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FRACTIONATION OF NUCLEIC ACIDS ISOLATED BY THE PHENOL- AND DEP-METHODS FROM LEAF TISSUES AT DIFFERENT STAGES OF DEVELOPMENT

GABRIELLA LÁZÁR, I. FEDORCSÁK and F. SOLYMOSY

Institute of Plant Physiology and Department of Genetics, Eötvös University, Muzeum krt 4/A, Budapest, Hungary

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Abstract—The phenol method and a new method based on the use of diethylpyrocarbonate (DEP) as a nuclease inhibitor were compared with respect to the yield of total nucleic acid extractable from barley and tobacco plants in different stages of development. The percentage distribution of different nucleic acid species in the extracts prepared by the two methods was also studied by sucrose density gradient centrifugation and MAK column chromatography. The nucleic acid yield in the extracts from first leaves of 11-day-old barley seedlings was higher with the DEP method than with the phenol method. With barley the same percentage distribution of the different nucleic acid species was obtained with both methods. By contrast, with tobacco (except for very young tissues) a preferential loss of hrRNA was observed when using the DEP method. The ageing of barley leaf tissues was characterized by a virtual increase in DNA apparently due to a preferential breakdown of rRNA. This pattern, characteristic of ageing tissues, was more pronounced with attached leaves than with detached, "artificially aged" tissues.

INTRODUCTION

RECENTLY in our laboratory a new method based on the use of diethylpyrocarbonate (DEP*) as a nuclease inhibitor has been developed for the extraction of undegraded nucleic acids from higher plant tissues (DEP method).[1] Some advantages of the new method over the phenol method[2] (more powerful protection against RNase, higher yields) were described previously.[1] The DEP method has been used so far only with young tissues or seedlings. In the present study the application of the method was extended to mature tissues. The experiments served two purposes. On the one hand the classical phenol and the DEP methods were compared using plant material of different ages. On the other hand alterations in the major nucleic acid fractions, including DNA, sRNA, hrRNA and lrRNA, were studied during the ageing of barley and tobacco leaves.

RESULTS AND DISCUSSION

Experiments with Barley

Total nucleic acid content and the relative amounts of the major nucleic acid fractions (DNA, sRNA, hrRNA and lrRNA) were determined in first leaves of barley seedlings during an 11-day period of leaf development. For a better characterization of the trend of changes,

* Abbreviations used: DEP, diethylpyrocarbonate; RNase, ribonuclease; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; sRNA, soluble RNA; hrRNA, heavy ribosomal RNA; lrRNA, light ribosomal RNA; MAK, methylated albumin coated kieselguhr; TCA, trichloroacetic acid.

[1] F. SOLYMOSY, I. FEDORCSÁK, A. GULYÁS, G. L. FARKAS and L. EHRENBERG, *European J. Biochem.* **5**, 520 (1968).

[2] T. ITOH and T. HIRAI, *Ann. Phytopathol. Soc. Japan* **32**, 227 (1966).

TABLE 1. DRY MATTER, PROTEIN, CHLOROPHYLL, AND NUCLEIC ACID CONTENTS IN BARLEY LEAVES AT DIFFERENT STAGES OF DEVELOPMENT

Stage of development (days)	Dry weight (mg/g fresh weight)	Protein (mg/g fresh weight)	Chlorophyll (A_{665} units/g fresh weight)	Total nucleic acid content			Nucleic acid (mg/g fresh weight) extracted by the		Per cent,* nucleic acid extracted by the	
				mg/g fresh weight	mg/g dry weight	mg/g protein	Phenol method	DEP method	Phenol method	DEP method
4	57·2	22·5	11·2	2·2	38	0·097	1·4	1·8	62·4	83·9
5	68·6	26·3	58·9	2·4	35	0·091	1·6	1·8	65·7	73·6
6	70·2	38·5	62·3	2·5	36	0·065	1·4	1·7	56·6	68·1
10	62·1	37·2	66·7	1·1	18	0·029	0·4	0·8	35·8	74·3
11	60·4	35·8	65·7	1·0	16	0·027	0·3	0·7	34·7	72·4

* Total nucleic acid content = 100 per cent.

the alteration of the amount of various fractions was related to changes in dry matter, protein, and chlorophyll content.

It may be seen from Table 1 that dry matter, protein and chlorophyll contents expressed on a fresh weight basis increased during leaf growth and reached a plateau at about the sixth day. By contrast, total nucleic acid content expressed on a fresh or dry weight basis remained more or less constant until the sixth day and decreased thereafter. This is most clearly shown if the changes in nucleic acid content are expressed on a protein basis (Table 1). The results are in a good agreement with those published by Rhodes and Yemm.[3]

TABLE 2. EFFECT OF DETACHMENT ON DRY MATTER, PROTEIN, CHLOROPHYLL, AND NUCLEIC ACID CONTENTS IN BARLEY LEAVES

Treatment	Stage of development (days)	Dry weight (mg/g fresh weight)	Protein (mg/g fresh weight)	Chlorophyll (A_{665} units/g fresh weight)	Total nucleic acid content			Nucleic acid (mg/g fresh weight) extracted by the		Per cent,* nucleic acid extracted by the	
					mg/g fresh weight	mg/g dry weight	mg/g protein	Phenol method	DEP method	Phenol method	DEP method
Intact	6	70·2	38·5	62·3	2·5	36	0·065	1·4	1·7	56·6	68·1
	10	62·1	37·2	66·7	1·1	18	0·029	0·4	0·8	35·8	74·3
Excised leaves from 6-day-old plants floated for 4 days on distilled water	10	54·1	20·5	17·9	1·5	28	0·074	0·4	0·9	27·6	60·5

* Total nucleic acid content = 100 per cent.

The extractability of undegraded nucleic acids (expressed in per cent of the total nucleic acid content) did not change considerably during the development of the leaf tissues, as assayed with the DEP method, whereas the phenol method used in the present work gave smaller yields with increasing leaf age (Table 1). Thus, the already established superiority of the DEP method over the phenol method with respect to the yield was particularly conspicuous in the case of older tissues.

To compare, from the above point of view, the normal process of ageing with that artificially induced by detachment, protein and nucleic acid contents were determined in 6-day-old attached leaves ("zero-time"), in the leaves of the same batch of plant material after

[3] M. J. C. RHODES and E. W. YEMM, *Nature* **200**, 1077 (1963).

4 days of further development ("intact leaves") and after 4 days of detachment ("excised leaves"), respectively. Leaves of 6-day-old plants were chosen as starting material for these experiments because dry weight, protein and total nucleic acid contents reach a plateau at about this time, and the dramatic drop of nucleic acid content follows thereafter (Ref. 3).

As shown in Table 2 the dry weight, protein and chlorophyll contents decreased upon detachment by about 22, 46 and 75 per cent, respectively, as compared to the "zero-time" control. Total nucleic acids decreased by 40 per cent. The extractability by the phenol method of undegraded nucleic acids decreased from 56·6 to 27·6 per cent, and by the DEP method from 68·1 to 60·5 per cent.

A comparison of the data, "intact" vs. "excised" leaves, revealed striking differences in the pattern of changes of proteins and nucleic acids. Whereas the protein content of the leaf tissues dropped to about the half during 4 days of detachment, and remained practically unchanged during the same period of time in the attached leaves, nucleic acids decreased in both cases but the decrease (as compared to the "zero-time" control) was somewhat more pronounced in the attached leaves. It remains to be elucidated why the often observed correlation between RNA and protein content is so markedly altered during ageing in attached barley leaves in contrast to the more "normal" relationship in detached leaf tissues where total proteins and RNA decrease more or less simultaneously.[4]

To obtain information on the relative contribution of various nucleic acid fractions to the changes outlined above, the nucleic acids extracted by the phenol and DEP methods, respectively, were fractionated by sucrose density gradient centrifugation and MAK column chromatography.

Sucrose density gradient sedimentation patterns and MAK column elution profiles of 4-, 6-, and 11-day-old first leaves of barley seedlings are presented in Fig. 1. Quantitative evaluation of the profiles presented is given in Table 3. It may be seen from Fig. 1 and from the values for MAK chromatography in Table 3 that there is a relative increase in the DNA fraction over ribosomal RNA and sRNA during leaf development. A quantitative evaluation of the sedimentation patterns of nucleic acids proved difficult in view of the overlap of the nucleic acid fractions. With 4- and 6-day-old leaves only the lrRNA and DNA fractions were not resolved (Fig. 1A, B and Table 3) whereas with 11-day-old leaves the resolution was too poor to make any quantitative evaluation possible (Fig. 1C and Table 3). Therefore, in

TABLE 3. PERCENTAGE DISTRIBUTION OF DIFFERENT NUCLEIC ACID FRACTIONS IN NUCLEIC ACID EXTRACTS PREPARED BY THE PHENOL AND DEP METHODS, RESPECTIVELY, FROM BARLEY LEAVES AT DIFFERENT STAGES OF DEVELOPMENT

Method of fractionation:		Sucrose density gradient centrifugation						MAK column chromatography		
Stage of development of leaf tissues:		4 day old		6 day old		11 day old		4 day old	6 day old	11 day old
Method of extraction:		DEP	Phenol	DEP	Phenol	DEP	Phenol	DEP	DEP	DEP
Nucleic acid fraction, per cent	hrRNA	51·8	50·9	50·6	44·4	—	—	—	—	—
	lrRNA+DNA	36·5	35·7	36·0	37·5	—	—	—	—	—
	sRNA	11·7	13·4	13·4	18·1	—	—	—	—	—
	hrRNA+lrRNA	—	—	—	—	—	—	70·1	67·2	57·2
	DNA	—	—	—	—	—	—	15·3	19·6	28·0
	sRNA	—	—	—	—	—	—	14·6	13·2	14·8

[4] D. J. OSBORNE, *Plant Physiol.* **37**, 595 (1962).

the following, the possible reasons for the distortion of the "normal" sedimentation profile were analysed. To this end the method described previously[1] for the separate estimation of DNA and RNA in sucrose density gradients was applied (see Experimental). It may be seen (Fig. 2) that DNA can be located as a distinct peak in the density gradient profile and that by subtracting the absorbance values for DNA in the individual fractions from the respective values in the original profile a normal sedimentation pattern is arrived at. This indicates that

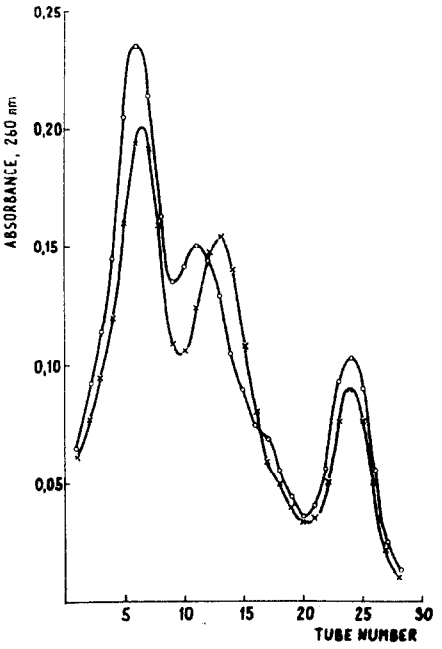

FIG. 1A

FIG. 1. SUCROSE DENSITY GRADIENT SEDIMENTATION PATTERNS AND MAK-CHROMATOGRAPHY PROFILES OF NUCLEIC ACIDS EXTRACTED FROM FIRST LEAVES OF BARLEY SEEDLINGS AT DIFFERENT STAGES OF LEAF DEVELOPMENT.

Sedimentation patterns: 4-day- (1A), 6-day- (1B), and 11-day-old (1C) leaf tissues. Nucleic acids were extracted by the phenol method (○—○—○) and by the DEP method (×—×—×), respectively. In each experiment 8 A_{260} units of nucleic acids were layered on a 5–20 per cent sucrose gradient and centrifuged for 4 hr in the SW 39 rotor of the Beckman L 50 preparative ultracentrifuge. 0·14 ml fractions were collected. Each fraction was diluted with 1·0 ml of distilled water and the absorbance of the fractions was measured at 260 nm in cells with a light path of 0·5 cm.

MAK-chromatography profiles: 4-day- (1D), 6-day- (1E), and 11-day-old (1F) leaf tissues. Nucleic acids were extracted by the DEP method. In each experiment 15–20 A_{260} units of nucleic acids were applied to the MAK column and 3·7 ml fractions were collected. The absorbance of the fractions was measured at 260 nm.

the "abnormality" of the sedimentation profile of the nucleic acids from old (11-day-old) leaves is at least partly due to the relatively large amount of DNA present in the extracts. This observation is in agreement with the results represented by the MAK profiles (Fig. 1, D–F).

The effect of detachment (artificial ageing) on the nucleic acid *fractions* was also studied. The experimental material and set-up were identical with those described above for the determination of changes in total nucleic acids, protein etc. during detachment. Representative sedimentation and MAK profiles of nucleic acids isolated from leaf tissues floated on distilled water for 4 days are shown in Fig. 3A and B. The respective data of "zero-time"

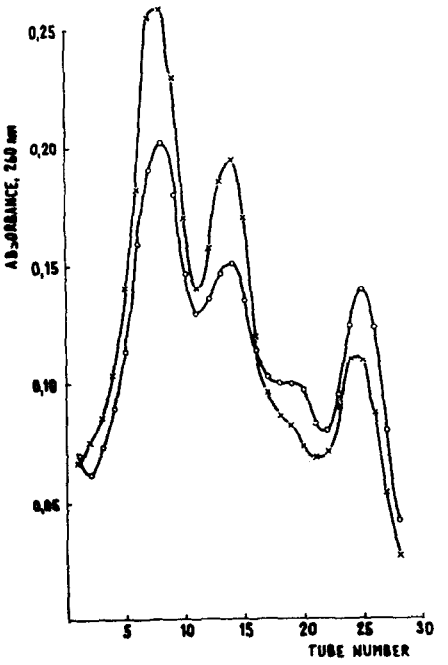
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FIG. 1B

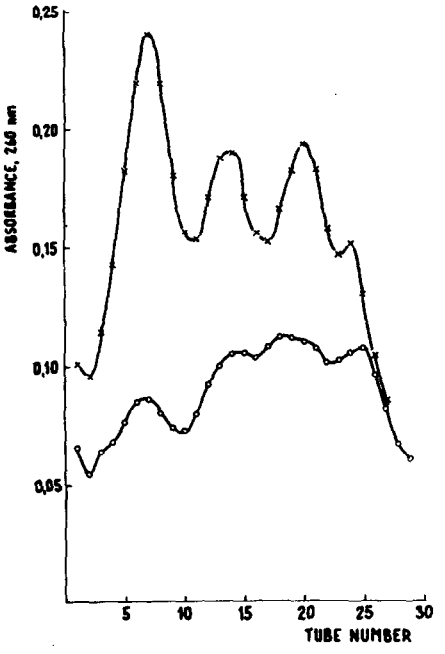
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FIG. 1C

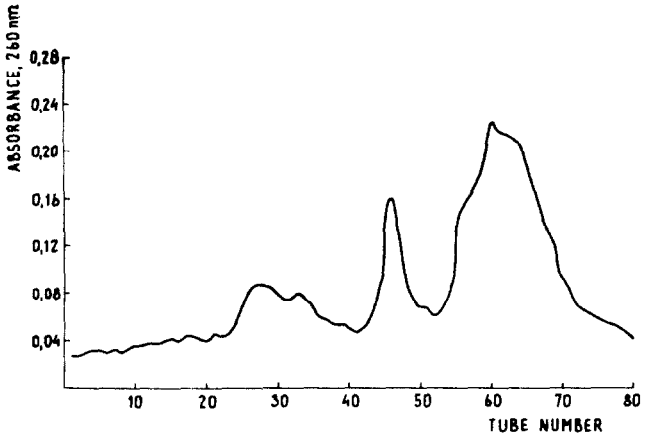

FIG. 1D

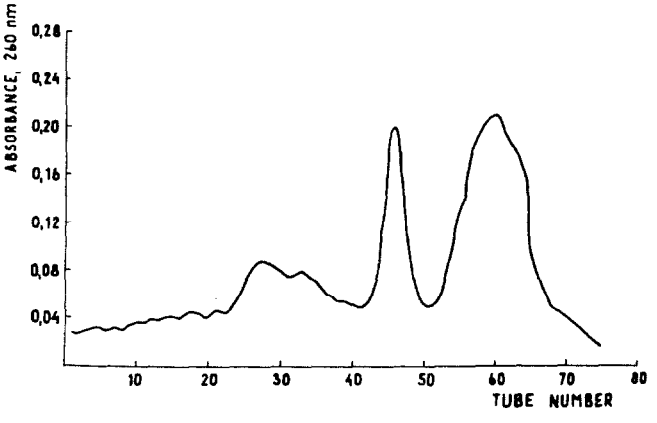

FIG. 1E

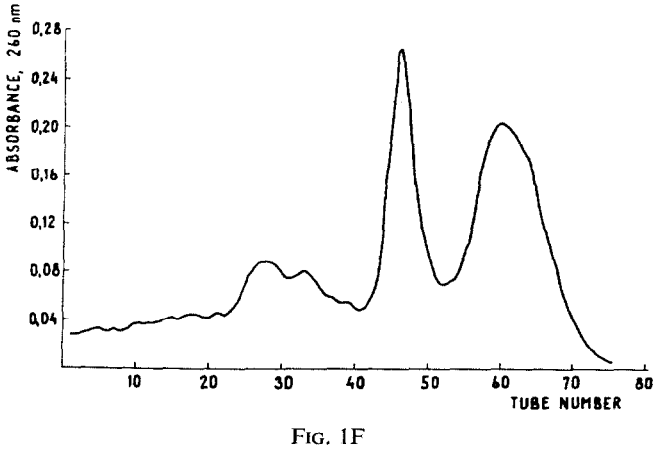

FIG. 1F

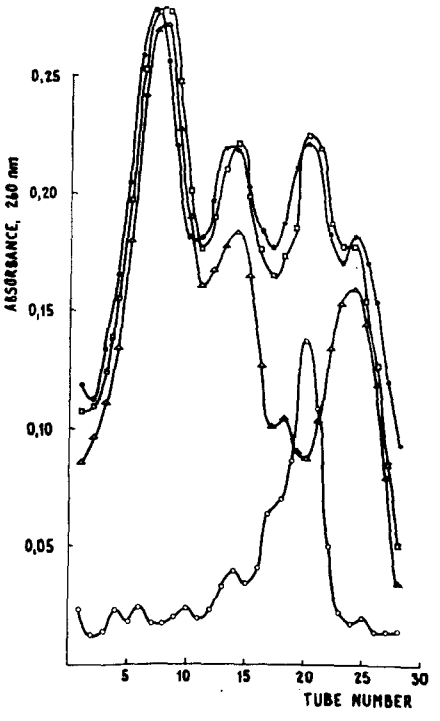

FIG. 2. DETERMINATION OF THE POSITION OF DNA IN A SUCROSE DENSITY GRADIENT PROFILE.
Sedimentation profile of total nucleic acid extracted by the DEP method from 11-day-old first seedling leaves of barley: ●—●—●; DNA content of the fractions determined after removal of RNA: ○—○—○; RNA content of the fractions, determined after removal of DNA: △—△—△; DNA hydrolysate and RNA hydrolysate combined: □—□—□. Sedimentation analysis of nucleic acids was carried out as described in the legend to Fig. 1. For the details of the separate determination of RNA and DNA in the fractions, see Experimental.

controls are presented in Figs. 1B and 1E. It is clear that the most pronounced trend which occurred in the tissues upon detachment was a relative increase in the DNA fraction.

It may be concluded from the results that both in intact and in detached leaves a relative shift in the amount of nucleic acid fractions takes place during ageing. The breakdown of ribosomal RNAs during ageing is particularly conspicuous. This leads to a "virtual" increase in the DNA fraction in both MAK chromatography and sucrose sedimentation profiles. Thus, in ageing plant tissue, the percentage of DNA in total nucleic acids might reach unexpectedly high values (around 30 per cent or above). These observations are in line with earlier reports on the nucleic acid metabolism of ageing plant tissues.[4–8]

Experiments with Tobacco

The experiments on barley seedlings gave essentially similar results with the phenol and DEP methods. By contrast, marked differences were obtained in the sedimentation profiles of nucleic acids extracted by the phenol and DEP method, respectively, from fully developed

[5] A. WOOD and J. W. BRADBEER, *Biochem. J.* **98**, 32P (1966).
[6] R. WOLLGIEHN, *Symp. Soc. Exp. Biol.* **21**, 231 (1967).
[7] B. I. S. SRIVASTAVA and R. K. ATKIN, *Biochem. J.* **107**, 361 (1968).
[8] R. K. ATKIN and B. I. S. SRIVASTAVA, *Physiol. Plantarum* **21**, 1234 (1968).

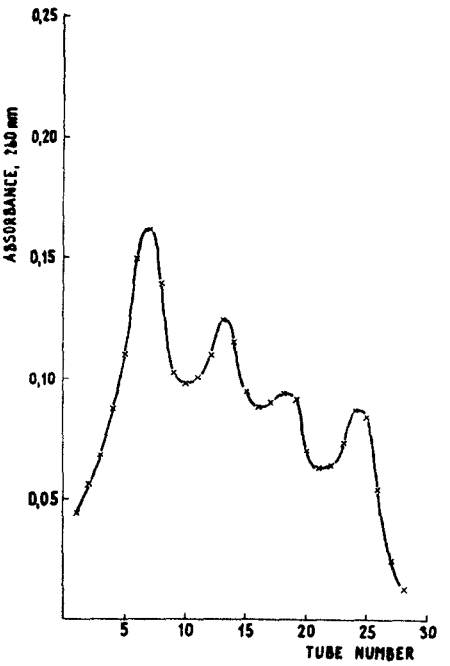

FIG. 3A

FIG. 3. SUCROSE DENSITY GRADIENT SEDIMENTATION PATTERN (A) AND MAK COLUMN CHROMATOGRAPHY PROFILE (B) OF NUCLEIC ACIDS EXTRACTED BY THE DEP METHOD FROM 11-day-old FIRST SEEDLING LEAVES OF BARLEY FLOATED FOR 4 days ON DISTILLED WATER IN THE DARK.

The leaves were detached at an age of 6 days. For further details, see the legend to Fig. 1.

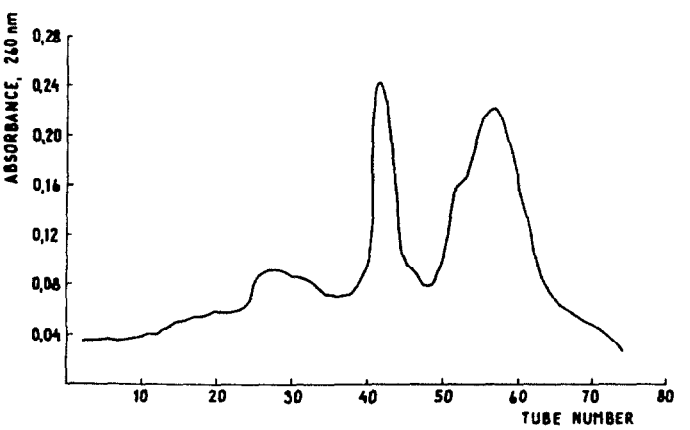

FIG. 3B

leaves of 3-month-old tobacco plants (Fig. 4C, D). The most conspicuous difference between the two profiles was the markedly lower amount of hrRNA extracted by the DEP as compared to the phenol method. In contrast, no such difference was found with leaf tissues of 3-week-old tobacco seedlings (Fig. 4A, B).

Upper (young, actively growing) leaves of 3-month-old tobacco plants yielded nucleic acid preparations with sedimentation properties similar to those of the fully developed leaves, i.e. they contained low amounts of hrRNA with the DEP but not with the phenol method (Fig. 4E, F).

We can conclude that the classical phenol and the new DEP methods on barley yielded essentially similar results as far as the sucrose density sedimentation and MAK column elution profiles of the extracted nucleic acids are concerned (Fig. 1, Table 3). The previously

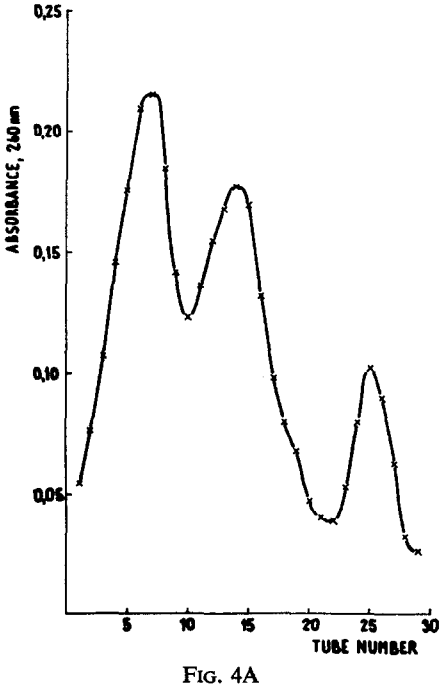

FIG. 4A

FIG. 4. SUCROSE DENSITY GRADIENT SEDIMENTATION PATTERNS OF NUCLEIC ACIDS EXTRACTED FROM TOBACCO LEAF TISSUES.

3-week-old seedlings; nucleic acids extracted by the DEP method (A), and the phenol method (B), respectively. Fully developed leaves of 3-month-old plants; nucleic acids extracted by the DEP method (C) and by the phenol method (D), respectively. Upper (young) leaves of 3-month-old plants; nucleic acids extracted by the DEP method (E) and by the phenol method (F), respectively. For further details, see legend to Fig. 1.

reported superiority of the DEP method over the phenol method with respect to the yields was confirmed irrespective of the age of the leaf tissues investigated (Table 1). With mature tobacco leaves, however, the DEP method gave consistently lower yields of hrRNA as compared to the phenol method (Fig. 4C and D). The reason for this discrepancy is not clear. It might indicate differences in the binding of hrRNA to proteins in the ribosomes of seedling and mature tobacco leaf, respectively. Interestingly, the young, growing leaves of several-months-old tobacco plants yielded nucleic acid preparations similar to those of fully developed leaves of the same plants if extracted by either method (Fig. 4E and F). At the same time identical sedimentation patterns were obtained with nucleic acids extracted from seedling tobacco leaves by either the phenol or DEP methods (Fig. 4A and B). Further studies are

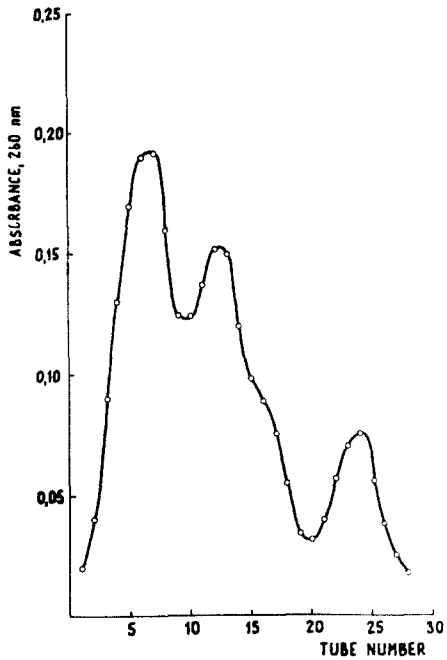
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FIG. 4B

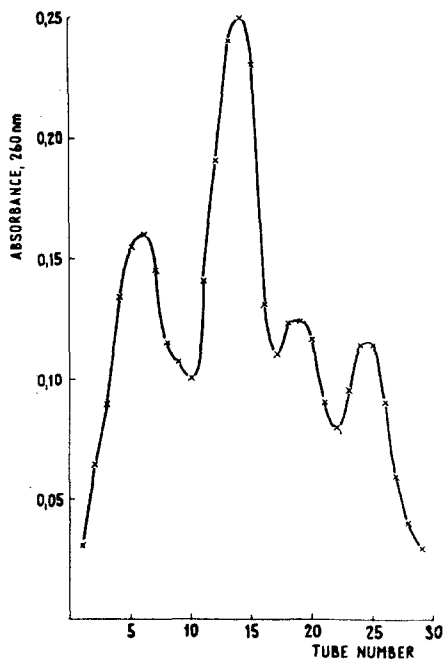
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FIG. 4C

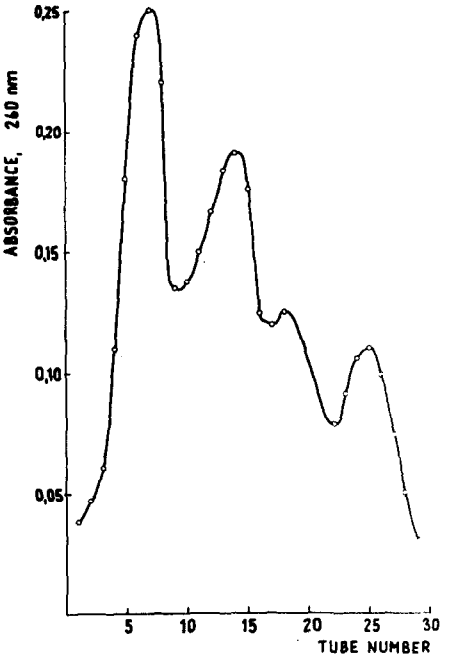
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FIG. 4D

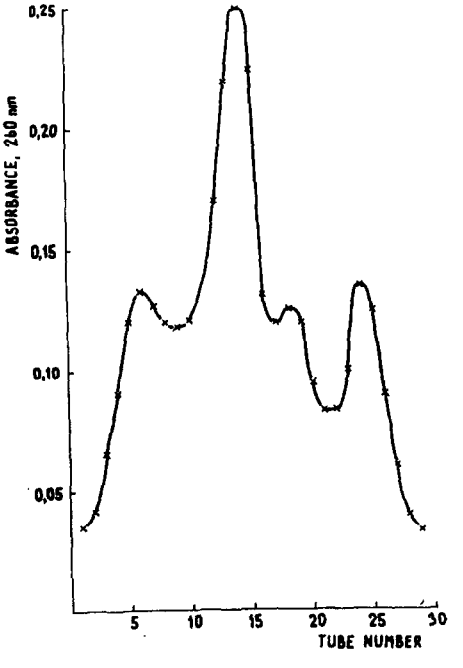
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FIG. 4E

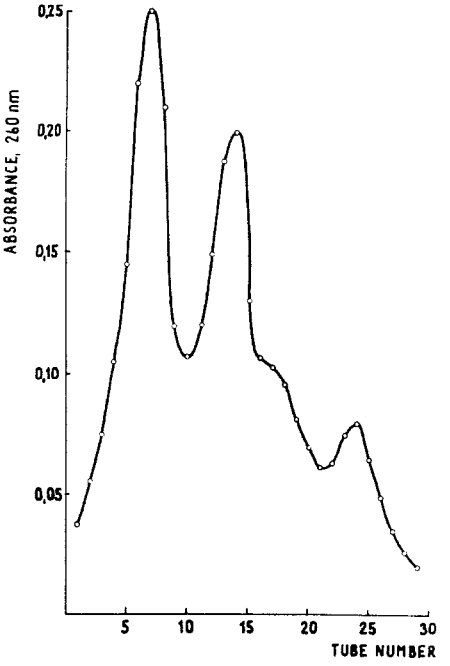

FIG. 4F

required; they might contribute to our understanding of the state of RNAs in the ribosomes in different stages of leaf development. The results also indicate that there is no single universal method which can be applied successfully to all plant tissues and/or all developmental stages.[9]

EXPERIMENTAL

Plant Material

Barley plants, *Hordeum vulgare* L. cv. MFB-104, were grown under controlled conditions (12 hr darkness, 12 hr light 6000 lux, day temperature 28°, night temperature 22°) in sand cultures irrigated at regular intervals with Knopp's nutrient solution. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in soil under ordinary greenhouse conditions. Leaves were harvested at time intervals as indicated in the text. The leaf material used for all subsequent extractions was taken from the middle portion of the leaves (both with barley and tobacco), the basal and apical parts being discarded.

Determination of Dry Weight, Protein, Chlorophyll and Total Nucleic Acid Contents

2 g of leaf tissue were homogenized in ice cold 96% ethanol in a motor-driven Potter–Elvehjem type all-glass tissue grinder (800 rev/min.). The homogenate was centrifuged at 3000 × *g* in the cold and the sediment was washed with 96% ethanol until it became colourless. The supernatants were pooled, diluted to a final ethanol concentration of 80 per cent and used for spectrophotometric chlorophyll determination. Chlorophyll contents are expressed as absorbance units* at 665 nm.

The colourless sediment was washed three times with boiling ether and dried in a boiling water bath. The weight of the dry powder obtained was taken as the dry weight of the leaf tissue. The dry powder was suspended in 5 ml of ice cold 5% TCA and centrifuged in the cold at 20000 × *g*. The sediment was washed once more with 5 ml of ice cold 5% TCA. The nucleic acids in the sediment were hydrolyzed with 5% TCA at 90° for 15 min. After centrifugation at 20,000 × *g* for 30 min the supernatant fluid was used for the estimation of total nucleic acid content and the sediment for assaying total protein content.

* One absorbance unit is that amount of material which gives a reading of 1 at the given wavelength in the spectrophotometer in a cell of 1 cm light path.

[9] J. INGLE and R. G. BURNS, *Biochem. J.* **110**, 605 (1968).

An aliquot of the supernatant fluid was diluted with 5% TCA and the absorbance of the solution was read at 268·5 nm against a similarly treated blank. The amount of nucleic acid was estimated by assuming the molar extinction coefficient with respect to nucleotide phosphate to be $\epsilon_{(P)}=9{\cdot}850\ M^{-1}\ cm^{-1}$ at 268·5 nm[10] and the average molecular weight of nucleotides to be 320.

The sediment was taken up in 5 ml of 1 N NaOH and kept at 37° for 48 hr. The hydrolysate was centrifuged at 16,000 × *g* for 15 min and the supernatant, after proper dilution with distilled water, was used for the determination of protein content according to Lowry *et al.*[11]

Extraction of Undegraded Nucleic Acids

This was carried out according to the phenol method[2] and the DEP method[1] as described previously.

Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation and the determination of the DNA content in the material from the individual fractions were done as described previously[1] with the following modification. After the alkaline hydrolysis of RNA in the fractions and centrifugation of the acid-precipitated material, the supernatant was used for the estimation of RNA content. The amounts of RNA in the individual fractions were calculated by assuming a hyperchromic effect of 27 per cent which arose upon alkaline hydrolysis, as shown experimentally.

MAK Column Chromatography

This was carried out essentially according to Mandell and Hershey.[12] The nucleic acid sample (15 to 20 absorbance units at 260 nm) was loaded on the column (100 × 8·5 mm) in 16 ml of 0·005 M phosphate buffer, pH 6·8, containing 0·4 M NaCl. Elution was carried out by using a linear gradient from 0·4 M NaCl to 1·5 M NaCl in 0·005 M phosphate buffer, pH 6·8. The volumes were 150 ml in both the reservoir and the mixing chamber. About eighty fractions were collected, 3·7 ml each, at a flow rate of 1 ml per min.

[10] J. E. LOGAN, W. A. MANNELL and R. J. ROSSITER, *Biochem. J.* **51**, 480 (1952).
[11] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[12] J. D. MANDELL and A. D. HERSHEY, *Anal. Biochem.* **1**, 66 (1960).