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Studies on Ribosomes from Barley Leaves. Changes During Senescence[1]

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Summary. The effect of sucrose, Mg^{2+} and deoxycholate on the yield of ribosomes from barley leaves was determined and the changes in the amount and the composition of ribosomes during senescence of intact and excised first seedling leaves were examined.

The extraction medium containing 20 mM tris-HCl, 0.25 M sucrose, 1 mM $MgCl_2$ and 0.5 % deoxycholate (pH 7.8) gave the maximum yield of polyribosomes and ribosomes. That polyribosomes were not non-specific aggregates was suggested by their capacity to synthesize nascent protein. During senescence of both intact and excised leaves polyribosomes and ribosomes were lost and the ribosomes-polyribosomes which originally contained 48 % protein and 52 % RNA showed substantial decline in the protein content during senescence indicating the degradation of ribosomes and the loss of their function.

Excised green leaves, floated on water, senesce rapidly which is manifested by fall in chlorophyll, RNA and protein content and a decline in the capacity to synthesize RNA and protein (21). The intact first seedling leaf of barley is also induced to senesce when the plant is about 7 days old and the second leaf starts to grow. Due to a direct relationship between protein synthesis and the polyribosome content it was suggested (18) that decline in polyribosome level may mark the initiation of senescence. Shaw and Manocha (17) and Barton (2) later showed that 1 of the first changes in senescing leaf cells was the swelling of endoplasmic reticulum followed by a disappearance of endoplasmic reticulum and ribosomes. No analytical studies dealing with quantitative changes in polyribosomes and ribosomes of senescing leaves have, however, been made. Therefore, in the present paper the properties of ribosomes of barley leaves have been studied and the changes in polyribosomes and ribosomes during senescence of excised and intact leaves have been examined.

Materials and Methods

Barley (*Hordeum vulgare* L. Var. Wolfe) plants were grown in soil in pots on greenhouse benches and given 16 hours of light per day. For studying ribosome changes during senescence of intact leaves the first seedling leaves from 7, 9, 11, 15 and 19 day old plants were analyzed. On the other hand, for examining ribosome changes in senescing excised leaves the first seedling leaves from 7 day old plants were excised, washed with sterile deionized distilled water and floated in 5 g batches on 500 ml of sterile distilled water in sterile Pyrex trays at 25° in the dark. Samples of fresh leaves and of leaves floated on water for 2, 4, 6 and 8 days were analyzed.

Extraction and Fractionation of Ribosomes. In order to obtain maximum yield of polyribosomes extraction media containing sucrose and different concentrations of Mg^{2+} and sodium deoxycholate were tested, when 20 mM tris-HCl, (pH 7.5) containing 0.25 M sucrose, 1 mM $MgCl_2$ and 0.5 % deoxycholate (final pH 7.8) gave the best results. The incorporation of nuclease inhibitors such as bentonite, polyvinyl sulfate (5), zinc (1) or copper (8) in the above extraction medium did not give any improvement.

For the extraction of polyribosomes and ribosomes 5 g leaves were homogenized with 5 g acid-washed sand and 8 ml of freshly prepared extraction medium (20 mM tris-HCl, 0.25 M sucrose, 1 mM $MgCl_2$, 0.5 % deoxycholate, pH 7.8) using chilled mortar and pestle. The temperature during extraction and throughout subsequent operation was maintained close to 0 to 1°. The homogenate was squeezed through 2 layers of Miracloth and the brei was centrifuged at 21,600 × *g* for 20 minutes. The supernatant obtained was recentrifuged for 30 minutes at 28,700 × *g*. The ribosomes were sedimented by centrifuging the 28,700 × *g* supernatant for 1 hour at 144,000 × *g* in the SW-50 rotor of the Spinco Model L ultracentrifuge. The ribosomal pellet was gently suspended in 0.6 ml of buffer (20 mM tris-HCl, 1 mM $MgCl_2$, 0.25 M sucrose, pH 7.5) and clarified by centrifugation at 30,000 × *g* for 30 minutes. The clarified ribosomal suspension (0.8 – 1.0 ml) was layered on 27 ml of a 10 to 34 % linear sucrose gradient prepared in 20 mM tris-HCl, 1 mM $MgCl_2$, pH 7.5

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buffer. The gradients were spun at 25,000 rpm for 3 hours in the SW 25-1 rotor and allowed to stop without braking. Twenty drop fractions were collected by bottom puncture of the tubes, diluted with 2.5 ml of 20 mM tris-HCl, 1 mM $MgCl_2$, (pH 7.5) buffer and read at 260 mμ in a Beckman Model DU-2 spectrophotometer. The approximate aggregate size of major ribosomal components was determined with reference to ferritin standard (65S) and the data of Howell, Loeb and Tomkins, (9).

Estimation of RNA and Protein Content of Ribosomes. After determining the OD_{260}, the fractions containing ribosomes and polyribosomes (ca. fractions 20–90) were pooled and after adding enough cold 50 % (w/v) trichloroacetic acid to give a trichloroacetic acid concentration of 5 % the contents were left overnight at 1°. The precipitate formed was recovered by centrifugation (15,000 × *g*, 30 mins), washed once with cold 95 % ethanol and air dried. The residue was completely solubilized in 2 ml of 0.3 N NaOH and the solution obtained was incubated for 16 hours at 37° to hydrolyse RNA. On an aliquot of the hydrolysate the protein was estimated by the procedure of Lowry et al. (14). Another aliquot of the hydrolysate, after the removal of Na^+ by Dowex 50 H^+, was loaded on a column (0.5 × 15 cm) of Dowex 1 (200–400 mesh)Cl^-. After washing the column with 0.01 M NaCl the nucleotides were eluted with 50 ml of 0.9 N HCl plus 0.6 N NaCl. From the OD_{260} of the nucleotides the amount of RNA in the sample was calculated taking 34 units of OD_{260} as equivalent to 1 mg of RNA nucleotides per ml (6).

Protein Synthesis by Ribosomes. In order to demonstrate that polyribosomal units were the site of nascent protein synthesis the segments (0.5 mm) from 5 g of first seedling leaves from 7 day old barley plants were vacuum infiltrated for 5 minutes in 40 ml of medium containing 20 mM tris-HCl, 1 mM $MgCl_2$ (pH 7.6), 40 μg/ml of chloramphenicol (25) and 0.3 μc/ml of L-alanine-U-^{14}C and subsequently incubated for 25 minutes on a shaker at 25°. After incubation the leaf segments were quickly rinsed with cold distilled water. Ribosomes from the leaf segments were extracted with 20 mM tris-HCl, 1 mM $MgCl_2$, 0.25 M sucrose, 0.5 % deoxycholate (pH 7.8) medium and fractionated as described earlier except that 40 drop fractions were collected. After reading the OD_{260} of each fraction 50 μg crystalline bovine serum albumin and 1 ml 25 % (w/v) trichloroacetic acid were added to each fraction. The precipitates formed were collected on discs (2.5 cm diam.) of Bac-T-Flex membrane filters (type B_6) and washed 4 to 5 times with cold 5 % (w/v) trichloroacetic acid. The discs containing the precipitates were dried, transferred to standard scintillation vials containing 15 ml of scintillation fluid [4 g 2,5-diphenyloxazole and 0.1 g *p-bis*-((2-5-(phenyloxazolyl))-benzene per liter of toluene] and counted in a Nuclear Chicago Series 720 scintillation counter.

Stability of Polyribosomes and Ribosomes. In order to examine the stability of polyribosomes and ribosomes the extracts obtained by squeezing the leaf homogenates through Miracloth were allowed to stand for 0, 1 or 2 hours at 1° before proceeding to isolate and fractionate the ribosomes.

In other experiments the purified ribosomal suspension from 15 g leaves was divided into 3 equal parts. One part was used directly for density gradient centrifugation whereas the other 2 parts were treated with varying amounts of crystalline pancreatic ribonuclease before layering on the gradients.

Isolation and Fractionation of RNA from Ribosomes. The RNA from the ribosomes was isolated by phenol-sodium lauryl sulfate procedure and fractionated by density gradient centrifugation as described by Srivastava (20).

Estimation of Chlorophyll. The chlorophyll from the leaves was extracted with 80 % (w/v) boiling ethanol and the OD_{665} of the extract determined.

Results and Discussion

The data presented in table I show that both the excised barley leaves floated on water and the intact first seedling leaves of barley lost chlorophyll during senescence.

The data presented in figure 1 illustrate that both sucrose and magnesium were required for the stability of the polyribosomes and in the absence of Mg^{2+} the polyribosomes were degraded beyond monomer stage. In barley leaves the maximum yield of ribosomes was obtained at 1 to 2 mM Mg^{2+} and at 5 mM Mg^{2+} the yield of ribosomes declined considerably (fig 2). Similar requirements for Mg^{2+} for other plant ribosomes have been reported (4, 11, 16). Furthermore, the inclusion of deoxycholate in the extraction buffer increased the yield of ribosomes (fig 3). Therefore, for the final ribosome extraction buffer containing sucrose, 1 mM Mg^{2+} and 0.5 % deoxycholate was used.

The duplicate analysis of the ribosomes using the finally selected buffer showed similar sucrose gradient distribution profiles and only 3 % variation from the mean in the yield of ribosomes. The ribosomes from

Table I. *The Chlorophyll Content of Excised and Intact Senescing Barley Leaves*

Excised leaves floated on water		Intact leaves	
Days of floating	Chlorophyll content*	Age of leaves days	Chlorophyll content*
0**	99.0	7	90.0
2	52.0	11	93.0
4	40.0	15	79.5
6	17.0	19	52.6
8	10.0	—	—

* $OD_{665(1\,mm)}$ per g fresh weight.

** Zero day leaves in this and other tables refer to leaves from 7 day old plants and were obtained from different batch of plants as the intact leaves.

7 day old barley leaves had a 260/235 ratio of 1.66 ± 0.03 and a 260/280 ratio of 1.98 ± 0.02 (n = 13). When barley leaf segments were incubated for short time with L-alanine-U-^{14}C following

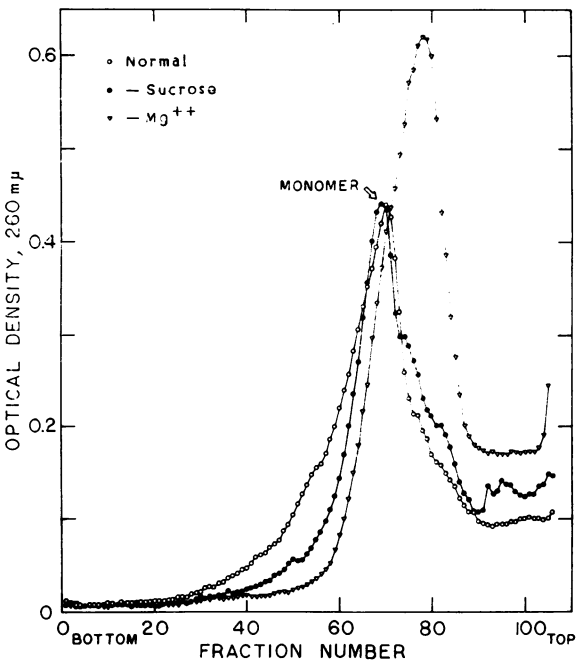

FIG. 1. The effect of sucrose and Mg^{2+} on the yield of ribosomes from first seedling leaves of 8 day old barley plants.

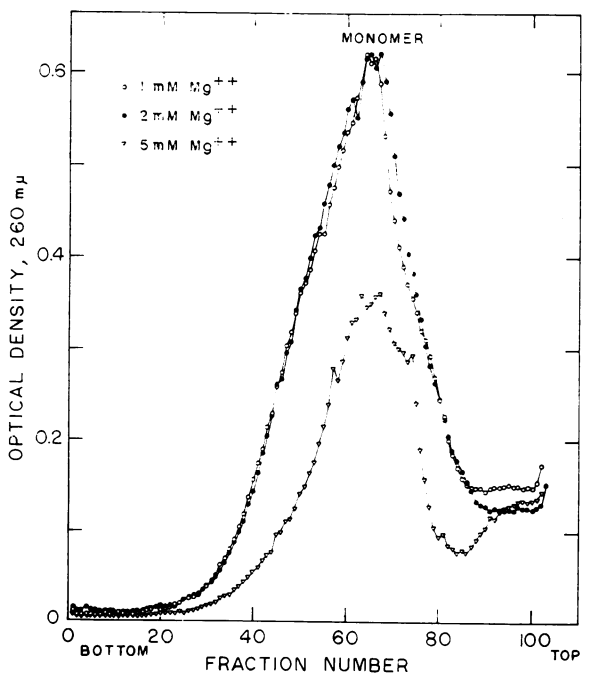

FIG. 2. The effect of different concentrations of Mg^{2+} on the yield of ribosomes from first seedling leaves of 7 day old barley plants.

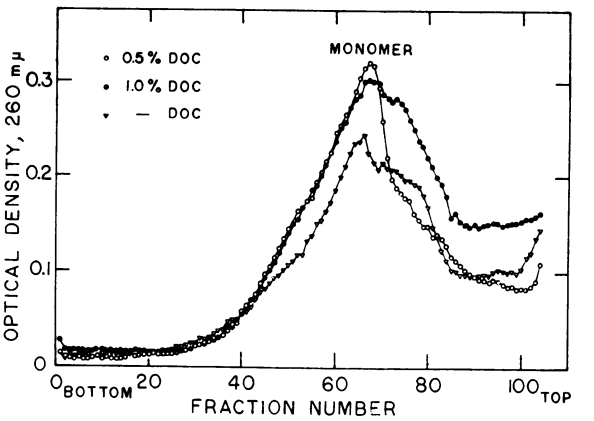

FIG. 3. The effect of different concentrations of deoxycholate on the yield of ribosomes from first seedling leaves of 9 day old barley plants.

which the ribosomes were isolated and fractionated, most of the radioactivity was detected in the polyribosome region (fig 4) indicating that the ribosomal aggregates were active in protein synthesis.

The sedimentation diagrams of the ribosomes before and after treatment with pancreatic ribonuclease presented in figure 5 do not show the general expected shift of optical density from polyribosome region to monosome region. Ribonuclease at low concentration (0.05 μg) had little effect on the ribosome profile whereas at high concentration (10 μg)

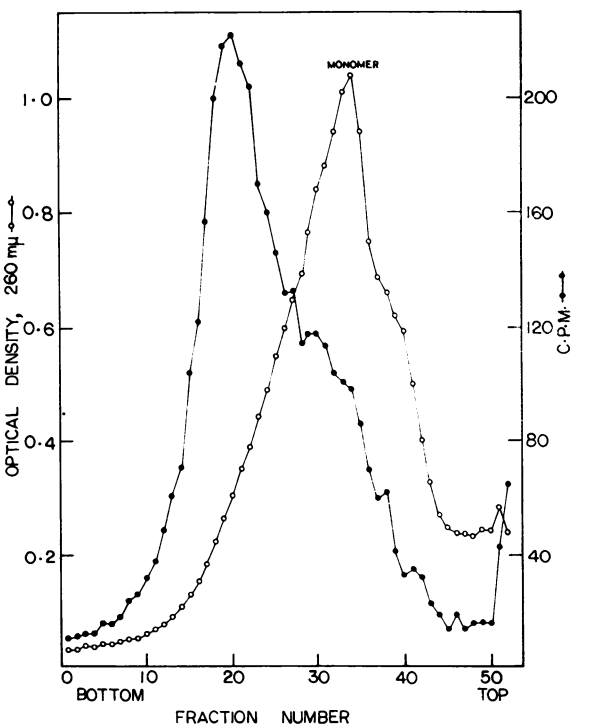

FIG. 4. Sucrose density gradient pattern of ribosomes isolated from barley leaf sections incubated with L-alanine-U-^{14}C.

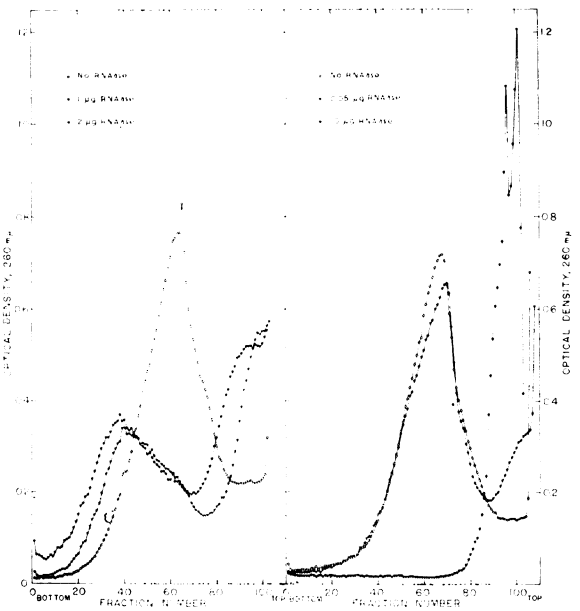

FIG. 5. Effect of ribonuclease on the sedimentation profile of ribosomes from leaves of 7 day old barley plants. Ribosomes were incubated with ribonuclease for 10 minutes at 37°.

it degraded the ribosomes completely. At medium concentration (1–2 μg) of ribonuclease optical density in fractions 10 to 40 and 80 to 100 increased at the expense of fractions 41 to 79 indicating both aggregation and degradation. These results may suggest that particles assumed to be polyribosomes may represent nonfunctional ribosomal aggregates. However, formation of aggregates on ribonuclease treatment of functional ribosomes has been observed in peas (24), pollen (13) and bacteria (10) and polyribosomes resistant to ribonuclease have also been found in pea seedlings (3) and *Brasscia* leaves (5). It has been suggested (10) that ribosomes resistant to ribonuclease constitute special group of ribosomes and that they form aggregates with other ribosomes as a

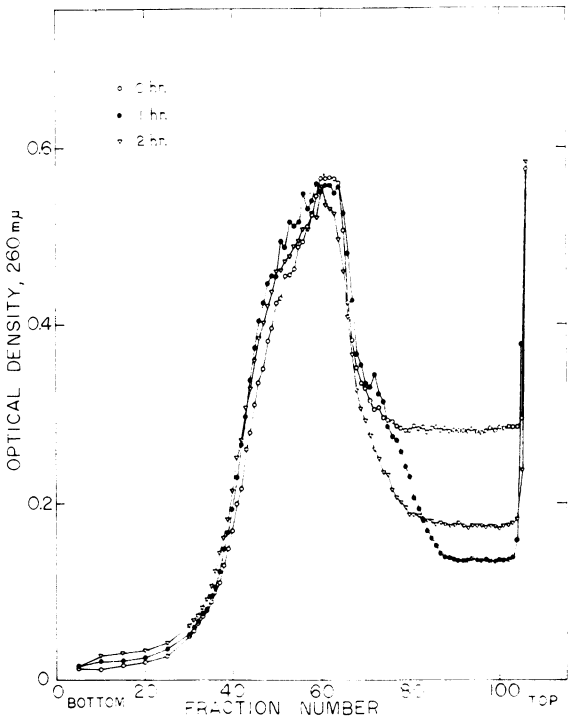

FIG. 6. Effect of time of standing of the homogenate (from leaves of 7 day old barley plants) on the sedimentation profile of ribosomes.

Table II. *The Changes in the Amount* of Different Ribosomal Components in Intact and Excised First Seedling Leaves of Barley*

The data are the means of 2 separate series of experiments.

Treatment	Total ribosomal components OD	%	Polyribosomes (Fractions 15–62) OD	%	Monosomes (Fractions 63–69) OD	%	Breakdown product A (Fractions 70–91) OD	%	Breakdown product B (Fractions 92–101) OD	%
A) Intact leaves, age, days										
7	76.9	100.0	36.4	47.5	14.7	19.0	20.2	26.0	5.7	7.5
11	20.5	27.0	6.3	30.5	4.9	24.0	5.5	27.0	3.8	18.5
15	14.5	19.0	4.9	34.0	2.4	17.0	1.7	12.0	5.4	37.0
19	6.4	8.5	1.8	29.0	0.5	7.5	0.4	6.0	3.7	57.5
B) Leaves floated on water, days										
0	47.8	100.0	21.1	44.0	9.0	18.5	13.3	28.0	4.5	9.5
2	43.5	91.0	17.3	40.0	9.9	23.0	11.5	26.0	4.8	11.0
4	30.8	64.5	11.4	37.0	6.9	22.5	5.7	18.5	6.7	22.0
6	22.8	48.0	8.1	35.5	5.5	24.0	4.6	20.0	4.7	20.5
8	12.0	25.0	3.0	25.0	2.5	20.5	2.3	19.0	4.2	35.5

* $OD_{260(1ml)}$ per g fresh weight.

protection against further action of ribonuclease. It is possible that ribonuclease sensitive polyribosomes were predominantly converted to monosomes and other lower forms during grinding and these were the ribonuclease resistant polyribosomes which survived grinding. This may also explain the good reproducibility of the ribosomal pattern and the stability of the ribosomes in the homogenate when it was allowed to stand at 1° for 1 to 2 hours (fig 6). If ribonuclease action could be prevented during the isolation of ribosomes a much higher yield of polyribosomes from barley leaves may be obtained; about 90% of the ribosomes in *Brassica* leaves are supposed to occur as polyribosomes (5). Unfortunately this has not been possible and the incorporation of ribonuclease inhibitors (bentonite, polyvinyl sulfate, Zn, CU) into the extracting medium has been ineffective. That ribosomes were not severely damaged during their isolation is indicated by 23s and 16s major peaks in RNA from the ribosomes (fig 7) although the presence of some material smaller than 16s suggests some breakdown.

Since particles assumed to be polyribosome in this paper were capable of nascent protein synthesis (as indicated by the high specific activity of the polyribosomal region) we feel that they represent polyribosomes. The possibility that some nonfunctional

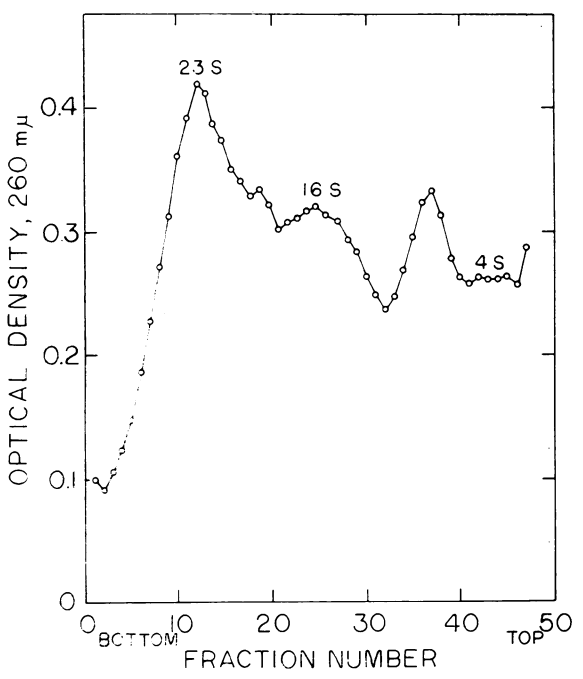

FIG. 7. The sedimentation profile of nucleic acids from ribosomes of barley leaves. Nucleic acids were sedimented for 15 hours at 25,000 rpm in SW 25-1 rotor of a Spinco Model L ultracentrifuge through 5 to 20% linear sucrose gradients containing 0.05 M sodium phosphate buffer, pH 6.8, and 1 mM $MgCl_2$. Optical density refers to 40 drop fractions diluted with 3 ml of water.

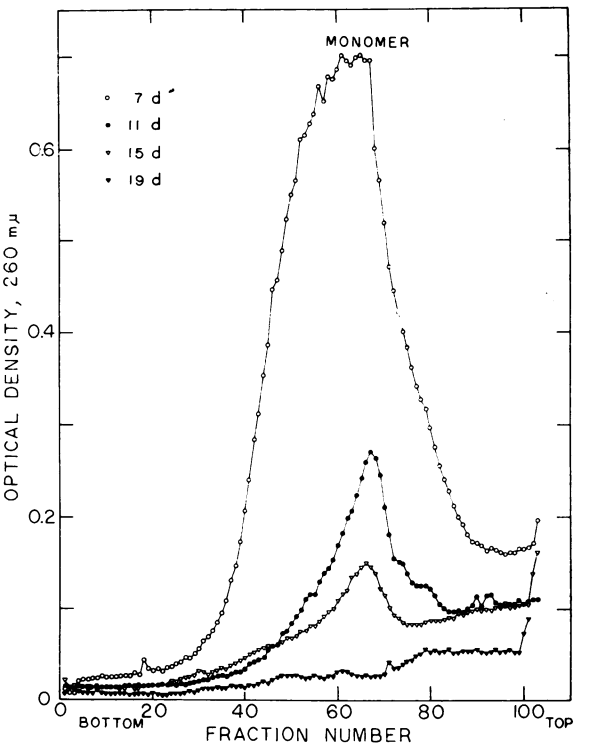

FIG. 8. Sucrose density gradient pattern of ribosomes isolated from first seedling leaves of 7, 11, 15 and 19 day old barley plants.

ribosomal aggregates were formed during isolation of ribosomes can, however, not be excluded. Nevertheless, it should be noted that ribosomal aggregates formed by the action of pancreatic ribonuclease appeared in fractions 10 to 40 where only little ribosomal material was detected in normal extractions.

The sucrose gradient distribution profiles of ribosomes from intact barley leaves of increasing age and from excised barley leaves floated on water are presented in figures 8 and 9 respectively, and the amounts of different ribosomal components are presented in table II.

The data presented in table II show that in both intact and excised leaves senescence caused a decline in the level of polyribosomes and as the senescence progressed the leaves progressively lost polyribosomes, monosomes and other ribosomal components. Since ribonuclease activity in excised barley leaf tissue floated on water does not increase (22) and since decline in ribosomal RNA (isolated directly from the leaves by phenol procedure and fractionated by MAK chromatography) in barley leaves floated on water has been noted (19) the loss of ribosomes in excised leaves reported here is considered real and not due to any artifacts of ribosome isolation procedure. Although ribonuclease activity in intact aging barley leaves has not been determined the loss of ribosomal RNA in aging barley leaves similar to the loss of ribosomes noted here has been found (unpublished data). A parallel loss of ribosomes and ribosomal

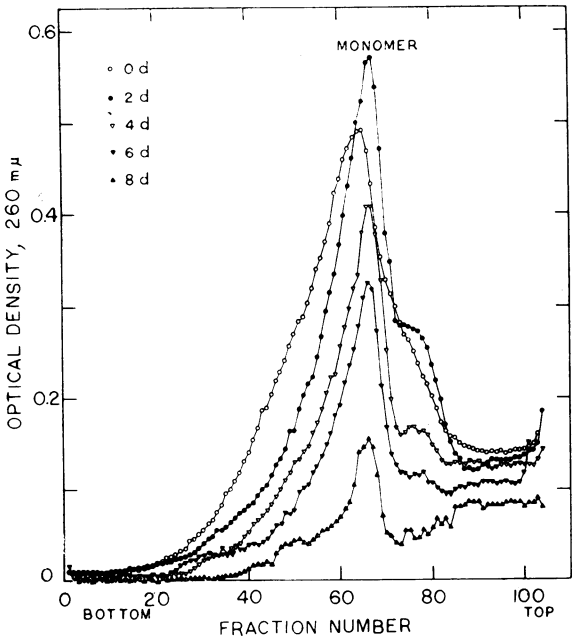

FIG. 9. Sucrose density gradient pattern of ribosomes isolated from first seedling leaves of 7 day old barley plants (0 day) and from excised barley leaves floated on water for 2, 4, 6 and 8 days in the dark.

RNA during senescence suggests that the RNA formed during ribosome breakdown must be degraded. This is in agreement with the finding of Giles and Myers (7) who have reported that in *Lupinus albus* hypocotyl the RNA of ribosomes produced by breakdown of polyribosomes was unstable. It is interesting to note that in excised barley leaves loss of polyribosomes was closely followed by decline in the level of chlorophyll (table I) but in intact leaves although leaves lost 73 % of the total ribosomal components and 82 % of the polyribosomes by the eleventh day the chlorophyll level of the leaves had not declined. In any event the present results support the electron microscopic observations of Shaw and Manocha (17) and Barton (2) who have reported loss of ribosomes in excised leaves floated on water. A similar loss of polyribosomes and ribosomes as observed here has also been reported (24) in endosperm of castor bean seeds during ripening.

The protein and RNA content of the ribosomes from 7 day old barley leaves (table III) was typical of ribosomes from other sources (12, 15, 16). During senescence of both excised and intact leaves the protein content of the ribosomes progressively declined indicating the degradation of ribosomes and the loss of their function. The above results clearly demonstrate that senescence in first seedling leaves of barley either induced by the growth of the second leaf or by leaf excision is accompanied by a loss of ribosomes and thus decline in the capacity to synthesize proteins.

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Table III. *The RNA and Protein Content of Ribosomes (and Polyribosomes) from Excised and Intact Senescing Barley Leaves*

The data are the means of 2 separate series of experiments.

Excised leaves floated on water			Intact leaves		
Floating time Days	RNA %	Protein %	Age of leaves Days	RNA %	Protein %
0	52	48	7	51	49
2	58	42	11	64	36
4	70	30	15	65	35
6	71	29	19	86	14
8	82	18	...	...	...

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