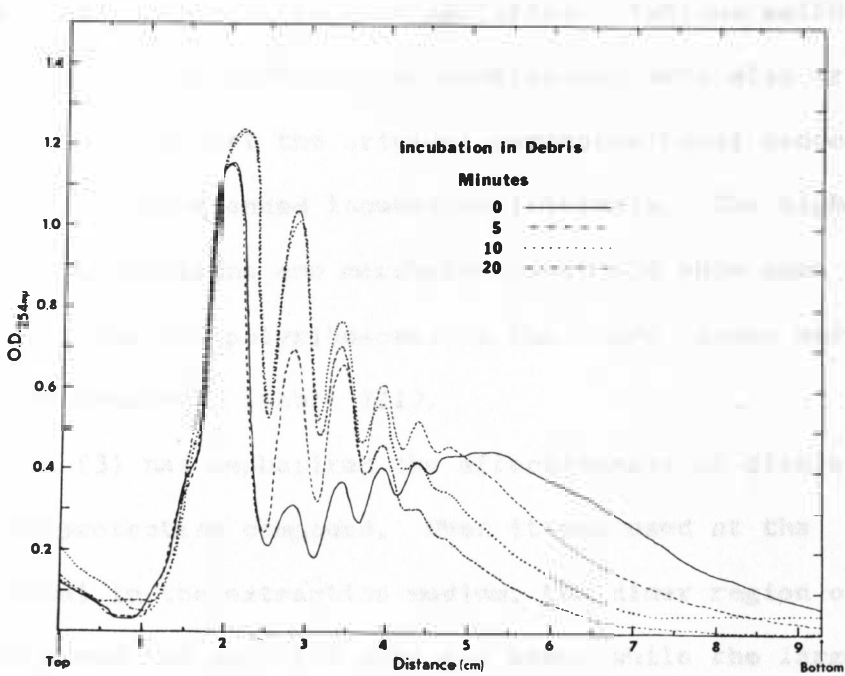

Fig. 8. A time-course study of the influence of cell debris on stability of polyribosomes from barley shoots. Other conditions were the same as reported in fig. 7. The data showed that stability of the polyribosome fraction was related to the duration of exposure to the cell debris, with the 20 minute incubation period having the greatest detrimental effect (see fig. 2).

ascorbic acid was investigated, but it was found to be ineffective (table III) as determined by its failure to prevent the formation of dimers at the expense of heavier particles. Various sulfhydryl-protecting compounds at different concentrations were also tried, since it was possible that the original mercaptoethanol concentration was insufficient for extended incubation intervals. The highest concentrations of cysteine and mercaptoethanol did show some improvement in stabilizing the polyribosomes in the crude tissue extract during the 25° treatment (table III).

Cleland (3) has emphasized the effectiveness of dithiothreitol (DTT) as a SH-protecting compound. When it was used at the 1×10^{-2} M level in the extraction medium, the dimer region of the gradient accounted for only 8.7 area per cent, while the larger than dimer region accounted for 65 area per cent, after a 20 minute incubation of polyribosomes in the cell debris (table III). These values reflect the approximate stability of polyribosomes found under normal extraction conditions without incubation. The results showed that adequate levels of SH-protectants did stabilize the polyribosome fraction, when incubated with the debris in 0.40 M KCl.

The question arose as to whether the observable effects of high KCl level and DTT on polyribosome stability were similar. If this were true, it might be possible to extract stable polyribosomes from barley tissue at lower levels of KCl, provided that adequate amounts of DTT were present. The data shown in fig. 9 demonstrate that the KCl requirement was reduced considerably, when

Table III. The Contribution of Various Redox Systems in the Extraction Medium to Polyribosome Stability During Their Incubation in the Presence of the Total Cell Extract

Additive[1]	Particle Size	Concentration (M) 10^{-4}	10^{-3}	10^{-2}	10^{-1}
		Area per cent[2]			
Ascorbic acid					
	Dimer		20.0	20.0	20.8
	>Dimer		42.0	39.0	37.0
Cysteine					
	Dimer		21.5	22.0	12.0
	>Dimer		38.0	34.0	58.0
Mercaptoethanol					
	Dimer		22.0		19.3
	>Dimer		31.0		44.2
Dithiothreitol					
	Dimer	25.0	16.0	8.7	
	>Dimer	29.0	38.0	65.0	

1 Materials were dissolved in or added to the extraction medium to give final concentrations as indicated. Total homogenate was incubated for 20 minutes at 25°.

2 Conditions for centrifugation of cell extracts and calculation of particle distribution were the same as indicated in table I.

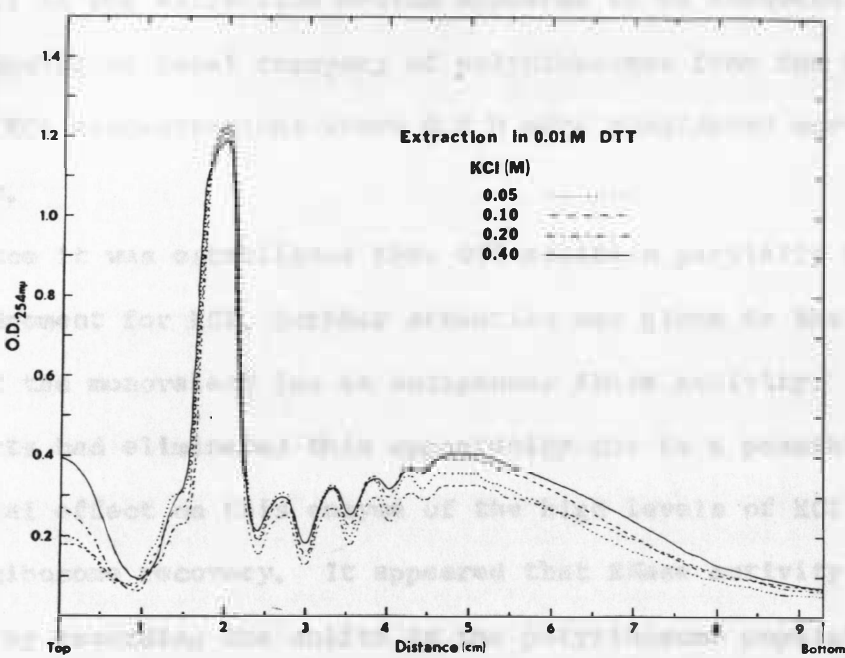

Fig. 9. The compensating effect of 1 x 10^{-2} M DTT in the extraction medium on barley polyribosomes prepared under conditions of varying KCl concentration. Other preparative conditions same as in fig. 7, except that no incubation period was employed. As determined by the criteria set forth in fig. 2, DTT appreciably reduced the need for high salt concentrations to stabilize polyribosomes.

1×10^{-2} M DTT was added to the extraction medium. As judged by the level of dimer to total area under the curve for the polyribosomes, 0.05 M KCl in the extraction medium appeared to be adequate. From the standpoint of total recovery of polyribosomes from the tissue, however, KCl concentrations above 0.2 M were considered more desirable.

Once it was established that DTT addition partially replaced the requirement for KCl, further attention was given to the specific effect of the monovalent ion on endogenous RNase activity. Earlier experiments had eliminated this opportunity due to a possible detrimental effect on this enzyme of the high levels of KCl required for polyribosome recovery. It appeared that RNase activity could be measured by recording the shifts in the polyribosome population. To determine their sensitivity to RNase, a commercial preparation of RNase was added to either the homogenate following the removal of cell debris or to a resuspension of the polyribosome pellet. The results shown in table IV indicated about a 10-fold increase in sensitivity to RNase, when it was added to the 1 ml volume of the resuspended polyribosomes as compared to a similar RNase addition to the strained homogenate. As previously indicated, increasing levels of the dimer reflected activity of the enzyme. This assay system could easily detect levels of enzyme down to 1×10^{-2} μg for a 10-minute incubation interval. The difference in sensitivity recorded was most likely explained by the difference in volumes and

Table IV. A Comparison of the Effect of Varying Levels of RNase, Added at Two Different Stages in the Preparation, on the Stability of Polyribosomes from Barley Tissue

Stage of Preparation	Particle Size	RNase added (μg)				
		0.10	0.1	1.0	10.0	100.0
				Area per cent[3]		
144,000 x g pellet[1]	Dimer	12.4	20.9	22.3	(28.0)	
	> Dimer	58.0	36.7	11.9	7.6	
Minus cell debris[2]	Dimer		7.5	16.8	24.7	16.3
	> Dimer		69.7	53.1	18.7	3.5

[1] RNase was added at the final step in polyribosome preparation, after resuspension of the pellet in 1 ml of gradient buffer.

[2] RNase was added to the cell suspension, after removal of debris by straining. Total volume per treatment at this step was 10.8 ml.

[3] Calculations were made as described in table I.

proximity to substrate sites. The incubation of polyribosomes provided an assay system for RNase much more sensitive than did the method of Tuve and Anfinsen using the transfer RNA assay (25).

The data in fig. 9 established that stable polyribosomes could be extracted from barley with lower levels of KCl, if DTT was present in adequate amounts. Table IV showed that low levels of RNase resulted in appreciable changes in the particle distribution of the polyribosome fraction. These two observations permitted a closer evaluation of the specific effect of KCl upon RNase. As suggested by Wilson's data (28), a high monovalent ion level may be essential for the removal of degrading enzymes from this cell fraction.

The contribution of KCl to the protection of polyribosomes from endogenous RNase was first investigated by extracting barley tissue in buffer (pH 8.0) containing 0.10 M KCl and 1×10^{-2} M DTT. Following the straining step, to remove cell debris, KCl was added to the suspension of two samples to a level of 0.4 M. The stability of these samples was compared to two additional tissue extracts in which the 0.10 M KCl level was not changed; all were incubated at 25° for 20 minutes before density gradient centrifugation (fig. 10). To eliminate *in vitro* protein synthesis during this incubation period, which also results in a loss of polyribosomes (such a system presumably lacks the capacity for mRNA production), 10 µg of cycloheximide ml (21) was added initially to one each of the 0.1 and 0.40 M KCl samples. The two higher salt samples retained a low level of dimer indicating good polyribosome stability whereas both samples incubated with 0.10 M KCl showed a marked shift into the light region of the gradient. This evidence suggested that 0.10 M KCl-extracted polyribosomes contained a level of RNase which resulted in polyribosome breakdown, but that 0.40 M KCl prevented this from happening. The data also showed some stabilizing effect of cycloheximide which suggested that these polyribosomes were capable of *in vitro* protein synthesis.

A more direct demonstration was needed to show the effect of KCl concentration of RNase. To accomplish this a low level of RNase was added to polyribosomes extracted in buffer containing 0.10 M KCl, 1×10^{-2} M DTT and cycloheximide (fig. 11). After removing the cell

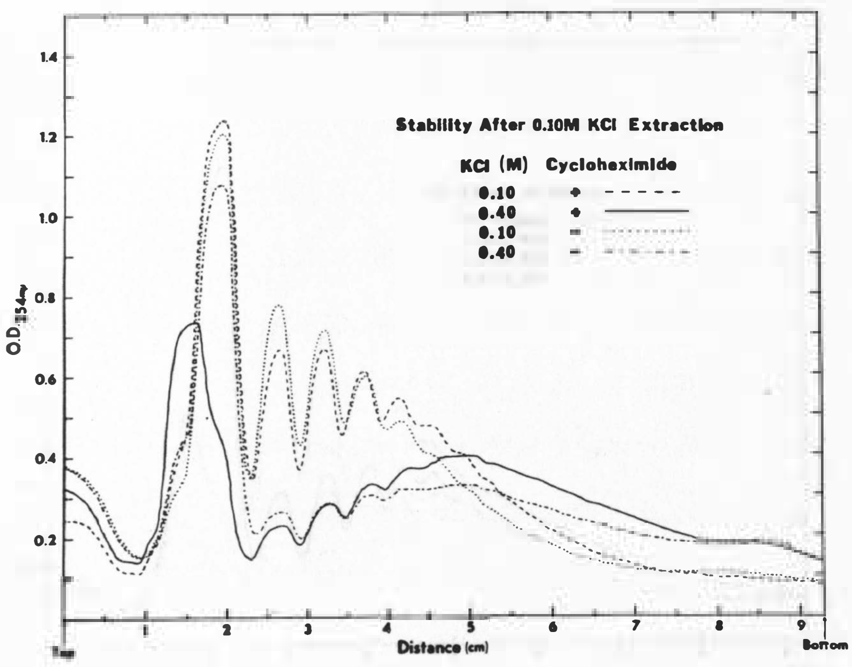

Fig. 10. The stability of polyribosomes extracted with 0.10 M KCl and 1 x 10^{-2} M DTT and incubated 20 minutes with and without cycloheximide, following removal of cell debris and adjustment of KCl to 0.4 M (2 samples). The tissue used was 4-day barley shoots. The 0.40 M KCl treatment retained a low level of dimer indicating good polyribosome stability, whereas both samples incubated with 0.10 M KCl showed a marked shift into the lighter region of the gradient. This evidence suggested that 0.10 M KCl extracted polyribosomes containing a level of RNase sufficient to produce polyribosome breakdown and that 0.40 M KCl prevented this from occurring. The data also showed some stabilizing effect on polyribosomes by the addition of 10 μg of cycloheximide per ml of extraction medium. A peak shift in the monomer region of the 0.40 M KCl treatment with cycloheximide was observed but not explainable.

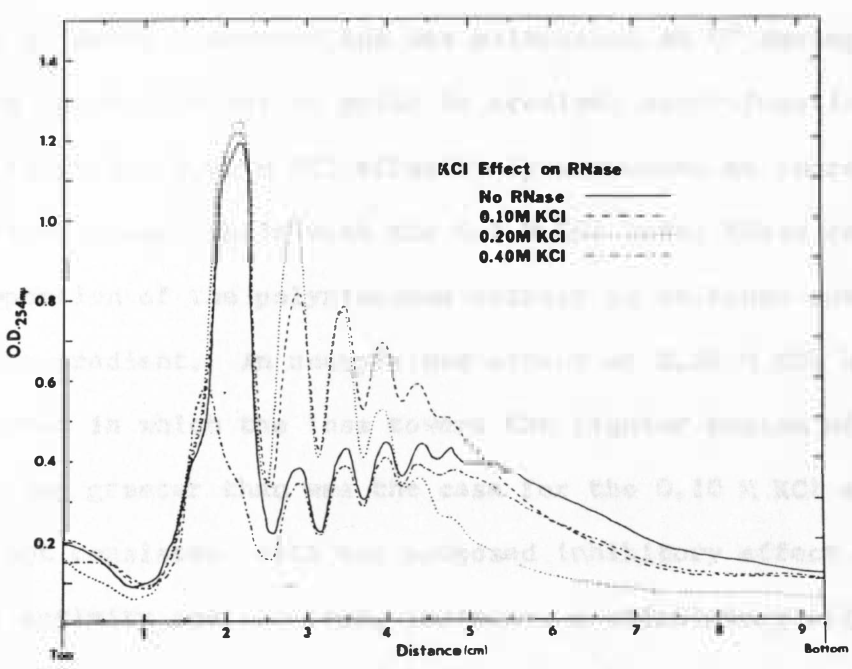

Fig. 11. The stability of polyribosomes extracted with 0.10 M KCl and incubated with 0.1 μg RNase in different levels of KCl. The tissue was extracted as described in fig. 10 with cycloheximide present. The suspension, after removal of debris by straining, was adjusted to 0.10, 0.20 or 0.40 M KCl and 0.1 μg of RNase was added; they were incubated for 20 minutes. One sample, extracted like the rest, was held at 0° as a control. The 0.40 M KCl effectively prevented an increase in the level of dimers that was observed in the samples incubated in 0.10 or 0.20 M KCl. The apparent stimulation of RNase shown by 0.20 M KCl treatment, as compared to 0.10 KCl, cannot be explained with present data.

debris with cheesecloth, the KCl concentration was readjusted to 0.10, 0.20 or 0.40 M and 0.1 μg RNase was added to each of these samples; they were incubated as described for fig. 10. One sample received no RNase treatment and was maintained at 0° during the 20 minute incubation period prior to gradient centrifugation. As before (fig. 10), 0.40 M KCl effectively prevented an increase in the level of dimer, while with the 0.1 M KCl level RNase caused a high proportion of the polyribosome extract to be found toward the top of the gradient. An unexplained effect of 0.20 M KCl on RNase was observed in which the loss toward the lighter region of the gradient was greater than was the case for the 0.10 M KCl sample. This is not consistent with the proposed inhibitory effect of KCl on RNase activity and, in fact, indicated a stimulatory effect at this concentration. Additional information is needed to explain the results shown for the effect of KCl concentration in fig. 10 and 11. Another unexplained result was observed in the collection of data for these figures. Incubation of polyribosomes in 0.40 M KCl containing cycloheximide resulted in a loss of the monomer with an apparent increase in the ribosomal subunit level. As was observed in preparations extracted in 0.60 M KCl, cycloheximide may have caused a dissociation of monomers into subunits or prevented formation of monomers.

CONCLUSIONS

The need for an adequate level of monovalent cation to stabilize polyribosomes during extraction from cereal tissue was clearly evident in these studies. The salt content alone was not the only factor which resulted in stable preparations since it was shown that increasing the pH to 8.2 could partially compensate for KCl when it was added at suboptimal levels. As judged by the physical method used in this investigation, for optimum isolation of stable polyribosomes from barley tissue, a combination of 0.40 M KCl and a pH of 8.0, in addition to the other components of the extraction medium was desirable. Increasing the KCl content up to 0.40 M KCl did have the specific effect, however, of increasing the 260/280 mμ ratio, suggesting that binding of extraneous protein may have been a factor contributing to instability. The possibility that a portion of the protein may have been RNase was indicated by the fact that polyribosomes extracted in 0.10 M KCl were unstable upon incubation, but when the KCl content of the suspension was adjusted upward to 0.4 M prior to incubation, shifts into the lighter region of the gradient were absent. Proof of the inhibitory effect of 0.40 M KCl on RNase was specifically demonstrated by the addition of a low level of RNase to the polyribosomes after KCl adjustment and prior to incubation.

The beneficial effect of DTT on polyribosome stability requires further study before definite conclusions can be drawn. DTT has been

shown to effectively replace part of the requirement for KCl in the polyribosome preparations. However, DTT did not provide the same degree of stability against RNase in conjunction with 0.10 M KCl that was found in extracts prepared with 0.40 M KCl minus DTT. Tamaoki and Miyazawa (22) have provided evidence that SH-groups have an important role in the association of 30S and 50S subunits of Escherichia coli, and Wang and Matheson (26) have observed a SH-dependent association for dimers. Additionally, Temmermann and Lebleu (23) have reported on a RNA-protein complex which was tentatively identified as a mRNA-protein complex; this complex was shown to have a high amount of accessible SH-groups which react with iodoacetate. Others (2,10,15) have reported isolations of similar RNA-protein complexes from the polyribosomes of various tissues. An adequate thiol reducing system, such as would be provided by DTT, appears to be necessary to stabilize these components of the polyribosomes. The function of KCl is not clearly defined; however, ionic strength (in conjunction with pH) could contribute to the stabilization of this protein system.

It is reasonable to conclude, therefore, that relatively high levels of KCl during extraction contributed to the removal of endogenous RNase or inhibited this enzyme resulting in stable preparations. The relationship between KCl, pH and DTT on polyribosome stability has not been resolved. Without considering the experiments involving incubation in cell debris, an increase in the pH of the extraction medium or the addition of DTT to it were not as

satisfactory as 0.40 M KCl. Therefore, the possibility exists that factors other than endogenous RNase contribute to instability of polyribosomes and that KCl performs more than a single function.

Perhaps the ultimate criterion of characterizing polyribosome preparations is relating such preparations to the in vivo status of cells. At present controversy exists on this subject and most of the disagreement rests upon whether on completion of peptide synthesis, ribosomes are detached from mRNA as monomers or subunits. Colombo et al. (5) presented a model in which a monomer pool results following the termination of a peptide molecule. The monomer was conceived of a breaking down into subunits just prior to reforming on a new strand of mRNA. Subunit formation was conceived of as an energy-dependent dissociation of the monomer. This model is contrasted with that suggested by Phillips et al. (18), who found that different chloride salts produced different gradient profiles. Their model reflects a subunit pool; they obtained profiles low in monomers with increased levels of subunits from bacterial cells extracted with Li^+ or Na^+ and concluded that monomers were a result of cell lysis. The experiments with barley and rye, which are reported here, showed no differences in the effect of various chloride salts used, as a normal amount of monomer was found with Li^+, Na^+, NH_4^+ or K^+ amendments. This discrepancy between the data reported here and that of Phillips et al. would tentatively favor the model proposed by Colombo et al.

LITERATURE CITED

1. Britten, R. J. and R. B. Roberts. 1960. High-resolution density gradient sedimentation analysis. Science 131: 32-33.

2. Cartouzou, G., J.-C. Attali and S. Lissitzky. 1968. Acides ribonucleiques messagers de la glande thyroide. I. RNA a' marquage rapide des noyaux et des polysomes. European J. Biochem. 4: 41-54.

3. Cleland, W. W. 1964. Dithiothreitol, a new protective reagent for SH groups. Biochemistry 3: 480-482.

4. Cohen, G. H. and R. M. Iverson. 1967. High-resolution density-gradient analysis of sea urchin polysomes. Biochem. Biophys. Res. Commun. 29: 349-355.

5. Colombo, B., C. Vesco and C. Baglioni. 1968. Role of ribosomal subunits in protein synthesis in mammalian cells. Proc. Natl. Acad. Sci. 61: 651-658.

6. Earl, D. C. N. and A. Korner. 1965. The isolation and properties of cardiac ribosomes and polysomes. Biochem. J. 94: 721-734.

7. Earl, D. C. N. and H. E. Morgan. 1968. An improved preparation of ribosomes and polysomes from cardiac muscle. Arch. Biochem. Biophys. 128: 460-469.

8. Hayes, D. H., M. Grunberg-Manago and M. F. Guerin. 1966. Interactions between homopolyribonucleotides and bacterial ribonucleic acid in solutions of high ionic strength. Part I. J. Mol. Biol. 18: 477-498.

9. Hayes, D. H., F. Hayes and M. F. Guerin. 1966. Association of rapidly labeled bacterial RNA with ribosomal RNA in solutions of high ionic strength part II. J. Mol. Biol. 18: 499-515.

10. Henshaw, E. C. 1968. Messenger RNA in rat liver polyribosomes: Evidence that it exists as ribonucleoprotein particles. J. Mol. Biol. 36: 401-411.

11. Heywood, S. M., R. M. Dowben and A. Rich. 1967. The identification of polyribosomes synthesizing myosin. Proc. Natl. Acad. Sci. 57: 1002-1009.

12. Infante, A. A. and M. Nemer. 1967. Accumulation of newly synthesized RNA templates in a unique class of polyribosomes during embryogensis. Proc. Natl. Acad. Sci. 58: 681-688.

13. Kenefick, D. and E. Whitehead. 1969. Growth and related nucleic acid changes in a hardy and nonhardy winter barley following acclimation. Proc. Second International Barley Genetics Symposium. Washington State University Press, Pullman, Wash.

14. Lin, C. Y. and J. L. Key. 1967. Dissociation and reassembly of polyribosomes in relation to protein synthesis in the soybean root. J. Mol. Biol. 26: 237-247.

15. Perry, R. P. and D. E. Kelly. 1968. Messenger RNA-protein complexes and newly synthesized ribosomal subunits: Analysis of free particles and components of polyribosomes. J. Mol. Biol. 35: 37-59.

16. Petermann, M. L. and A. Pavlovec. 1961. Ribonucleoprotein from a rat tumor, the Jensen sarcoma II. Complexes formed with hemoglobin. J. Biol. Chem. 236: 3235-3239.

17. Petermann, M. L. and A. Pavlovec. 1967. The effect of temperature on the magnesium binding and ultracentrifugal properties of rat liver ribosomes. Biochemistry 6: 2950-2958.

18. Phillips, L. A., B. Hotham-Iglewski and R. M. Franklin. 1969. Polyribosomes of Escherichia coli. I. Effects of monovalent cations on the distribution of polysomes, ribosomes and ribosomal subunits. J. Mol. Biol. 40: 279-288.

19. Srivastava, B. I. S. and C. Arglebe. 1967. Studies on ribosomes from barley leaves. Changes during senescence. Plant Physiol 42: 1497-1503.

20. Salami, A. U. 1968. The influence of zinc and tryptophan on polysome stability and growth response of corn seedlings. Ph.D. Thesis, South Dakota State University.

21. Taber, R. L., Jr. and W. S. Vincent. 1969. Effects of cycloheximide on ribosomal RNA synthesis in yeast. Biochem. Biophys. Res. Commun. 34: 488-494.

22. Tamaoki, T. and F. Miyazawa. 1967. Dissociation of Escherichia coli ribosomes by sulfhydryl reagents. J. Mol. Biol. 23: 35-46.

23. Temmerman, J. and B. Lebleu. 1969. Evidence for the detachment of a ribonucleoprotein messenger complex from EDTA-treated rabbit reticulocyte polyribosomes. Biochim. Biophys. Acta 174: 544-550.

24. Tissières, A., J. D. Watson, D. Schlessinger and B. R. Hollingworth. 1959. Ribonucleoprotein particles from *Escherichia coli*. J. Mol. Biol. 1: 221-233.

25. Tuve, T. W. and C. B. Anfinsen. 1960. Preparation and properties of spinach ribonuclease. J. Biol. Chem. 235: 3437-3441.

26. Wang, J. and A. T. Matheson. 1966. The possible role of sulfhydryl groups in the dimerization of 70S ribosomes from *Escherichia coli*. Biochem. Biophys. Res. Comm. 23: 740-744.

27. Wettstein, F. O., T. Staehelin and H. Noll. 1963. Ribosomal aggregate engaged in protein synthesis: Characterization of the ergosome. Nature 197: 430-435.

28. Wilson, C. M. 1968. Plant Nucleases. I. Separation and purification of two ribonucleases and one nuclease from corn. Plant Physiol. 43: 1332-1338.

29. Yang, P. C., K. Hamada and R. Schweet. 1968. Studies on salt-treated reticulocyte ribosomes. Arch. Biochem. Biophys. 125: 506-513.