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# THE NUCLEIC ACIDS

# OF

# VICIA FABA (L)

by

D.M. Lonsdale, B.Sc. (Dunelm)

A thesis submitted in accordance
with the requirements for the degree
of Doctor of Philosophy in the
University of Durham

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# ABSTRACT

Seeds of *Vicia faba* (L) become fully mature in about 130 days from the fertilization of the ovule. After an initial phase of cell division, storage protein is synthesised in the cotyledons. During the stage of storage protein synthesis, rRNA synthesis occurs concomitant with synthesis of ER, suggesting that storage protein synthesis is mediated on membrane bound ribosomes by mRNA(s). Analysis of the nucleic acids during cotyledon development did not lead to the identification of any non-ribosomal RNA species.

A fourth rRNA was identified, which had a molecular weight of 52,000 daltons and was hydrogen bonded to the 25S rRNA species.

Attempts to identify rRNA precursor molecules in cotyledons and roots were unsuccessful, though in the latter the bacterial precursor molecule to the 16S rRNA was identified.

Isolated polyribosomes, from cotyledons at various stages of development, had similar profiles and their constituent nucleic acids were degraded, indicating that they were cleavage products of larger *in vivo* units. The rRNA cleavage products arising during polyribosome isolation were characterised by their molecular weights.

The properties of diethylpyrocarbonate as a nuclease inhibitor were reinvestigated and it was found to be incapable of effectively inhibiting high concentrations of nucleases. The extraction of polyribosomes in the presence of diethylpyrocarbonate effectively protected the polyribosome against RNase during the later stages of the extraction procedure, but not during the initial stages resulting in marked rRNA cleavage and the formation of ribosomal subunits.

The use of antilegumin as a method in the identification of a mRNA is discussed.

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## INTRODUCTION

Nucleic acid was first isolated in the 19th Century by Miescher (1844-95) who obtained a compound of high molecular weight, with a phosphorus content of 9.59%, from nuclei which he termed nuclein. It is now known that nuclein was nucleic acid; a term coined by Altmann in 1889.

Following the chemical analysis of nucleic acid by Miescher, Kossel, Neumann, Levene and others, two types were recognised. One was characteristic of thymus nucleic acid and contained the sugar, deoxyribose and the bases, adenine, guanine, cytosine and thyamine. The other, characteristic of yeast nucleic acid, contained the sugar, ribose and the bases, adenine, guanine, cytosine and uracil. Both types contained phosphoric acid. These nucleic acids have been termed deoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) respectively. As the nucleic acids isolated from animals resembled DNA, and those from plants RNA, the assumption was made that the distinction represented a valid classification. This classification, however, did not last long when it was found that RNA had a widespread occurrence in both animal and plant tissues, as had DNA, which was localised in the nucleus. The possible connection between DNA and genetic inheritance had been realised soon after the original discovery of nucleic acids by Miescher. However, it was not until 1944 that positive proof linked DNA to genetic inheritance. Avery, Macleod and McCarty (1944), following up the work of Griffith (1928), showed that the transformation of non-encapsulated pneumococci to a capsulated form could be brought about with an extract from capsulated pneumococci, and that the extract was chemically identical to DNA. Further proof was forthcoming in 1952, when Hershey and Chase demonstrated that virtually only phage DNA entered the bacterium and was responsible

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for the production of new phage particles. The chemical explanation of the ability of DNA to store genetic information and to divide and so duplicate the information was formulated by Watson and Crick in 1953 (a and b).

The intracellular distribution of nucleic acids was first investigated by Brachet (1940), using RNase coupled with basophilic dyes, and by Caspersson (1941), using ultraviolet photography in conjunction with the Feulgen reaction (Feulgen and Rossenbeck, 1924). They showed that DNA was localised in the nucleus, RNA mainly located in the cytoplasm, and that the concentration of RNA was greater in dividing cells. The RNA was localised in basophilic granules which, when isolated by centrifugation, consisted of phospholipid ribonucleoprotein complexes. These were termed microsomes (Bensley, 1937; Claude, 1941). Tracer experiments, in which Hultin (1950) used young chicks and glycine; Borsook _et_ _al_.,(1950) used guinea pigs and glycine, histidine, leucine and lysine, Keller (1951) used rats and cystine, showed that, following the injection of the labelled amino acid, the microsome fraction of the liver had a higher specific activity than any other fraction. These experiments identified the microsome fraction with the site of protein synthesis. In 1955, Littlefield _et_ _al_. demonstrated that the microsomes were separable into a deoxycholate-soluble fraction and a deoxycholate-insoluble fraction. The latter consisted of particles having a diameter of about 240 A$^{o}$ and a RNA to protein ratio of approximately 1. These ribonucleoprotein particles, or ribosomes, were the initial site of amino acid incorporation into protein. Prior to the description of messenger RNA the occurrence of ribosome complexes (Watson, 1959) was ascribed to the non-specific aggregation of ribosomes. The importance of the ribosome aggregate in protein synthesis was then demonstrated (Warner _et_ _al_., 1962; Marks _et_ _al_., 1962; Gierer, 1963), and the concept of the polysome arose (Wettstein _et_ _al_., 1963; Marks _et_ _al_., 1963). The mechanism of protein synthesis is now well documented (Konings, 1969; Boulter, 1970).

Ribosomes, now accepted as characteristic organelles of the living cell, fall into two size classes. Those of prokaryotic organisms have a sedimentation coefficient of about 70S, while those of eukaryotic organisms have a sedimentation coefficient of about 80S (Taylor and Stork, 1964). An exception to this occurs in the eukaryote *Dictyostelium discoideum*, whose ribosomes have a sedimentation coefficient of 70S; though they do resemble eukaryotic ribosomes in their magnesium stability (Ashworth, 1966). Eukaryotic cells, in addition to the cytoplasmic 80S ribosomes, possess 70S ribosomes. These are localised within the mitochondria and chloroplast. They are typically bacterial-like, not only in their sedimentation coefficient, but also in their magnesium stability (Boardman *et al.*, 1966; Kuntzel, 1969) and other properties. All ribosomes consist of two unequal subunits, 30S and 50S in 70S ribosomes (Tissieres and Watson, 1958) and 40S and 60S in 80S ribosomes (Chao, 1957).

Most of the early techniques used in the isolation of RNA resulted in the degradation of the molecules to fragments of lower molecular weight. As the isolation techniques improved, so the molecular weight of the RNA obtained increased (Grinnan and Mosher, 1951; Kirby, 1965). The early analysis of bacterial rRNA's resulted in Midgley (1965, a; b; c) suggesting that the 30S ribosomal subunit contained a 16S RNA, while the 50S ribosomal subunit contained a 16S RNA dimer having a sedimentation coefficient of 23S. This was apparently supported by the work of Godson and Butler (1964), who observed that the 16S RNA became labelled prior to the 23 RNA. However, in 1965, Stanley and Bock showed that both the 23S and 16S RNA's were single polynucleotide chains. It is now generally accepted that one large RNA molecule is localised in each of the two ribosomal subunits.

The classification of ribosomes on size and magnesium stability into two groups also reflects the size of their constituent RNA molecules. The two major RNA's present in the ribosomes of prokaryotic organisms, mitochondria and chloroplasts, have molecular weights of 1.1 x $10^6$ and 0.56 x $10^6$ daltons respectively. The rRNA's of eukaryotic organisms, on the other hand, show some variation in size. The larger of the two rRNA's in the animal kingdom for example, is in HeLa cells 1.75 x $10^6$ daltons; *Xenopus* 1.51 x $10^6$ daltons and *Drosophila* 1.40 x $10^6$ daltons. In the plant kingdom, the size of the larger of the rRNA's is relatively constant at 1.3 x $10^6$ daltons. In most eukaryotic organisms the smaller of the two rRNA's is approximately 0.70 x $10^6$ daltons; except in *Amoeba* and *Euglena*, which have values of 0.89 x $10^6$ and 0.85 x $10^6$ daltons respectively (Loening, 1968).

The presence of a low molecular weight RNA of approximately 5S, in ribosome preparations from *Escherichia coli*, was first described by Rosset and Monier (1963). Since then it has been shown that the 5S RNA is a natural component of both the 70S and 80S ribosomes, being located in the large subunit (Comb and Katz, 1964; Combe and Zehavi-Willner, 1967; Marcot-Queiroz *et al*., 1965; Bachvaroff and Tongur, 1966; Brown and Littna, 1966; Knight and Darnell, 1967; Li and Fox, 1969). However, Lizardi and Luck (1971) have demonstrated that the ribosomes of *Neurospora crassa* do not possess a 5S RNA molecule. The complete sequence of the 5S RNA from *E. coli* (Brownlee *et al*., 1968), and from human carcinoma (KB) cells (Forget and Weissman, 1969), is known, though as yet the exact nature of its function is unclear. Siddique and Hosokawa (1969), have suggested that the 5S RNA may play a role in the specific binding of transfer RNA to ribosomes.

Transfer RNA's were first postulated by Crick in 1957 and were subsequently demonstrated by Hoagland *et al*. (1957). Transfer RNA has since been purified from a wide range of sources and there is at least one specific ${}_{t}$RNA for each of the 20 common amino acids used in protein biosynthesis. The first primary structure of a ${}_{t}$RNA species to be elucidated was alanine ${}_{t}$RNA from yeast (Holley *et al.*, 1965). Since then the nucleotide sequence of a wide range of ${}_{t}$RNA species has been determined (Dayhoff, 1969), and all are very similar; having a molecular weight of approximately 25,000 daltons, and containing a large proportion of unusual bases, the majority of which are methylated. From the many published secondary structures, X-ray diffraction studies have indicated that the most probable model is the clover leaf model originally suggested by Holley *et al.* (1965), and that the tertiary structure is a compact elongate molecule, the axis of which is a double helix, the loops being tightly accommodated against the central axis (Abraham, 1971).

Prior to Jacob and Monod in 1961 (see later), the template upon which specific proteins were made was envisaged to be rRNA. This would necessitate a high rate of turnover for the cellular RNA, and for rRNA to be heterogeneous. The evidence against rRNA being informational was that it was not heterogeneous, and that its base composition was relatively constant, having a high cytidylic and guanylic acid content, which did not reflect the base composition of DNA; and further that it was stable (Davern and Meselson, 1960). In 1961, Jacob and Monod proposed that genetic information was transferred from the genes to the ribosomes by transient molecules of RNA, which they termed messenger RNA. Evidence for the existence of such RNA's had previously been published by Volkin and Astrachan (1956), Brenner *et al.* (1961), Gros *et al.* (1961) and Ycas and Vincent (1960). A critical appraisal of the papers by Brenner *et al.* (1961) and Gros *et al.* (1961) is given by Harris (1968). Volkin and Astrachan (1956) identified a rapidly labelled RNA fraction in T2 infected *E. coli* which had a base composition similar to T2 DNA. This was later shown to be complementary to T2 DNA by Hall and Spiegelman (1961). Ycas and Vincent (1960) detected a rapidly labelled RNA fraction, with a base composition similar to DNA, in yeast. They suggested that it may be a primary gene product acting as an agent for the transmission of genetic information from DNA to protein. However it would seem more probable that it corresponded to rapidly labelled nuclear RNA, rather than messenger RNA.

Since this early work, the occurrence of transient molecules which are involved in information transfer has been established in bacterial and phage infected bacterial systems. However, in eukaryotic cells, although messenger RNA is an accepted concept, the position is more complex than in bacteria, due to the presence of a nucleus and the rapidly labelled nuclear RNA's, from which cytoplasmic messenger RNA's must be distinguished.

First attempts to characterise messenger RNA activity in eukaryotic cells were by using experiments designed to demonstrate the ability of separated RNA fractions to stimulate amino acid incorporation in a cell-free system. Using this approach, so called messenger RNA has been demonstrated in rabbit reticulocytes (Drach and Lingrel, 1964; Arnstein _et_ _al_., 1965), rat liver cytoplasm (Hoagland and Askonas, 1963; DiGirolamo _et_ _al_., 1964), nuclei (Barondes _et_ _al_., 1962; Brawerman _et_ _al_., 1963; DiGirolamo _et_ _al_., 1964), sheep thyroid (Cartouzou _et_ _al_., 1965) and calf lens (Konings and Bloemendal, 1969). It is clearly evident from these and other papers (see review; Konings, 1969), that RNA will enhance amino acid incorporation in a cell-free system: (a) if it has a lower guanylic-cytidylic acid content than rRNA, and (b) if it has been extracted at either extremes of pH or at temperatures greater than 30°C. In other words, any RNA which does not possess, or has lost, a highly ordered secondary structure will enhance amino acid incorporation. Such RNA does not code for specific proteins, but either codes for heterogeneous peptidyl material, or enhances the endogeneous activity, natural or poly-U directed, of the system (Drach and Lingrel, 1966; Hunt and Wilkinson, 1967;

Konings, 1969).

Rapidly labelled RNA, following precursor incorporation, which becomes associated with polysomes has been ascribed to messenger RNA; though in some cases it has been termed DNA-like RNA (DRNA) owing to its similarity to DNA in its base composition (Loening, 1965; Lin et al., 1966). This type of rapidly labelled RNA is polydisperse (Kumar, 1968) and can be accounted for by the view that it represents nuclear RNA which has been released into the cytoplasm from nuclei damaged during the preparation of the polysomes (Flagemann, 1969). It is only in a relatively few cases that specific rapidly labelled RNA's, isolated from polysomes, have been implicated in the synthesis of specific proteins. Probably the best characterised of these are the messenger RNA's for haemoglobin which were first isolated by Marbaix and Burny (1964; 1965). The physical characteristics of these messengers seem dependent upon the isolation procedure used. Marbaix and Burny (1964; 1965) assigned to them a sedimentation coefficient of 9S and a molecular weight of approximately 150,000. Labri (1969) proposed values of 10S and a molecular weight of 190,000. Gaskill and Kabat (1971) and Bloebel (1971) assigned to them a molecular weight of approximately 220,000 corresponding to a sedimentation coefficient of 11.3S. Marcoun et al. (1971) showed that there were two forms of the messenger RNA's; one with a sedimentation coefficient of 17S, the other being 9S. Both apparently had the same molecular weight and therefore only differed in conformation (see Bramwell and Harris, 1967). There is little doubt now that these particular RNA's, when added to a reticulocyte cell-free system, code for the protein haemoglobin (Adamson et al., 1968; Zucker and Schulman, 1968; Housman et al., 1970; Gilbert et al., 1970). Although Laycock and Hunt (1969) claimed the synthesis of haemoglobin in an E. coli system, it would appear that the system made a haemoglobin-like protein, rather than haemoglobin itself. They claimed protein initiation was by N-acetyl-valine tRNA, whereas, in vivo and in the reticulocyte system, initiation is by methionine - $RNA^{MET.F}$ (Wilson and Dintzis, 1970; Gonano and Baglioni, 1969; Housman et al., 1970; Jackson and Hunter, 1970).

Another messenger RNA which has been characterised is the 26S RNA coding for the large subunit of myosin, (Heywood and Nwagwu, 1968a; 1968b; 1969) which was isolated from the heavy polysomes, containing 60 to 80 ribosomes. The light myosin chains are produced on light polysomes, containing 4-9 ribosomes (Low et al., 1971), though the characteristics of the RNA coding for the light chains is not yet known. Heywood and Nwagwu demonstrated a 10-12S RNA associated with the light polysome fraction but were unable to demonstrate that it directed the synthesis of light myosin subunit chains in vitro.

The haemoglobin and myosin messenger RNA's are the only cases, so far, in which a eukaryotic cytoplasmic RNA has been shown to direct the synthesis of a specific protein in a cell-free system. Other claimed messenger RNA's are those coding for the light and heavy antibody chains (Keuchler and Rich, 1969a, b), and the messenger RNA(s) coding for the histone proteins in sea urchins (Nemer and Lindsay, 1969; Daigneault et al., 1970; Kedes and Birnsteil, 1971). In both these cases, the identification of the RNA with a messenger function is based on circumstantial evidence, rather than their ability to direct the synthesis of a specific protein in a cell-free system.

Indirect evidence for the existence of messenger RNA's in eukaryotic cells comes from the use of the antibiotic actinomycin D, which inhibits RNA transcription though not template translation. There are numerous reports relating to cases in which the synthesis of a protein has been initiated in the presence of actinomycin D, or in which synthesis of a protein persists and continues to be regulated in the presence of this antibiotic. A review of the animal literature is given by Harris (1968). Actinomycin D has also been used on germinating plant embryos to demonstrate polysome formation and the synthesis of protein in the absence of detectable RNA synthesis, thus indicating the existence of preformed messenger RNA in the dry seed; which must have been synthesised during embryogenesis (Dure and Waters, 1965; Ihle and Dure, 1969; Holloman, 1969). In the absence of actinomycin D, similar conclusions as to the presence of a masked messenger RNA in plant embryos, which is activated by imbibition, were reached by Marcus and Feely (1964, 1966), Chen _et al._ (1968) and Weeks and Marcus (1971). Actinomycin D is in itself a toxic compound causing changes in cell metabolism, some aspects of which are directly linked to protein synthesis (Soeiro and Amos, 1966); also it causes a differential inhibition of the synthesis of different RNA species in some cell types (Ellem, 1967; Kay and Cooper, 1969) and may induce degradation of the RNA (Wiesner _et al._, 1965). The experiments which purport to demonstrate messenger RNA by using actinomycin D are open to the above criticisms and also that the inhibitor did not reach its suggested site of action (Neumann, 1964; Abdul-Baki, 1969). It is concluded that, in general, the evidence for long-lived messenger RNA in seeds is unreliable, though it is still possible that the drugs main _in vitro_ effect on DNA transcription may be its _in vivo_ action. More reliable evidence for the existence of stable templates has come from the study of enucleated cells, such as _Acetabularia_, sea urchin and amphibian oocytes (Brachet, 1967). It would therefore seem reasonable to conclude, firstly that, whereas the messenger RNA's of bacteria exhibit a rapid turnover, there exists in eukaryotic cells a class of messenger RNA's which are relatively stable. And secondly, that the time at which a particular gene is transcribed into RNA has no immediate connexion with the time at which this RNA is translated into protein.

Yet another technique which purports to separate messenger RNA or at least the rapidly labelled RNA in eukaryotic cells, is methylated albumin kieselguhr chromatography. The rapidly labelled RNA's are normally eluted after the ribosomal RNA and consist of three fractions, which have been termed $Q_1$-, $Q_2$-, and TD-RNA. $Q_1$-RNA has been identified as ribosomal precursor RNA (Yoshikawa-Fukada et al., 1965; Yoshikawa-Fukada, 1967) whereas $Q_2$- and TD-RNA are DNA-like in their base composition (Yoshikawa-Fukada et al., 1965; Ellem, 1966), and have mean sedimentation coefficients of 50S and 16S respectively. $Q_2$-RNA would appear to be a precursor to TD-RNA on labelling kinetics, the latter according to Ellem (1966) being messenger RNA. Therefore, $Q_2$-RNA would represent the polycistronic nuclear precursors of the messenger RNA; the existence of which has been suggested by Penman et al. (1963) and Scherrer and Marcaud (1968). Rapidly labelled RNA associated with polysomes (see earlier discussion) elutes with the nuclear $Q_2$- and TD-RNA's (Miller et al., 1968). The elution factor therefore, cannot be size, as nuclear $Q_2$-RNA has a mean sedimentation coefficient of 50S while polysomal $Q_2$-RNA has a mean sedimentation coefficient of 16S, but is more likely base composition and/or secondary structure. Miller et al. (1968) suggested that polysomal $Q_2$-RNA represents the cytoplasmic fragmented counterpart of messenger RNA present in the nucleus as giant polycistronic molecules, which is contrary to the precursor-product relationship suggested to exist between $Q_2$- and TD-RNA by Ellem (1966). It would therefore appear, that to draw any analogy between the fractions eluted off methylated albumin kieselguhr columns and cytoplasmic messenger RNA would be hazardous.

There are in the cell, in addition to rRNA, $_t$RNA and mRNA's, ribosomal precursor molecules, an example being the $Q_1$ fraction of methylated albumin kieselguhr chromatography. These RNA's form the major part of the nuclear RNA's in eukaryotic organisms and also occur in bacteria (Marrs and Kaplan, 1970). They are now well characterised in mammalian cells (Craig et al., 1968; Amalric et al., 1969; Ab and Malt, 1970; Quagliarotti et al., 1970; Egawa et al., 1971), *Xenopus laevis* (Loening et al., 1969), *Chironomus tentans* (Edstrom and Daneholt, 1967), sea urchins

(Sconzo *et al.*, 1971); *Urechis caupo* (Dus *et al.*, 1970), *Amoeba proteus* (Goldstein, 1971), *Dictyostelium discoideum* (Iwabuchi *et al.*, 1971), yeast (Taber and Vincent, 1969; Van den Bos and Planta, 1971), *Gyrodinium cohnii* (Rae, 1970), *Tetrahymena pyriformis* (Kumar, 1970) and higher plants (Leaver and Key, 1970; Rogers *et al.*, 1970; Gheng and Hagen, 1971; Chen *et al.*, 1971).

In eukaryotes the synthesis and the subsequent processing of the ribosomal precursor molecule occurs in the nucleolus (Birnsteil, 1967). The precursor is transcribed in the nucleolar core (Macgregor, 1967; Simard *et al.*, 1969; Amalric *et al.*, 1969; Dus *et al.*, 1970) and transferred to the nucleolar cortex concomitant with the cleavage of the molecule and its association with protein; following which the immature ribosomal subunits are transferred to the cytoplasm, where they mature by a process involving the loss of protein (Perry, 1967; Vaughan *et al.*, 1967; Izawa and Kawashima, 1968; Liau and Perry, 1969; Chen *et al.*, 1971).

The site of rRNA synthesis and the mechanism of ribosome assembly and transport from the nucleus to the cytoplasm is now relatively well understood. In contrast, little is known concerning the mechanism of mRNA transcription and its subsequent transport from the nucleus to the cytoplasm which is partly due to the elusiveness of mRNA in the first place. Ribonucleoprotein particles or particle complexes have been identified in various types of cells (see Table 1) and have been associated by these authors as involved in mRNA transport. Spirin and Nemer (1965), and Nemer and Infante (1965) have also described discrete cytoplasmic ribonucleoprotein particles containing rapidly labelled RNA, which have been termed informosomes. In contrast, the nuclear 30S particles have been termed informofers by Samarina *et al.* (1966, 1968). Similarities between the protein components of nuclear and cytoplasmic particles have been established by Lissitsky *et al.* (1970) and Schweiger and Hannig (1970), though a definite precursor-product relationship has

## TABLE 1

### Ribonucleoprotein Particles.

| Reference | Source | Particle characteristics | RNA mean S.value | RNA $\frac{A+U}{G+C}$ | $\frac{RNA}{protein}$ | Comments |
|---|---|---|---|---|---|---|
| Henshaw *et al.*, (1965) | Rat liver cytoplasm | 45S | 18S | 1.13 | | stimulates E. coli cell-free system |
| McConkey and Hopkins, (1965) | HeLa cell cytoplasm | 45S | 15-16S | - | - | represents 40S ribosomal subunit precursor with mRNA attached |
| Samarina *et al.*, (1966) | Ehrlich carcinoma nuclei | 30S | 12-18S | - | - | pre-existing particles bind newly transcribed mRNA |
| Samarina *et al.*, (1968) | Rat liver nuclei | 30S<br>180 x 180 x 80 Å | 8.5S | 1.26 | 1 : 4 | particles form polysome-like complexes with RNA |
| Moule and Cheuveau, (1968) | Rat liver nuclei | 40S | 3.4S | 1.00 | 1 : 8 | - |
| Perry and Kelley, (1968) | L-cells cytoplasm | Polydisperse | 10-12S | - | 1 : 1.5 | - |
| Cartouzou *et al.*, (1969) | Sheep thyroid nucleus | Polydisperse<br>15S monomer? | - | 1.25 | 1 : 4 | stimulates E. coli cell-free system |
| Ishikawa *et al.*, (1969) | Rat liver nuclei | 45S | 14S | 1.05 | 1 : 5.6 | - |
| Lissitzsky *et al.*, (1970) | Sheep thyroid cytoplasm | 400-1000S<br>Polydisperse | 12S to 30S | 1.25 | - | - |

not yet been demonstrated to exist between any of the nuclear and cytoplasmic particles described. The RNA of these ribonucleoprotein particles stimulates amino acid incorporation in a cell-free system (Cartouzou et al., 1969; Henshaw et al., 1965) and exhibits a base composition different from that of ribosomal RNA, and therefore has been identified with mRNA (see earlier). Criticism of experiments purporting to demonstrate ribonucleoprotein particles involved in mRNA transport can be based on: (1) a confusion with ribosome subunit precursors; (2) RNA contamination from disrupted nuclei and mitochondria; (3) the ability of native RNA to form stable complexes with basic proteins (Perry and Kelley, 1968); (4) on the fact that until it can be demonstrated that ribonucleoprotein particles contain an RNA which acts as a template for the translation of a biologically functional molecule, their status must remain in doubt.

Experiments designed to dissociate reticulocyte polyribosomes and release the haemoglobin mRNA have not clarified the informosome problem. Holder and Lingrel (1970), using tetra-sodium pyrophosphate, localised the haemoglobin mRNA on the 40S ribosomal subunit (CF. McConkey and Hopkins, 1965). Temmerman and Lebleu (1969), Burny et al. (1969) and Lebleu et al. (1971), using disodium EDTA, characterised the haemoglobin mRNA as a discrete ribonucleoprotein particle. Bloebel (1971), using KCl and puromycin, demonstrated that the haemoglobin messenger RNA was a native molecule. Complete identification of the released RNA fraction with the haemoglobin mRNA was not attempted in any of the above experiments, and therefore identification of the haemoglobin mRNA must remain in doubt.

In the developing seeds of Vicia faba, Payne (1968) has shown that rapid RNA synthesis precedes slightly the initiation of protein synthesis. This developing system, in which the synthesis of two characterised proteins, namely legumin and vicilin, is switched on at a precise time in seed development, would appear to offer excellent experimental material for the search for mRNA.

Before any attempt to identify any mRNA could be made it was first necessary to separate and characterise the types of RNA molecules present in the developing bean during its different stages of development, and secondly to identify all the ribosomal RNA breakdown products, either occurring naturally in the developing bean or produced during the extraction of subcellular particles. Separation and characterisation of RNA molecules forms the major part of the work of this thesis in which the highly resolving technique of polyacryamide gel electrophoresis has been employed. Attempts to identify an RNA involved in legumin synthesis were based on tracer studies, and the use of antilegumin to specifically precipitate labelled polysomes engaged in the synthesis of legumin polypeptides.

# MATERIALS

## 1. Biological materials

Seeds of *Vicia faba* (L) var. Triple White were obtained from the Tyneside Seed Company, Gateshead.

Seeds of *Phaseolus aureus* were obtained from Anglo-Continental Supplies Ltd., Grove Street, Newcastle.

## 2. Chemicals and Reagents

With the exceptions listed below, chemicals were obtained from British Drug Houses Ltd., Poole, Dorset; and from Hopkin and Williams Ltd., Chadwell Heath, Essex. They were of analytical grade when necessary.

p-Dimethylaminobenzaldehyde
Trizma base
*Torula* RNA; Grade VI
DNA; Type III, highly polymerised from salmon
DNase I; stock No. DN-EP
were obtained from Sigma Chemical Co. Ltd., London.

1,4 - Di [2- (5-phenoxazolyl)] benzene
2,5 - Diphenyloxazole
DNase I; 450 Kunitz units/mg
were obtained from Koch-Light Laboratories, Colnbrook, Buckinghamshire.

DEAE-cellulose; DE52 (Whatman Ltd.)
True-touch gloves
were obtained from Macfarlane Robson Ltd., Blaydon-on-Tyne, Durham.

Sephadex; G100 and G75

were obtained from Pharmacia Ltd., Uppsala, Sweden.

Zeocarb 225 (200-400 mesh)

was obtained from Permutit Co. Ltd., London.

Sucrose; RNase-free

was obtained from Mann Research Laboratories, Liberty Street, New York, U.S.A.

DNA; A grade

was obtained from Calbiochem Ltd., London.

Lyphogel

was obtained from Hawksley and Sons, Lancing, Sussex.

$[^{32}P]$-orthophosphate

was obtained from the Radiochemical Centre, Amersham.

Diethylpyrocarbonate

was a generous gift from Bayer Chemicals Ltd., Richmond, Surrey.

Antilegumin; purified by molecular sieve and affinity chromatography, in 0.1M-phosphate, pH 7.0 was a generous gift from Dr. T.A. Graham, Department of Botany, The Queen's University of Belfast, Northern Ireland.

# METHODS

## 1. Growth of biological materials

### (i) The developing seed.

Seeds of Vicia faba (L) var. Triple White were grown in the University of Durham botanic gardens; in the open during the summer and in heated greenhouses during the winter. The seeds were sown in heated greenhouses in February and the young plants planted out at the end of March. Flowers were labelled on the first day on which they were observed to be fully open, the age of the developing seed being calculated from the day of labelling. For the age required, beans of an average cotyledon weight were used (fig. 1). Plants grown during the winter period were subjected to an artificial regime of 12 hours of light and 12 hours of dark; within the temperature range of 13°C to 20°C. Pod set and bean development were extremely variable under these conditions.

### (ii) Germinating seeds.

#### (a) Method I

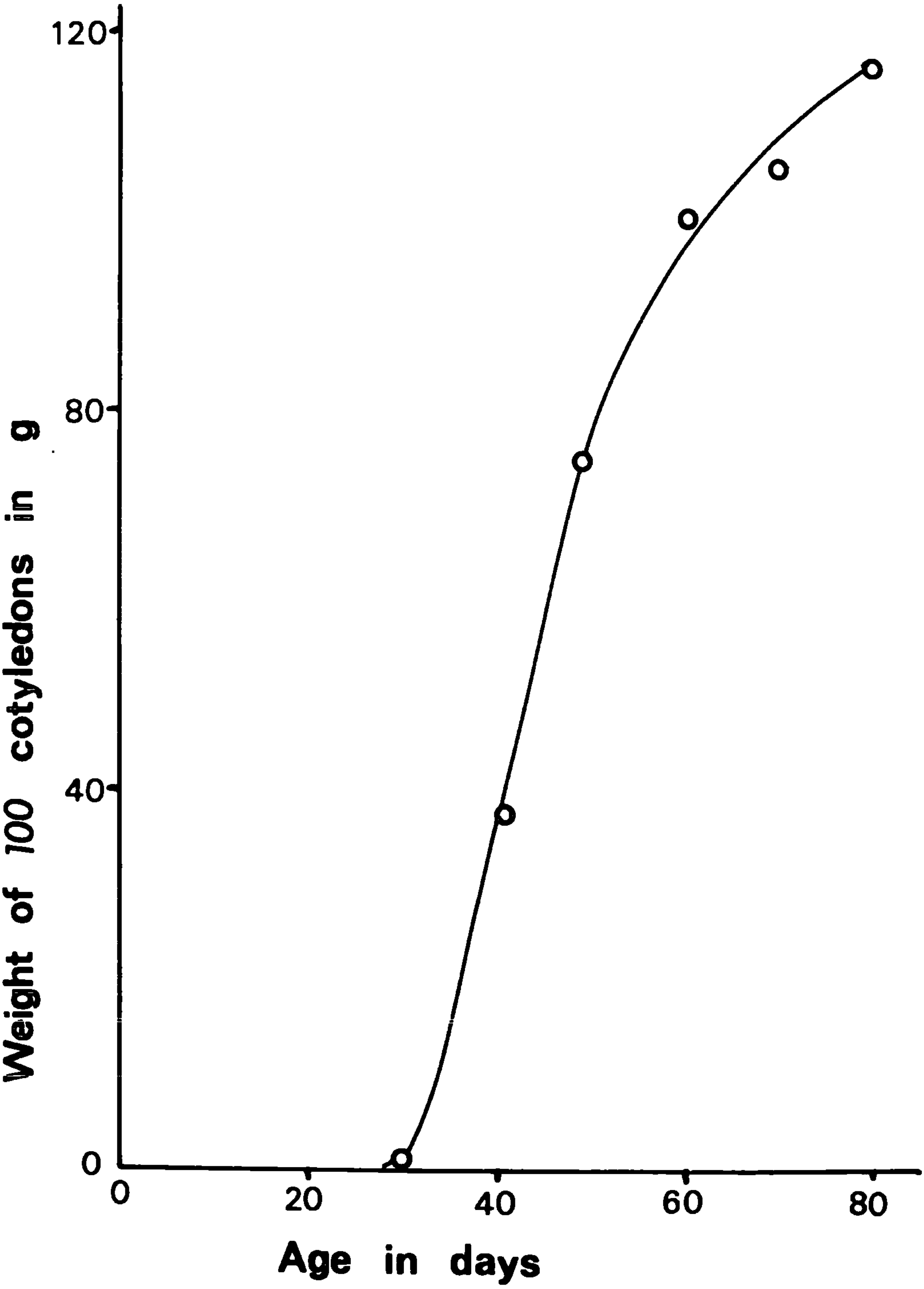
Vicia faba seeds were imbibed in running tap water for 24 hours. They were then surface sterilized by washing in freshly prepared and filtered 10% (w/v) calcium hypochlorite for one minute. The seeds were then washed in distilled water to remove the calcium hypochlorite and sown in seed trays in vermiculite. The seeds were grown at 25°C for periods up to 2 weeks in a growth room.

#### (b) Method II

Vicia faba seeds were surface sterilized by washing in absolute ethanol for 1 min, followed by soaking in 10% (w/v) calcium hypochlorite for 10 min. The seeds were then imbibed overnight in distilled water. The testas were removed from the imbibed beans, and any seed with an

Fig. 1.

The average increase in fresh weight of developing cotyledons collected during the 1969 season.


Weight of 100 cotyledons in g
120
80
40
0
0
20
40
60
80
Age in days

infected cotyledon was discarded. The seeds were then washed 10 times in distilled water and sterilized by immersion for 1 min in absolute ethanol, then for 2 min in 10% (w/v) calcium hypochlorite. The seeds were then washed 6 times in sterile distilled water, and planted in sterile vermiculite under sterile conditions. The seeds were allowed to germinate for a period of 3 days in a sterile inoculating room.

### 2. Preparation of lithium lauryl sulphate

The method used to prepare LLS was as previously described (Noll and Stutz, 1968). The resin, Zeocarb 225 (200-400 mesh) was regenerated by stirring overnight in hot 2M-NaOH. The resin was then washed with an excess of distilled water. It was then resuspended in distilled water and the pH adjusted to less than 1 with concentrated $H_2SO_4$ and equilibrated overnight. The sulphuric acid was removed and the resin washed with distilled water. The resin was then resuspended in 2M-LiCl and equilibrated for 3 h. The resin was washed with an excess of distilled water and following resuspension in distilled water, was packed into a 2 cm column, to a height of 15 cm. The column was washed with two column volumes of distilled water prior to the application of 150 ml of 20% (w/v) SDS. The column eluant was monitered with a sugar refractometer (Bellingham and Stanley Ltd., London) to detect the schlieren produced by the ion front. The first 250 ml was collected, and diluted to 300 ml. The resulting solution was stored in a dark bottle at 4°C. A sample was compared with a solution of 10% (w/v) SDS with regard to its cooling properties, to determine whether the exchange of $Na^+$ for $Li^+$ had taken place.

### 3. Preparation of sodium bentonite

The sodium bentonite was prepared by a modification of the method of Watts and Mathias (1967). 100 g bentonite was suspended in 2 l of distilled water by stirring overnight. The suspension was centrifuged at 230 g max. for 20 min in the 4 x 380 ml rotor of the MSE Mistral 4L. The pellet was discarded and the supernatant was recentrifuged at 2,100 g max. for 30 min. The pellets were made up to 700 ml by suspension in distilled water; and allowed to stand for 30 min. The sediment was discarded, and the remaining suspension was centrifuged at 1450 g max. for 30 min. The supernatant was discarded and the pellet was suspended in 350 ml 0.1M-disodium EDTA. This was then diluted to 700 ml with distilled water and stirred overnight. The suspension was centrifuged at 520 g max. for 15 min and the resulting pellet discarded. The supernatant was recentrifuged at 1,450 g max. for 30 min. The resulting supernatant was discarded and the pellet was suspended in 350 ml distilled water and stirred for a minimum of 2h. This washing and pelleting was repeated 4 times. The final pellet was suspended in 10 ml distilled water, and the sodium bentonite concentration determined by drying 0.5 ml aliquots at 105°C till constant weight was attained.

## 4. Extraction of nucleic acids

### (i) Extraction from whole tissue.

The material, normally 2 g, was homogenised in 5 vol. of Kirby's buffer: 1% (w/v) TNS, 6% (w/v) sodium 4-aminosalicylate, 1% (w/v) NaCl and 6% (v/v) phenol-cresol (Kirby, 1965; Parish and Kirby, 1966; Loening, 1969), in an MSE homogeniser for 30-60s at 14,000 rev./min. An equal vol. of phenol-cresol (redistilled phenol, 500 g; redistilled cresol 70 ml; 8-hydroxyquinoline, 0.5 g; and distilled water to saturation) was added and mixed, using a whirlimixer (Fissons Scientific Apparatus Ltd., Loughborough). The phases were separated by centrifugation in an MSE Mistral 4L centrifuge at 4,000 g max. or an MSE Minor centrifuge at 2,750 g max. for 15 min at 4°C. The phenol phase was removed and $^1/_{10}$ vol. of 5M-NaCl was added to the interface material and aqueous layer, which were then extracted with an equal vol. of phenol-cresol. The phases were separated by centrifugation, as previously detailed, and the aqueous layer was then removed without disturbing the interphase material and re-extracted with an equal vol. of phenol-cresol. The final centrifugation was for 30 min. The nucleic acids were precipitated from the aqueous phase by the addition of 2 vol. of absolute ethanol and by overnight storage at -20°C. The precipitated nucleic acids were pelleted by centrifugation (as previously detailed); the supernatant was decanted off, and the residual pellet was dried under vacuo. The nucleic acids were then taken up in a small vol. of 0.15M-sodium acetate·acetic acid, 0.5% (w/v) SDS, pH 6.0 and precipitated by the addition of 2 vol. of absolute ethanol and by overnight storage at -20°C. The precipitated nucleic acids were pelleted by centrifugation and the pellet washed twice with 80% (v/v) ethanol, 10mM-NaCl and stored at -20°C under absolute ethanol.

Rat liver RNA was extracted from livers, obtained from freshly killed female rats (kindly supplied by the Department of Zoology, University of Durham), by the above technique.

RNA was also extracted from Brome Grass Virus by the above method. The virus was a generous gift from Dr. J.B. Bancroft (Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana, U.S.A.).

All solutions were normally kept at 4°C. The sodium salts, chloride, acetate and dodecyl sulphate were later replaced by the corresponding lithium salts. The advantage of this change was an increase in low temperature and ethanol solubility.

The nucleic acids prepared by this method were extremely stable during storage, appearing unchanged in electrophoretic mobility after 1 year at -20°C.

### (ii) Extraction of 65° RNA.

After extraction of the total nucleic acids outlined in section (i), the first two phenol phases, including the interphase material, were combined and an equal vol. of extractant, 0.3% (w/v) SDS, 0.14M-NaCl, 50mM-sodium acetate•acetic acid, pH 5.0 (Floyd et al., 1966), was added. The mixture was shaken for 1 min and then incubated at 65°C for 10 min in a water bath; this was followed by shaking at 20°C for a further 30 min. The phases were separated by centrifugation at 4,000 g max. for 20 min at 20°C. The aqueous layer was removed and re-extracted with an equal volume of phenol-cresol for 15 min at 20°C. The phases were separated, as described above, and the nucleic acids precipitated from the aqueous layer by the addition of 2 vol. absolute ethanol. The method was continued as described in section 4.(i).

### (iii) Extraction from the mitochondrial pellet.

The nucleic acids were extracted from the crude mitochondrial pellet (see section 7.(ii)) by a modification of the above method. The pellet was homogenised in an MSE homogeniser with 1 vol. pH 5.0 extractant for 1 min at 14,000 rev./min. An equal vol. phenol-cresol was added. The mixture was shaken for 1 min at 20°C; incubated at 65°C for 10 min and then shaken for a further 30 min at 20°C. The phases were separated by centrifugation at 16,000 g max. for 10 min at 4°C in the 8 x 50 ml rotor of the MSE High Speed 18. The aqueous phase was removed and re-extracted twice with phenol-cresol; the nucleic acids being precipitated from the aqueous phase as described in section 4.(i) above.

### (iv) Extraction from polysomes and supernatant fractions.

#### (a) Method I

This procedure was applied to the post-mitochondrial supernatant, polysome suspensions and the high speed supernatant fractions. The suspended polysomes (section 7.(ii)) or the supernatant fractions (section 7.(ii)) were made 1% (w/v) with respect to LLS, and an equal vol. of phenol-cresol added. The phases were mixed by shaking for 1 min and then separated by centrifugation at 4,000 g max. for 15 min at 0°C. The aqueous phase was re-extracted with an equal vol. of phenol-cresol, and the nucleic acids precipitated from the final aqueous phase as detailed in section 4.(i). All the operations were at 0°C.

(b) Method II

This procedure was a modification of the method described by Oda and Joklick (1967); it was used on DEP-extracted microsome and polysome suspensions. The suspensions were made 1% (w/v) with respect to LLS and 0.2M with respect to LiCl; shaken for 1 min at room temperature and then precipitated by the addition of 2 vol. absolute ethanol and by overnight storage at -20°C. The precipitate was pelleted at 4,000 g max. for 15 min at 4°C. The pellet was resuspended in a minimum vol. of 1 ml (1 ml/5 mg particles) 50mM-sodium acetate•acetic acetic, 10mM-$Na_2$EDTA, pH 5.1, and made 1% (w/v) with respect to SDS and 0.5M with respect to sodium perchlorate. An equal vol. of chloroform-isoamyl alcohol (24:1) was added, and the mixture was shaken for 1 min. The mixture was then chilled for 15 min on ice. The phases were separated by centrifugation at 4,000 g max for 15 min at 4°C. The organic phase was removed, and the aqueous phase was re-extracted with chloroform-isoamyl alcohol. After the second extraction the aqueous phase was removed and made 0.2M with respect to NaCl. The nucleic acids were precipitated by the addition of 2 vol. of absolute ethanol and overnight storage at -20°C.

(v) Extraction from nuclei.

The nuclei prepared as described (section 7.(i)) were extracted by the method outlined in section 4.(i).

(vi) Extraction for the subsequent isolation of low molecular weight RNA.

To obtain milligram quantities of LMW RNA species, large amounts of tissue were necessary. The tissues used in the extraction procedures outlined below were either etiolated stems and leaves of 14 day germinated seeds, 2-day germinated seeds, or 50 to 60-day old developing cotyledons of Vicia faba (see methods I.(i) and (ii),(a)). The extraction procedures described are listed in their order of development and use.

### (a) Method I

Approximately 1 kg fresh weight of tissue was blended in a Townson and Mercer top drive macerator for 2 min with an equal vol. of redistilled 90% (v/v) phenol and extractant, 0.1M-Tris•HCl, 24mM-KCl, 3mM•$MgCl_2$, pH 7.5, in the ratio of 2 vol. phenol to 1 vol. extractant. The homogenate was stirred in an ice bath for 1 h. The aqueous phase was separated by centrifugation at 2100 g max. for 15 min at 4°. The aqueous phase was removed and the nucleic acids were precipitated by the addition of 2 vol. absolute ethanol and by overnight storage at -20°C.

### (b) Method II

The procedure was a modification of Method I. The extractant was supplemented with 1% (w/v) disodium naphthalene-1,5-disulphonate, and used in a 1:1 ratio with the 90% (v/v) phenol. After the initial phase separation, the aqueous phase was removed and $^1/_{10}$ vol. of 5M-NaCl added, followed by an equal vol. of 90% (v/v) phenol. The phases were separated by centrifugation at 2100 g max. for 30 min at 4°C and the nucleic acids were precipitated from the resulting aqueous phase as described in Method I.

### (c) Method III

The procedure was a modification of Method II. The phenol was water saturated and the extractant of Method I was supplemented with 1% (w/v) TNS, 6% (w/v) sodium 4-aminosalicylate and used in the ratio of 1:2 with water saturated phenol. Stirring the homogenate in an ice bath for 1 h (Methods I and II) was omitted, and the homogenate was treated as described in Method II.

(d) Treatment of RNA obtained by Methods I to III.

The precipitated RNA was pelleted by centrifugation at 2,100 g max. for 15 min at 4°C. The pellets were dried under vacuo and then dissolved in 0.15M-lithium acetate•acetic acid, 0.5% (w/v) LLS, pH 6.0. The insoluble material (starch?) was pelleted by centrifugation at 2,100 g max. for 30 min at 4°C and discarded. The RNA was precipitated from the supernatant by the addition of 2 vol. absolute ethanol and by overnight storage at -20°C.

## 5. The fractionation and purification of low molecular weight RNA

DEAE-cellulose and salt precipitation were used to separate the high molecular weight RNA's from the low molecular weight RNA's. The latter were purified by chromatography on Sephadex G100 and G75.

All column parameters are given in the legends to the figures.

(i) Preparation of DEAE-cellulose.

DEAE-cellulose (DE52) was equilibrated in excess 50mM-Tris•HCl, pH 7.5. The pH was readjusted to 7.5 with HCl, and the resin allowed to settle. The fines were removed, and the resin washed twice in excess fresh buffer. The resin was poured as a thick slurry into the column; allowed to settle, and then packed under hydrostatic pressure. The column was washed with buffer prior to sample loading.

(ii) Preparation of Sephadex G100 and G75.

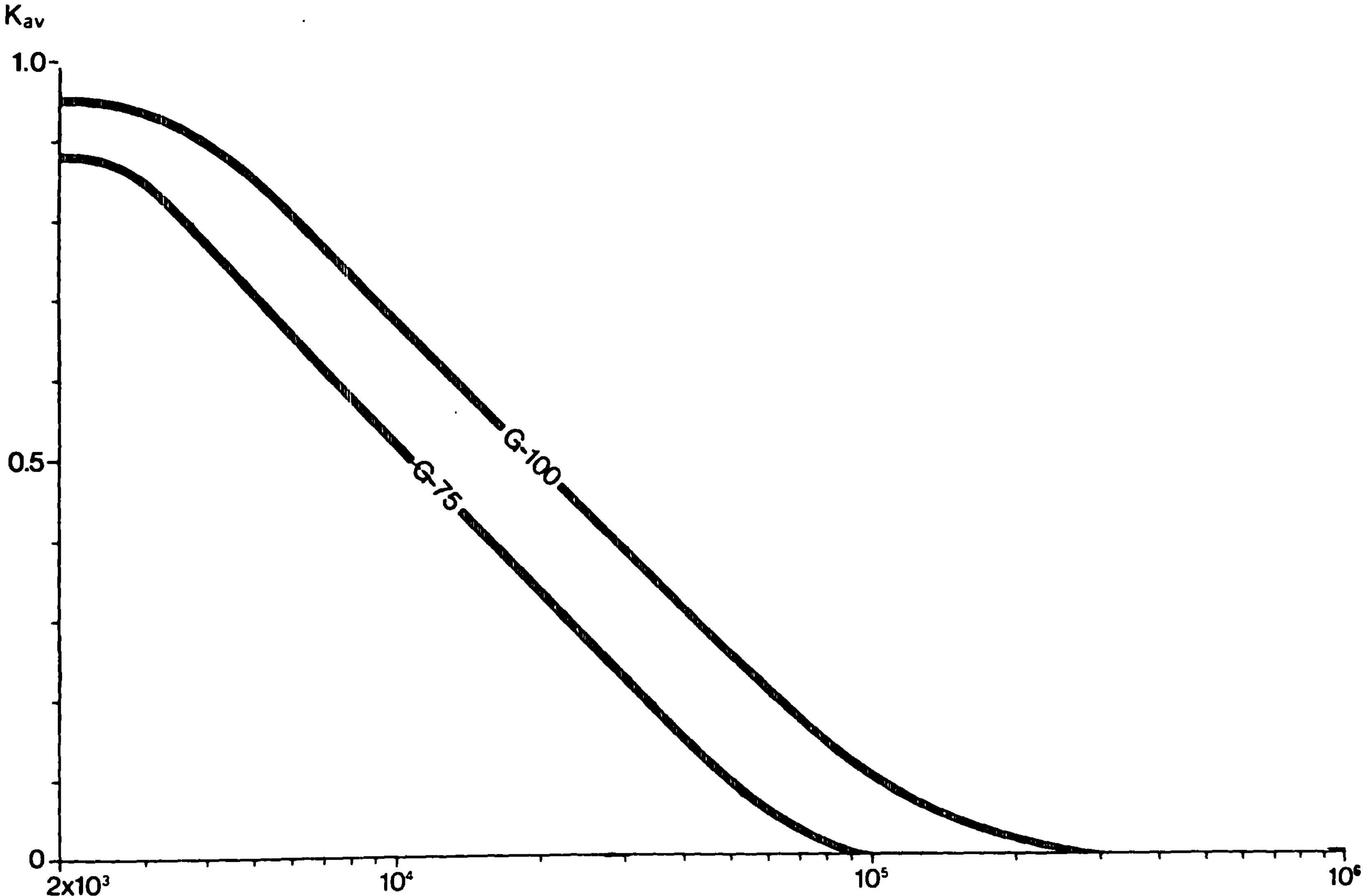
The resins were swollen in 50mM-sodium acetate•acetic acid or 50mM-ammonium acetate•acetic acid, pH 5.1 at room temperature. The swollen resins were washed with excess fresh buffer and the fines removed.

(iii) Preparation and monitoring of columns.

All columns were poured at room temperature and allowed to settle

Fig. 2.

Relationship between the partition coefficient ($K_{av}$) and molecular weight for G75 and G100 columns; taken from "Gel Filtration in Theory and Practice" published by Pharmacia Ltd., Uppsala, Sweden.



$K_{av}$
1.0
0.5
0
G-100
G-75
$2 \times 10^3$
$10^4$
$10^5$
$10^6$
Molecular weight

under gravity. The columns were equilibrated at 4°C, and packed under hydrostatic pressure.

The column eluant was continuously monitored at 258nm, using an Isco optical unit (Model UA) and analyser system connected to a Servoscribe potentiometric recorder. Fractions were collected using an LKB fraction collector, and their extinction of 260nm measured in an SP.800 spectrophotometer.

### (iv) Molecular weight determinations.

The approximate molecular weights of fractions eluted off Sephadex columns were calculated from the correlation between the partition coefficient (Kav) and molecular weight (Fig. 2).

The partition coefficient was determined from the equation:

$$\mathrm{Kav} = \mathrm{Ve} - \mathrm{Vo}/_{\mathrm{V_t}} - \mathrm{Vo}$$

where Vo = void volume, Ve = elution volume and $\mathrm{V_t}$ = total volume of the gel bed.

### (v) Salt fractionation.

#### (a) 1.0M-LiCl

The RNA sample was dissolved in 50mM-Tris•HCl, pH 7.5 at 4°C and $\frac{1}{4}$ vol. 5M-LiCl added. The sample was left overnight at 4°C and the resulting precipitate pelleted by centrifugation at 2,750 g max. for 15 min at 4°C. The pellet was resuspended in 2 vol. 1.0M-LiCl, 50mM-Tris•HCl, pH 7.5 and repelleted by centrifugation. The washed pellet was dissolved in 50mM-Tris•HCl, pH 7.5 and the nucleic acids precipitated by the addition of 2 vol. absolute ethanol. The supernatants were treated as described in section 5(vi)

#### (b) 0.6M-LiCl

The RNA was suspended in 0.6M-LiCl, 50mM-Tris•HCl, pH 7.5 at 4°C. Any insoluble material was pelleted by centrifugation at 2,750 g max.

for 15 min at 4°C. The resulting supernatant was centrifuged at 115,000 g av. for 4.5 h at 2°C in the 10 x 10 ml titanium rotor of the MSE Super Speed 65. The resulting pellets were discarded and the supernatant was treated as described in section 5.(vi) below.

(vi) Recovery of RNA from supernatant fractions.

If the $E^{260}_{1.0cm}$ had a value greater than 1.0, the RNA was precipitated directly from solution by the addition of 2 vol. absolute ethanol and by overnight storage at -20°C. If the $E^{260}_{1.0cm}$ was less than 1.0, then the solution was concentrated by lyphogel or the sample was absorbed onto a 1 x 2 cm DEAE-cellulose column and eluted with 1M-LiCl. Once concentrated, the RNA was collected by ethanol precipitation.

## 6. Methods for the separation and characterization of nucleic acids.

### (i) Spectrophotometric estimation of DNA, RNA and ribosome concentration.

The concentration was determined from the $E^{260}_{1cm}$ with a distilled water blank, using a SP.800 spectrophotometer (Unicam Instruments, Ltd.).

The following extinction coefficients were used:

DNA : $E^{260}_{1\%} = 210$ (Calbiochem Data).

RNA : $E^{260}_{1\%} = 240$ (Yarwood, 1968).

Ribosomes : $E^{260}_{1\%} = 113$ (Tso and Vinograd, 1961).

Sample purity was determined from $^{260}/_{230}$ and $^{260}/_{280}$ ratios.

### (ii) Polyacrylamide gel electrophoresis.

(a) Method

Acrylamide and bis-acrylamide were recrystallised from chloroform and acetone respectively, and stored as stock solutions at 4°C (Loening, 1967). All reference to gel concentration is made with respect to the acrylamide concentration only. The gel mixtures were prepared and polymerised according to Loening (1968). For gels greater than 5% (w/v) acrylamide, the solution was cooled to 0°C prior to degassing and polymerisation, so as to increase the polymerisation time and prevent geling during the transfer of the solution into the gel tubes. The gels were prepared in 10 cm tubes, with an internal diameter of 0.65 cm, and were supported by 3 mm tygon tubing inserts, which were supplemented with millipore filter disks for gels of concentration less than 2.4% (w/v) acrylamide. The electrophoresis buffer (36mM-Tris, 30mM-$NaH_2PO_4$, ImM-$Na_2EDTA$, pH 7.7 to 7.8 at 20°C) was prepared as 5 times and 10 times concentrated stock solutions. The former was used for gel preparation, and the latter after dilution was made 0.2% (w/v) with respect to SDS and used as the electrophoresis buffer. Gels were pre-electrophoresed at 5mA/tube for 1 h, prior to sample loading, to remove the excess ammonium persulphate. The nucleic acid sample was dissolved in sterile electrophoresis buffer, not containing SDS, plus 5% (w/v) sucrose (autoclaved 0.7 Kg/$cm^2$ for 15 min) to a final concentration not exceeding 2 mg/ml. Normally, 20 µg of nucleic acids were loaded/gel and electrophoresed at 5mA/tube for 3 h (exceptions are noted in figure legends). Following electrophoresis, gels were blown from the tubes with a syringe and soaked for 1 h. Gels with a concentration less 5% (w/v) acrylamide were soaked in electrophoresis buffer. Gels with a concentration greater than 5% (w/v) acrylamide were soaked in 40% (v/v) ethoxyethanol to prevent swelling. The gels were scanned at 265 nm in a Joyce Loebel chromoscan, fitted with a 265 nm interference filter and a liquid filter (15 mg p-dimethylamino benzaldehyde/100 ml methanol). The slit width was 36 µm and the focussing lens was adjusted to give maximum resolution. A linear expansion factor of 3 was used for all gels. The vertical expansion was varied as required using the cam system of the chromoscan.

Areas under peaks were determined using the Integrator/calculator, Model UAG (Technicon Ltd.).

### (b) Gel slicing and counting of $\left[^{32}P\right]$-RNA

After scanning the gel at 265 nm, it was placed in an aluminium foil trough, with the top of the gel against a cork stopper. A second cork was placed against the bottom of the gel, and the distance between the two cork stoppers adjusted, so as to correspond to the length of the gel as scanned. The trough was then placed on powdered solid $CO_2$, and the gel allowed to freeze (Loening, 1967). When frozen the gel was sliced into 1 mm thick sections with a gel slicer (Mickle Laboratory Engineering Company, Gomshall, Surrey). The gel slices were dried on to filter paper strips (LKB type 3276) using an infra-red lamp. The radioactivity present in the gel slices was then determined by counting each gel slice, plus approximately 1 $cm^2$ filter paper in 10 ml of toluene scintillation fluid (4.5 g 2,5-diphenyloxazole and 0.1 g 1,4-di$\left[2\text{-(5-phenyloxazolyl)}\right]$ benzene/l toluene) in a Beckman liquid scintillation counter, model LS-200B. Background was automatically subtracted, and no corrections for either decay or for quenching were attempted with this isotope. Samples were counted for 2 min, twice, and the counts averaged.

### (c) Molecular weight and sedimentation coefficient determinations.

Molecular weights were determined from a logarithmic plot of molecular weight against mobility using standard markers. Sedimentation coefficients were determined from a linear plot against mobility.

The molecular weights and sedimentation coefficients of the standards used are given in Table 2. The molecular weights quoted in this thesis were determined from a minimum of five determinations and are subject to a variation not exceeding ± 0.02 daltons, except where stated.

### (iii) Sucrose density gradient centrifugation.

Gradients were prepared using the apparatus described by Henderson (1969). The mixing chamber had an internal height of 6.0 cm and an

## TABLE 2

RNA Standards.

| Species | MW and sedimentation coefficients of RNA | References |
|---|---|---|
| *Vicia faba* | 1.29 and 0.70 x $10^6$<br>(25S) (18S) | This thesis. |
| Rat liver | 1.75 and 0.70 x $10^6$<br>(28S) (16S) | Loening (1968) |
| *Escherichia coli* | 1.08 and 0.56 x $10^6$<br>(23S) (16S) | Loening (1968) |
| Bromegrass virus | 1.07, 0.76 and 0.33 x $10^6$<br>(26.8S)(22.3S) (14S) | Loening (1969)<br>Bockstahler and Kaesberg (1965) |
| Plant '5S' RNA | 0.379 x $10^5$<br>(4.48S) | Payne and Dyer (1971)<br>Soave *et al*. (1970) |
| Plant '4S' RNA | 0.250 x $10^5$<br>(3.95S) | Boedtker and Kelling (1967) |

internal diameter of 2.70 cm. The apparatus was used to produce convex gradients. The gradients were prepared in 23 ml polycarbonate centrifuge tubes at 4°C, and centrifuged in the 3 x 23 ml rotor in the MSE Super Speed 65 at 4°C. The gradients were fractionated at 0.5 ml/min with 50% (w/v) sucrose, 0.5% (w/v) sodium benzoate, using an Isco density gradient fractionator, Model 180, fitted with a tube piercing device (Fig. 3). The gradient $E^{258}_{0.5cm}$ was monitored with an Isco optical unit, Model UA, attached directly to the top of the centrifuge tube by a flow cell adaptor unit; and recorded on a Servoscribe potentiometric recorder, Model RE.511.20, at a chart speed of 12 cm/h.

The sucrose, used in gradient preparation, was of Analar grade and sterilized at 0.7 $Kg/cm^2$ for 15 min in an autoclave; or was RNase free (Mann Research Laboratories, New York).

Sucrose volumes, slope of gradient, centrifugation parameters and buffers are given in the legends to the figures.

## 7. The isolation of sub-cellular components

### (i) Nuclei.

Seeds of *Vicia faba* were germinated for 5 days, as described (section 1.(ii),(a)). The root systems were excised and washed in distilled water. They were then sterilized by immersion in 10% (w/v) calcium hypochlorite for 30 s, and then washed in distilled water. 100 g of roots were homogenised at 4°C in a Townson and Mercer top drive macerator for 1 min with 500 ml Keuhl's medium:20mM-Tris·acetic acid, 0.1M-sucrose, 2mM-$CaCl_2$, 4% (w/v) gum acacia, 4mM-n-octanol, pH 7.6 (Keuhl, 1964). The homogenate was filtered through 4 layers of muslin and then centrifuged at 850 x g max. for 15 min at 4°C. The pellet was suspended in 20 ml Keuhl's medium and filtered through silk with an average pore diameter of approximately 80 um. The filtrate was centrifuged at 850 g max. for 15 min at 4°C. The resulting pellet was suspended in 18 ml Keuhl's medium and made 1% (v/v) with respect to triton x-100,

Fig. 3.

Tube piercing device for the Isco density gradient fractionator (Model 180).

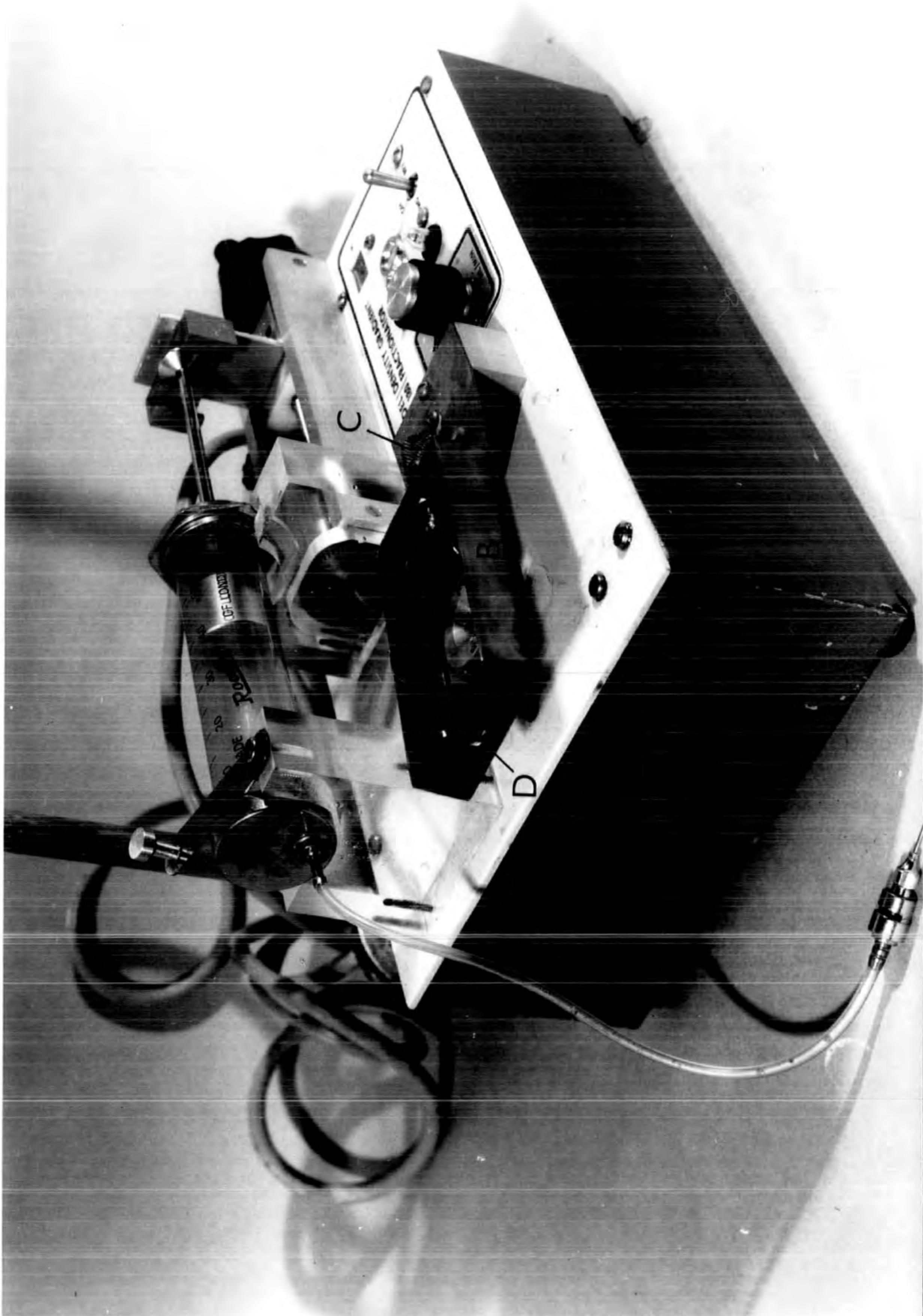
A : Fixed plate.

B : Movable puncturing assembly.

C : Thumb screw; positioned by means of a nut welded on to the puncturing assembly.

D : Hole to position luer lock adaptor.

The apparatus is set up as described in the manual with the tube to be fractionated in position. The luer lock adaptor, attached to the hypodermic needle, is positioned so that the tip of the needle is resting on the tube seal inside the guide hole and the luer lock adaptor is positioned in hole D. The pump is switched on (0.5 ml/min) in order to remove the entrapped air in the guide hole. With the pump still on, the thumb screw (C) is turned against the fixed plate (A), so pulling the puncturing assembly towards it, and causing the needle to pierce the tube.


C
B
D

by the addition of 2 ml 10% (v/v) triton x-100, 50mM-Tris•HCl, pH 7.5. The suspension was kept at 0°C for 5 min and then centrifuged at 250 g max for 10 min at 4°C (D'Alessio and Trim, 1968). The nucleic acids were extracted from the resulting pellet according to the method described (section 4.(v)).

Samples were removed at each stage during the preparation and visually monitored for the presence and appearance of nuclei by staining with either saffranin, methylene blue or acetocarmine. Phase contrast and electron micrographs were taken of some of the preparations.

Further purification of the nuclei by the methods of Dick (1968) and D'Alessio and Trim (1968) was attempted.

The isolation of nuclei from imbibed cotyledons of Vicia faba by sucrose-calcium (Johri and Varner, 1968) and citric acid (Douce, 1955) techniques was attempted.

## (ii) Ribosomes and Polysomes.

The tissue was homogenised with a mortar and pestle at 4°C in 3 vol. of 50mM-Tris•HCl, 0.5M-sucrose, 16mM-KCl, 5mM-$MgCl_2$, pH 7.5; containing sodium bentonite to a final concentration of 1 mg/ml. Sodium bentonite was omitted if DEP was added. DEP was added to a final concentration of 1% (v/v), and the Tris concentration was adjusted to 0.1M by the addition of $^1/_{39}$ vol. 2M-untitrated Tris (Weeks and Marcus, 1969). The brei was filtered through silk, having a pore diameter of approximately 80 um and the filtrate was centrifuged at 38,000 g max for 15 min at 4°C in the 8 x 50 ml rotor of the MSE High Speed 18. For the isolation of microsomes, the 38,000 g supernatant was recentrifuged at 105,000 g av. for 90 min at 2°C in the 10 x 10 ml titanium rotor of the MSE Super Speed 65. For the isolation of ribosomes the 38,000 g supernatant, was made 4% (v/v) with respect to triton x-100 by the addition of 0.4 vol. 10% (v/v) triton x-100, 50mM-Tris•HCl, 16mM-KCl, 5mM-$MgCl_2$, pH 7.5, or 0.2% (w/v) with respect to sodium deoxycholate, and then centrifuged at 105,000 g av. for 90 min at 2°C. The resulting supernatant was carefully decanted and the centrifuge tube walls were dried with absorbant paper. The pellets were then washed once with resuspending medium, (10mM-Tris·HCl, 25mM-KCl, 1mM-$MgCl_2$, pH 7.6) and finally suspended in a small volume of resuspending medium. 10μl was taken to estimate the particle concentration; the remaining particles were either (i) stored at -70°C, (ii) immediately analysed on sucrose gradients, or (iii) the RNA extracted as described (4.(iv),(a) and (b)).

Prior to the analysis of microsomes on sucrose gradients, the suspension was adjusted to 4% (v/v) triton x-100, by the addition of 0.4 vol. 10% (v/v) triton x-100 in resuspending medium.

The nucleic acids were extracted from the 38,000 g max. pellet, namely, the mitochondrial pellet; the 38,000 g max. supernatant, namely, the post-mitochondrial supernatant and the 105,000 g av. supernatant as described in the methods (section 4.(iv),(a)).

## 8. Preparation of ribonuclease from developing beans

5g of 40-day old developing beans were homogenised in 5 ml, 50mM-Tris·acetic acid, 2mM-magnesium acetate, pH 7.4 at 0°C. The homogenate was centrifuged at 2,750 g max. for 30 min at 4°C. The supernatant was kept at 0°C prior to use.

## 9. Enzyme incubation systems

Commercial enzymes were dissolved in 50mM-Tris·acetic acid, 2mM-magnesium acetate, pH 7.4, to a concentration of 0.5 mg/ml and stored frozen in 0.1 ml aliquots at -20°C. The *Vicia faba* RNase was freshly prepared before use (section 8).

2 to 3 aliquots of DEP were transferred from the storage container in Quickfit stoppered test tubes in a dry $N_2$ atmosphere, in order to exclude water vapour.

The enzymes and DEP were kept at 0°C prior to use.

(i) Incubation system I: products analysed by polyacrylamide gel electrophoresis.

The nucleic acids extracted from 38-day old developing cotyledons (section 4.(i)), were dissolved in a small volume of 50mM-Tris•acetic acid, 2mM-magnesium acetate, pH 7.4 and adjusted to a concentration of 2 mg/ml. 0.5 ml aliquots of substrate were then transferred to 12 ml Pyrex tubes (Catalogue No. 1680/02) and 50 $\mu$l enzyme, DEP or LLS added. All incubations had a final volume of 0.6 ml. The mixture was incubated for 30 min at 0°C. The reaction was stopped by the addition of 2 vol. absolute ethanol, and the nucleic acids were allowed to precipitate at -20°C overnight. The precipitate was pelleted by centrifugation at 2,100 g max. for 15 min at 4°C and the pellets were dried under vacuo. The dry pellets were dissolved in 2 ml Kirby's buffer, and extracted with an equal vol. phenol-cresol. The phases were separated at 2,100 g max. for 30 min at 4°C, and the nucleic acids were precipitated from the aqueous phase by the addition of 2 vol. absolute ethanol and by overnight storage at -20°C. Before analysis on acrylamide gels, the nucleic acids were reprecipitated from 0.15-M lithium acetate, 0.5% (w/v) LLS, pH 6.0, and washed twice with 80% (v/v) ethanol, 0.1-M LiCl.

(ii) Incubation system II: quantitative determination of enzyme activity.

This system was designed primarily for use with DNase I, but was also used as a RNase assay. The system was based on Incubation System I, and the methods of Fedrocsak and Ehrenberg (1960) and Uchida and Egami (1967). The DNA was prepared as a 2 mg/ml solution ($E^{260}_{1.0cm}$ = 1.38 mg/ml) by weight in 50mM-Tris•acetic acid, 2mM-magnesium acetate, pH 7.4 The RNA, prepared as described (section 4.(vi),(a)) and fractionated with 1M-LiCl to remove the LMW RNA (section 5.(v),(a)), was dissolved in

Tris-magnesium-acetate buffer and adjusted to 4 mg/ml.

Incubation mixture, total vol. 1 ml containing:

0.5 ml substrate, 1 mg DNA or 2 mg RNA
0.5 - (x + y) ml Tris-magnesium-acetate buffer
(x) ml enzyme
(y) ml DEP.

The substrate and buffer were equilibrated at 25°C prior to use. Buffer 0.5 ml was added to each tube (Pyrex, Catalogue No. 1680/02), and an amount equal in volume to the enzyme and DEP to be added was removed. The substrate, enzyme and DEP were then added in the required order and incubated for 15 min at 25°C. The DEP was added directly or as a 10% (v/v) solution in ethanol. The reaction was stopped by the addition of 0.25 ml uranyl reagent (25% (v/v) perchloric acid, 0.75% (w/v) uranyl acetate). The resulting precipitate was pelleted by centrifugation for 10 min in a Piccalo bench centrifuge. The supernatant was transferred to a clean tube (12 x 100 mm) and recentrifuged for a further 2 min. 0.2 ml of the supernatant was added to 3 ml distilled water, and the $E^{260}_{1.0cm}$ determined against a distilled water blank, using a Uvispeck H 700 spectrophotometer.

Mixing of the incubation components was achieved using a whirlimixer. All incubations were duplicated and the $E^{260}_{1.0cm}$ averaged.

### 10. The incorporation of $[^{32}P]$-orthophosphate into roots of *Vicia faba*

The $[^{32}P]$-orthophosphate, pH 2-3, was adjusted to between pH 5.5 - 7.0 with 10mM-NaOH.

(i) Excised root tips.

The beans were germinated as described (section 1.(ii),(a)), and the terminal 3-4 mm root segment excised into sterile 2% (w/v) sucrose. The root tips were washed twice with sterile 2% (w/v) sucrose, and then transferred to 2.5 ml sterile 2% (w/v) sucrose containing 100 µCi/ml $[^{32}P]$-orthophosphate. Approximately 100 root tips were used in each incubation. Incubation periods of 15, 30, 60 and 120 min were carried out at 25°C in a shaker water bath (Rogers et al., 1970). At the end of each incubation, the root tips were drained; washed twice with 2% (w/v) sucrose, 50mM-$Na_3PO_4$, pH 7.0, and the RNA extracted as described (section 4.(i)).

(ii) Intact roots.

The beans were grown as described (section 1.(ii),(b)) and following harvesting were washed with sterile distilled water. The cotyledons were sliced in a plane perpendicular to the junction of the two cotyledons, leaving intact a small part of each cotyledon attached to the embryo by the cotyledon petiole. 60 embryos were incubated by clipping their roots into 43 ml distilled water containing 58 µCi/ml $[^{32}P]$-orthophosphate (Rogers et al., 1970). The incubation period was 30 min, followed by a 15 min chase in sterile distilled water. Following the incubation and chase periods, the terminal 3-5 mm segment of the root was excised on ethanol sterilized aluminium foil at 0°C, and the RNA extracted as described (section 4.(i)).

## 11. The incorporation of $^{32}P$ -orthophosphate into the cotyledons of Vicia faba

(i) In vitro.

Following the removal of the testa and radical, the cotyledons were sliced into 1-2 mm slices in a plane perpendicular to the junction of the cotyledons. The slices were weighed and then washed with sterile culture medium. 1 g of cotyledon slices were incubated in 10 ml sterile culture medium, containing 100 µCi/ml of $[^{32}P]$-orthophosphate (neutralized with 10 mM-NaOH), at 25°C in a shaker water bath. The incubation period was 30 min, followed by a 15 min chase in cold culture medium. Following the incubation, the samples were drained and washed twice with cold culture medium, and the RNA extracted as described (section 4.(i)).

The culture media were:

(i) 2% (w/v) sucrose

(ii) a modification of Hildebrant's (1962) sunflower medium; from which the $Na_2HPO_4$ was omitted and the glycine was replaced by an equimolar mixture of the 20 protein amino acids (Bailey et al., 1970).

(ii) In vivo.

$[^{32}P]$-orthophosphate was dried under vacuo and dissolved in distilled water to an activity of 5mCi/ml.

(a) For the subsequent extraction of total nucleic acids.

The selected pod was surface sterilized with absolute ethanol. A hypodermic needle was inserted into the pod airspace. 0.2 ml (1mCi) $[^{32}P]$-orthophosphate was then injected into the pod airspace. Both hypodermic needles were removed, and the area sterilized with ethanol and sealed with silicone grease.

Three injections per pod were given at 12 h intervals. The pod was harvested 12 h after the last injection; and the nucleic acids of the testa and cotyledon extracted as described (section 4.(i)).

(b) For the subsequent isolation of cotyledon polysomes.

Injections of 1mCi $[^{32}P]$-orthophosphate, 0.2 ml, were administered at 24 h intervals for 2 days and then at 12 h intervals for 1 day. 4 injections totalling 4 mC were administered. The pod was harvested 12 h following the last injection. The testa was removed and the polysomes were extracted from the cotyledon as described (section 7.(ii)).

# RESULTS

## 1. Temperature characteristics of SDS and LLS

The temperature characteristics of the 10% (w/v) LLS prepared, as described in the methods (section 2), were compared with those of a 10% (w/v) solution of SDS, by cooling in an ice-salt bath. The SDS began to precipitate from solution at 12°C whereas the LLS did not precipitate until the temperature was -5.5°C. The precipitation was immediate causing a temperature rise of 5.3°C. A mixed solution, containing 1 vol. 10% (w/v) SDS and 1 vol. of LLS solution could be cooled to -2.0°C before precipitation occurred. The precipitation was followed by a temperature rise of 1.8°C.

## 2. The action of DEP on DNase and RNase

### (i) Incubation system I.

Analysis of the nucleic acids from 38-day old developing cotyledons of *V. faba* showed that they contained three major components on 2.6% acrylamide gels: DNA, 25S RNA and 18S RNA. When these nucleic acids were incubated for 30 min at 0°C, no breakdown was observed, indicating the absence of endogenous nuclease activity (Fig. 4a). The addition of electrophoretically purified DNase I (Sigma Chemicals) resulted in the complete removal of the DNA (Fig. 5). The addition of DNase I (Koch-Light) resulted in the complete removal of DNA and RNA (Fig. 6) showing that the DNase possessed a contaminating component with RNase activity. The crude nuclease extract from developing cotyledons of *V. faba* contained RNase activity, but no detectable DNase activity (Fig. 7a). The addition of LLS to a final concentration of 0.8% (w/v) completely inhibited both DNase and RNase activity since the gel traces obtained were all essentially the same as the control (see Fig. 4a). The addition of DEP to a final concentration of 8% (v/v) had no apparent effect on the DNase activity of either the pure Sigma DNase or the impure Koch-Light DNase, and traces

Fig. 4.

38-day old cotyledon nucleic acids. The nucleic acids were incubated for 30 min at 0°C as described in the methods, section 9(i). The RNA species are notated by their sedimentation coefficient

(a) Electrophoresed in a 2.6% gel for 3h. Vertical expansion: cam A.

(b) Electrophoresed in a 7.5% gel for 3h. Vertical expansion: cam C.

Fig. 5.

38-day old cotyledon nucleic acids incubated with DNase I (Sigma Chemicals), as described in the methods, section 9(i). Electrophoresed on a 2.6% gel for 3h. Vertical expansion: cam A.

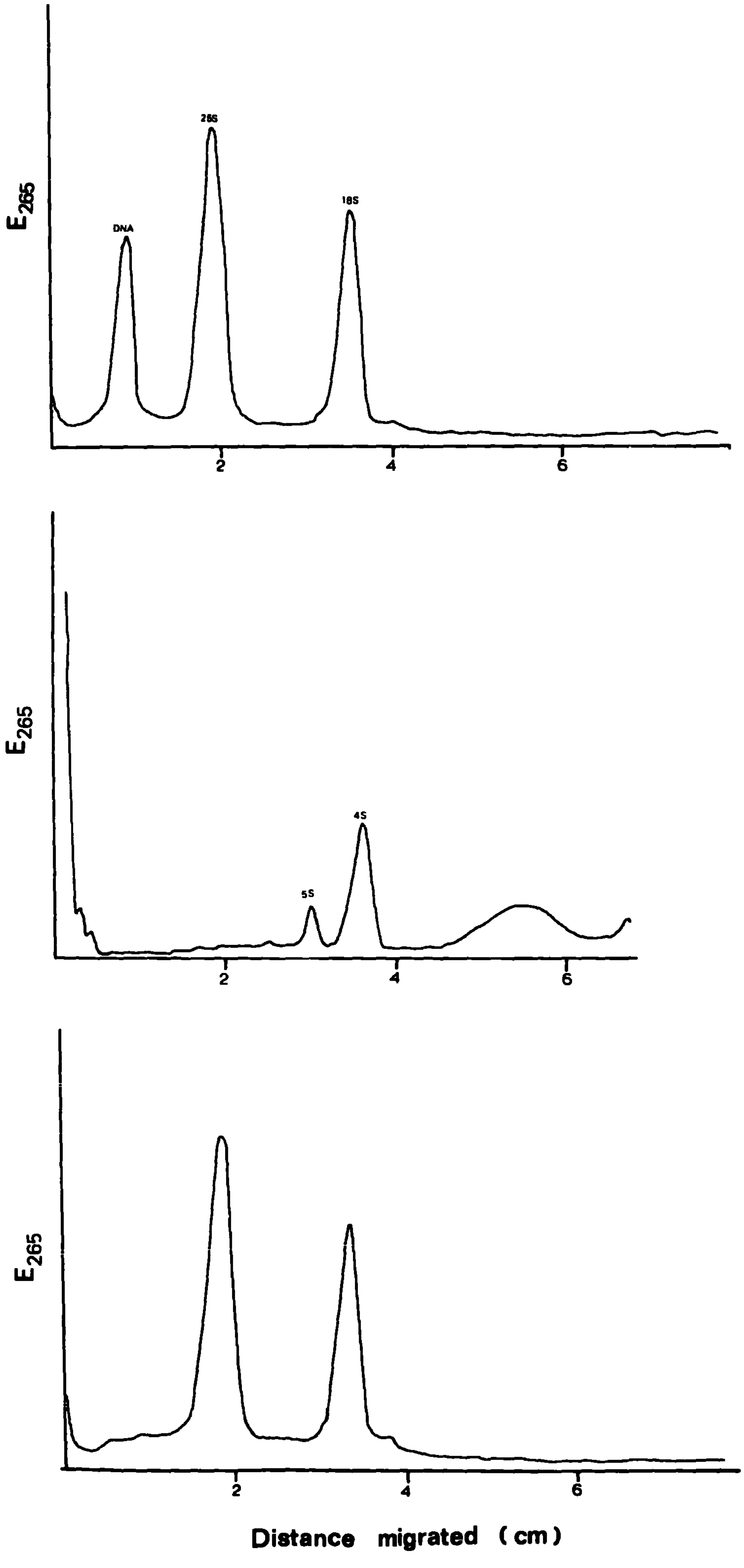

E$_{265}$
DNA
28S
18S
2
4
6
E$_{265}$
5S
4S
2
4
6
E$_{265}$
2
4
6
Distance migrated (cm)

Fig. 6.

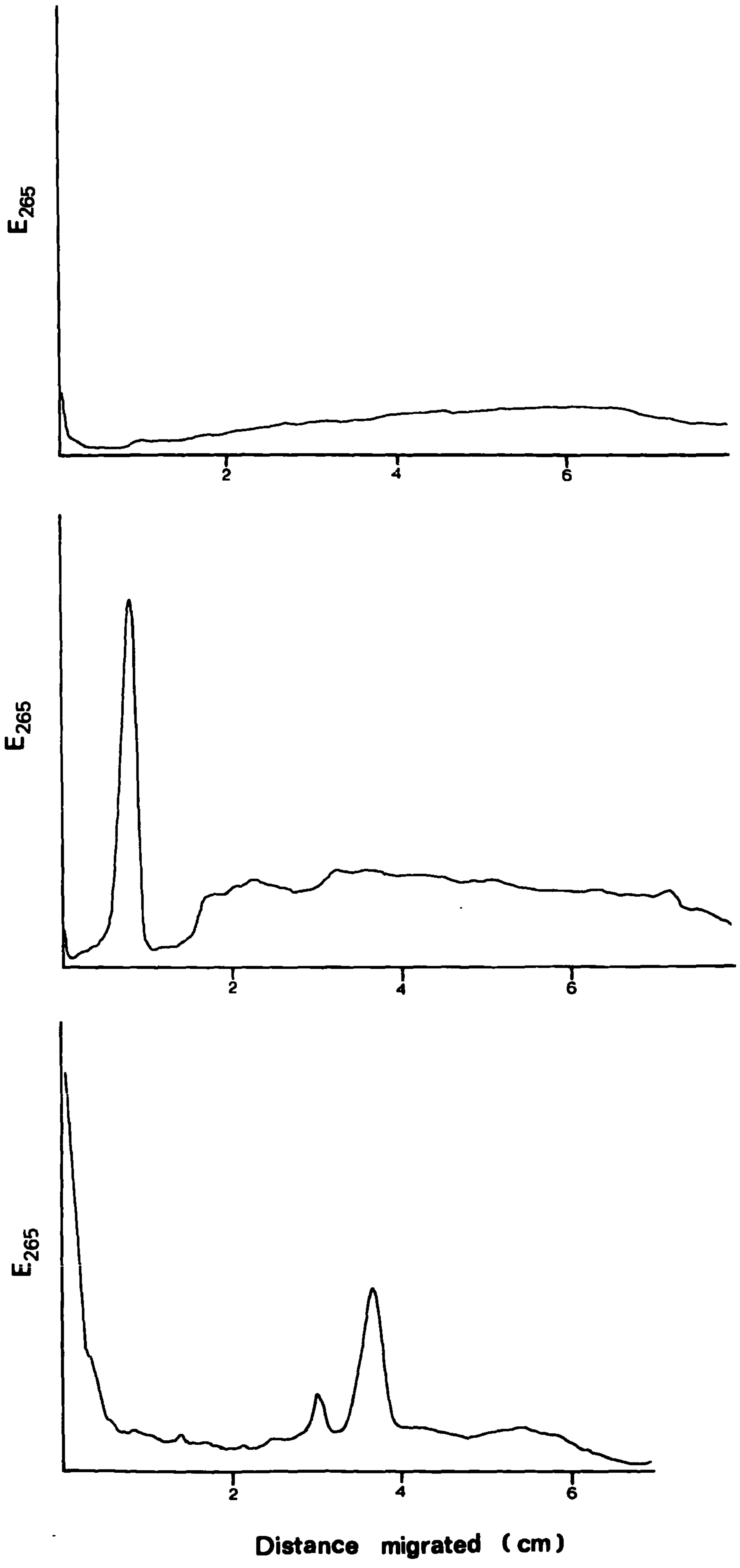
38-day old cotyledon nucleic acids incubated with DNase I (Koch-Light), as described in the methods, section 9(i). Electrophoresed on a 2.6% gel for 3h. Vertical expansion: cam A.

Fig. 7.

38-day old cotyledon nucleic acids incubated with _V. faba_ RNase, as described in the methods, section 9(i).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion: cam C.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion: cam C.


$E_{265}$
2
4
6
$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated (cm)

similar to Fig. 5 were obtained. However, the RNase present in the Koch-Light DNase must have been inhibited by the DEP, as no apparent degradation of the RNA was observed. Incubation of V. faba RNase with DEP resulted in the inhibition of RNase activity since a trace was obtained which was similar to the control (see Fig. 4a).

It was observed that the 5S and 4S RNA species were apparently not affected by a RNase concentration which caused the complete degradation of the 25S and 18S RNA's (compare Figs. 4a and 4b with 7a and 7b).

(ii) Incubation system II.

(a) Effect of DEP on DNase I

Preliminary experiments established linear relationships for the release of acid soluble digestion products against enzyme concentration and time (Figs. 8 and 9). From these results an enzyme concentration of 0.5 µg/ml and an incubation time of 15 min were selected to study the effect of DEP concentration on the initial rate of the reaction. A 2% (v/v) concentration of DEP completely inhibited DNase activity, and no significant different was observed when DEP was added as a 10% (v/v) solution in ethanol (Fig. 10). A two-phase incubation system was obtained when the DEP concentration was greater than 0.5% (v/v); at this concentration the observed inhibition of DNase activity was approximately 90%. Increasing the DEP concentration to 5% (v/v) and varying the enzyme concentration, showed that as the enzyme concentration was increased, the inhibition deceased (Fig. 11). Preincubating DNase with DEP showed a rapid inactivation of enzyme activity (Fig. 12).

(b) Effect of DEP on RNase

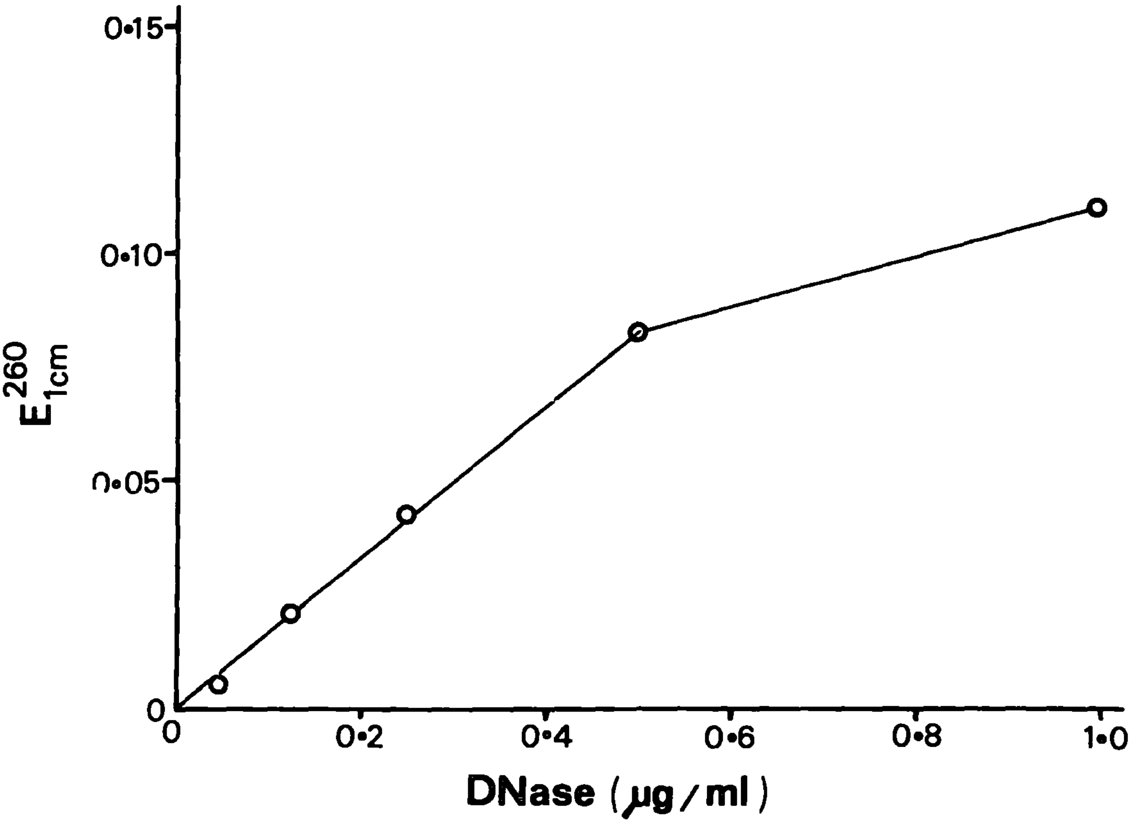
Previous experiments using incubation system I have demonstrated that RNase is inhibited by DEP, though the enzyme concentration in these experiments was not determined. If it is assumed that the observed effects of DEP on low concentrations of DNase are similar to the effects

Fig. 8.

The release of acid soluble digestion products with increasing enzyme concentration. Incubations were, carried out as detailed in the methods (section 9(ii)), at 25°C for 15 min.

Fig. 9.

The release of acid soluble digestion products with time. Incubations were, carried out as detailed in the methods (section 9(ii)), at 25°C and contained 0.5 µg/ml DNase I.


0•15
0•10
0•05
0
$E_{1cm}^{260}$
0
0•2
0•4
0•6
0•8
1•0
DNase (µg / ml)

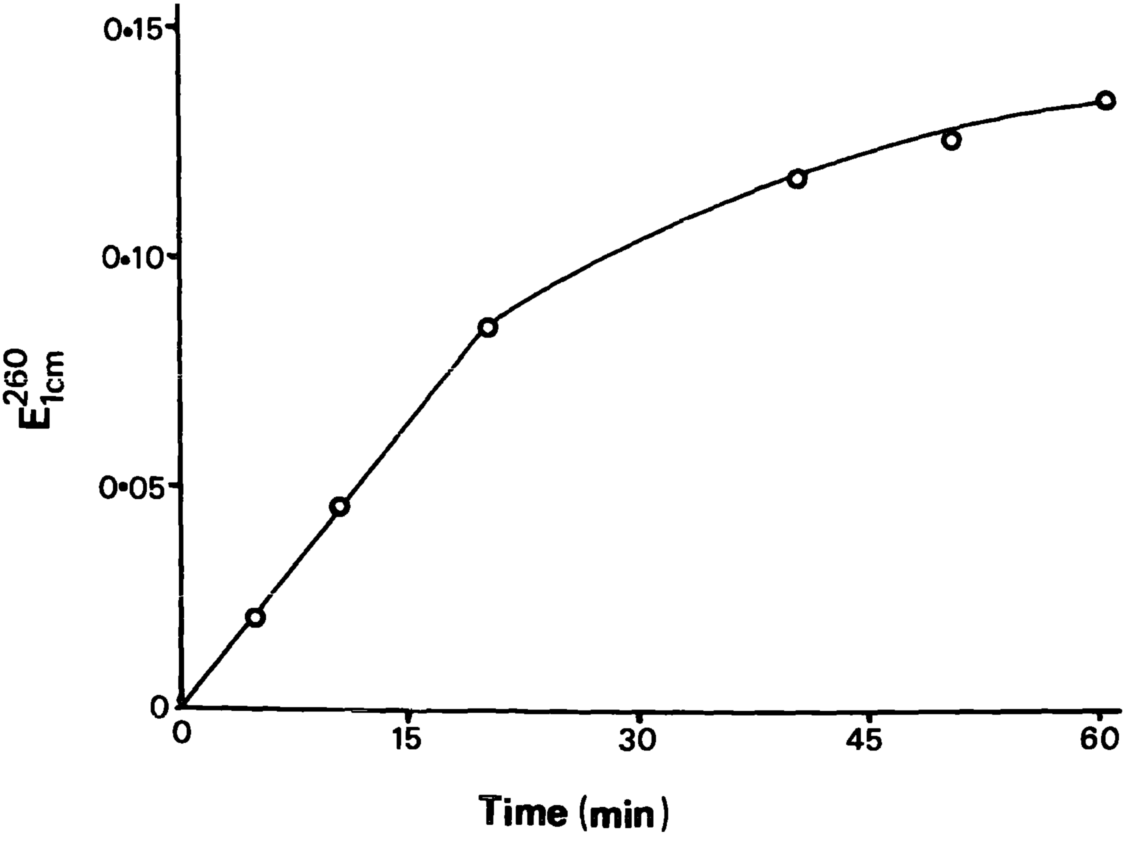

0•15
0•10
0•05
0
$E_{1cm}^{260}$
0
15
30
45
60
Time (min)

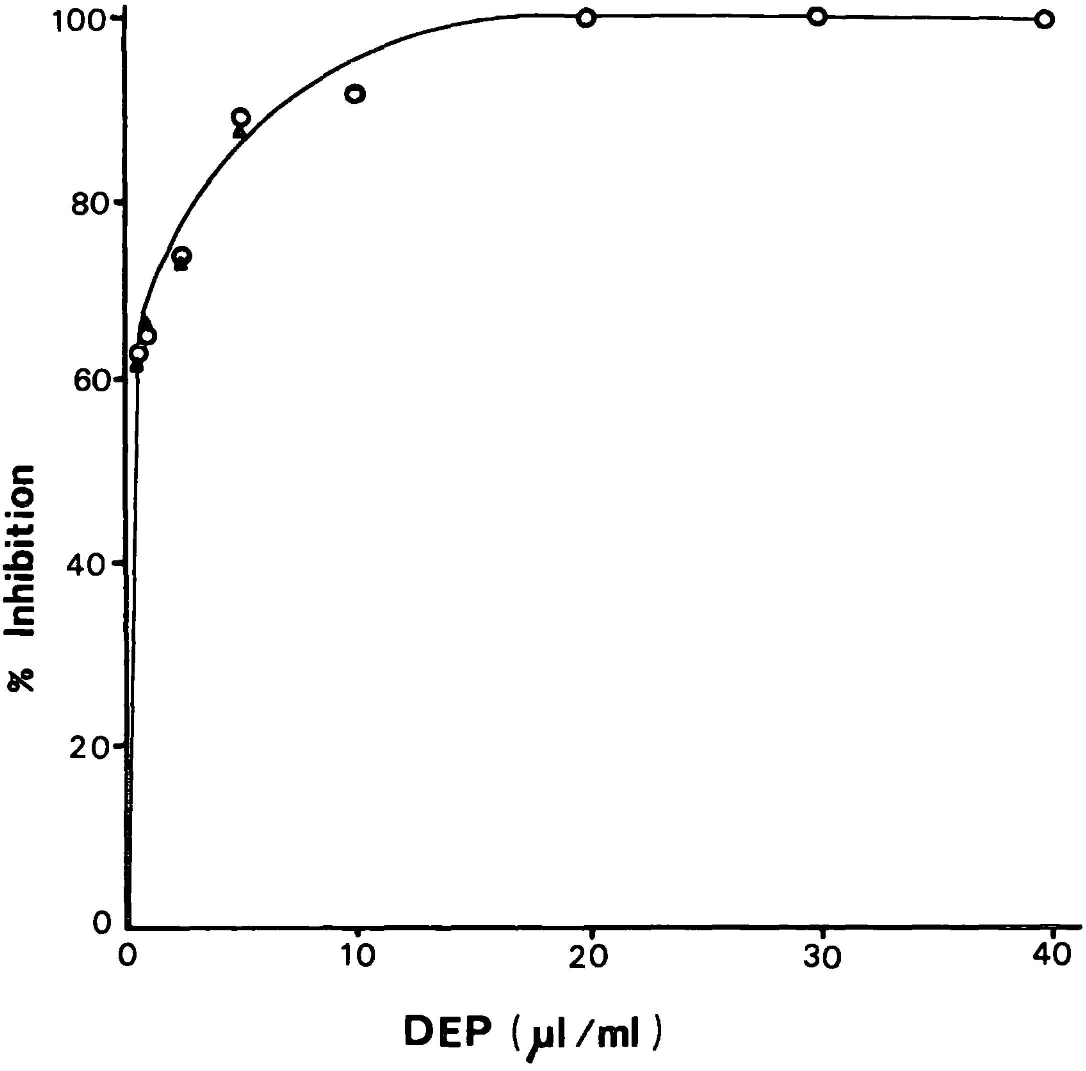
Fig. 10.

The action of DEP on the initial reaction rate of DNase I with DNA. Incubations were, carried out as detailed in the methods (section 9(ii)), at 25°C for 15 min and contained 0.5 µg/ml DNase I.

▲ : DEP added as a 10% (v/v) solution in ethanol.

Fig. 11.

The effect of increasing the DNase I concentration in the presence of excess DEP. Incubations were, carried out as detailed in the methods (section 9(ii)), at 25°C for 15 min and contained 50 µl/ml DEP.


% Inhibition
100
80
60
40
20
0
0
10
20
30
40
DEP (µl/ml)

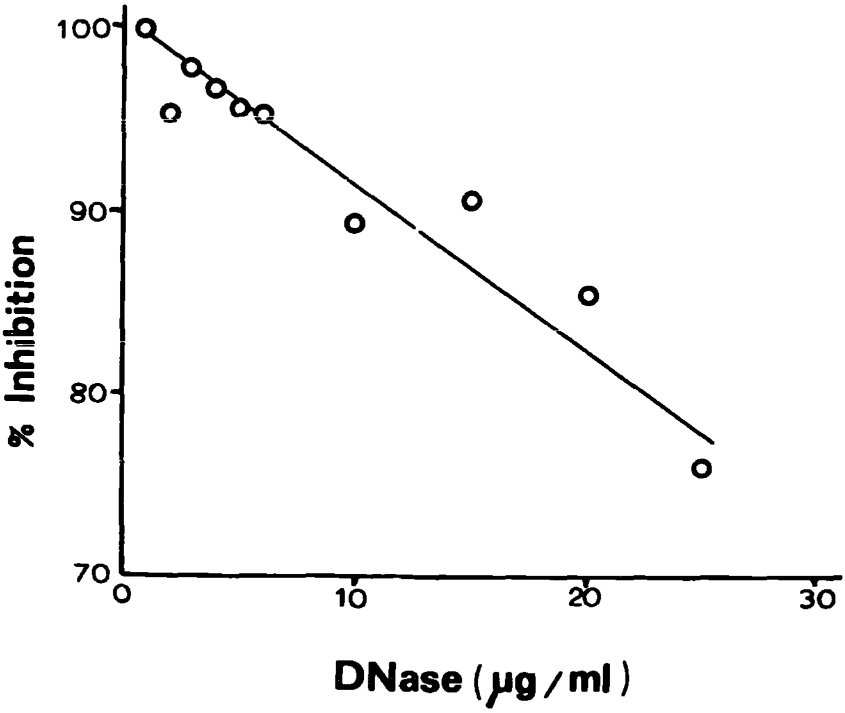

% Inhibition
100
90
80
70
0
10
20
30
DNase (µg/ml)

Fig. 12.

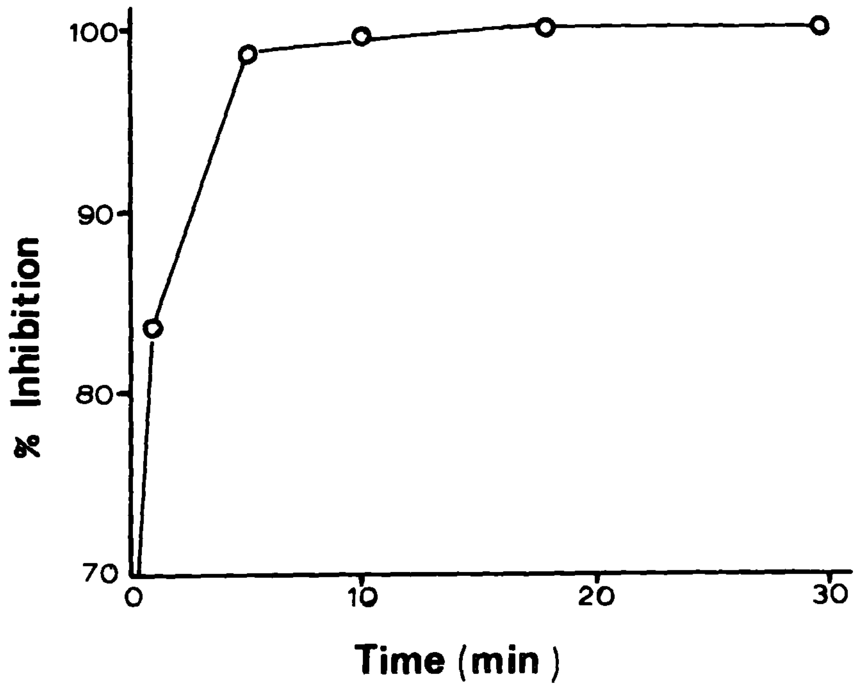
The effect of pre-incubating DNase with DEP. 12.5 µg DNase I was pre-incubated with 50 µl DEP in buffer, total volume 0.5 ml. The reaction was initiated by the addition of 0.5 ml (1 mg) DNA. Incubations were carried out as detailed in the methods, section 9(ii).

Fig. 13.

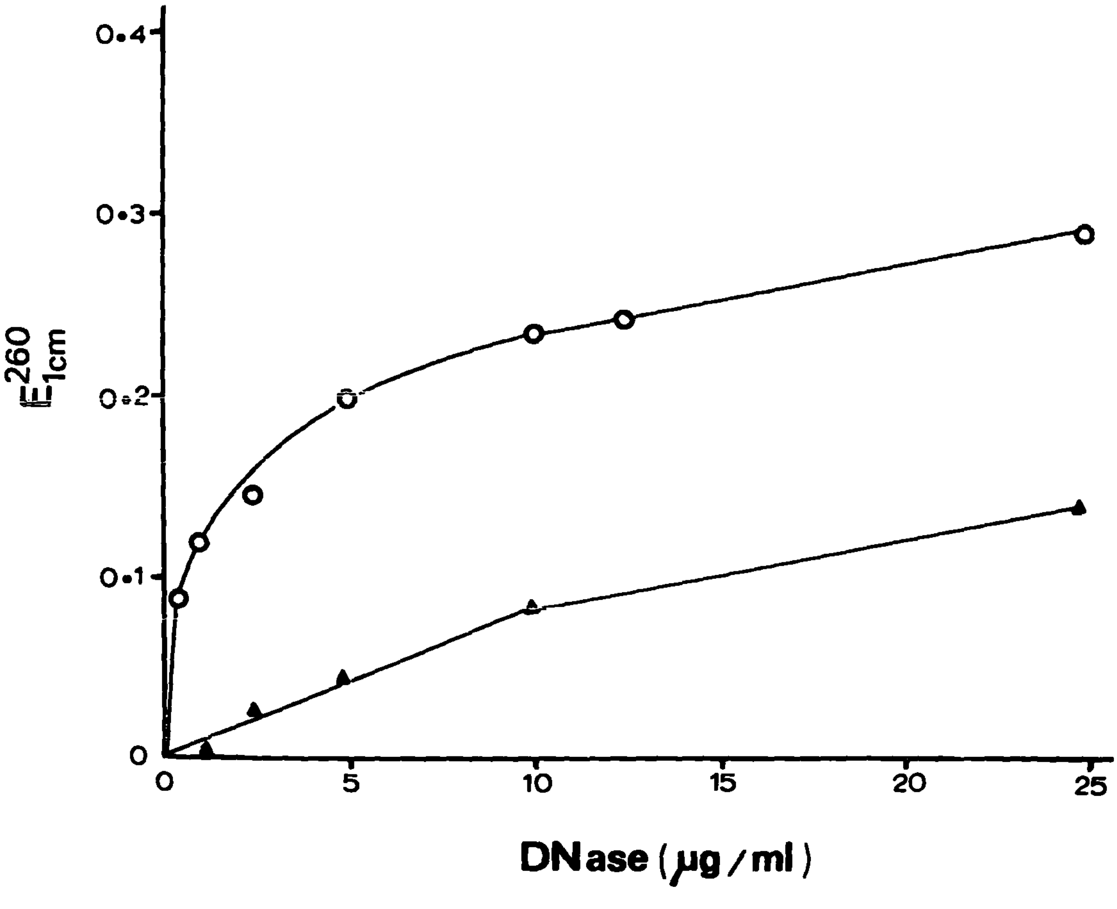
The effect of Tris concentration on DNase I activity. Incubations were carried out as detailed in the methods, section 9(ii).

○ : 50 mM-Tris.

▲ : 200 mM-Tris.



% Inhibition
100
90
80
70
0
10
20
30
Time (min)



$E^{260}_{1cm}$
0·4
0·3
0·2
0·1
0
0
5
10
15
20
25
DNase (µg/ml)

of DEP on low concentrations of RNase, then the apparent specific inhibition of RNase obtained in incubation system I can be explained. With excess RNase the observed inhibition of enzyme activity is 55% (see Table 3).

(c) Effect of Tris concentration on enzyme activity

Increasing the buffer concentration from 50mM- to 200mM-Tris markedly depressed DNase activity (Fig. 13), but had no effect on RNase activity. The substitution of Trizma base for Tris had no effect.

It was observed that the inhibition of DNase activity by DEP and buffer concentration were additive (see Table 3).

### 3. The purification of 4S and 5S RNA

Analysis of the nucleic acids, extracted as described in the methods (section 4.(vi),(a)), on 2.6% and 7.5% acrylamide gels showed little or no degradation. DNA was not detected in any of the samples (Figs. 14a and b).

4S RNA could be separated directly from the 25S, 18S and 5S RNA's, by chromatography on G100 (Fig. 36). The direct separation of 5S and 4S RNA from total nucleic acid samples by molecular sieve chromatography was not attempted as the theoretical yield would be 17% of the column capacity (see Table 4). The 25S and 18S RNA's were removed from the total nucleic acid sample by either molar-salt precipitation followed by DEAE-cellulose chromatography or directly by DEAE-cellulose chromatography.

(i) Molar-salt precipitation.

Analysis of the supernatant fraction on 2.6% and 7.5% acrylamide gels, following molar-salt precipitation, showed that a complete separation of the 25S and 18S RNA's from the 5S and 4S RNA's was not effected (Figs. 15 a and b). Washing the pellet with 2 vol. 1M-LiCl removed all entrapped 5S and 4S RNA as shown by analysis of the resulting pellet and supernatant fractions on 2.6% and 7.5% acrylamide gels (Figs. 16 a and b; 17 a and b).

TABLE 3

| Enzyme | | Tris | DEP | Inhibition |
|---|---|---|---|---|
| Type | µg/ml | concn. (mM) | % (v/v) | % |
| RNase | 25 | 50 | 0 | 0 |
| | | | 5 | 55 |
| | | 200 | 0 | 0 |
| DNase | 12.5 | 50 | 5 | 45 |
| | | 200 | 5 | 95 |

Incubations were carried out as detailed in the methods (section 9(ii)) at 25°C for 15 min.

# TABLE 4

Fractionation of 50-day cotyledon RNA by 1M-LiCl and/or DEAE-cellulose chromatography.

| | % 25S and 18S RNA | % 5S RNA | % 4S RNA | Molar ratio 4S to 5S RNA |
|---|---|---|---|---|
| Unfractionated RNA | 83 | 2.5 | 14.5 | 9.3 |
| 1M-LiCl supernatant RNA | 24 | 14.8 | 61.2 | 6.6 |
| 1M-LiCl supernatant RNA purified by DEAE-cellulose chromatography | Trace | 19.8 | 80.2 | 6.5 |
| 1M-LiCl DEAE-cellulose fraction | Trace | 22.3 | 77.7 | 5.6 |
| 1M-LiCl fraction following washing of 1M-LiCl pellet RNA | 71.6 | 2.6 | 25.9 | 15.9 |

Fig. 14.

50-day old cotyledon nucleic acids, extracted as described in the methods, section 4(vi).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion: cam A.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion: cam A.

Fig. 15.

1M-LiCl supernatant RNA. 50-day old cotyledon nucleic acids (see Fig. 14) were fractionated with 1M-LiCl as described in the methods, section 5(v)(a).

(a) Electrophoresed on a 2.6% gel for 3h . Vertical expansion: cam A.

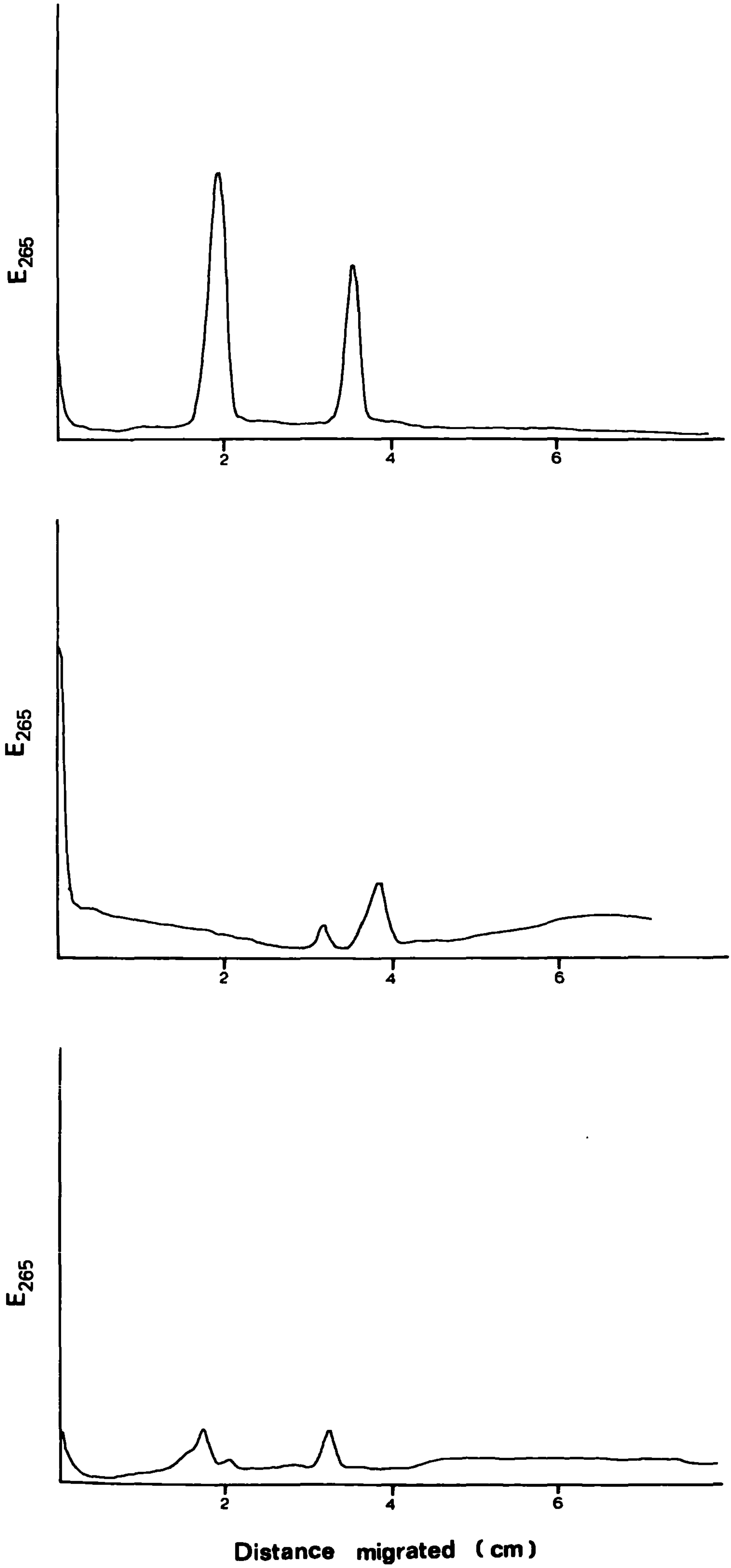

$E_{265}$
2
4
6
$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated (cm)

Fig. 15.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion: cam A.

Fig. 16.

The nucleic acids extracted from the 1M-LiCl pellet fraction with 1M-LiCl, following fractionation of 50-day cotyledon nucleic acids with 1M-LiCl, as described in the methods, section 5(v)(a).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion: cam A.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion: cam A.

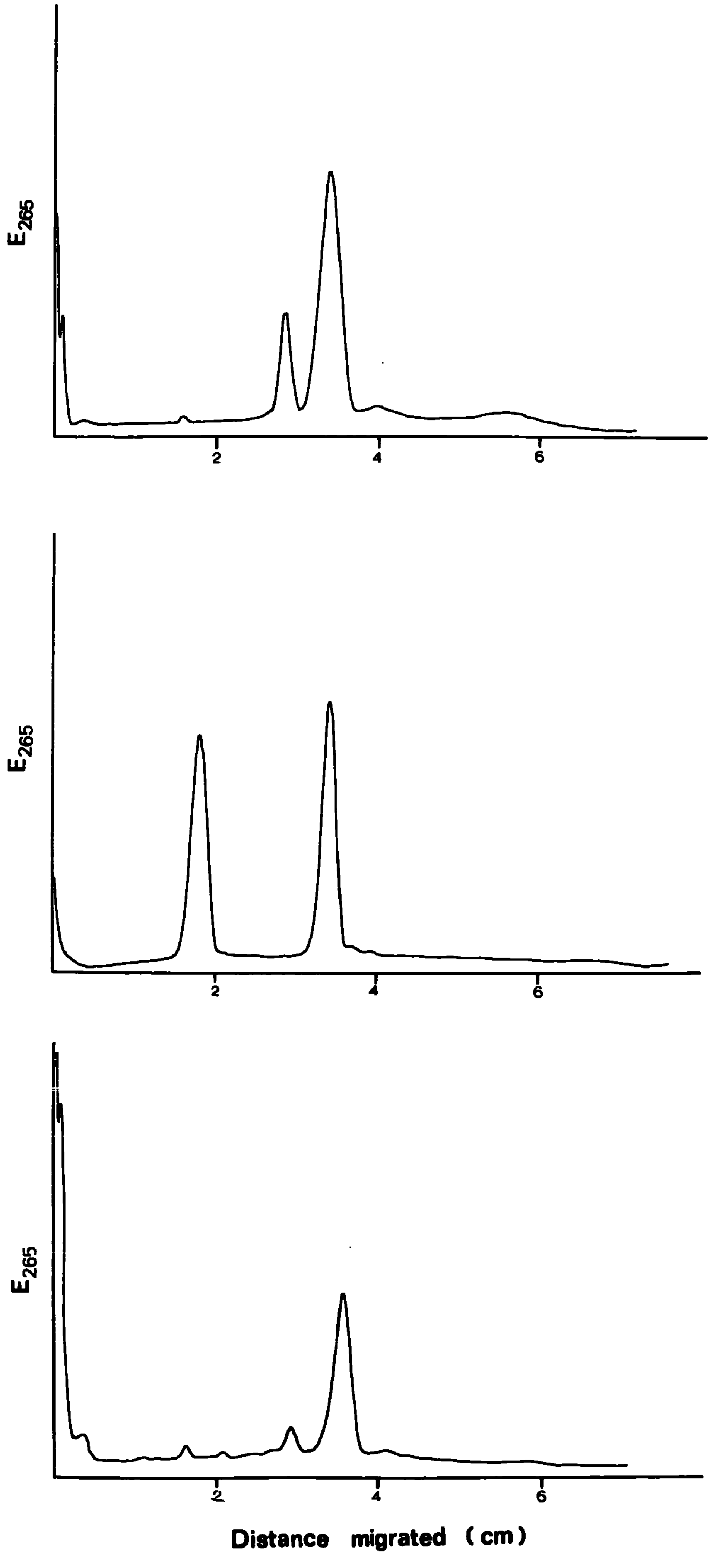
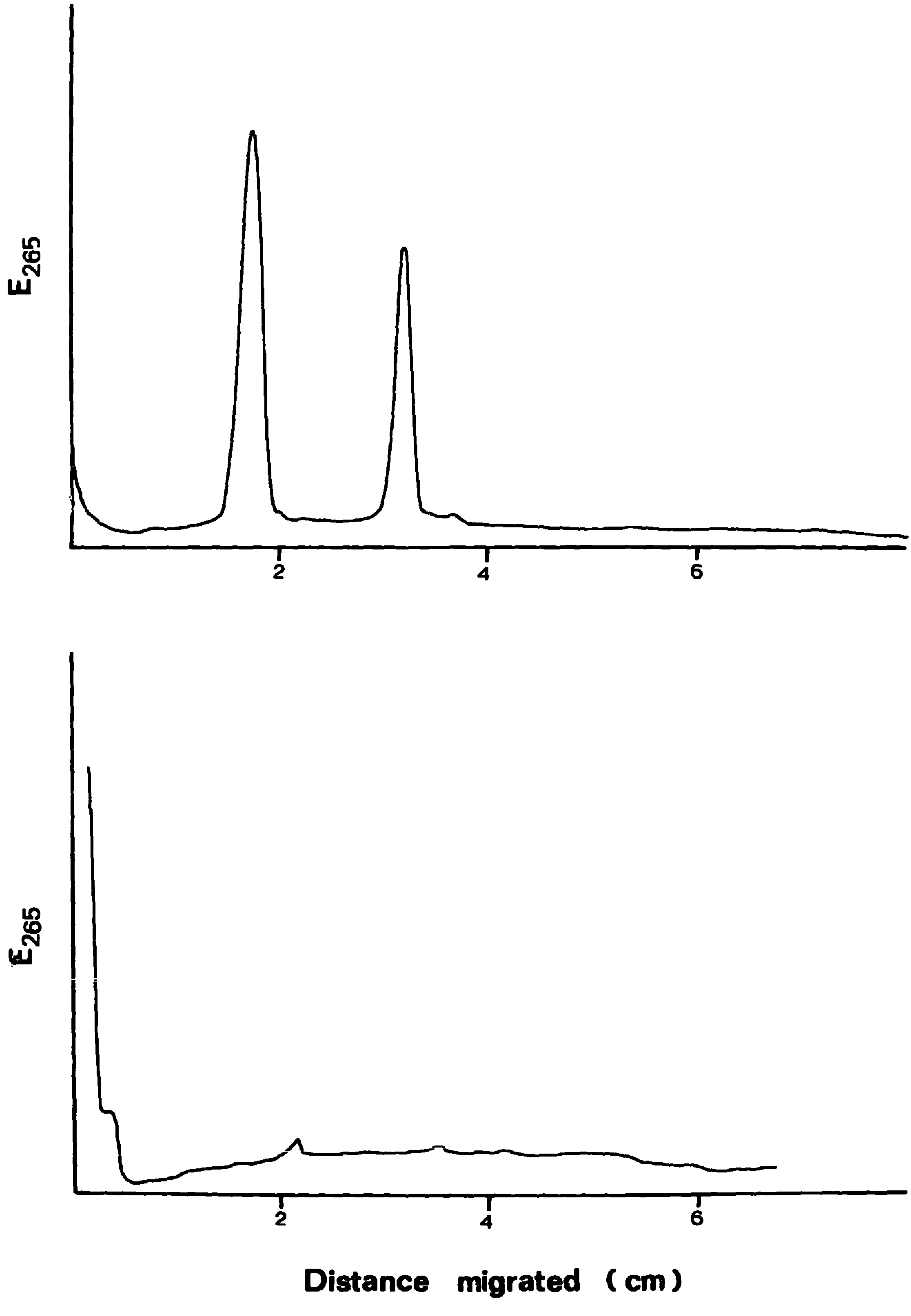

$E_{265}$
2
4
6
$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated (cm)

Fig. 17.

1M-LiCl pellet RNA. 50-day old cotyledon nucleic acids (see Fig. 14) were fractionated with 1M-LiCl as described in the methods, section 5(v)(a).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.


$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated ( cm )

However, the 25S and 18S RNA's were solubilised to some extent; the 18S RNA being in molar excess. It was also evident that the 4S RNA was preferentially entrapped during the molar-salt precipitation of the 25S and 18S RNA's (compare Figs. 15 b and 16 b; see Table 4).

The 25S and 18S RNA's present in the 1M-LiCl supernatant fractions were removed by chromatography on DEAE-cellulose (see below).

(ii) DEAE-cellulose chromatography.

Following the application of the RNA sample to the column, the 25S and 18S RNA's were eluted with 50mM-Tris·HCl, pH 7.5; the 5S and 4S RNA's were eluted with 1M-LiCl (Fig. 18). Analysis of the two fractions showed that a complete separation of the 25S and 18S RNA from the 5S and 4S RNA was achieved (Figs. 19 a and b).

A comparison of the techniques is given in Table 4.

(iii) Molecular sieve chromatography.

Separation of the 5S RNA from the 4S RNA was effected by chromatography on G100 (Fig. 20) and G75 (Fig. 21). The eluted fractions were analysed on 7.5% acrylamide gels. Fraction II (Fig. 23) and III (Fig. 24), G100 and G75, were shown to represent the 5S and the 4S RNA respectively. Fraction I (Fig. 22), G75 (G100 not analysed), was found to consist of one major species (0.90 x $10^5$ daltons) and three minor species (1.05, 0.78 and 0.68 x $10^5$ daltons).

Various characteristics of each fraction are given in Table 5. The purified 4S and 5S RNA's were used as markers on 5.0% and 7.5% acrylamide gels.

## 4. The total nucleic acids of V. faba

(i) The root.

Analysis of root nucleic acids on 2.2% acrylamide gels showed three major species: DNA, 25S RNA and 18S RNA. A minor RNA component,

## TABLE 5

Molecular weight and amino acid acceptor capacity of Sephadex fractions.

Sephadex G100; $V_o$ = 132 ml, $V_t$ = 421 ml (see Fig. 20)

| FRACTION | % of total | $V_e$ | $K_{av}$ | MW | $^{14}C$ -phenylalanine acceptor capacity |
|---|---|---|---|---|---|
| I | 3 | - | - | - | not tested |
| II | 14 | 203 ml | 0.246 | 40,000 | 0 |
| III | 83 | 245 ml | 0.390 | 28,000 | + |

Sephadex G75; $V_o$ = 105 ml, $V_t$ = 480 ml (see Fig. 21)

| FRACTION | % of total | $V_e$ | $K_{av}$ | MW | $^{14}C$ -phenylalanine acceptor capacity |
|---|---|---|---|---|---|
| I | 8.8 | 105 ml ($V_o$) | - | - | not tested |
| II | 9.4 | 144 ml | 0.10 | 45,000 | not tested |
| III | 81.8 | 213 ml | 0.28 | 26,000 | + |

The molecular weights were determined from the partition coefficient ($K_{av}$) using Fig. 2.

Fig. 18.

DEAE-cellulose chromatography of nucleic acids. The nucleic acids, extracted as detailed in the methods (section 4(vi)), were dissolved in 50mM-Tris•HCl, pH 7.5 and fractionated on a 1.5 x 15 cm column of DEAE-cellulose. The LMW RNA fraction was collected by washing the column with 50mM-Tris•HCl, 1M-LiCl, pH 7.5. The elution profile is representative of that obtained when nucleic acids were bulk fractionated by this technique.

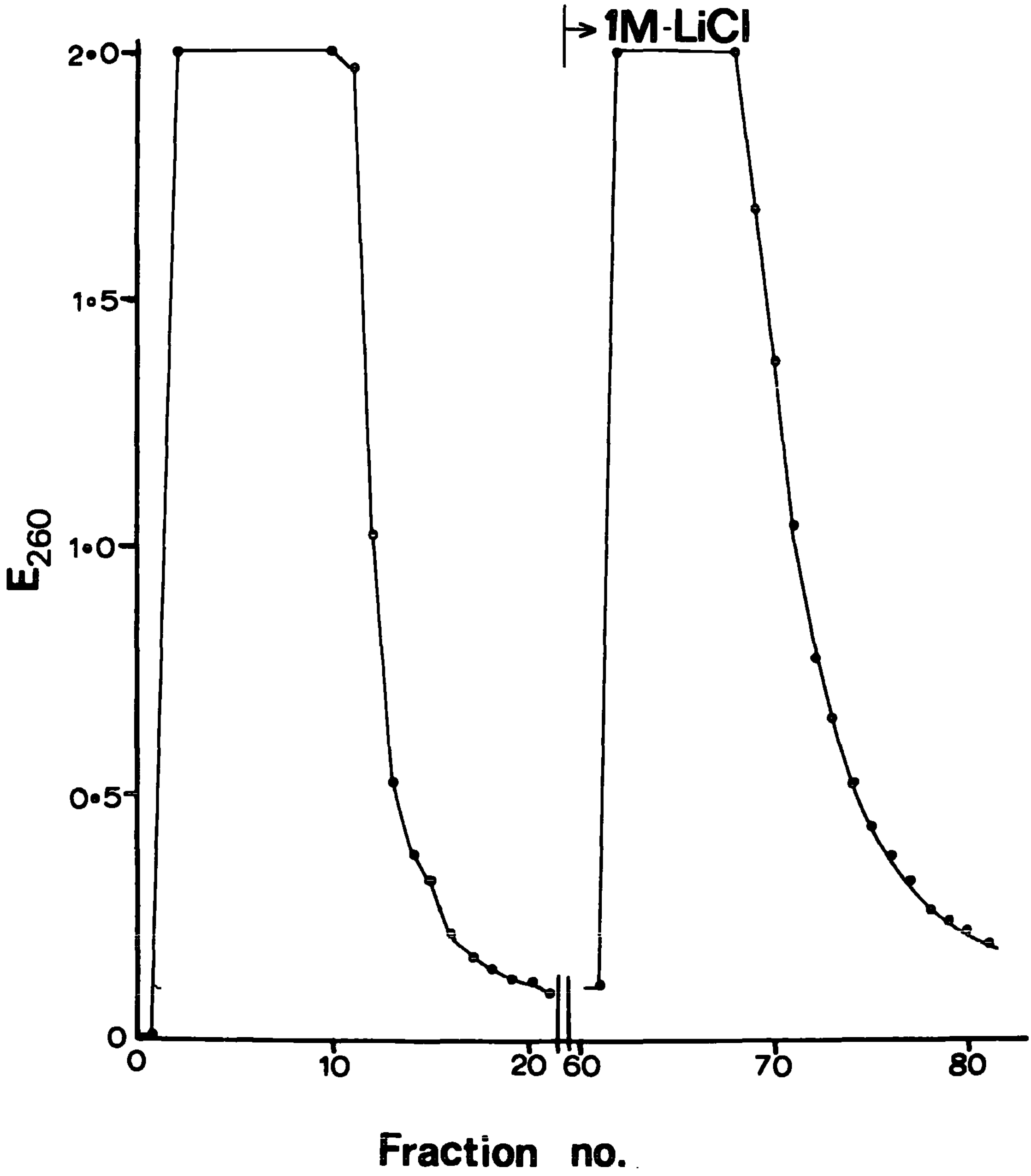

→1M-LiCl
2•0
1•5
1•0
0•5
0
$E_{260}$
0
10
20
60
70
80
Fraction no.

Fig. 19.

1M-LiCl fraction off DEAE-cellulose.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam D.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.

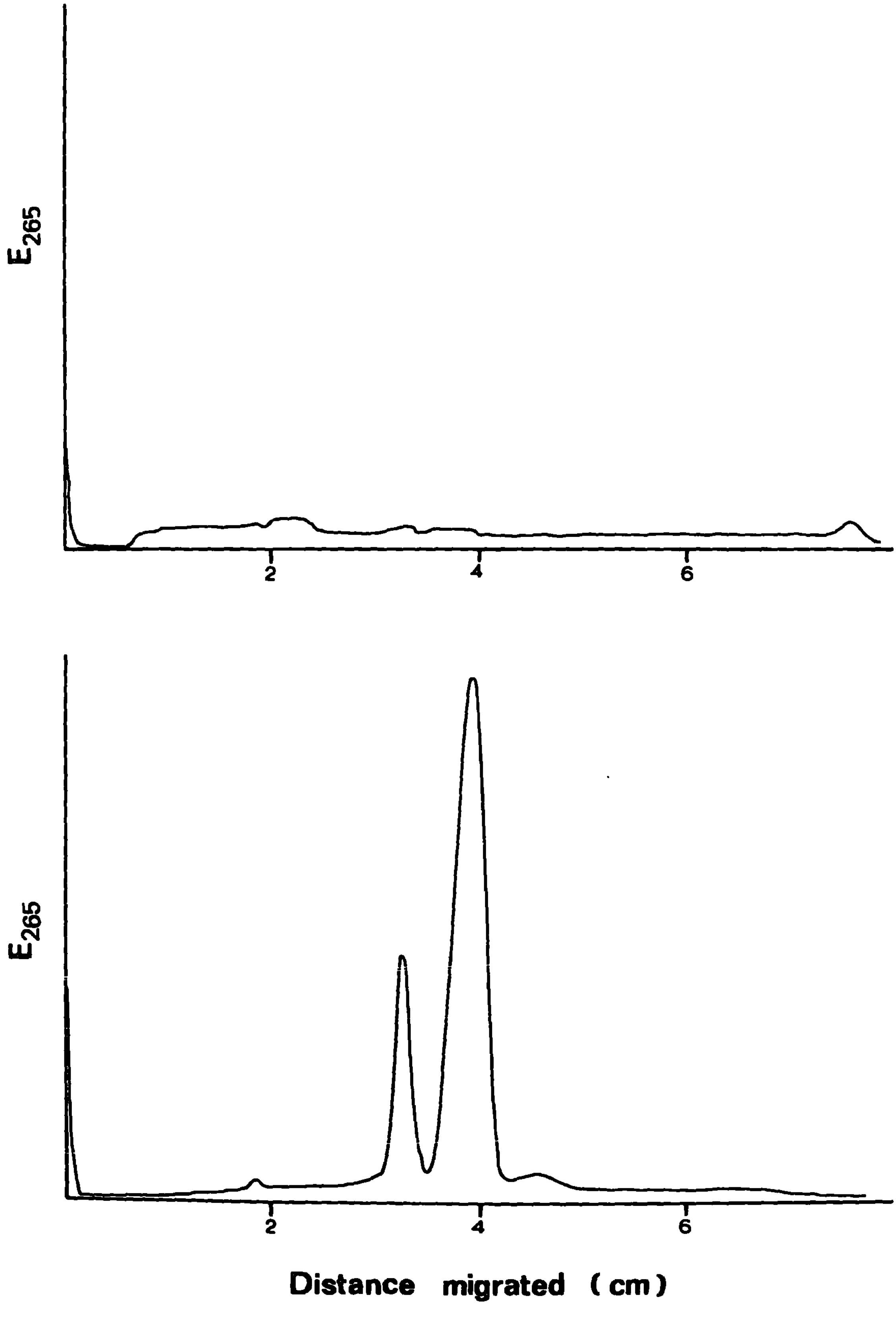
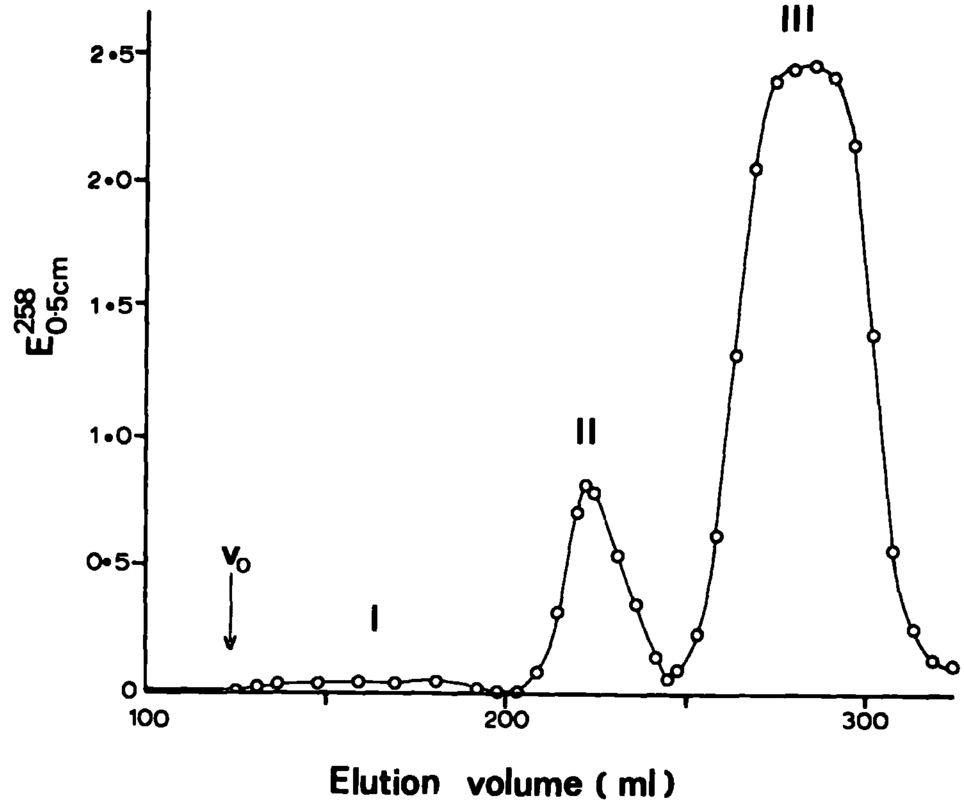

$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated (cm)

Fig. 20.

Sephadex G100 elution pattern of LMW RNA. The RNA was extracted (methods section 4(vi)(a)) from the etiolated stems of 2-week germinated seeds of V. faba and bulk fractionated on DEAE-cellulose (see legend to Fig. 18). 17 mg of the 1M-LiCl fraction was applied to the column. The column, 2 x 134 cm, was equilibrated with 50mM-ammonium acetate, pH 5.1. Roman numerals are used to indicate the fractions. The void volume ($V_0$) was determined with dextran blue 2000. Flow rate = 5 ml/h.

Fig. 21.

Sephadex G75 elution pattern of LMW RNA. The RNA was extracted (methods section 4(vi)(a)) from 50-day old cotyledons of V. faba and bulk fractionated on DEAE-cellulose (see legend to Fig. 18). 17 mg of the 1M-LiCl fraction was applied to the column. The column, 2 x 153 cm, was equilibrated with 50mM-sodium acetate, pH 5.1. The void volume ($V_0$) was determined with ribosomal RNA. Flow rate was 13.6 ml/h and fractions were collected every 30 min. Shaded areas are those fractions pooled for further analysis.


III
II
I
$V_0$
2·5
2·0
1·5
1·0
0·5
0
$E^{258}_{0\cdot5cm}$
100
200
300
Elution volume ( ml )

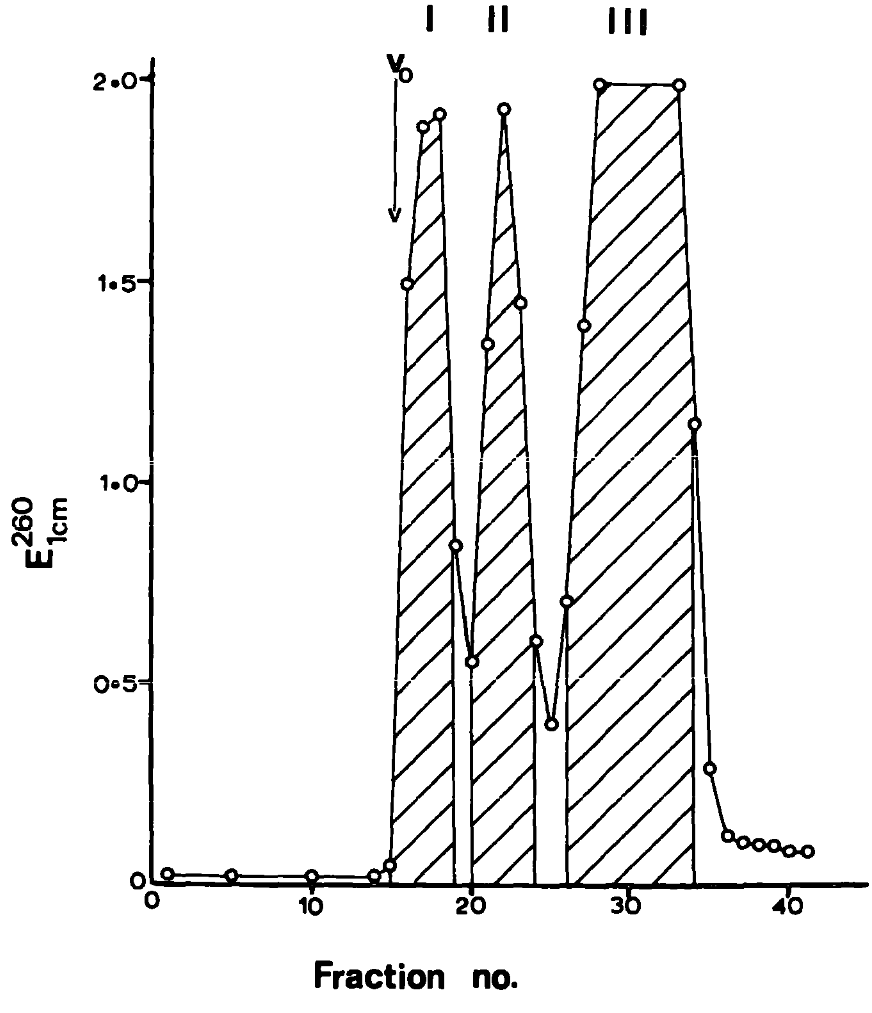

I
II
III
$V_0$
2·0
1·5
1·0
0·5
0
$E^{260}_{1cm}$
0
10
20
30
40
Fraction no.

Fig. 22.

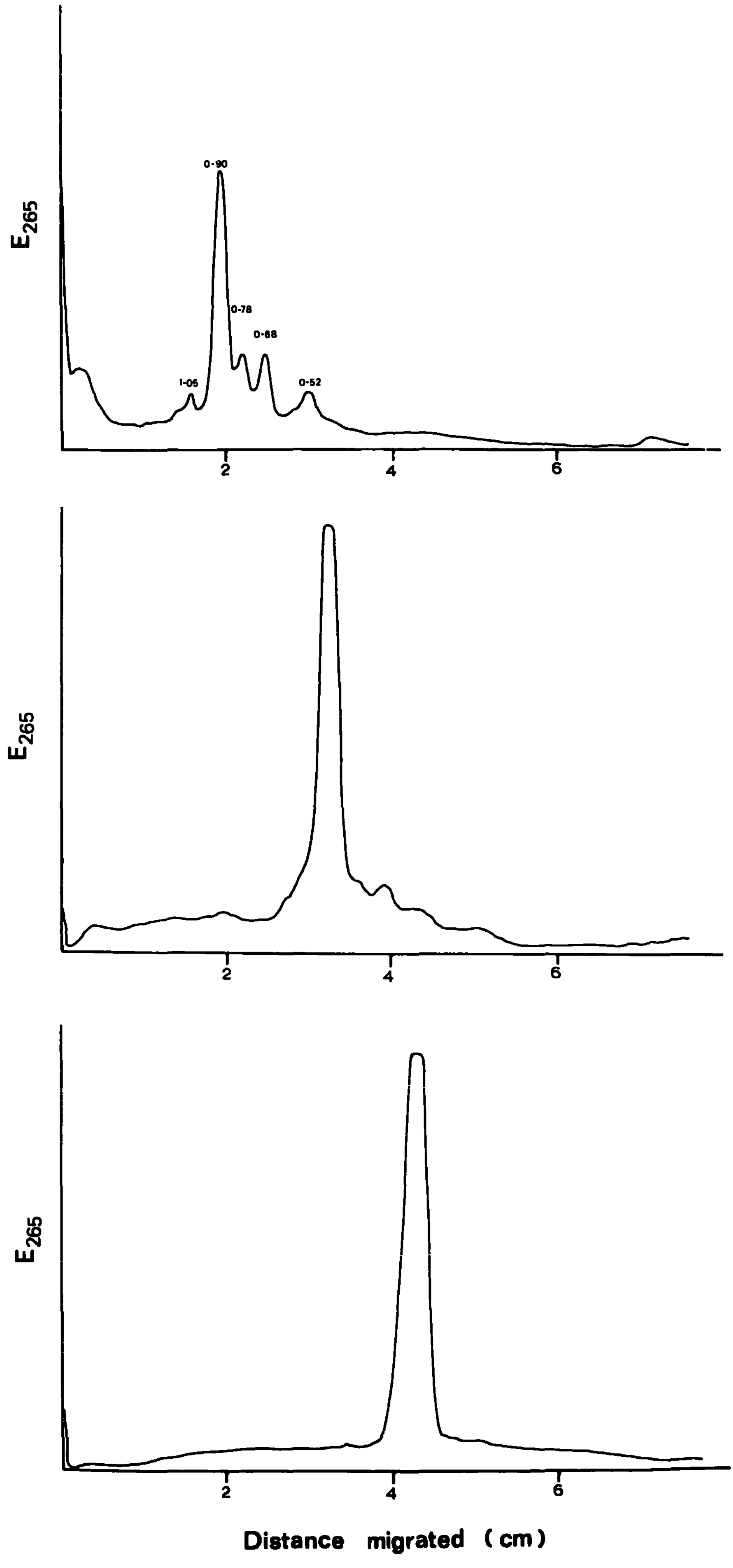
G75 fraction I (see Fig. 21). 10 μg RNA electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.

Fig. 23.

G75 fraction II (see Fig. 21). 10 μg RNA electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.

Fig. 24.

G100 fraction III (see Fig. 20). 10 μg RNA electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.


E265
0·90
0·78
0·68
1·05
0·52
2
4
6
E265
2
4
6
E265
2
4
6
Distance migrated (cm)

MW, $1.02 \times 10^6$ daltons, was present in all samples analysed (Fig. 25, see also section 8).

(ii) The leaf.

Analysis of leaf nucleic acids on 2.6% acrylamide gels (Fig. 26) showed 5 major species: DNA, 25S RNA, 23S RNA, 18S RNA and 16S RNA. The 23S and 16S RNA's had MW's of $1.1 \times 10^6$ and $0.56 \times 10^6$ daltons respectively, and were of chloroplastic origin. There was a molar excess of 16S chloroplast RNA over the 23S chloroplast RNA.

(iii) The developing cotyledon.

Analysis of the cotyledon nucleic acids on 2.6% acrylamide gels showed three major species: DNA, 25S RNA and 18S RNA (Fig. 27). During development of the seed rRNA was found to increase relative to DNA between day 20 and day 60 (Fig. 28). The 4S RNA was also found to increase relative to the 5S RNA (Fig. 29) during development, as determined by simple regression analysis.

The naturally-occurring RNA breakdown products were found to have a mobility greater than 4S RNA on gels. Their occurrence was negligible up to 60 days of development, but in older material they were accumulated (Figs. 30 and 31). The breakdown products had an average MW of $1.1 \times 10^4$ daltons.

(iv) The testa.

Analysis of testa nucleic acids on 2.6% acrylamide gels showed three major species: DNA, 25S RNA and 18S RNA.

Maximum development of the testa was judged to occur at about 30 days after flowering. This was based on its appearance, and the fact that, at this time the developing cotyledons completely occupied the space within the testa. From 30 days there was a gradual decline in the amount of rRNA relative to DNA (Fig. 32). In 30 day old testa material, the

Fig. 25.

Root nucleic acids. Electrophoresed on a 2.2% gel for 3h. Vertical expansion : cam A. The numbers above the peak refer to its MW x $10^6$.

Fig. 26.

Leaf nucleic acids. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C. The numbers above the peaks refer to their MW x $10^6$.

Fig. 27.

50-day old cotyledon nucleic acids. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

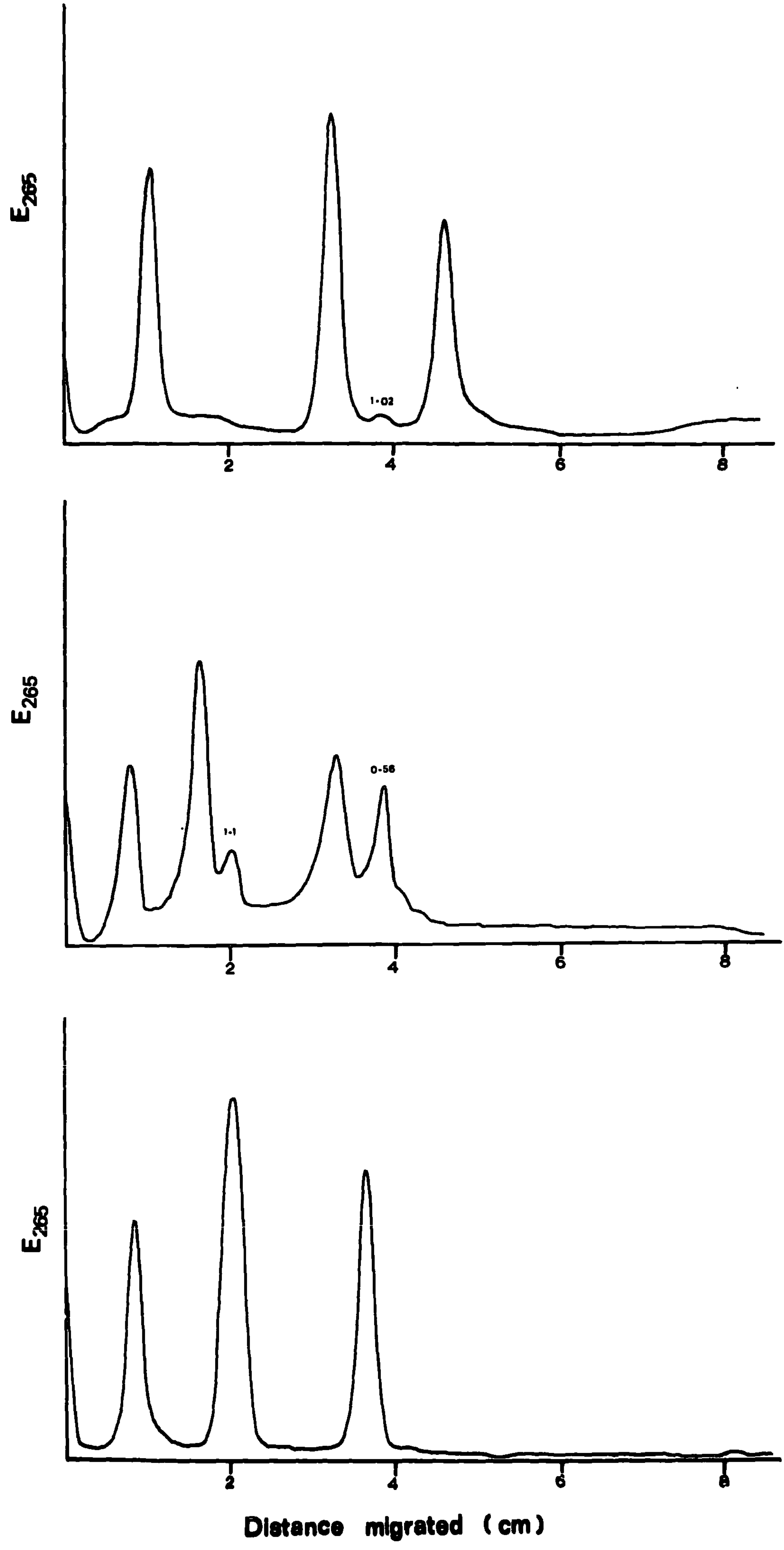
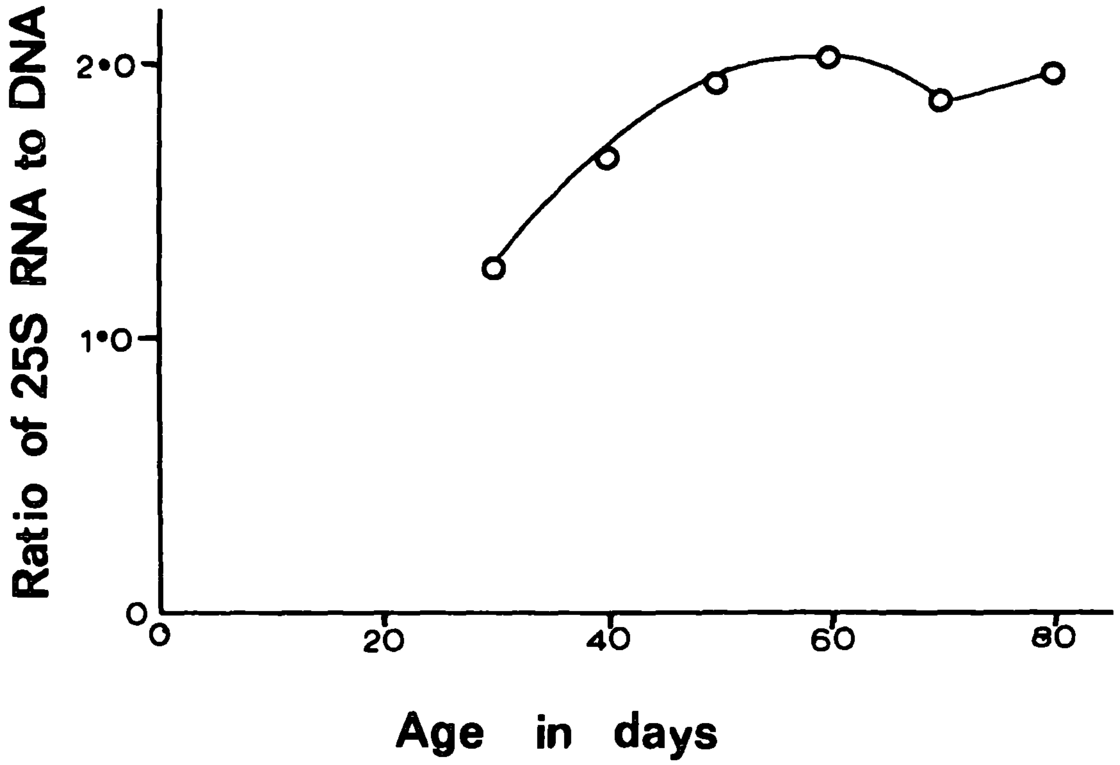

E265
1·02
2
4
6
8
E265
1·1
0·56
2
4
6
8
E265
2
4
6
8
Distance migrated (cm)

Fig. 28.

Change in the 25S rRNA/DNA ratio during development of the cotyledons. The relative amounts of 25S rRNA and DNA were determined from gel traces by integrating the peak areas. Results from the 1969 and 1970 season were averaged.

Fig. 29.

Change in the molar ratio of 4S to 5S RNA during development of the cotyledons. The relative amounts of 4S and 5S RNA were determined from gel traces by integrating the peak areas. Results from the 1969 and 1970 season were pooled. The slope, $y = 5.4138 + 0.0647\ (\pm\ 0.0243)(x)$, where $y$ = molar ratio of 4S to 5S RNA and $x$ = age in days, was determined by regression analysis. Both the correlation coefficient ($R = 0.4600$) and the t-distribution ($t = 0.2663$) were significant to $P = > 0.01$ but $< 0.02$.


Ratio of 25S RNA to DNA
2•0
1•0
0
0
20
40
60
80
Age in days

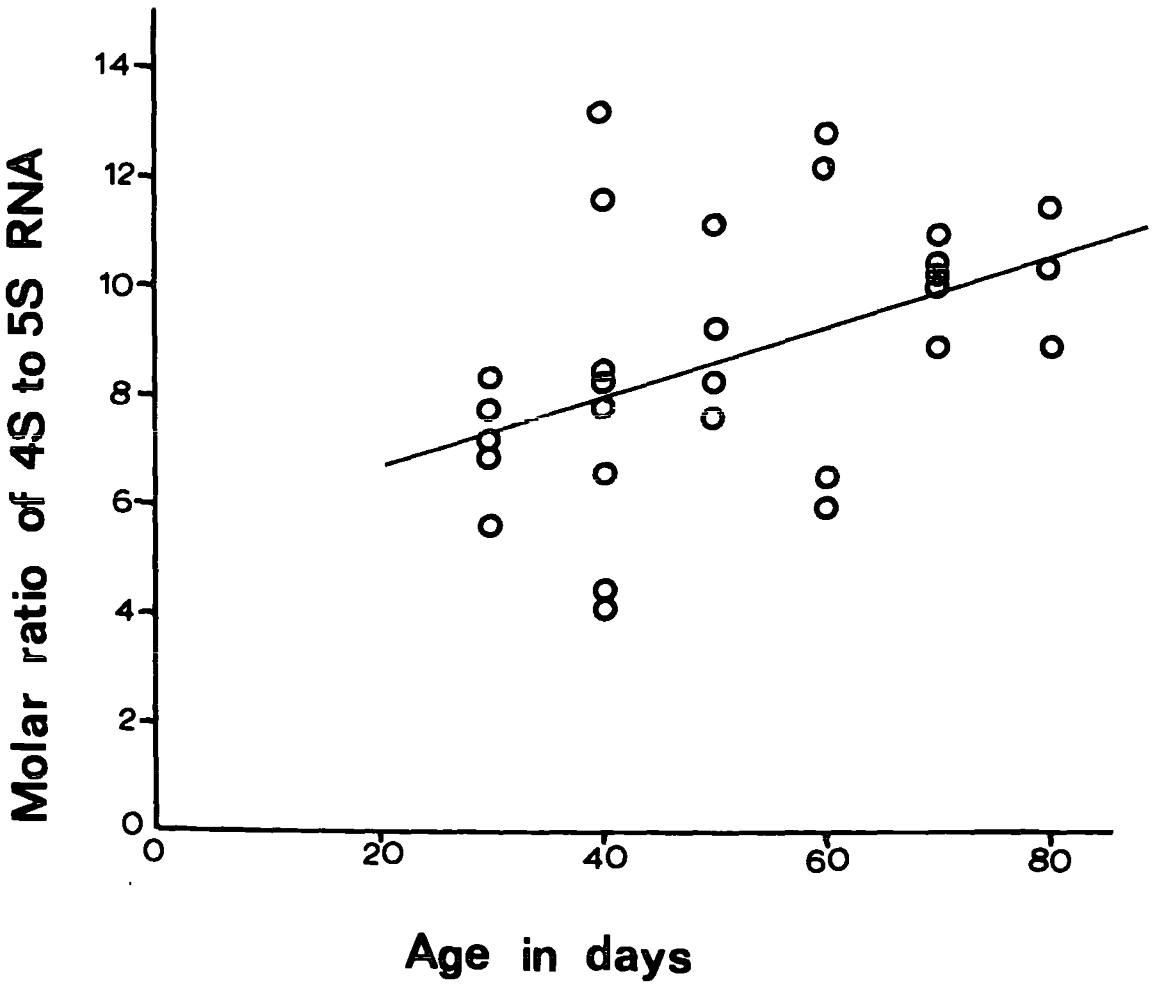

Molar ratio of 4S to 5S RNA
14
12
10
8
6
4
2
0
0
20
40
60
80
Age in days

Fig. 30.

70-day old cotyledon nucleic acids. Eletrophoresed on a 5% gel for 3h. Vertical expansion : cam B.

Fig. 31.

80-day old cotyledon nucleic acids. Electrophoresed on a 5% gel for 3h. Vertical expansion : cam B.

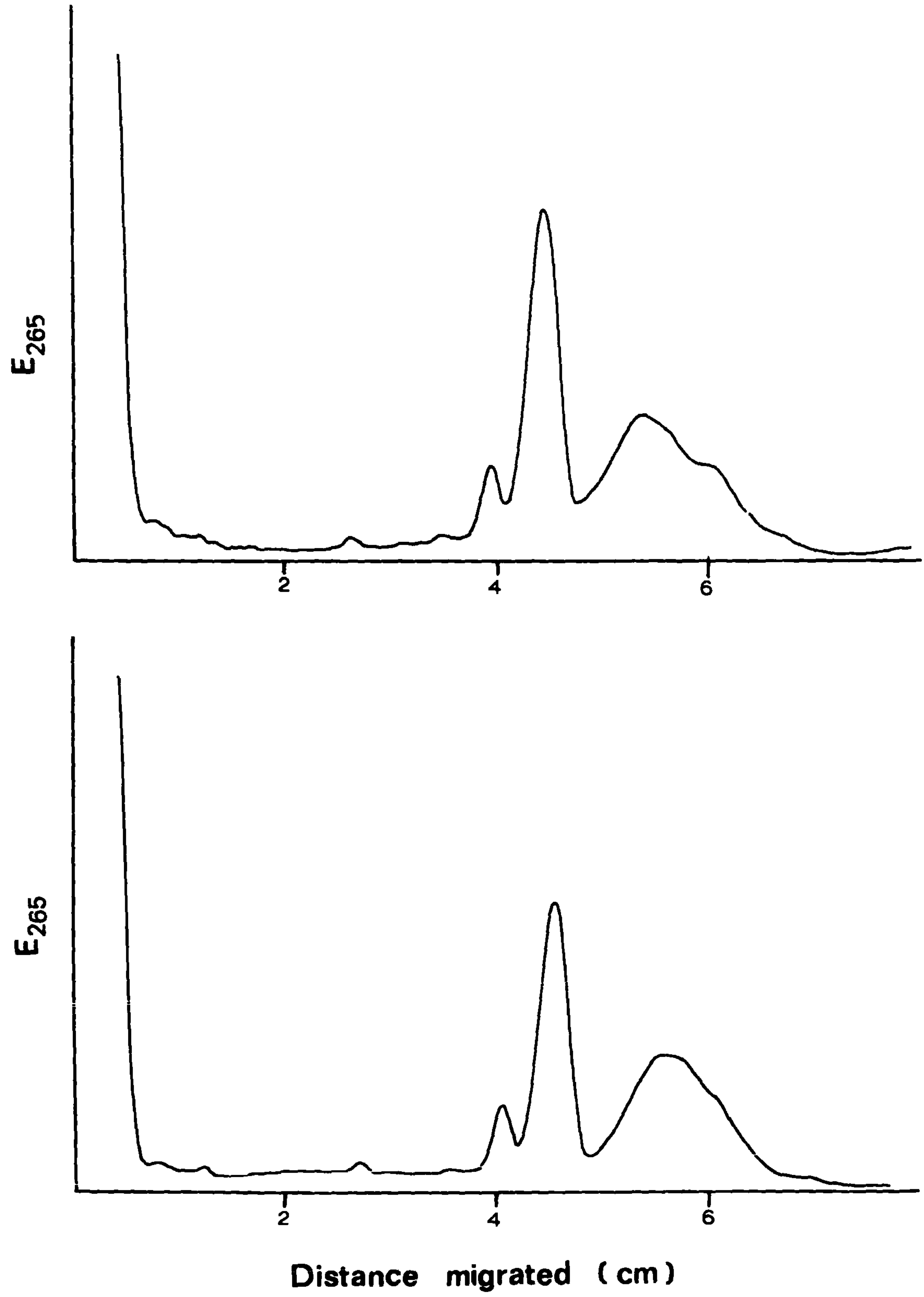
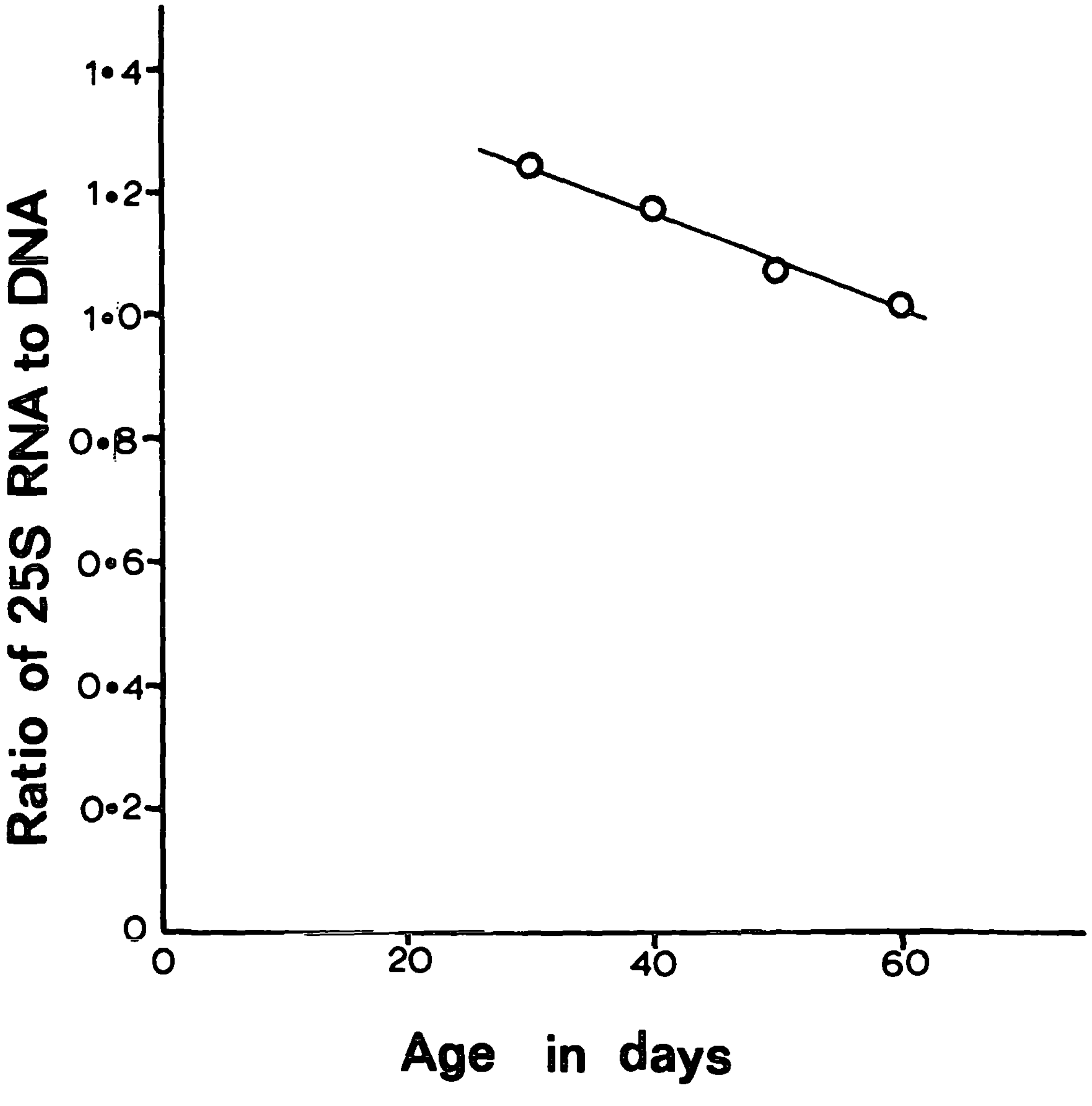

$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated (cm)

Fig. 32.

Change in the 25S rRNA/DNA ratio during maturation of the testa. The relative amounts of the 25S rRNA and DNA were determined from gel traces by integrating the peak areas.



Ratio of 25S RNA to DNA
1•4
1•2
1•0
0•8
0•6
0•4
0•2
0
0
20
40
60
Age in days

naturally-occurring RNA breakdown products were negligible. In older (40, 50 and 60 day) material, the breakdown products showed a marked increase (Figs. 33, 34 and 35). The breakdown products had an average MW of 0.55 x $10^4$ daltons. The differences in the MW distribution of the naturally-occurring breakdown products of the cotyledon and testa were sufficient to produce a separation during chromatography on G100 (Fig. 36).

It was noted that the cotyledons and testa were green, indicating the presence of chlorophyll; but no chloroplast rRNA's were detected in either cotyledon or testa nucleic acid samples.

(v) 65° RNA of the developing cotyledon and testa.

The extraction of the protein/phenolic interphase at pH 5.0 and 65°C as described (Methods, 4.(ii)) yielded on average a further 20% of nucleic acids. Analysis on 2.6% and 7.5% polyacrylamide gels normally showed 6 major species: DNA, 25S, 18S, 5.8S, 5S and 4S RNA (Figs. 37 a and b).

The molar ratio of 25S RNA to 18S RNA showed a wide variation (0.95 to 1.55) and the DNA/25S RNA ratio could not be calculated as in some extractions no DNA was present.

(vi) The mature seed.

Analysis of the seed nucleic acids on 2.6% acrylamide gels showed 3 major species: DNA, 25S RNA and 18S RNA. Three minor species were also discernible and had MW's of 1.02 x $10^6$, 0.52 x $10^6$ and 0.44 x $10^6$ daltons (Fig. 38a). Analysis on 7.5% acrylamide gels showed 5S and 4S RNA's and a group of LMW RNA's with a mobility less than the 5S RNA. These were calculated to have MW's of 0.78 x $10^5$, 0.68 x $10^5$, and 0.62 x $10^5$ daltons (Fig. 38b).

Fig. 33.

40-day old testa nucleic acids. Electrophoresed on a 5% gel for 3h. Vertical expansion : cam B.

Fig. 34.

50-day old testa nucleic acids. Electrophoresed on a 5% gel for 3h. Vertical expansion : cam D.

Fig. 35.

60-day old testa nucleic acids. Electrophoresed on a 5% gel for 3h. Vertical expansion : cam B.

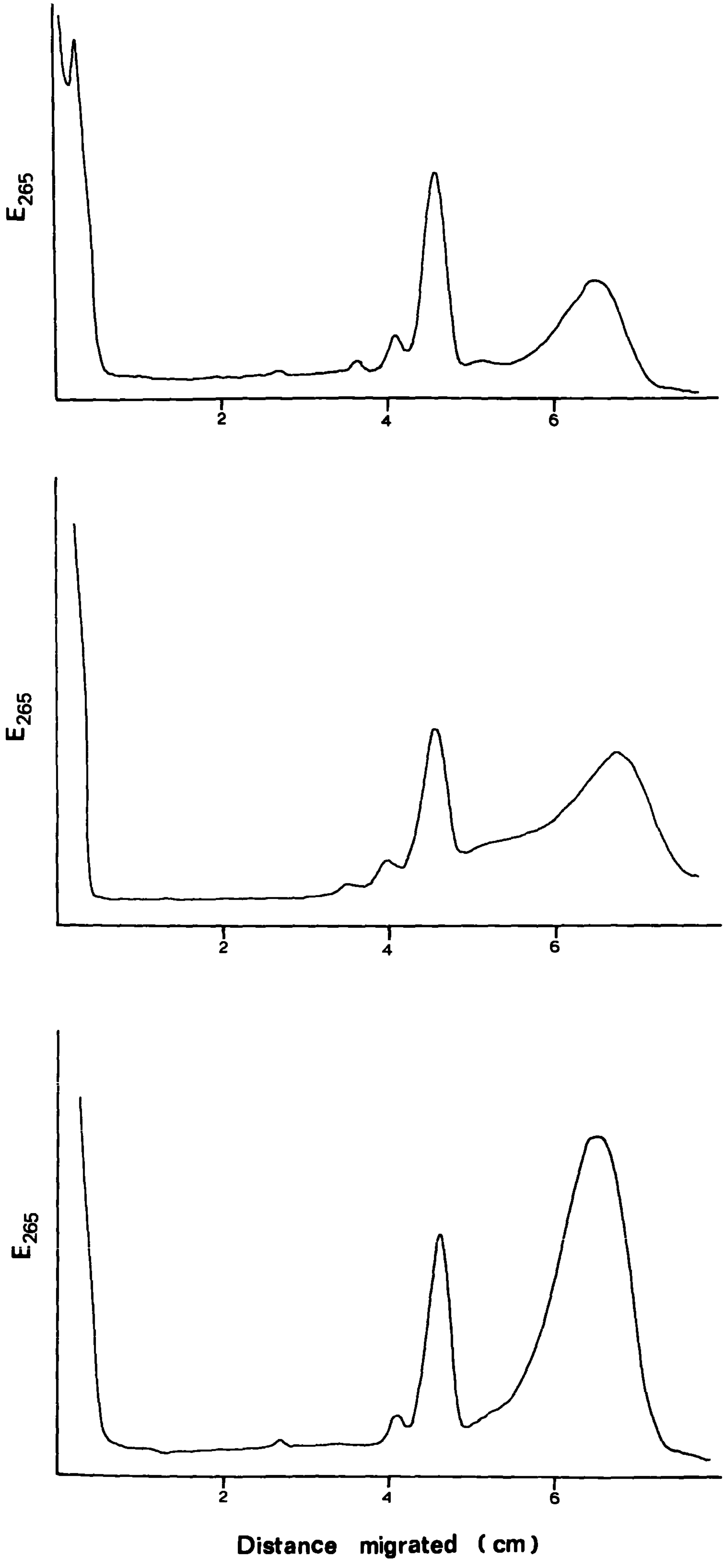

$E_{265}$
2
4
6
$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated (cm)

Fig. 36.

Sephadex G100 elution pattern of total nucleic acids from 40, 50 and 60-day old testa's and from 70 and 80-day old cotyledons. 15 mg of nucleic acids were applied to the column. The column, 2.5 x 80 cm, was equilibrated in 50mM-sodium acetate, pH 5.1. 12 ml fractions were collected.

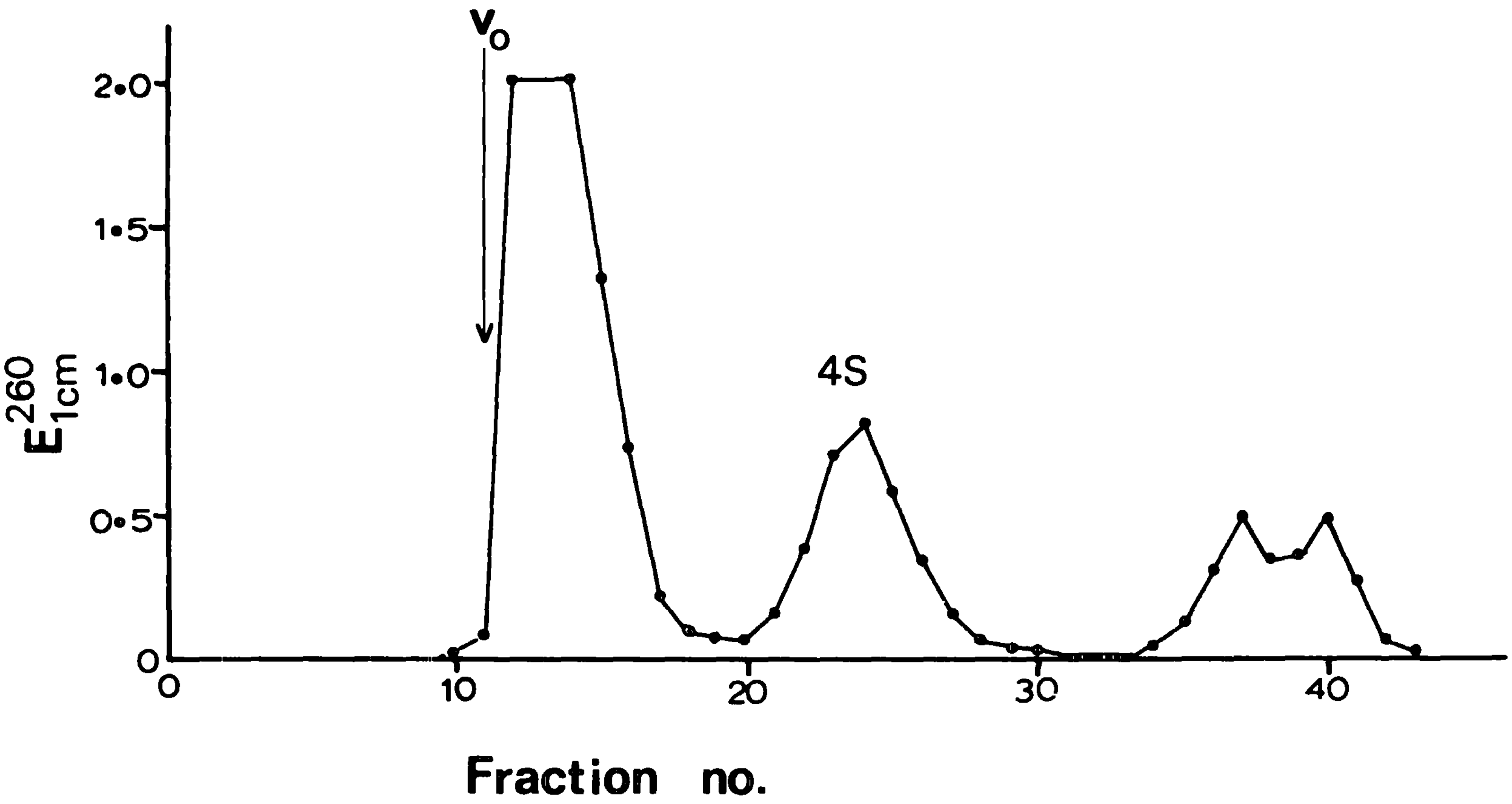

$V_0$
4S
$E^{260}_{1cm}$
2·0
1·5
1·0
0·5
0
0
10
20
30
40
Fraction no.

Fig. 37.

65° RNA from 40-day old cotyledons, extracted as described in the methods, section 4(ii).

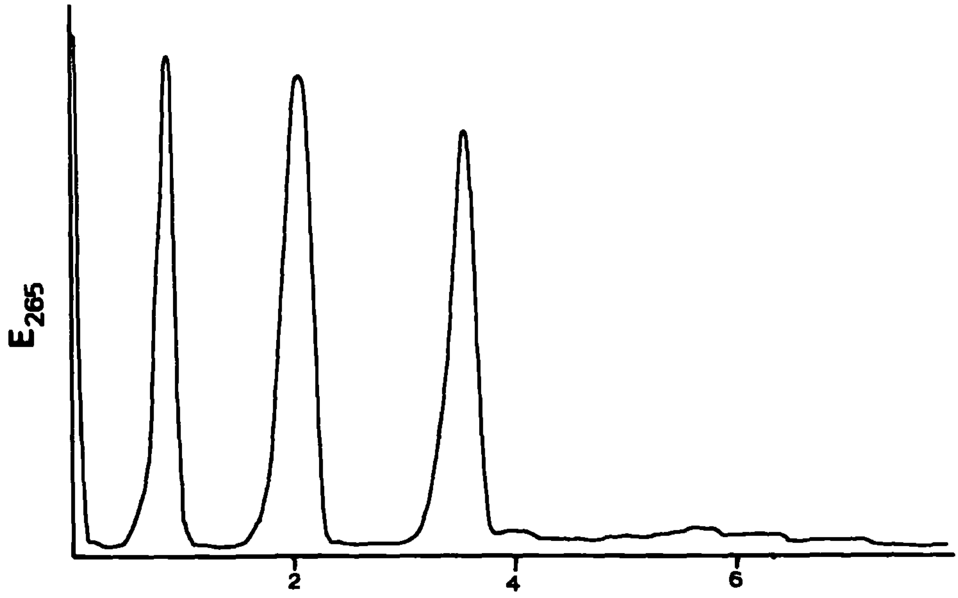
(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam D.

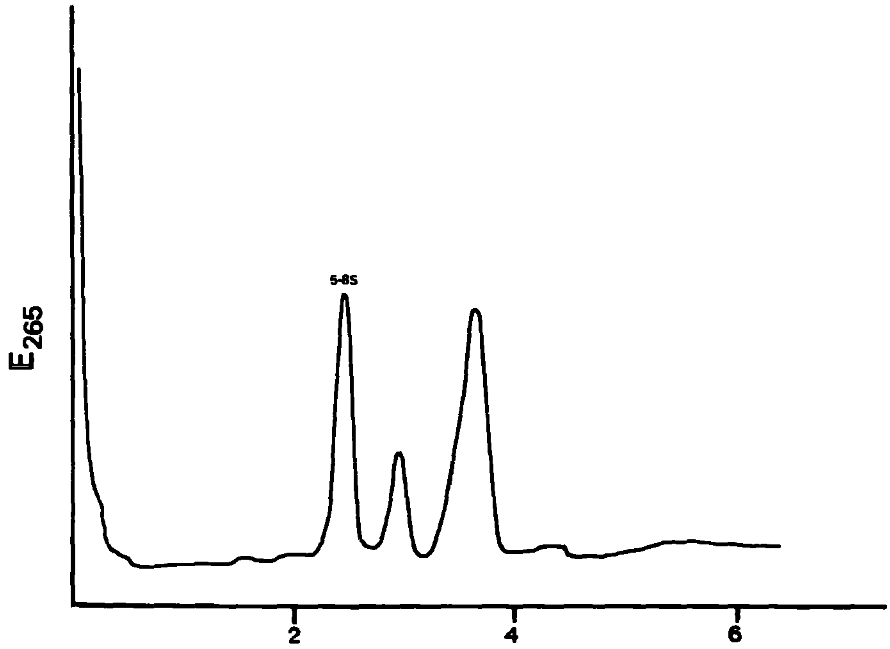
(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.

Fig. 38.

The nucleic acids of the dry seed.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A. The numbers above the peaks refer to their MW x $10^6$.


$E_{265}$
2
4
6


$E_{265}$
5·8S
2
4
6

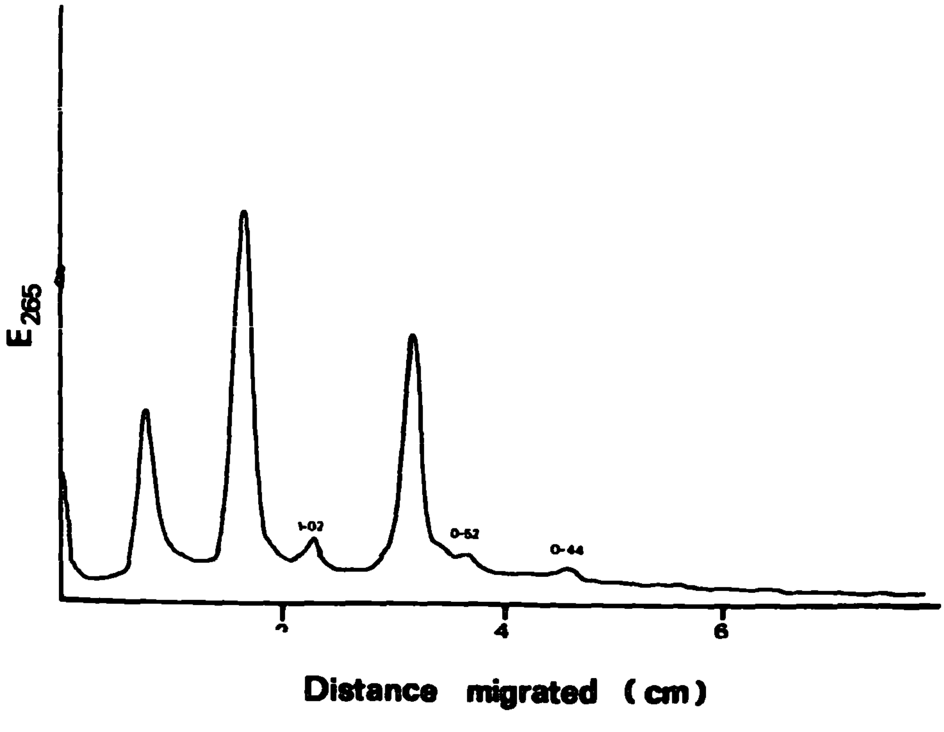

$E_{265}$
1·02
0·52
0·44
2
4
6
Distance migrated (cm)

Fig. 38.

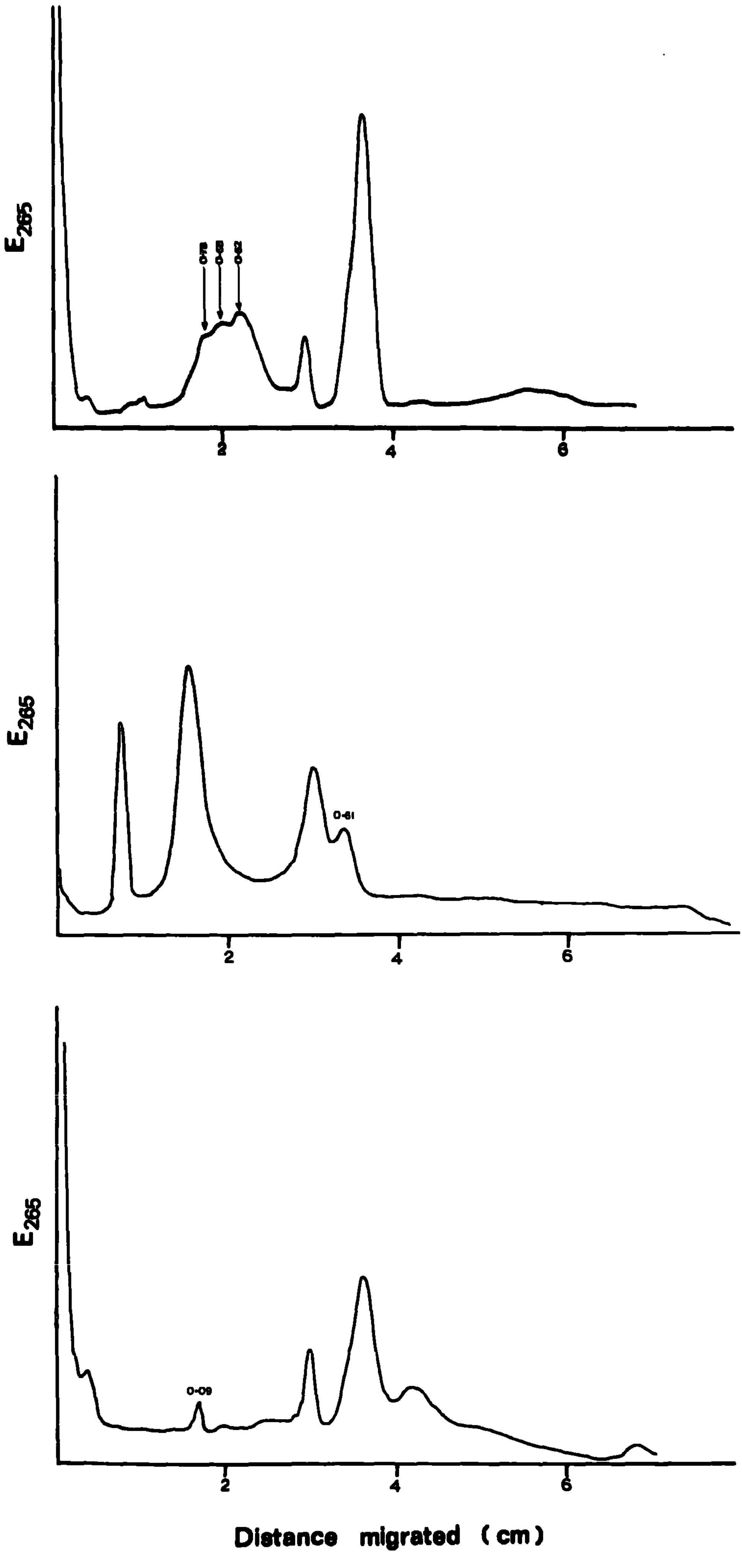
(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D. The numbers above the peaks refer to their MW x $10^5$.

Fig. 39.

40-day old cotyledon nucleic acids. The cotyledons were frozen in liquid $N_2$ and stored at -20°C for 6 months prior to extraction of the nucleic acids (methods section, 4(i)).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B. The numbers above the peak refer to its MW x $10^6$.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam C. The numbers above the peak refer to its MW x $10^5$.


E265
0·78
0·68
0·62
2
4
6
E265
0·61
2
4
6
E265
0·09
2
4
6
Distance migrated (cm)

(vii) The effect of storing beans at -20°C.

Following freezing in liquid $N_2$ and storage at -20°C, subsequent extraction and analysis of the nucleic acids on 2.6% and 7.5% polyacrylamide gels showed the breakdown of the 18S RNA into two fragments. The large fragment had a MW of $0.61 \times 10^6$. The small fragment had a MW of $0.90 \times 10^6$ (Figs. 39 a and b).

The sum of the MW's of the fragments is equal to the MW of the 18S RNA. Also the molar ratio, described by the equation:

$$\frac{\text{Area of 25S RNA} \times \text{MW of 18S RNA}}{(\text{Area of 18S RNA} + 0.61 \times 10^6 \text{ area}) \times \text{MW of 25S RNA}}$$

is approximately equal to 1. This indicates a precursor product relationship between the 18S RNA and the $0.61 \times 10^6$ daltons component.

The molar ratio of intact rRNA was determined from 30 samples of cotyledon and testa nucleic acids. The value obtained was 1.08 ± 0.14.

## 5. The 5.8S RNA

(i) Characterisation.

In addition to the major RNA's identified so far, an RNA species, having a MW of $0.52 \times 10^5$ daltons and a sedimentation coefficient of 5.8S, was identified in RNA samples extracted at 65°C (Figs. 37b and 64b) and RNA samples prepared from gradient purified monosomes (Fig. 71d). It also occurred in total nucleic acid samples extracted at 0°C and subsequently melted at 60°C for 10 min (Figs. 40 a to d). The effect of temperature on the release of the 5.8S RNA was determined by heating total RNA samples, extracted from 40-day old developing cotyledons, at temperatures between 20°C and 90°C for 10 min. After rapid cooling in liquid $N_2$ each sample was analysed on a 7.5% acrylamide gel and the ratio of 5.8S RNA to the 5S RNA determined (Fig. 41).

Ribosomal RNA extracted from pelleted ribosomes appeared to be undegraded on density gradient centrifugation (Fig. 42). Analysis of the

Fig. 40.

80-day old cotyledon nucleic acids.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C.

(b) RNA heated at 60°C for 10 min, then cooled rapidly. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C.

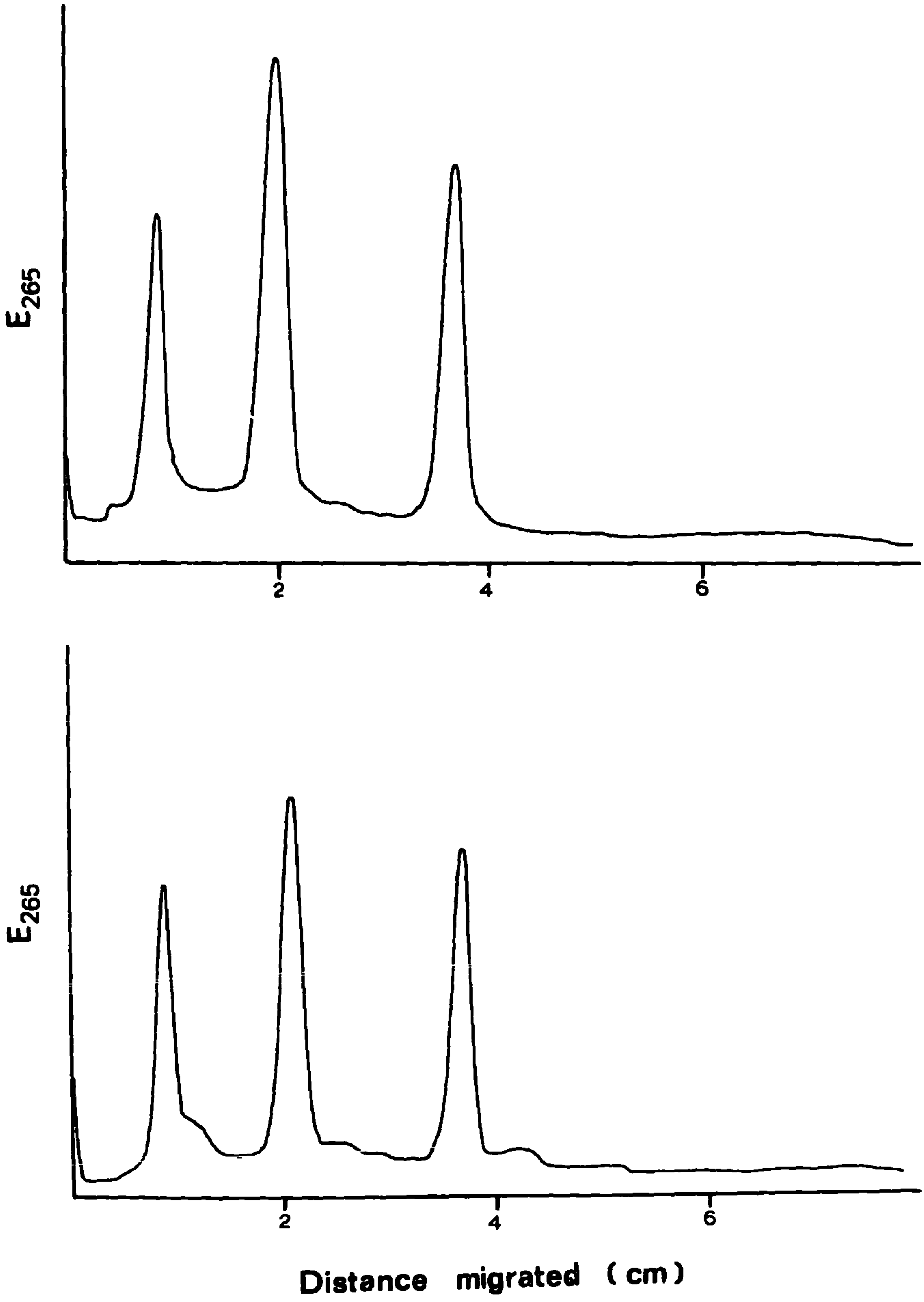
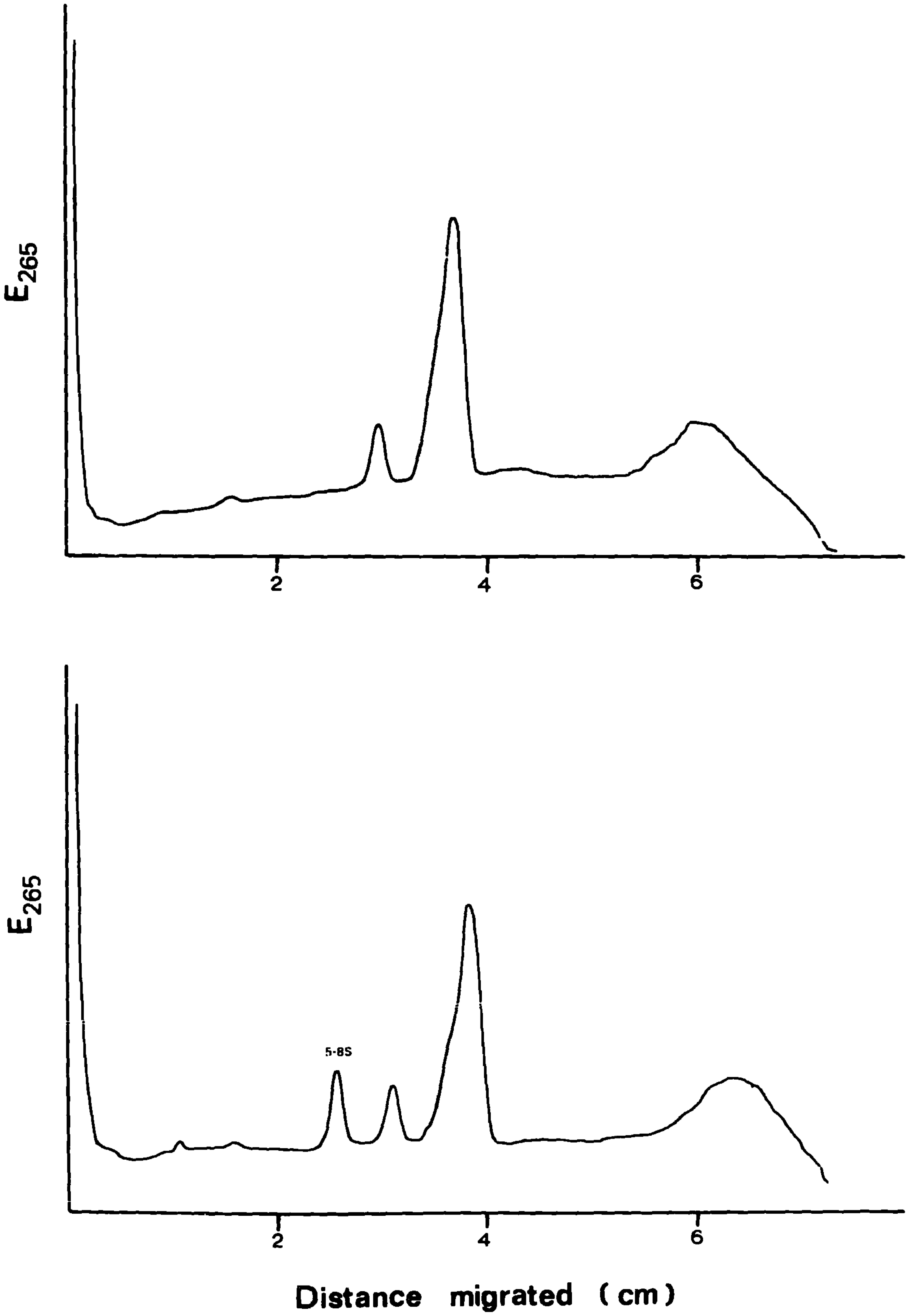

E265
2
4
6
E265
2
4
6
Distance migrated (cm)

Fig. 40.

(c) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.

(d) RNA heated at 60°C for 10 min, then cooled rapidly. Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.



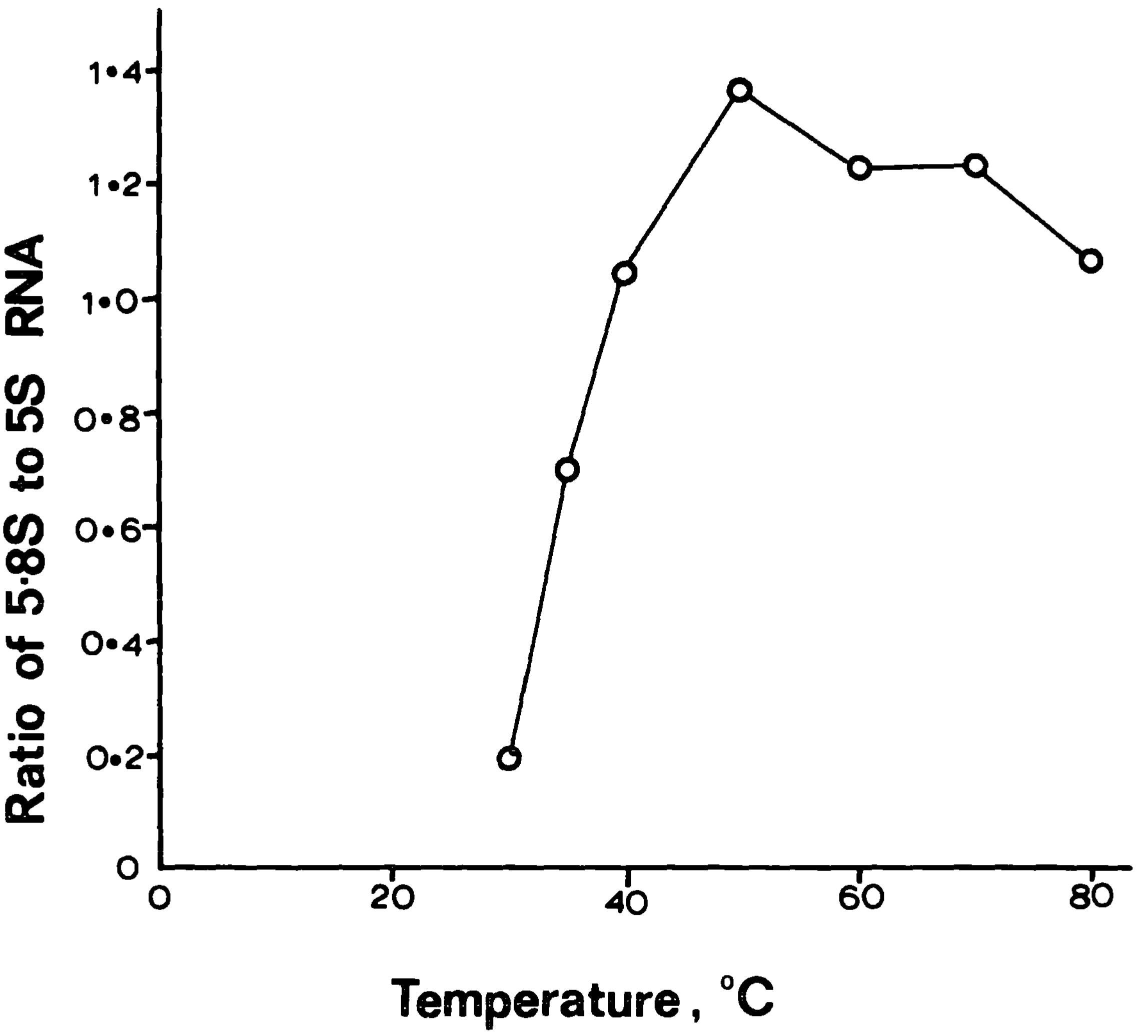
$E_{265}$
2
4
6
$E_{265}$
5·8S
2
4
6
Distance migrated (cm)

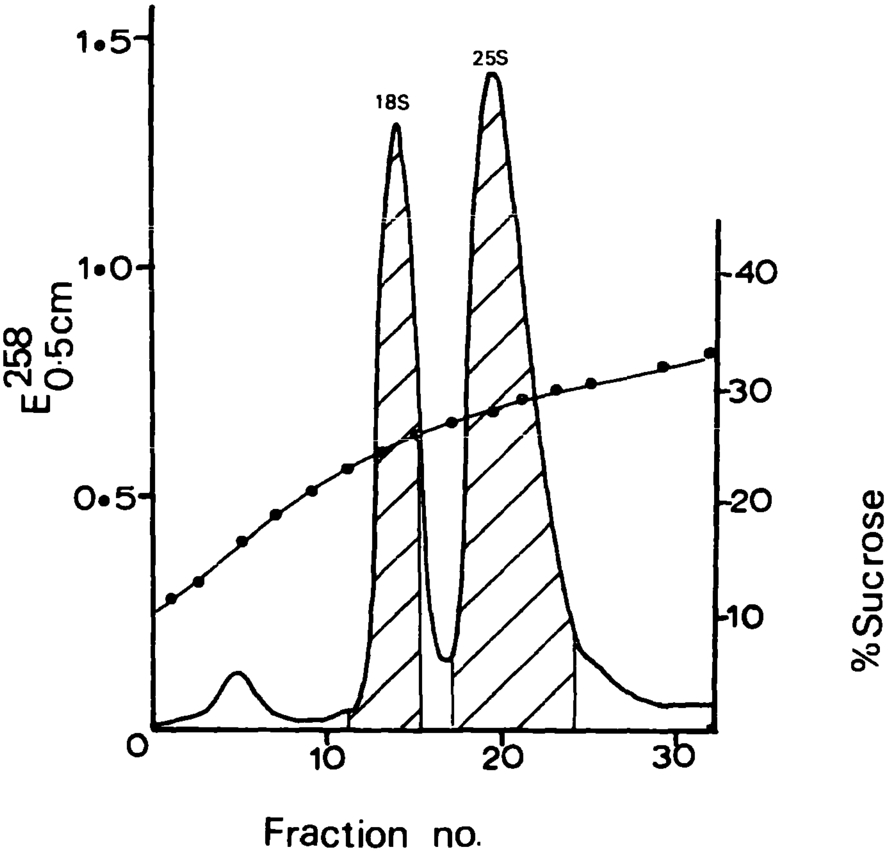
Fig. 41.

The effect of heat on the release of the 5.8S RNA. The RNA was dissolved in electrophoresis buffer containing 5% (w/v) sucrose at a concentration of 1 mg/ml. After heating for 10 min the RNA was cooled quickly and electrophoresed on a 7.5% gel for 3h. The relative amounts of 5.8S and 5S RNA were determined by integrating the peak areas.

Fig. 42.

Sucrose-gradient sedimentation profile of ribosomal RNA. The RNA was extracted from the ribosomal pellet, prepared from 50-day old cotyledons (methods section 7(ii)). The gradient was formed (methods section 6(iii)) from 10 ml 15% (w/v) sucrose and 19 ml 40% (w/v) sucrose, buffered with 10mM-sodium acetate•HCl, 100mM-NaCl, pH 5.1. 0.9 mg RNA was layered on to the gradient and centrifuged at 95,000 g av. for 24h at 4°C. 10 drop fractions were collected. Fractions were pooled (shaded) and the RNA collected by ethanol precipitation.


Ratio of 5·8S to 5S RNA
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
Temperature, °C


18S
25S
1·5
1·0
0·5
0
$E^{258}_{0·5cm}$
40
30
20
10
%Sucrose
10
20
30
Fraction no.

separated 25S and 18S RNA's on 2.6% and 7.5% acrylamide gels showed that the 18S RNA was degraded and that the 25S RNA contained some co-sedimenting 18S RNA (Figs. 43 a, b and 44). Following melting of the 25S and 18S RNA's at 60°C for 10 min, the 5.8S RNA was only detected in the 25S RNA fraction (Figs. 45 a and b, and 46 a and b).

Following the release of the 5.8S RNA from the 25S RNA there was a decrease in the 25S RNA peak area.

(ii) Attempted purification.

The 25S and 18S RNA's were purified by DEAE-cellulose chromatography and molar-salt precipitation (see previous section 3.(i) and (ii) and Figs. 17 a and b) and the 5.8S RNA released by heating at 60°C for 10 min (Figs. 47 a and b); this treatment caused the 25S and 18S RNA's to become aggregated.

The separation of the 5.8S RNA from the 25S and 18S RNA's by molecular sieve chromatography was not attempted as the theoretical yield of the 5.8S RNA would be 2.5% of the column capacity.

(a) Molar-salt precipitation

The heated preparation was extracted with 1M-LiCl and analysis of the 1M-LiCl supernatant fraction on 2.6% (trace not shown) and 7.5% acrylamide gels showed that separation of the aggregated 25S and 18S RNA's from the LMW RNA's was complete (Fig. 48). The components with a mobility greater than the 5.8S RNA had mobilities identical to 5S and 4S RNA. The components with a mobility less than the 5.8S RNA had MW's of 1.55, 1.05, 0.90, 0.78 and 0.68 x $10^5$ daltons respectively. Washing of the 1M-LiCl pellet with 2 vol. 1M-LiCl removed all entrapped LMW RNA's except for the 5.8S RNA, as shown by analysis of the resulting pellet and supernatant fractions on 7.5% acrylamide gels (Figs. 49 and 50).

An attempt to recover the 5.8S RNA from the pellet was made by high-speed centrifugation in 0.6M-LiCl. Analysis of the resulting supernatant

Fig. 43.

Sucrose-gradient purified 25S RNA (see Fig. 42).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.

Fig. 44.

Sucrose-gradient purified 18S RNA (see Fig. 42). Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A.

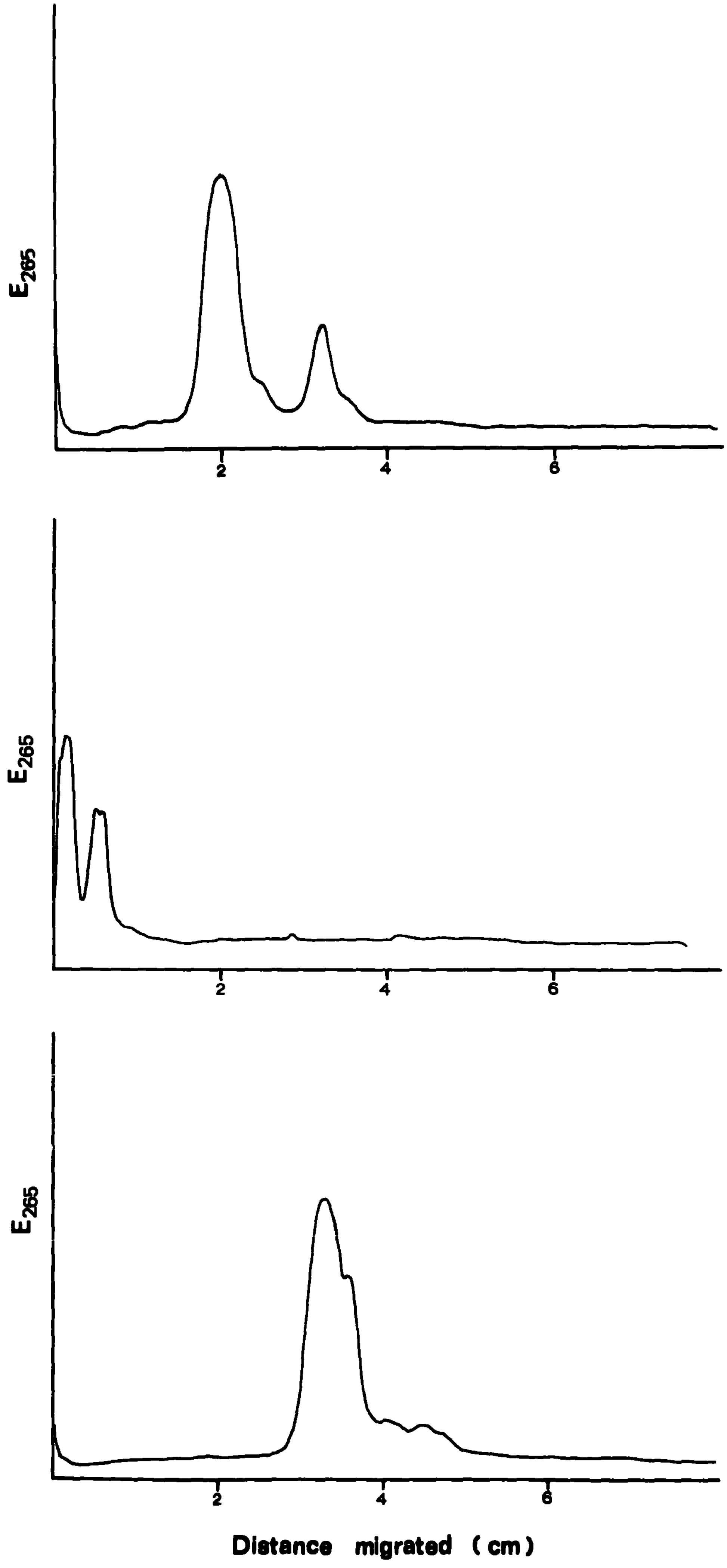

E265
2
4
6
E265
2
4
6
E265
2
4
6
Distance migrated (cm)

Fig. 45.

Release of the 5.8S RNA from sucrose-gradient purified 25S RNA (see Fig. 42). The RNA was heated at 60°C for 10 min then cooled rapidly, prior to loading on gels. The numbers above the peaks, except for the 5.8S RNA, refer to their MW x $10^6$.

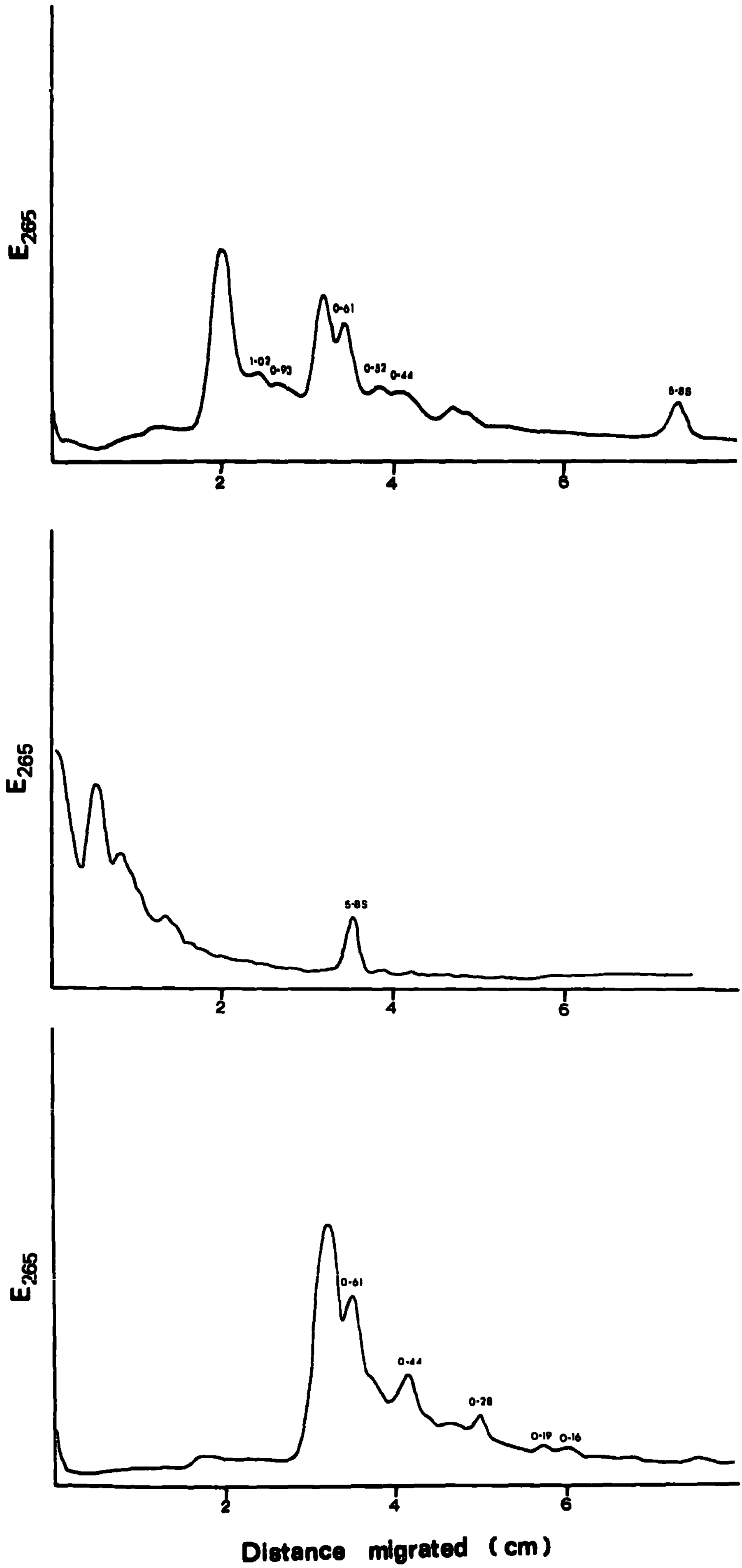
(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.

Fig. 46.

Sucrose-gradient purified 18S RNA (see Fig. 42), heated at 60°C for 10 min then cooled rapidly. The numbers above the peaks refer to their MW x $10^6$.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A.



E265
1·02
0·93
0·61
0·52
0·44
5·8S
2
4
6
E265
5·8S
2
4
6
E265
0·61
0·44
0·28
0·19
0·16
2
4
6
Distance migrated (cm)

Fig. 46.

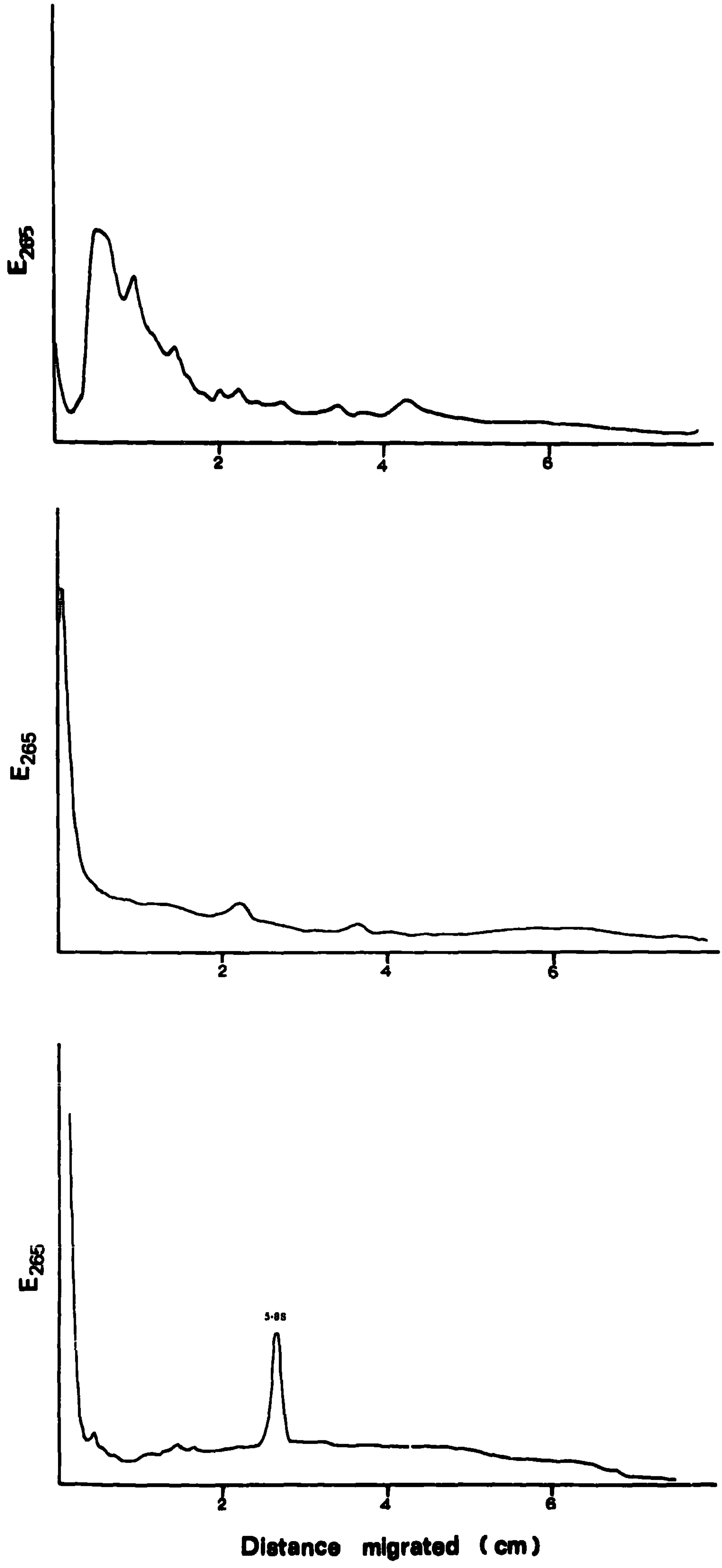
(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.

Fig. 47.

Release of the 5.8S RNA from purified 25S and 18S RNA. The 25S and 18S RNA's were purified by molar-salt precipitation (methods section 5(v)(a)) or by DEAE-cellulose chromatography (see Fig. 18) and heated at 60°C for 10 min, then cooled rapidly.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.



E265
2
4
6
E265
2
4
6
E265
5·8S
2
4
6
Distance migrated (cm)

Fig. 48.

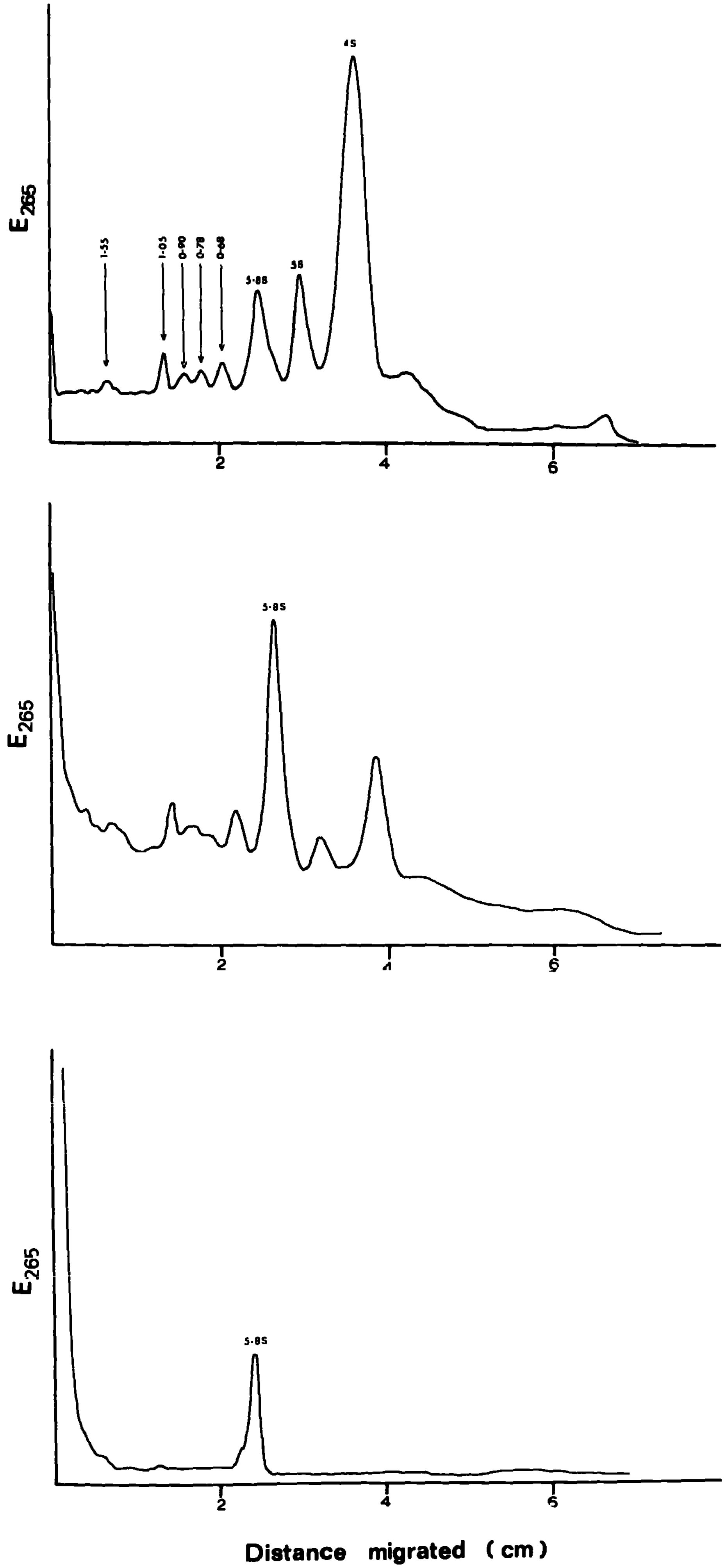
1M-LiCl supernatant RNA. The heat treated 25S and 18S RNA's (see Figs. 47a and b) were fractionated with 1M-LiCl as described in the methods, section 5(v)(a). Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam B. The numbers above the peaks refer to their MW x $10^5$, except for the 5.8S, 5S and 4S RNA's which are notated by their sedimentation coefficient.

Fig. 49.

The nucleic acids extracted from the 1M-LiCl pellet fraction with 1M-LiCl, following fractionation of heated 25S and 18S RNA's with 1M-LiCl, as described in the methods, section 5(v)(a). Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam B.

Fig. 50.

1M-LiCl pellet RNA. Heat treated 25S and 18S RNA's (see Figs. 47a and b) were fractionated with 1M-LiCl, as described in the methods, section 5(v)(a). Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.


E265
1·55
1·05
0·90
0·78
0·68
5·8S
5S
4S
2
4
6
E265
5·8S
2
4
6
E265
5·8S
2
4
6
Distance migrated (cm)

RNA on a 7.5% acrylamide gel (Fig. 51) showed the 5.8S RNA to be the main component but still impure.

(b) DEAE-cellulose chromatography

A similarly heated preparation to that used in the previous section was subjected to DEAE-cellulose chromatography and the 5.8S RNA fraction eluted with 1M-LiCl. When the 5.8S fraction was electrophoresed, there were 3 components on 7.5% acrylamide gels (Fig. 52). These were identified as 5.8S RNA, 5S RNA and 4S RNA.

Further attempts to purify the 5.8S RNA were based on modifying the initial RNA extraction procedure (see Methods 4.(vi),(b) and 4.(vi),(c)), in an attempt to reduce nuclease nicking of the 25S and 18S RNA molecules; and by molecular sieve chromatography of the 5.8S RNA fraction eluted from DEAE-cellulose on G75 (Fig. 54). Analysis of the fraction, on 7.5% acrylamide gels, eluting after the void volume showed the presence of low molecular weight fragments detected in earlier attempts (Fig. 53).

The yield of the 5.8S RNA fraction, by molar-salt precipitation and DEAE-cellulose chromatography, was approximately 1%.

DEAE-cellulose was normally used for the bulk separation of 25S and 18S RNA from the LMW RNA's; however, it was found that with smaller quantities of RNA, the 25S and 18S RNA's did not elute from the column (Fig. 55). The binding of the 25S and 18S RNA's to the DEAE-cellulose explained the presence of the 5S and 4S RNA contaminants in the 5.8S RNA fraction (Fig. 52), and further attempts to purify the 5.8S RNA were discontinued.

## 6. The isolation of polysomes and polysomal RNA

### (i) Effects of detergents.

Preliminary experiments were conducted on 2-day old seedlings of Phaseolus aureus. Sucrose density gradient analysis of isolated microsomes (Fig. 56) gave poor resolution of the ribosome units. The addition of

Fig. 51.

0.6M-LiCl supernatant RNA. The 1M-LiCl pellet obtained following 1M-LiCl fractionation of heated 25S and 18S RNA's was treated as described in the methods, section 5(v)(a). Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam B.

Fig. 52.

5.8S RNA fraction eluted off DEAE-cellulose with 1M-LiCl. Purified 25S and 18S RNA was heated at 60°C for 10 min, then rapidly cooled and bulk fractionated on DEAE-cellulose (see legend to Fig. 18). Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam A.

Fig. 53.

5.8S RNA fraction eluted off G75 (see Fig. 54). Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.

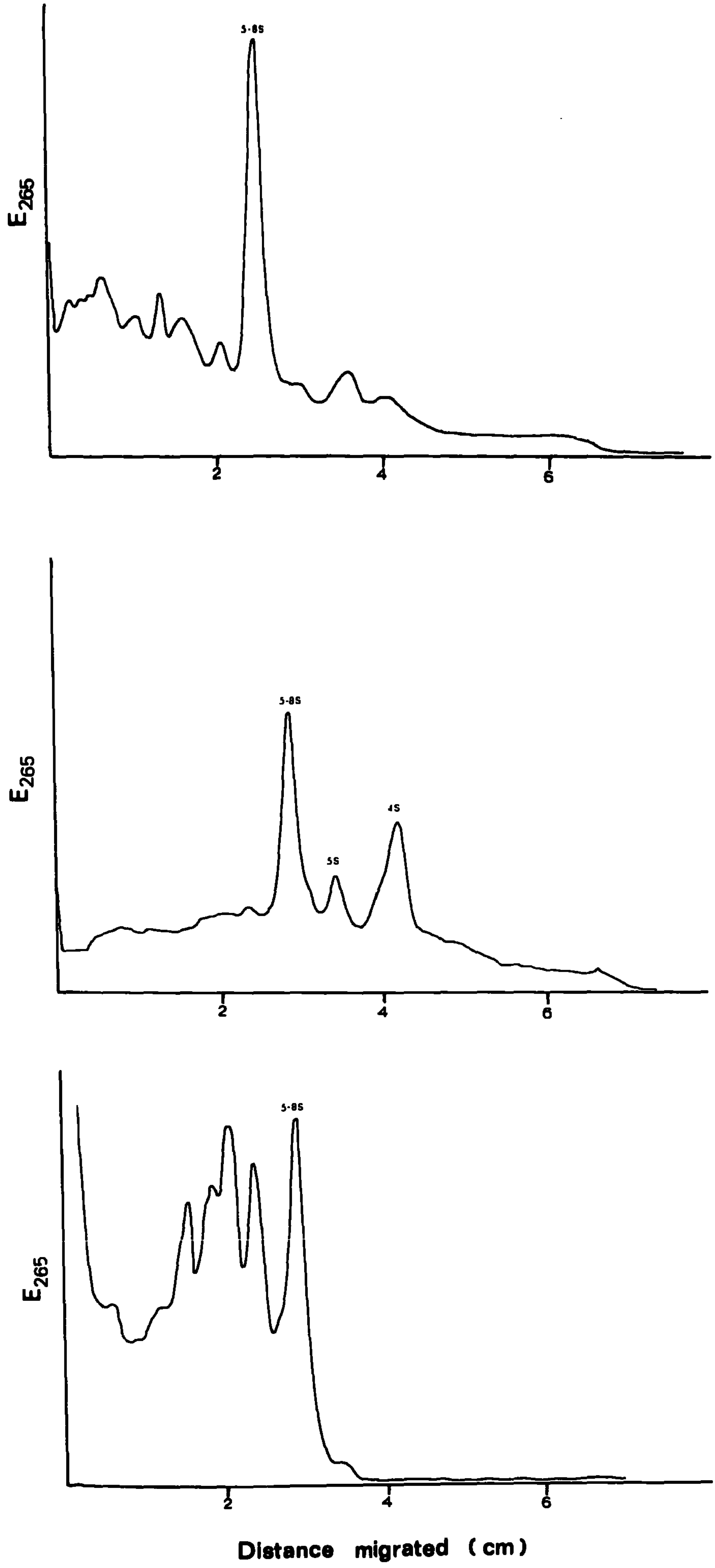
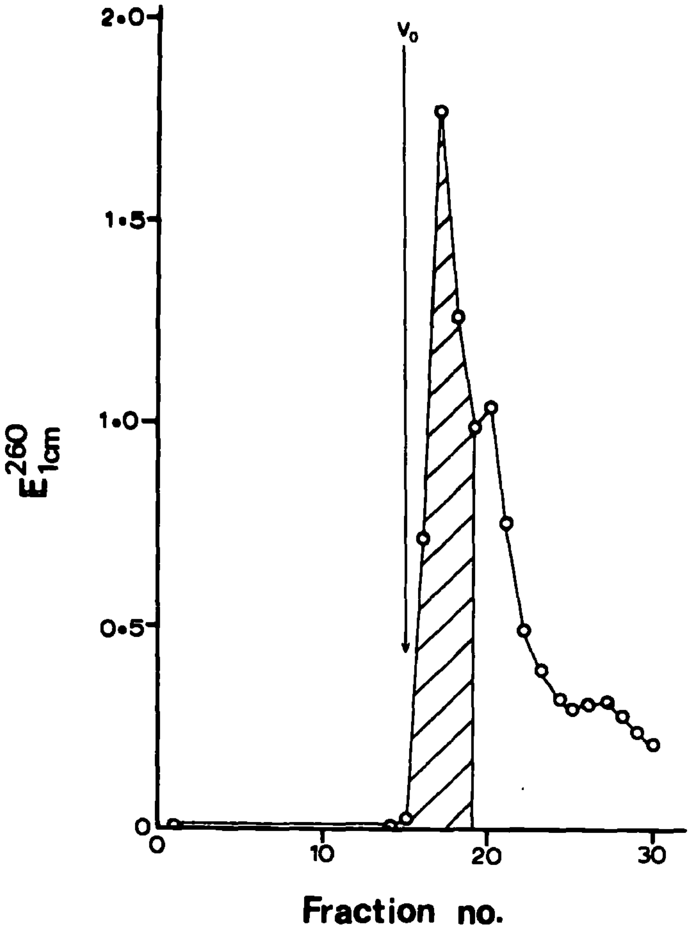

5·8S
E265
2
4
6
5·8S
5S
4S
E265
2
4
6
5·8S
E265
2
4
6
Distance migrated (cm)

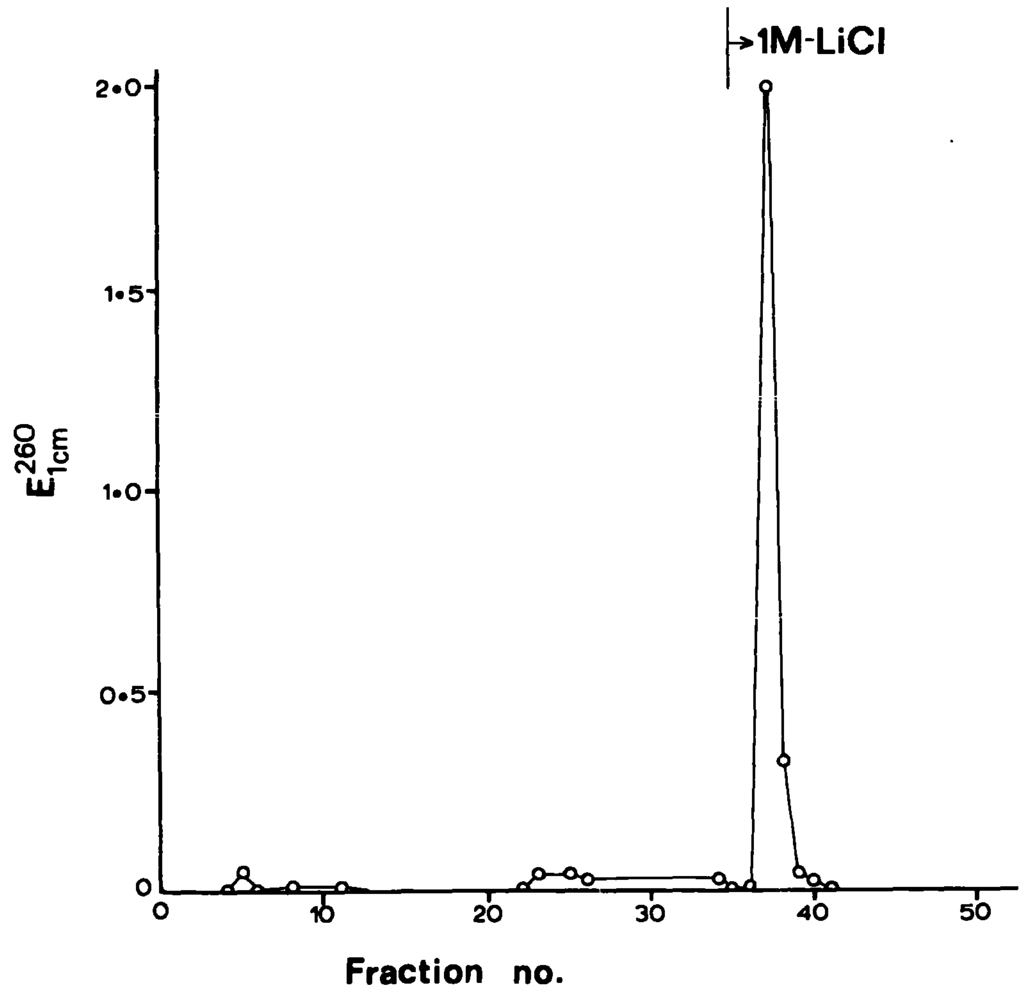
Fig. 54.

Sephadex G75 elution pattern of the 5.8S RNA fraction eluted off DEAE-cellulose with 1M-LiCl. 4 mg RNA was applied to the column. Column parameters are given in Fig. 21. The shaded area beneath the peak was collected for further analysis (see Fig. 53).

Fig. 55.

DEAE-cellulose elution profile of heated 25S and 18S RNA (see Figs. 47a and b). 10 mg RNA were applied to a 1 x 28 cm column of DEAE-cellulose. The low molecular weight RNA's were eluted with 1M-LiCl. 9 ml fractions were collected.


2·0
1·5
1·0
0·5
0
$E_{1cm}^{260}$
$V_0$
0
10
20
30
Fraction no.


→1M-LiCl
2·0
1·5
1·0
0·5
0
$E_{1cm}^{260}$
0
10
20
30
40
50
Fraction no.

sodium deoxycholate or triton x-100 to the post-mitochondrial supernatant improved the resolution of the ribosome units on the gradient (Figs. 57 and 58). The monomeric unit, the monosome, was assigned to the third peak from the top of the gradient on the basis of the results of other workers (Lin _et al._, 1966). Subsequent analysis of the nucleic acids present in this fraction showed the presence of both 25S and 18S RNA molecules (see section (v)). Particles having a greater sedimentation rate than the monosome, were assumed to be polysomes and the two peaks having a sedimentation rate less than the monosome peak were assumed to be the subunits.

Ribosomal subunits were present to varying extents in the different preparations. In the triton x-100 treated preparation (Fig. 58) they were probably hidden by the monosome peak. The ionic detergent sodium deoxycholate apparently caused a partial breakdown of polysomes and ribosomes with the formation of ribosomal subunits when compared to the non-ionic detergent triton x-100 (Figs. 57 and 58). Both sodium deoxycholate and triton x-100 produced identical effects on preparations from the developing cotyledons of _V. faba_ (Figs. 59 and 60).

The polysome profiles obtained from 40, 50 and 60-day developing cotyledons of _V. faba_ were similar, and are represented by one figure (Fig. 59).

The storage of polysome preparations at -70°C had no effect on their gradient profile, but breakdown was substantial on preparations which had been frozen and thawed for a second time (Fig. 61). The breakdown products were mainly ribosomal subunits.

The storage of microsomes at 0°C for 24 h resulted in the breakdown of polysomes into monosomes and subunits, as judged by a comparison of Figs. 62 with 59.

### (ii) The RNA's of the mitochondrial pellet.

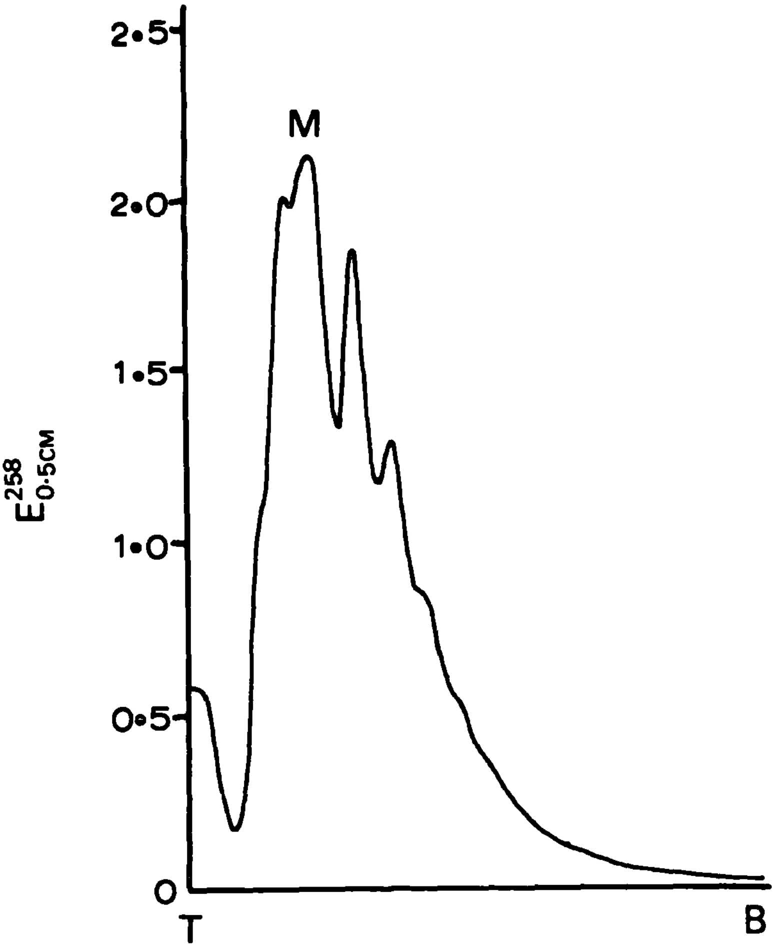
The extraction of the nucleic acids from the mitochondrial pellet

Fig. 56.

Sucrose-gradient sedimentation profile of *Phaseolus aureus* microsomes. The gradient was prepared from 10 ml 8% (w/v) sucrose and 20 ml 30% (w/v) sucrose, buffered with 10mM-Tris, 25mM-KCl, 1mM-$MgCl_2$, pH 7.6, as described in the methods, section 6(iii). 2 mg microsomes were layered on to the gradient and centrifuged at 65,000 g av. for 2h at 4°C. M = monosome. T and B = top and bottom of gradient respectively.

Fig. 57.

Sucrose-gradient sedimentation profile of sodium deoxycholate-treated polysomes from *P. aureus*. The polysomes were prepared as described in the methods (section 7(ii)), the post-mitochondrial supernatant being made 0.2% (w/v) with respect to sodium deoxycholate. 1.23 mg of suspended polysomes were layered on to the gradient. Gradient parameters are given in the legend to Fig. 56.


M
2·5
2·0
1·5
1·0
0·5
0
$E^{258}_{0\cdot5CM}$
T
B

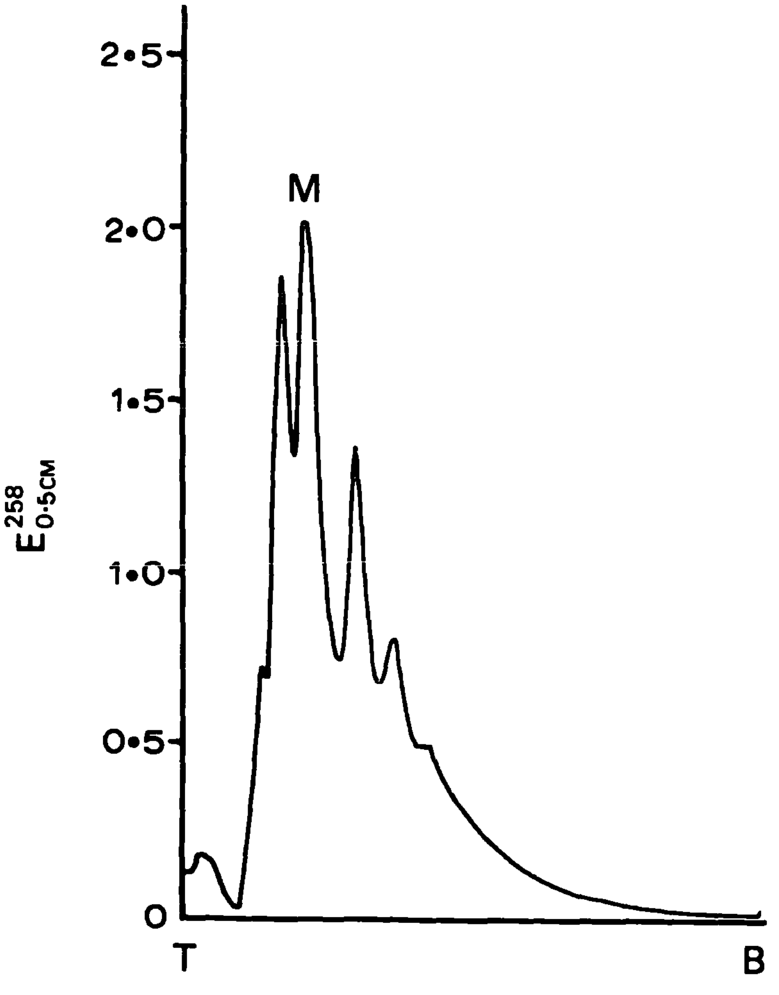
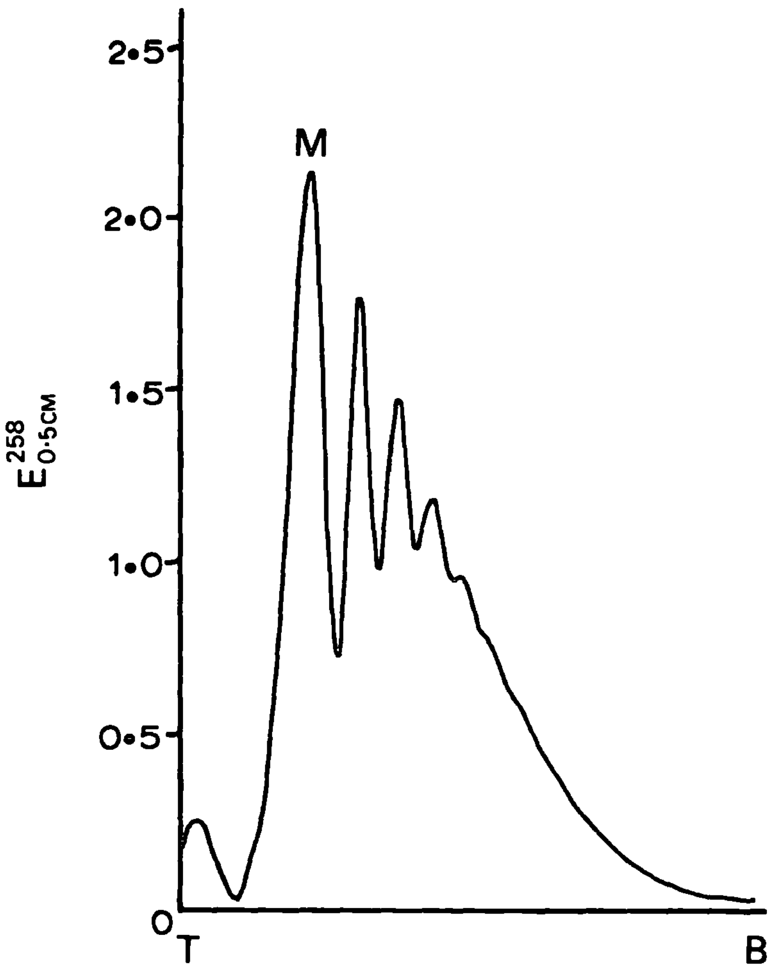

M
2·5
2·0
1·5
1·0
0·5
0
$E^{258}_{0\cdot5CM}$
T
B

Fig. 58.

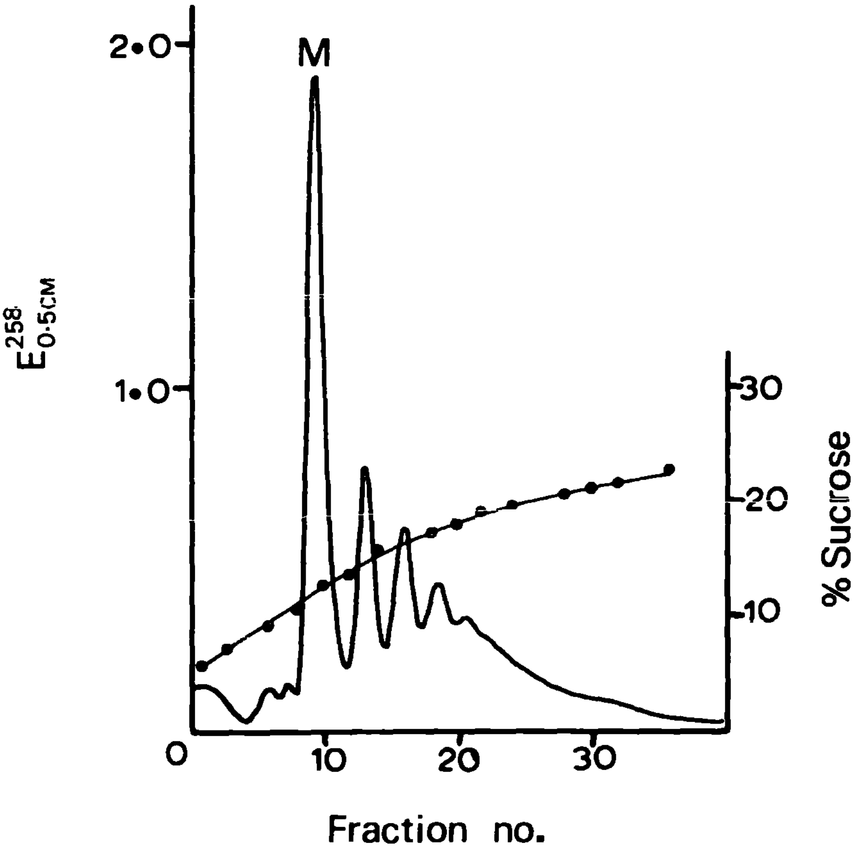
Sucrose-gradient sedimentation profile of triton X-100 treated polysomes from _P. aureus_. The polysomes were prepared as described in the methods (section 7(ii)), the post-mitochondrial supernatant being made 4% (v/v) with respect to triton X-100. 1.8 mg of suspended polysomes were layered on to the gradient. Gradient parameters are given in the legend to Fig. 56.

Fig. 59.

Sucrose-gradient sedimentation profile of triton X-100 treated polysomes from 40-day old cotyledons of _V. faba_. The polysomes were prepared as described in the methods (section 7(ii)), the post-mitochondrial supernatant being made 4% (v/v) with respect to triton X-100. 1.0 mg of suspended polysomes were layered on to the gradient. Gradient parameters are given in the legend to Fig. 56. The gradient was fractionated into 10 drop fractions.



2•5
2•0
1•5
1•0
0•5
0
$E^{258}_{0\cdot5CM}$
M
T
B



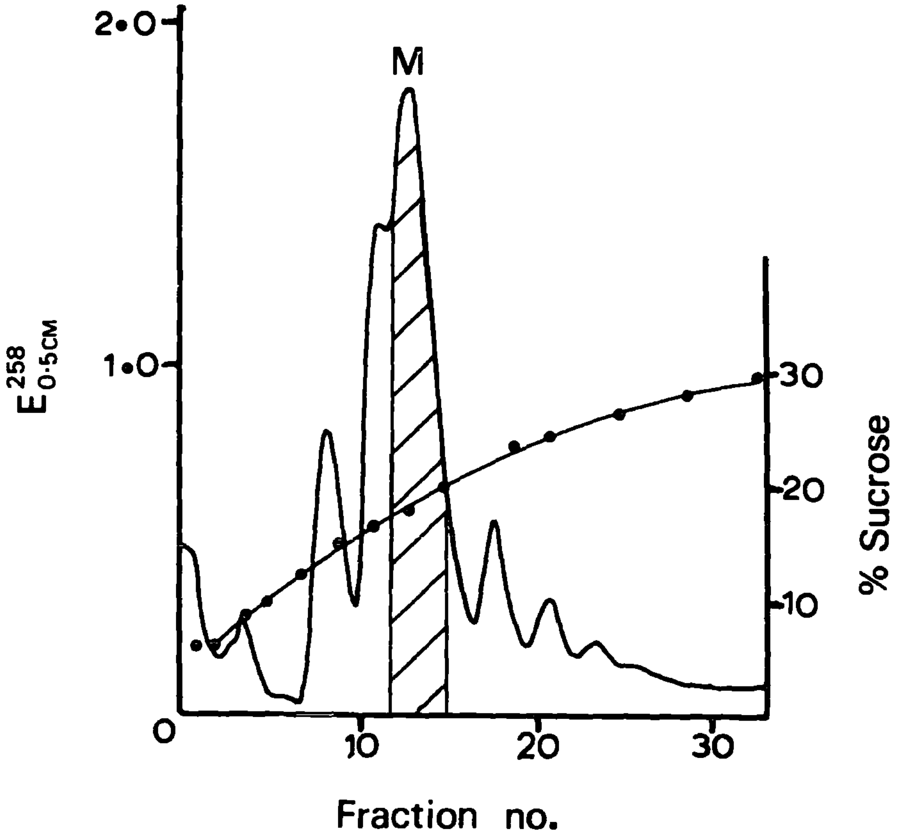
2•0
1•0
0
$E^{258}_{0\cdot5CM}$
M
10
20
30
Fraction no.
30
20
10
% Sucrose

Fig. 60.

Sucrose-gradient sedimentation profile of sodium deoxycholate-treated polysomes from 70-day old cotyledons of V. faba. The polysomes were prepared as described in the methods; the post-mitochondrial supernatant being made 0.2% (w/v) with respect to sodium deocycholate. The gradient was prepared from 10 ml 8% (w/v) sucrose and 19 ml 40% (w/v) sucrose, buffered with 10mM-Tris, 25mM-KCl, 1mM-$MgGl_2$, pH 7.6, as described in the methods, section 6(iii). 2.7 mg polysomes were layered on to the gradient and centrifuged at 95,000 g av. for 3h at 4°C. The gradient was fractionated into 10 drop fractions and the monosome peak (shaded) collected, and the RNA extracted as described in the methods, section 4(iv)(a).

Fig. 61.

Sucrose-gradient sedimentation profile of sodium deoxycholate-treated polysomes, frozen and thawed twice, from P. aureus. Gradient parameters are given in the legend to Fig. 56.


M
2•0
1•0
0
$E^{258}_{0\cdot5CM}$
10
20
30
Fraction no.
30
20
10
% Sucrose

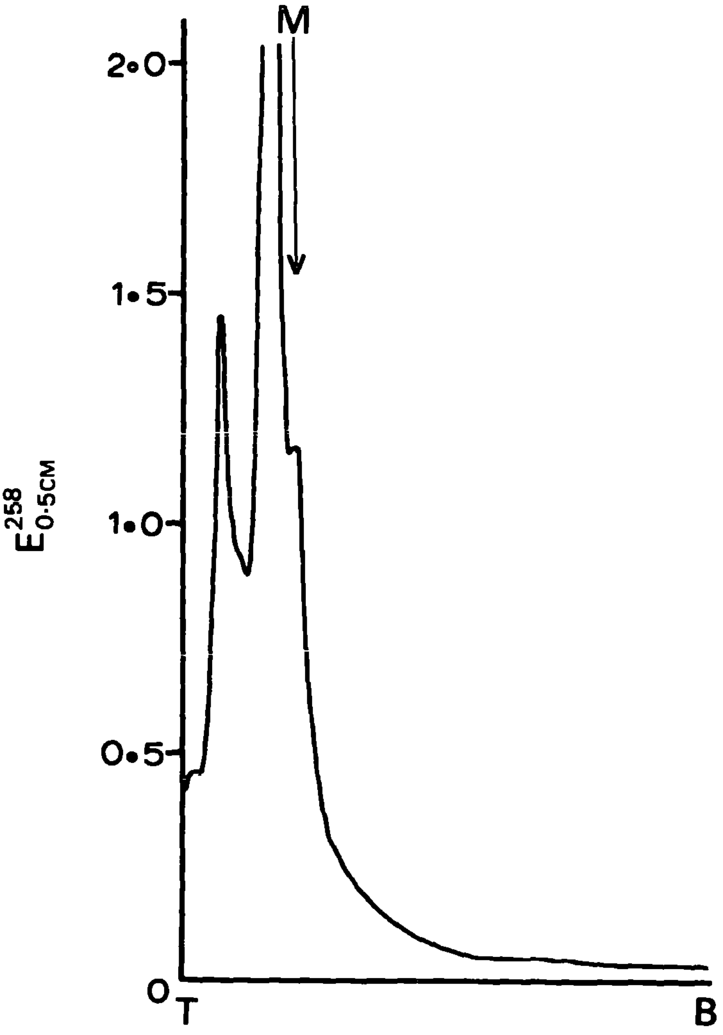
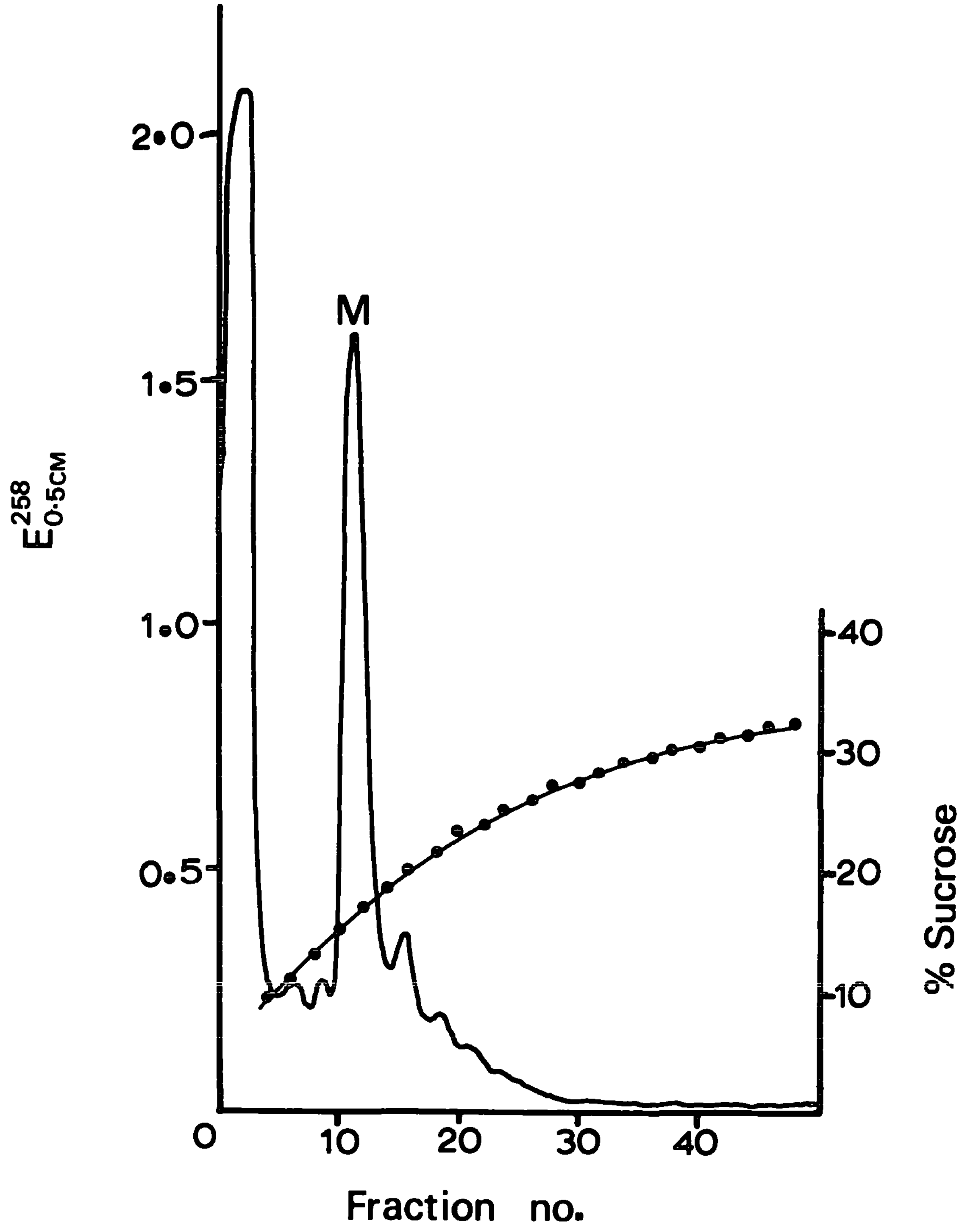

M
2•0
1•5
1•0
0•5
0
$E^{258}_{0\cdot5CM}$
T
B

Fig. 62.

Sucrose-gradient sedimentation profile of triton X-100 disrupted microsomes from 50-day old cotyledons of *V. faba*. The microsomes, extracted as described in the methods (section 7(ii)), were kept at 0°C for 24h. The gradient was prepared as described in the legend to Fig. 60 and fractionated into 0.38 ml fractions. 1 mg of triton X-100 disrupted microsomes were layered on to the gradient and centrifuged at 65,000 g av. for 2h at 4°C.


2•0
1•5
1•0
0•5
$E^{258}_{0\cdot 5CM}$
M
0
10
20
30
40
Fraction no.
40
30
20
10
% Sucrose

(Methods, section 4.(iii)) normally yielded RNA's in a high state of aggregation (Fig. 63). Melting, at 60°C for 10 min, released the aggregates and showed that they were degraded (Figs. 64 a and b). DNA and 5.8S RNA were detected in these samples.

(iii) The RNA's of the post-mitochondrial and 105,000 g supernatants.

Analysis of the nucleic acids, extracted, as described (Methods, section 4.(iv),(a)), on acrylamide gels showed four major components: 25S RNA, 18S RNA, 5S RNA and 4S RNA (Figs. 65, a and b; 66a and b). The 25S RNA and the 18S RNA were the main components of the post-mitochondrial supernatant, whereas the 4S RNA was the main component of the 105,000 g supernatant. The 18S RNA was degraded to a greater extent in the 105,000 g supernatant than in the post-mitochondrial supernatant (compare Figs. 65a and 66a).

(iv) The RNA's of the ribosomal pellet.

Analysis of the nucleic acids present in the ribosomal pellet on acrylamide gels showed that the rRNA's were degraded. The degree of breakdown varied with each preparation, and the maximum and minimum limits of the 18S RNA breakdown are illustrated (Figs. 67 and 68a).

The amount of 4S RNA present in the ribosomal pellet (Fig. 68b) was visibly lower than the amounts present in the post-mitochondrial and 105,000 g supernatants. The molar ratio of 4S RNA to 5S RNA was approximately 1.5.

The 5.8S RNA was detected in these preparations (Fig. 68b).

Following the separation of the ribosomal pellet components by sucrose density gradient centrifugation (Fig. 59), the gradient was fractionated into a light polysome fraction comprised of subunits, monomers, dimers, trimers and tetramers and a heavy polysome fraction, comprised of particles heavier than the tetramer. The rRNA's of both fractions were similar and degraded (Fig. 69) to a greater extent than the rRNA's extracted directly from the ribosomal pellet (Fig. 67).

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SECTION
LIBRARY

Fig. 63.

The nucleic acids of the mitochondrial pellet, extracted, as described in the methods (section 4(iii)), from 40-day old cotyledons. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam D.

Fig. 64.

The nucleic acids of the mitochondrial pellet, extracted from 40-day old cotyledons, heated at 60°C for 10 min then cooled rapidly.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.

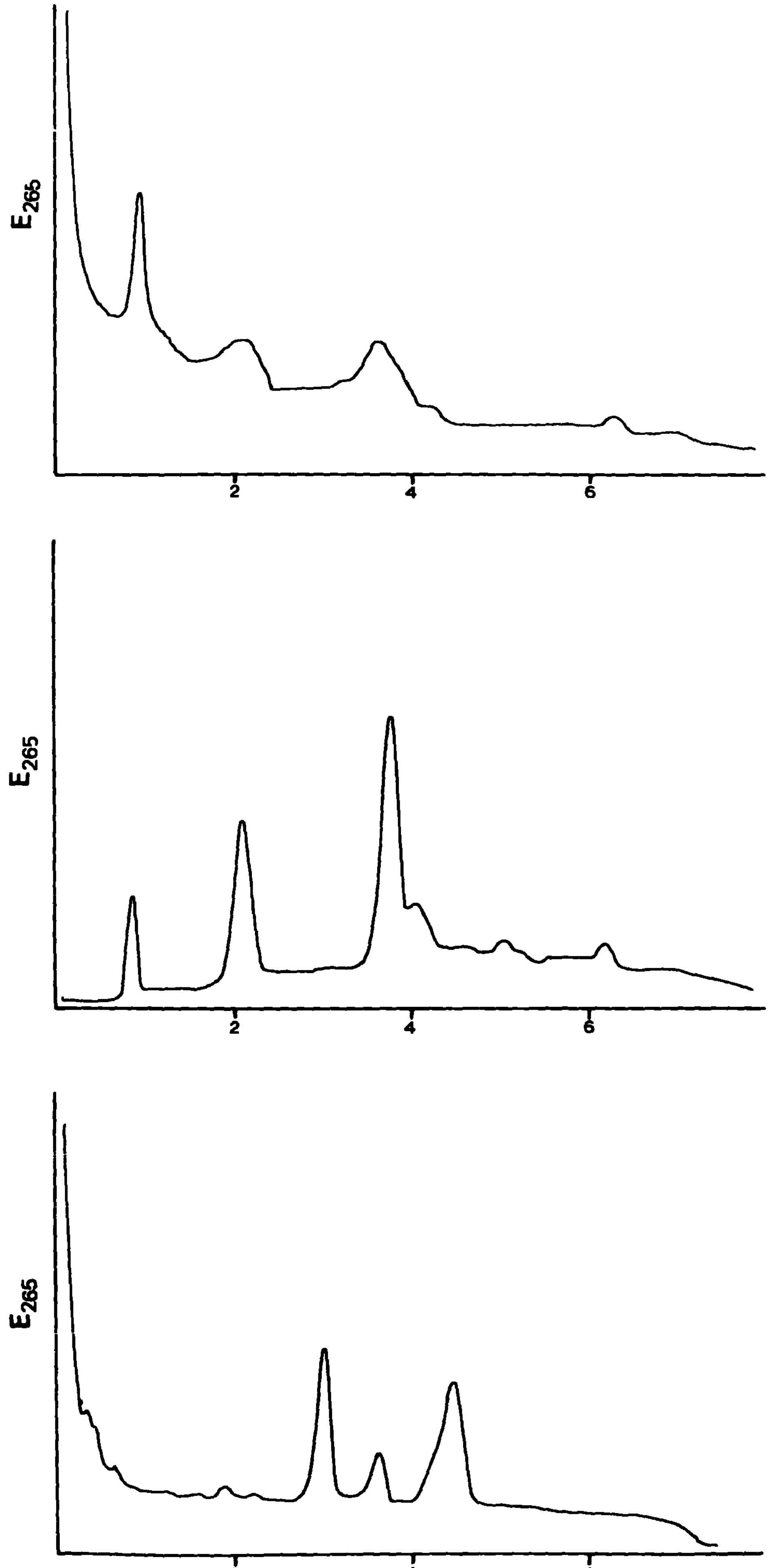

$E_{265}$
2
4
6
$E_{265}$
2
4
6
$E_{265}$
2
4
6
Distance migrated (cm)

Fig. 65.

The nucleic acids of the post-mitochondrial supernatant, extracted as described in the methods (section 4(iv)(a)), from 40-day old cotyledons.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A.

(b) Electrophoresed on a 5.0% gel for 3h. Vertical expansion : cam A.

Fig. 66.

The nucleic acids of the 105,000 g supernatant, extracted as described in the methods (section 4(iv)(a)), from 40-day old cotyledons.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

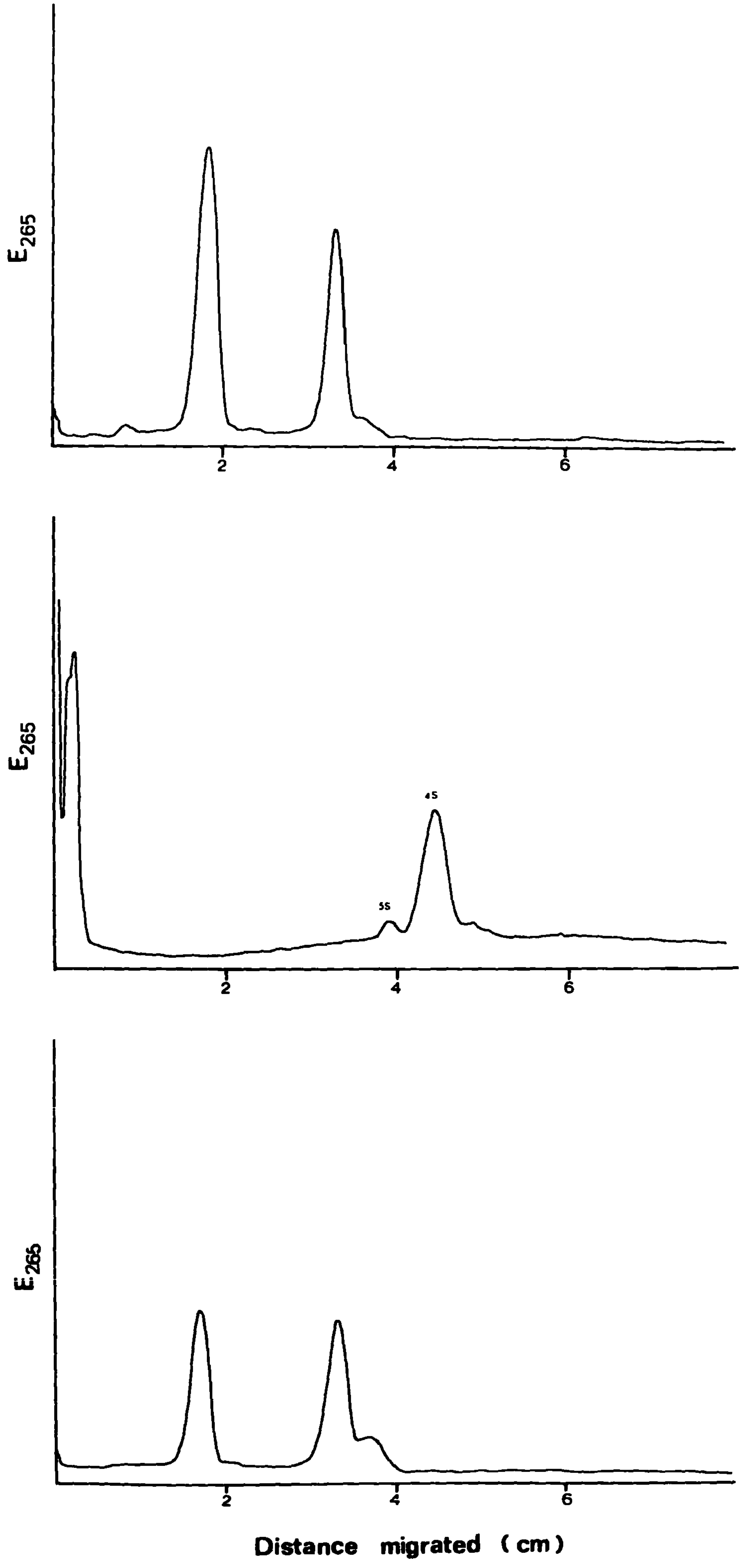

E$_{265}$
2
4
6
E$_{265}$
5S
4S
2
4
6
E$_{265}$
2
4
6
Distance migrated (cm)

Fig. 66.

(b) Electrophoresed on a 5.0% gel for 3h. Vertical expansion : cam B.

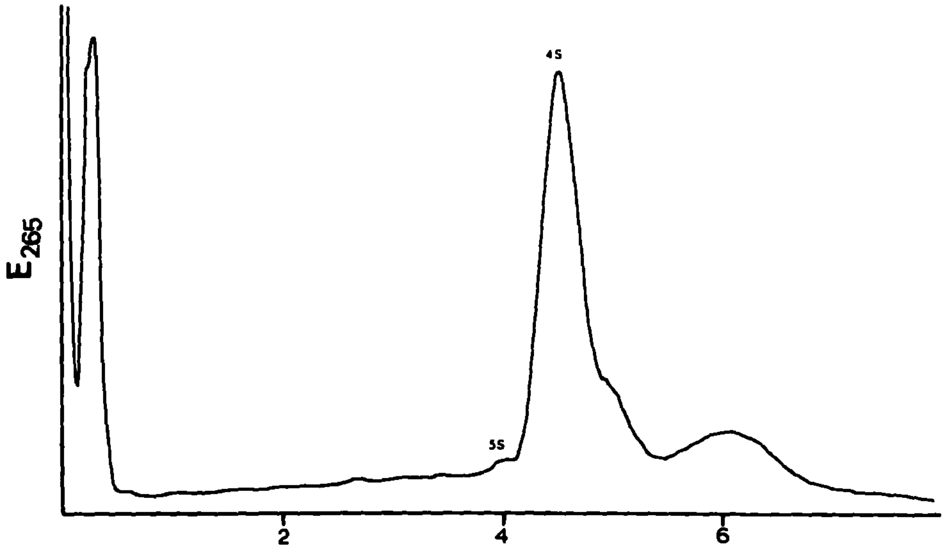
Fig. 67.

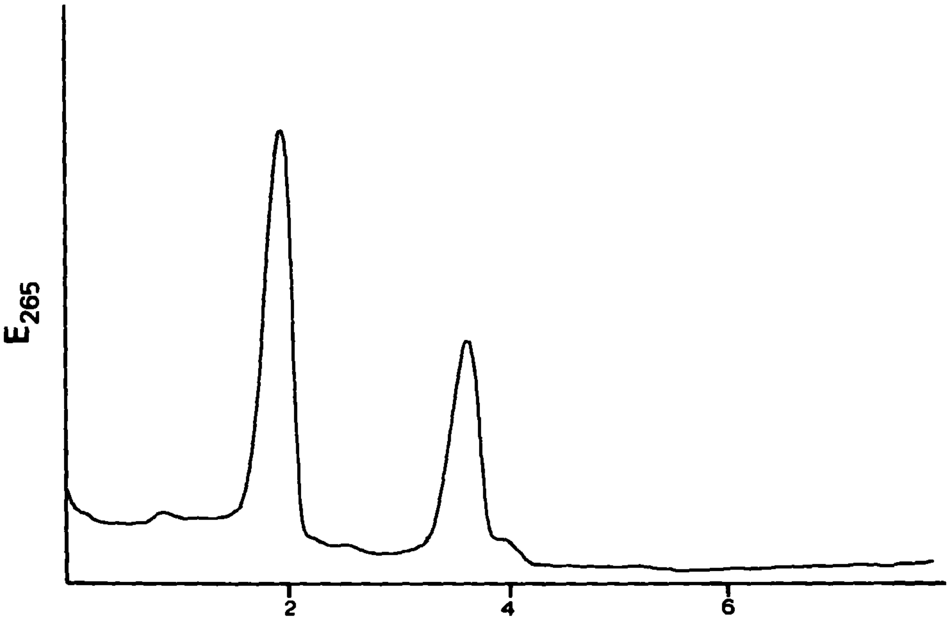
The nucleic acids of the ribosomal pellet, extracted as described in the methods (section 4(iv)(a)), from 50-day old cotyledons. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

Fig. 68.

The nucleic acids of the ribosomal pellet, extracted as described in the methods (section 4(iv)(a)), from 40-day old cotyledons.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B. The numbers above the peaks refer to their MW x $10^6$.


E$_{265}$
4S
5S
2
4
6


E$_{265}$
2
4
6

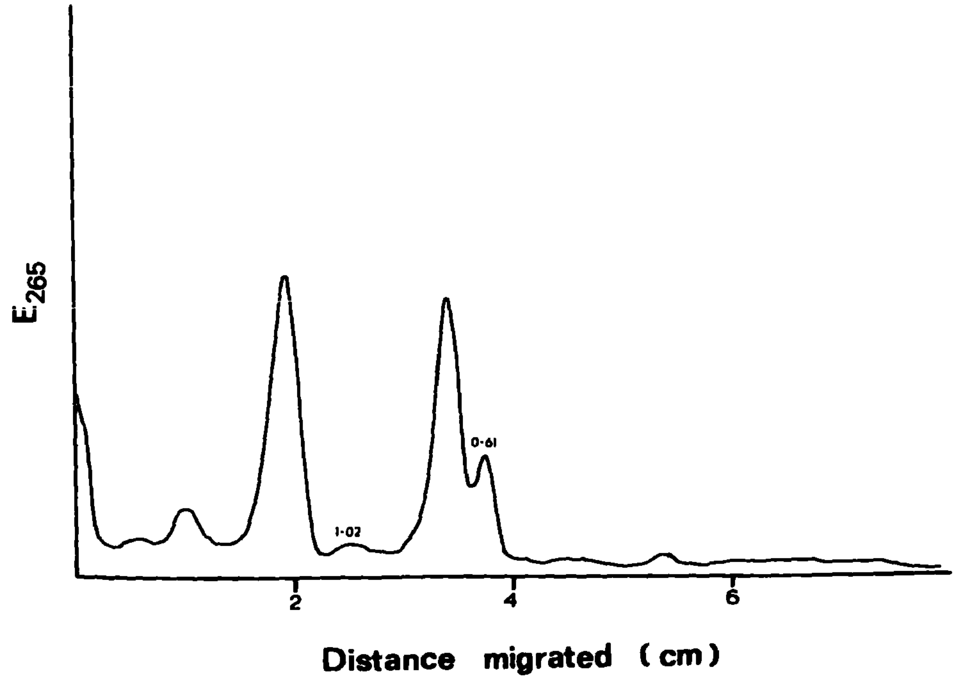

E$_{265}$
0·61
1·02
2
4
6
Distance migrated (cm)

Fig. 68.

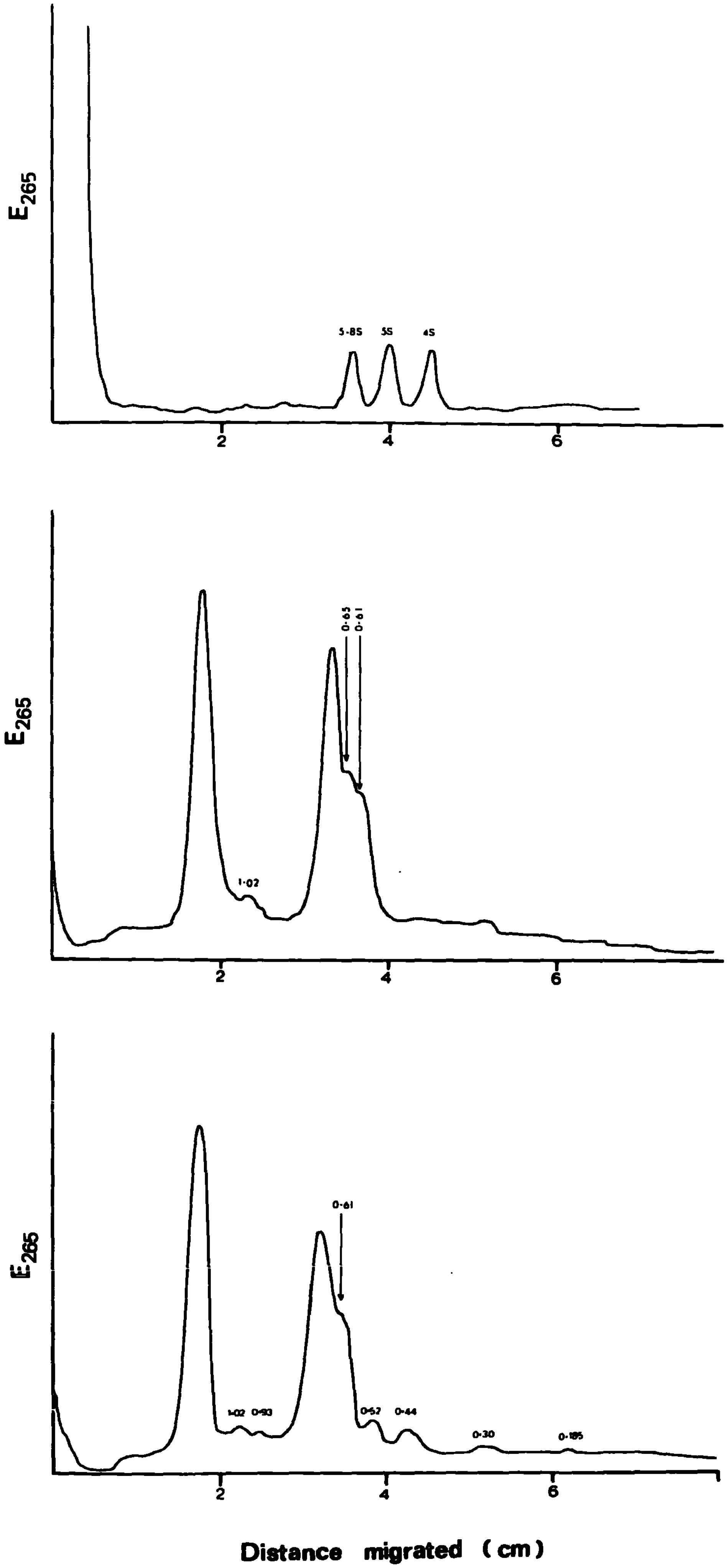
(b) Electrophoresed on a 5.0% gel for 3h. Vertical expansion : cam D.

Fig. 69.

The nucleic acids of the light-polysome fraction (see text), prepared from 40-day old cotyledons. The ribosome units were separated by sucrose-gradient centrifuge (see Fig. 59) and the nucleic acids extracted as described in the methods, section 4(iv)(a). Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam D. The numbers above the peaks refer to their MW x $10^6$.

Fig. 70.

The nucleic acids of microsomes, prepared from 50-day old cotyledons (see also Fig. 62). The nucleic acids were prepared,as described in the methods (section 4(iv)(b)), from microsomes which had been stored at $0^oC$ for 24h. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C. The numbers above the peaks refer to their MW x $10^6$.


E265
5·8S
5S
4S
2
4
6
E265
1·02
0·65
0·61
2
4
6
E265
0·61
1·02
0·93
0·52
0·44
0·30
0·185
2
4
6
Distance migrated (cm)

The nucleic acids extracted from the microsomal pellet (see Fig. 62), which had been stored at 0°C for 24 h, showed severe breakdown of the rRNA's (Fig. 70).

(v) Induced breakdown of ribosomal RNA.

There was little degradation of the rRNA when bentonite was used in the extraction of polysomes from the developing cotyledons of *V. faba* (see previous section). The breakdown products detected in these samples had MW's of 1.02, 0.93 (Fig. 70), 0.65 (Fig. 69), and $0.61 \times 10^6$ daltons respectively, and originated from the 25S and 18S rRNA's. Of the two rRNA molecules the 18S RNA was by far the most labile.

The extraction of polysomes in the absence of bentonite and in the presence of the ionic detergent sodium deoxycholate, resulted in a breakdown of polysomes, to monosomes and subunits (see section (i), and Fig. 60). The analysis of the monosome RNA's on 2.6% acrylamide gels showed substantial degradation of the rRNA's (Fig. 71a). The observed breakdown products were determined to have MW's of 1.02, 0.61, 0.44 and $0.31 \times 10^6$ daltons. The latter two components appeared to be heterogeneous in nature, and had previously been detected in the mitochondrial pellet RNA (Fig. 64a).

Melting of the monosome RNA at 60°C for 10 min resulted in the release of a large number of fragments (Fig. 71b) and an apparent molar excess of 18 rRNA (cf. Fig. 64a). The $0.44 \times 10^6$ daltons component, which was observed to be heterogeneous in previous samples, was resolved into two components having MW's of 0.44 and $0.42 \times 10^6$ daltons. The other breakdown product, having a MW of $0.31 \times 10^6$ daltons, which had also been observed previously to be heterogeneous, was not further resolved. Other fragments not previously detected were determined to have MW's of 1.13, 0.79, 0.52, 0.20 and $0.13 \times 10^6$ daltons respectively.

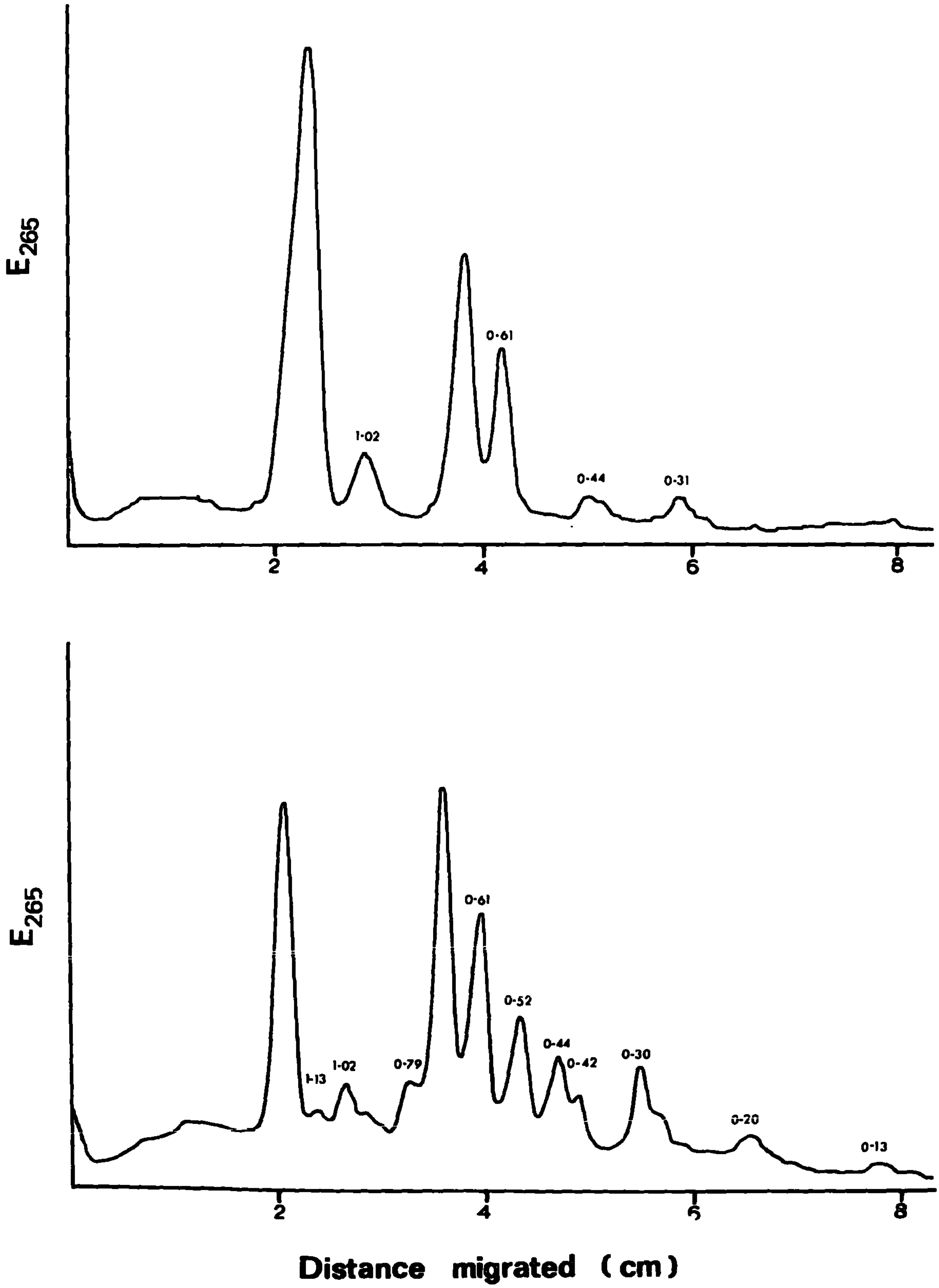
Analysis of the monosome RNA's on 7.5% acrylamide gels showed a

Fig. 71.

The nucleic acids of sucrose-gradient purified monosomes. The monosomes were prepared as detailed in the legend to Fig. 60. The nucleic acids were extracted as described in the methods, section 4(iv)(a).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C. The numbers above the peaks refer to their MW x $10^6$.

(b) RNA heated at 60°C for 10 min, then rapidly cooled. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C. The numbers above the peaks refer to their MW x $10^6$.

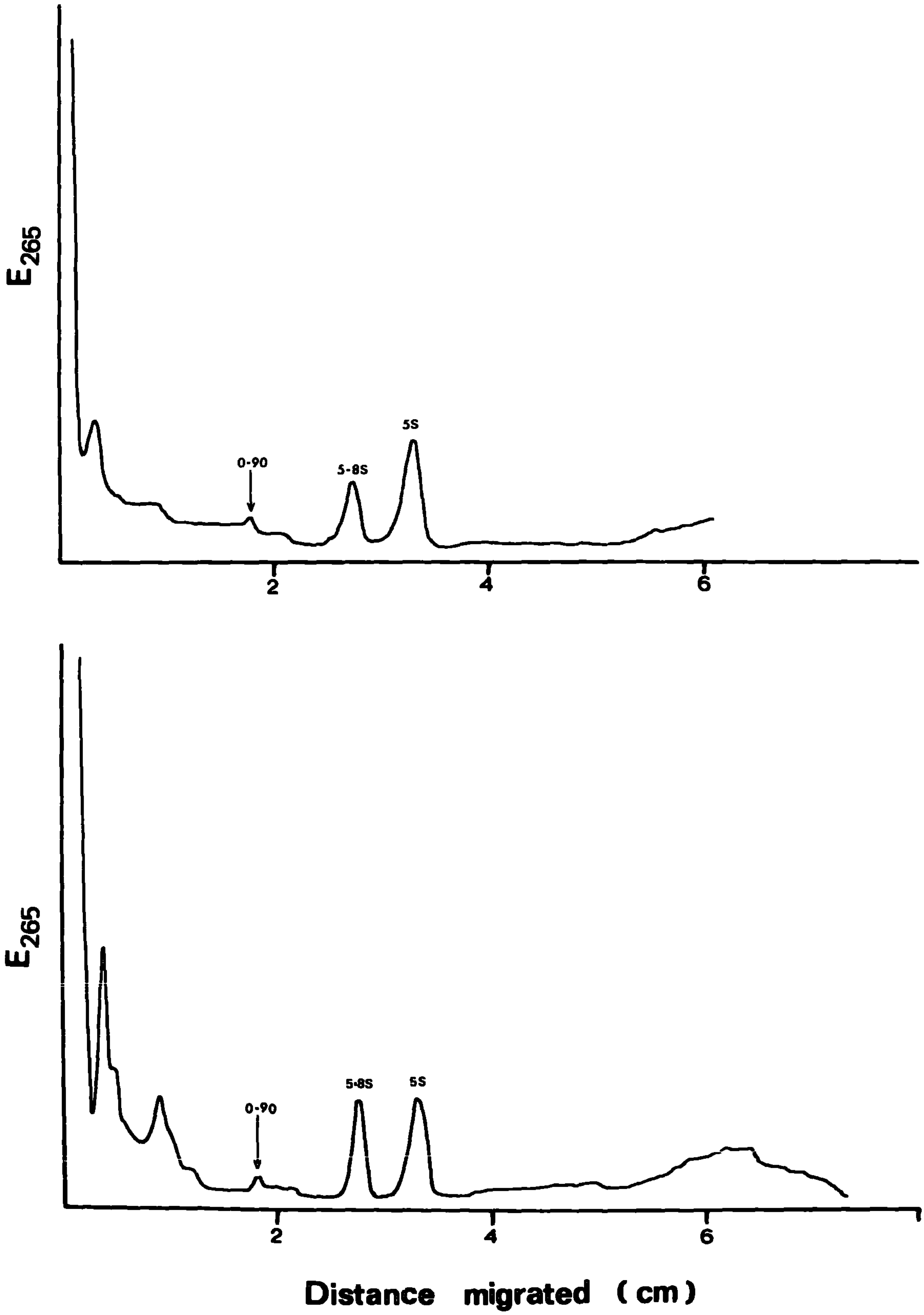


E265
1·02
0·61
0·44
0·31
2
4
6
8
E265
1·13
1·02
0·79
0·61
0·52
0·44
0·42
0·30
0·20
0·13
2
4
6
8
Distance migrated (cm)

Fig. 71.

(c) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D. The numbers above the peak refer to its MW x $10^5$.

(d) RNA heated at 60°C for 10 min, then cooled rapidly. Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D. The numbers above the peak refer to its MW x $10^5$.


$E_{265}$
0·90
5·8S
5S
2
4
6
$E_{265}$
0·90
5·8S
5S
2
4
6
Distance migrated (cm)

complete absence of 4S RNA (Figs. 71 c and d). The two RNA's present were identified as 5S RNA and 5.8S RNA by means of 4S and 5S RNA markers (not shown). There was no apparent increase of the 0.90 x $10^5$ daltons component following melting of the RNA; though there was an increase in the 5.8S RNA.

## 7. The isolation of nuclei and nuclear RNA

### (i) From cotyledons.

All methods of isolating nuclei from cotyledons failed, due to the high starch content of the tissue. The starch grains varied in size from a few microns up to 60 µm in diameter, with a concomitant variation in density. Some starch grains had densities greater than 1.355, as determined by their ability to sediment through 96% (w/v) sucrose. The yields of nuclei were visibly low in homogenates prepared from cotyledons when compared to root tissue homogenates. The yields were increased by digesting the cotyledon cell walls with pectinase and cellulase enzymes, though a complete loss of nuclei still occurred during the purification procedure, owing to co-sedimentation of the nuclei with the starch grains.

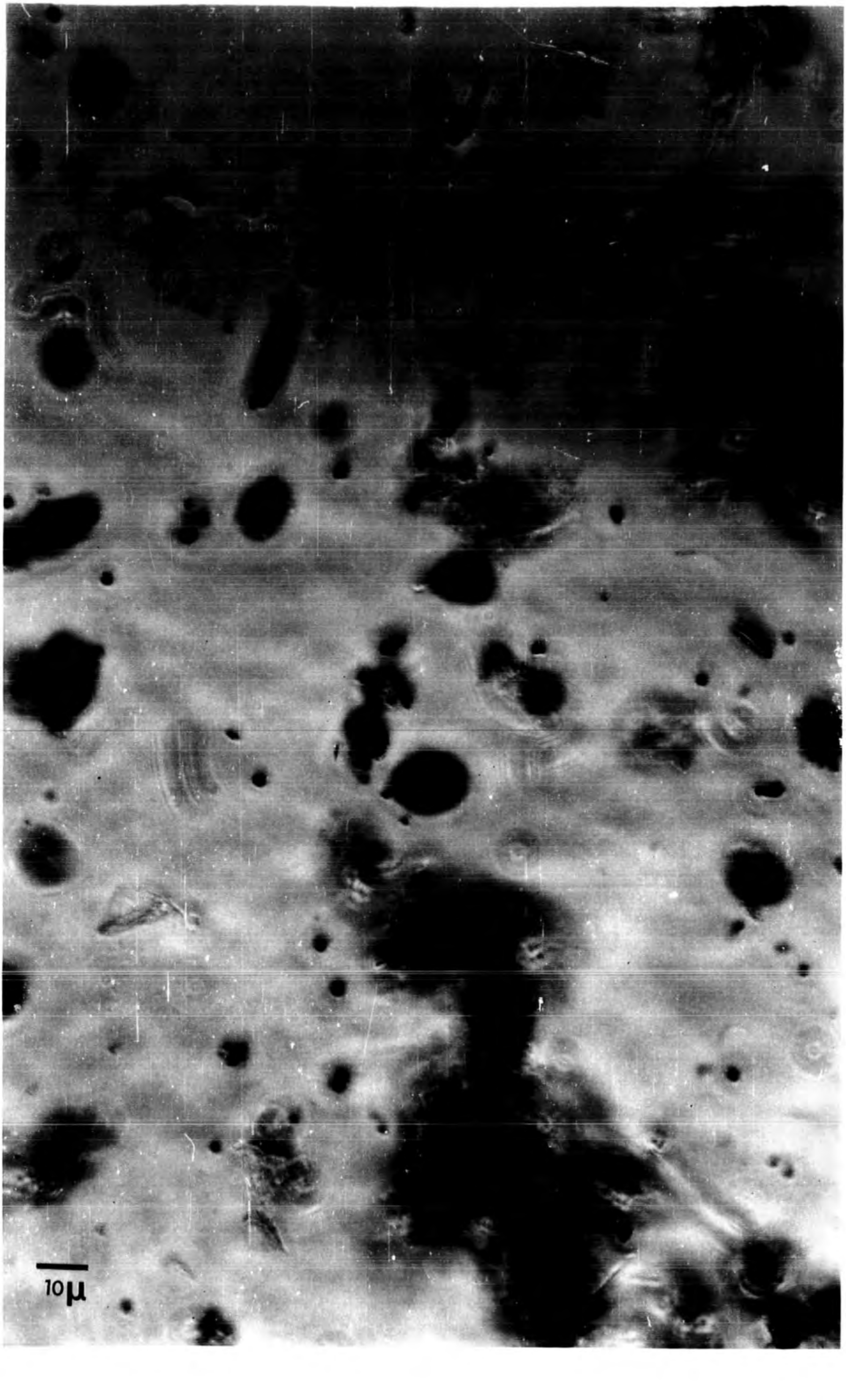
### (ii) From roots.

The nuclei prepared as described (Methods, 7.(i)), were relatively pure as judged by phase contrast microscopy (Plate 1), although electron microscopy showed the presence of fragments of cell wall and endoplasmic reticulum (Plate 2). Attempts to further purify the nuclei from this cell debris by centrifugation through continuous and non-continuous sucrose gradients (Dick, 1968; D'Alessio and Trim, 1968) were not successful.

Following the extraction of the nuclear nucleic acids, analysis on 2.2% and 7.5% acrylamide gels showed the sample to consist mainly of DNA (Fig. 72a). The DNA was removed by digestion with DNase (Methods, 9.(i)), and further analysis established the presence of 25S RNA and 18S RNA (Fig. 73). These could be accounted for by the presence of rough

## PLATE 1

Bean root nuclei isolated as described in the methods, section 7(i). Phase contrast. Magnification 1,000x


10µ

## PLATE 2

Electron micrograph of bean root nuclei, isolated as described in the methods, section 7(i). Magnification x 6,500.

ER : Endoplasmic reticulum

N : Nucleus

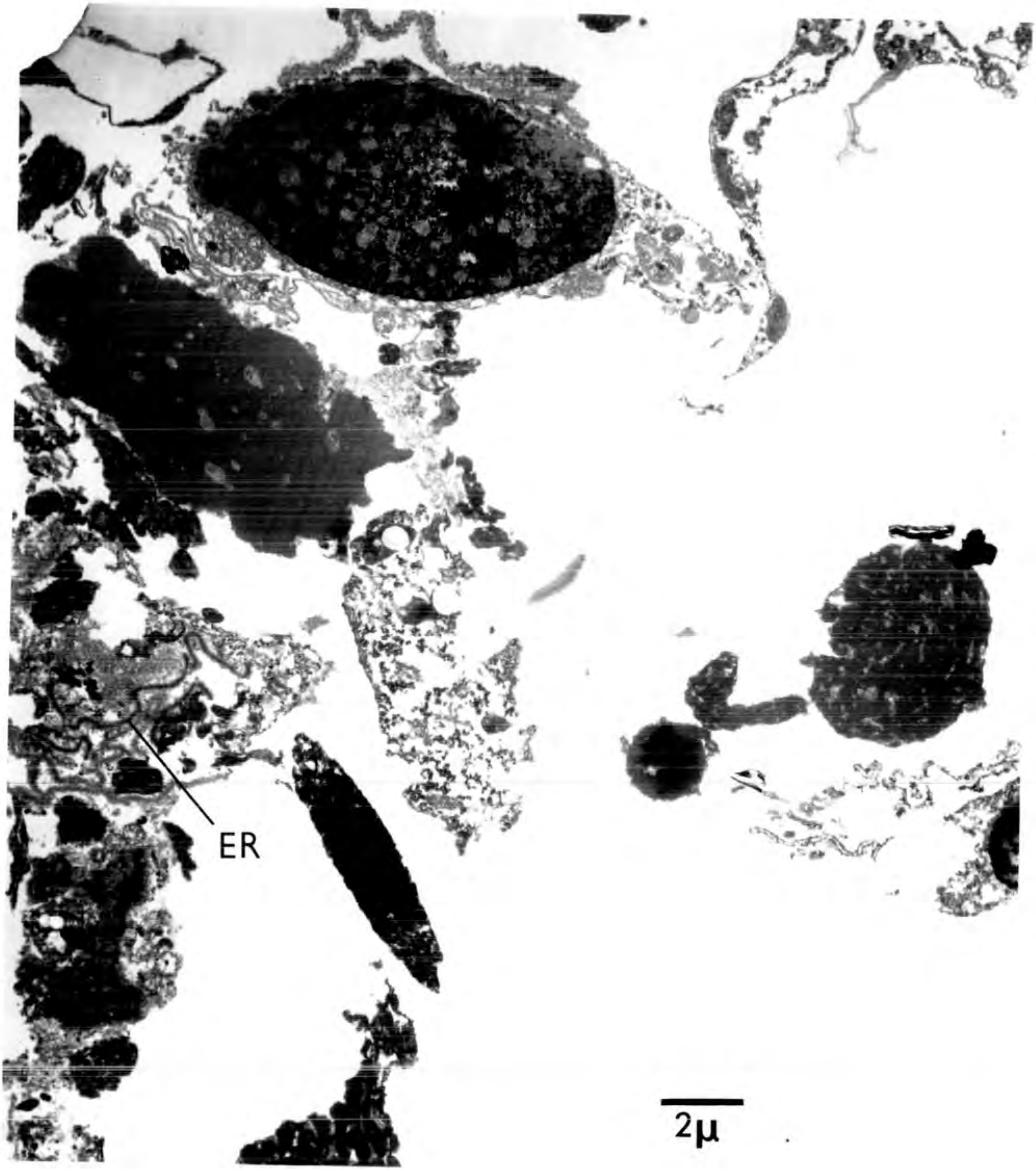

ER
2μ

Fig. 72.

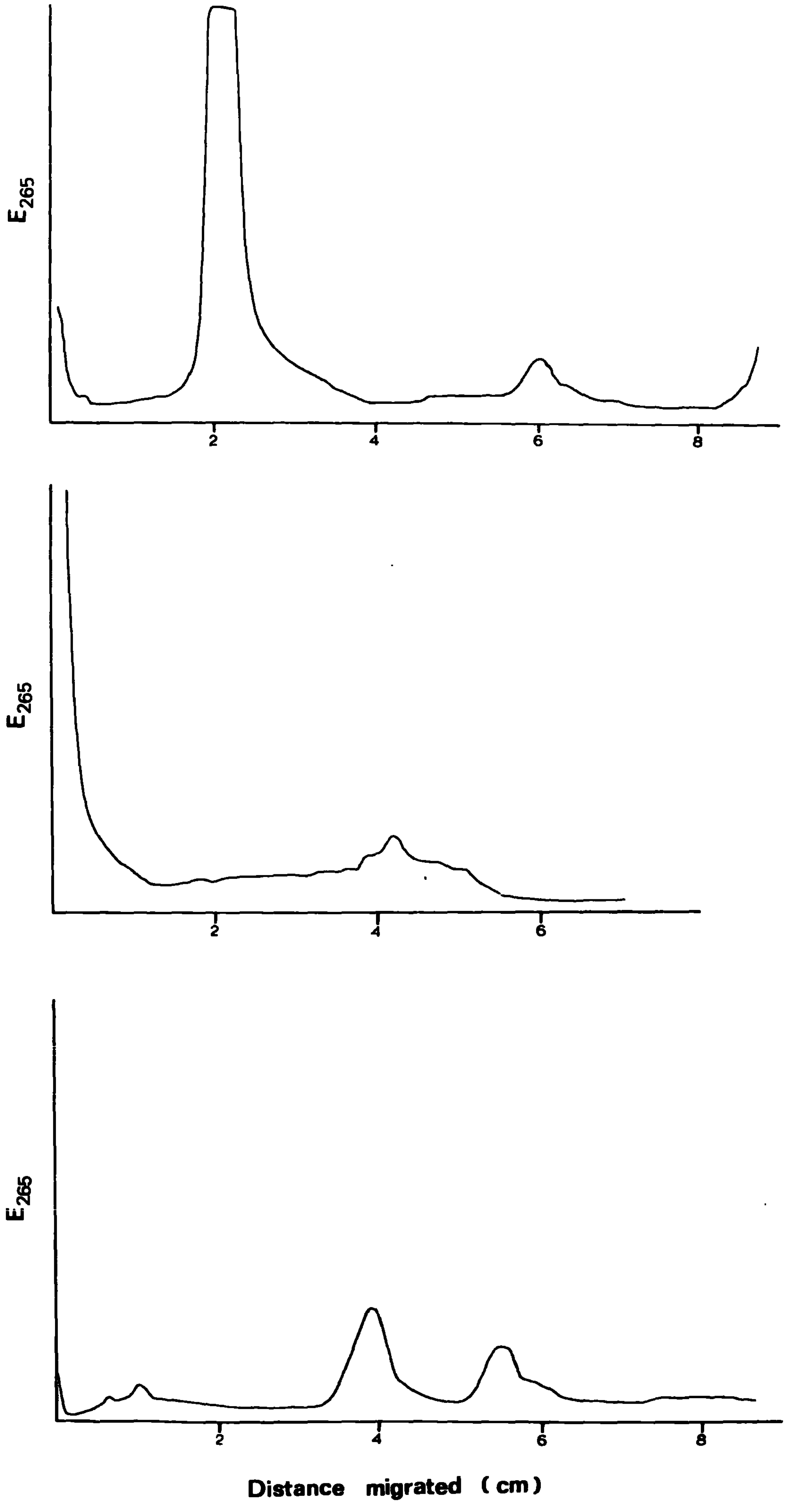
The nucleic acids of root nuclei. The nuclei, prepared from the roots of V. faba (methods, section 7(i)), were extracted as described in the methods, section 4(v).

(a) 25 µg electrophoresed on a 2.2% gel for 6h. Vertical expansion : cam D. Note: the 18S RNA has migrated off the gel.

(b) 25 µg electrophoresed on a 7.5% gel for 4h. Vertical expansion : cam D.

Fig. 73.

The RNA's of root nuclei. The total nucleic acids from root nuclei (details in legend to Fig. 72) were digested with DNase I (methods section 9(i)). 200 µg electrophoresed on a 2.2% gel for 3h. Vertical expansion : cam A.


$E_{265}$
2
4
6
8
$E_{265}$
2
4
6
$E_{265}$
2
4
6
8
Distance migrated (cm)

endoplasmic reticulum in the original sample.

Identification of the LMW RNA's was not attempted (Fig. 72b).

## 8. Incorporation of $[^{32}P]$-orthophosphate into roots

### (i) Excised root tips.

Primary analysis of the samples indicated incorporation of $[^{32}P]$-phosphate into RNA (Table 6). Subsequent analysis of the samples on 2.2% and 7.5% acrylamide gels showed that $[^{32}P]$-phosphate was incorporated into bacterial nucleic acids.

After 15 min incorporation, bacterial DNA and heterodisperse RNA were labelled (Fig. 74). After 60 min incorporation, four peaks of radioactivity were detected (Fig. 75). These corresponded to: DNA and RNA's of MW, 1.1, 0.63 and 0.36 x $10^6$ daltons. The latter was only detected in this sample. After 120 min incorporation, the $[^{32}P]$-phosphate was localised in DNA and RNA's of MW, 1.1, 0.63 and 0.56 x $10^6$ daltons (Fig. 76a).

Labelling of the 5S and 4S RNA components was detected after 30 min incorporation and by 120 min labelling was substantial (Fig. 76b). The apparent non-alignment between the radioactive profile and the optical density profile was obtained with all samples.

There was no evidence of incorporation of $[^{32}P]$-phosphate into the 1.3 and 0.70 x $10^6$ components.

### (ii) Intact roots.

The stringent sterilization procedure adopted with these samples had little effect, as incorporation of $[^{32}P]$-phosphate was only observed in bacterial nucleic acids (Fig. 77). The radioactive peaks correspond to: DNA and RNA's of MW, 1.1, 0.63 and 0.56 x $10^6$ daltons.

Labelling of the 5S and 4S RNA's was observed, on 7.5% acrylamide gels, and there was an apparent non-alignment of the radioactive and optical density profiles (trace not shown, as similar to Fig. 76b).

TABLE 6

The incorporation of [$^{32}$P]-orthophosphate into RNA. Activity was calculated prior to polyacrylamide gel electrophoresis.

| Sample | [$^{32}$P]-pulse | chase | cpm/μg RNA | culture medium |
|---|---|---|---|---|
| Cultured root tips | 15 min | - | 64,946 | 2% (w/v) sucrose |
| | 30 min | - | 44,443 | |
| | 60 min | - | 149,750 | |
| | 120 min | - | 171,254 | |
| Intact roots | 30 min | 15 min | 14,073 | $H_2O$ |
| Cotyledon slices | 30 min | 15 min | 743 | 2% (w/v) sucrose |
| | 30 min | 15 min | 4,449 | Sunflower medium |
| Cotyledon | - | - | 1,971 | *In vivo* |
| Testa | - | - | 9,914 | |

Fig. 74.

The nucleic acids of cultured root tips of V. faba. Excised root tips were labelled for 15 min with $[^{32}P]$-orthophosphate (methods section 10(i)). Radioactivity is plotted as a histogram. Electrophoresed on a 2.2% gel for 4h. Vertical expansion : cam A.

Fig. 75.

The nucleic acids of cultured root tips of V. faba. Excised root tips were labelled for 30 min with $[^{32}P]$-orthophosphate. Radioactivity is plotted as a histogram. Electrophoresed on a 2.2% gel for 4h. Vertical expansion : cam A. The numbers above the peaks refer to their MW x $10^6$.

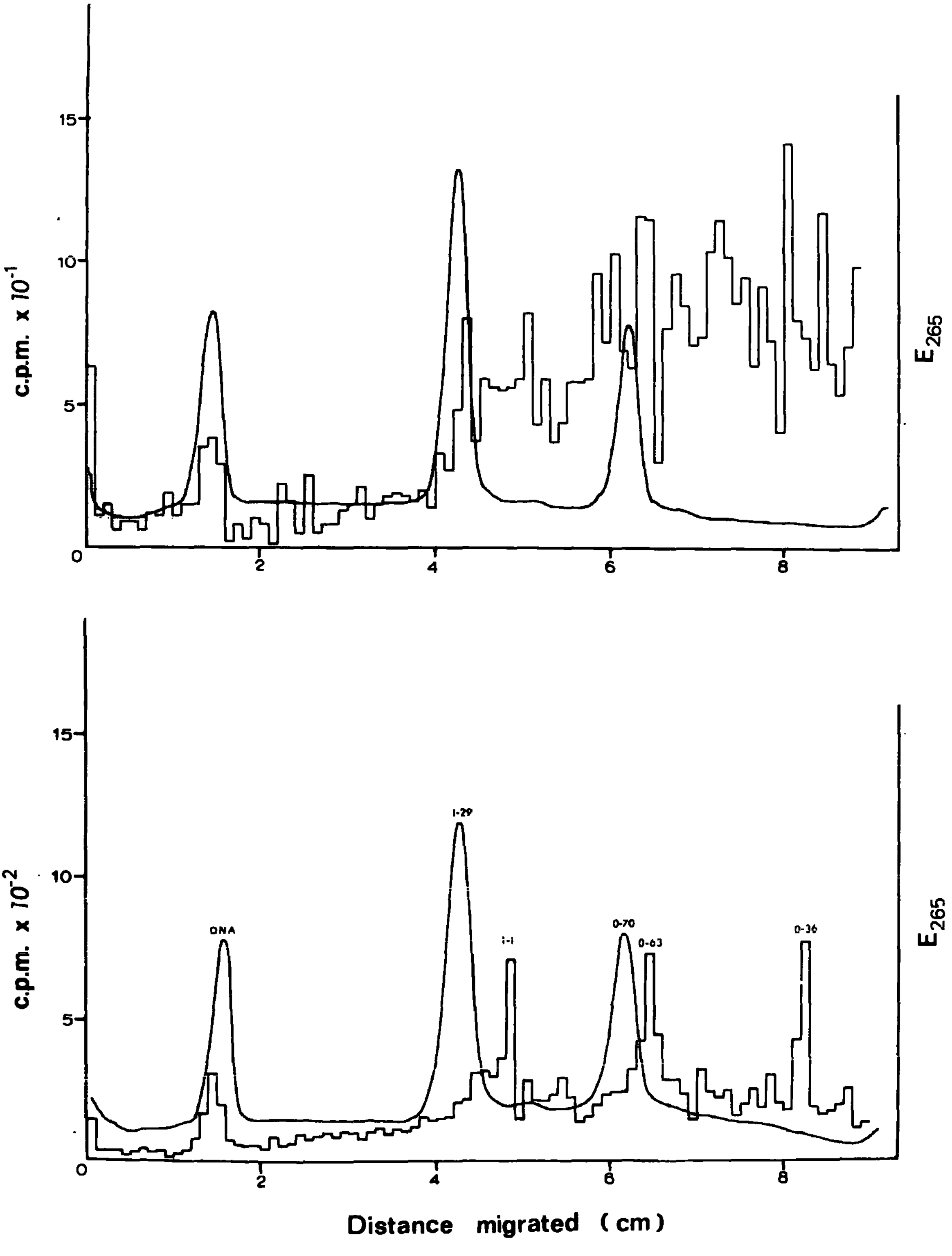

15
10
5
0
c.p.m. x $10^{-1}$
E$_{265}$
2
4
6
8
15
10
5
0
c.p.m. x $10^{-2}$
E$_{265}$
DNA
1-29
1-1
0-70
0-63
0-36
2
4
6
8
Distance migrated (cm)

Fig. 76.

The nucleic acids of cultured root tips of V. faba. Excised root tips were labelled for 120 min with $[^{32}P]$-orthophosphate. Radioactivity is plotted as a histogram. The numbers above the peaks refer to their MW x $10^6$.

(a) Electrophoresed on a 2.2% gel for 4h. Vertical expansion : cam A.

(b) Electrophoresed on a 7.5% gel for 3h. Vertical expansion : cam D.

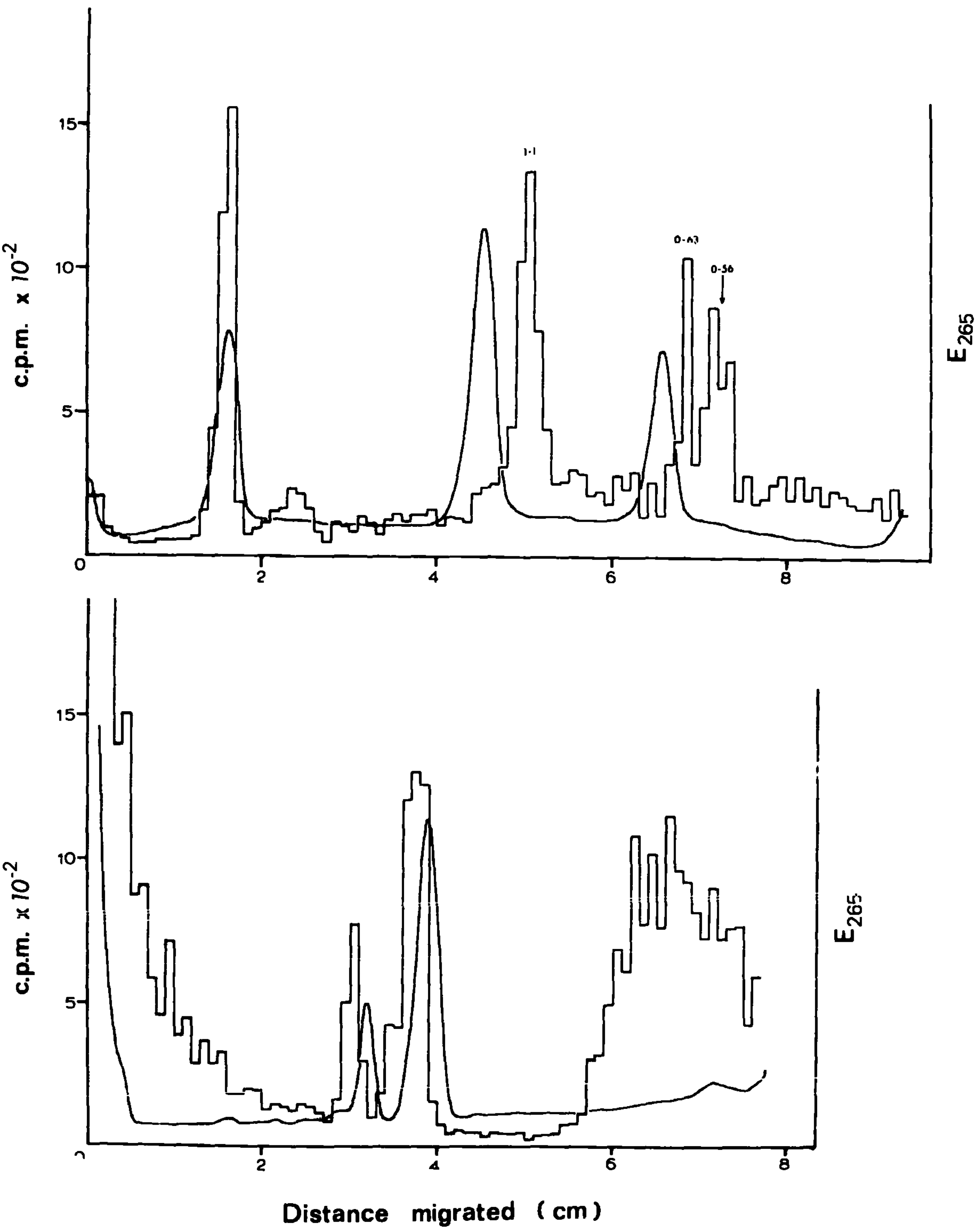
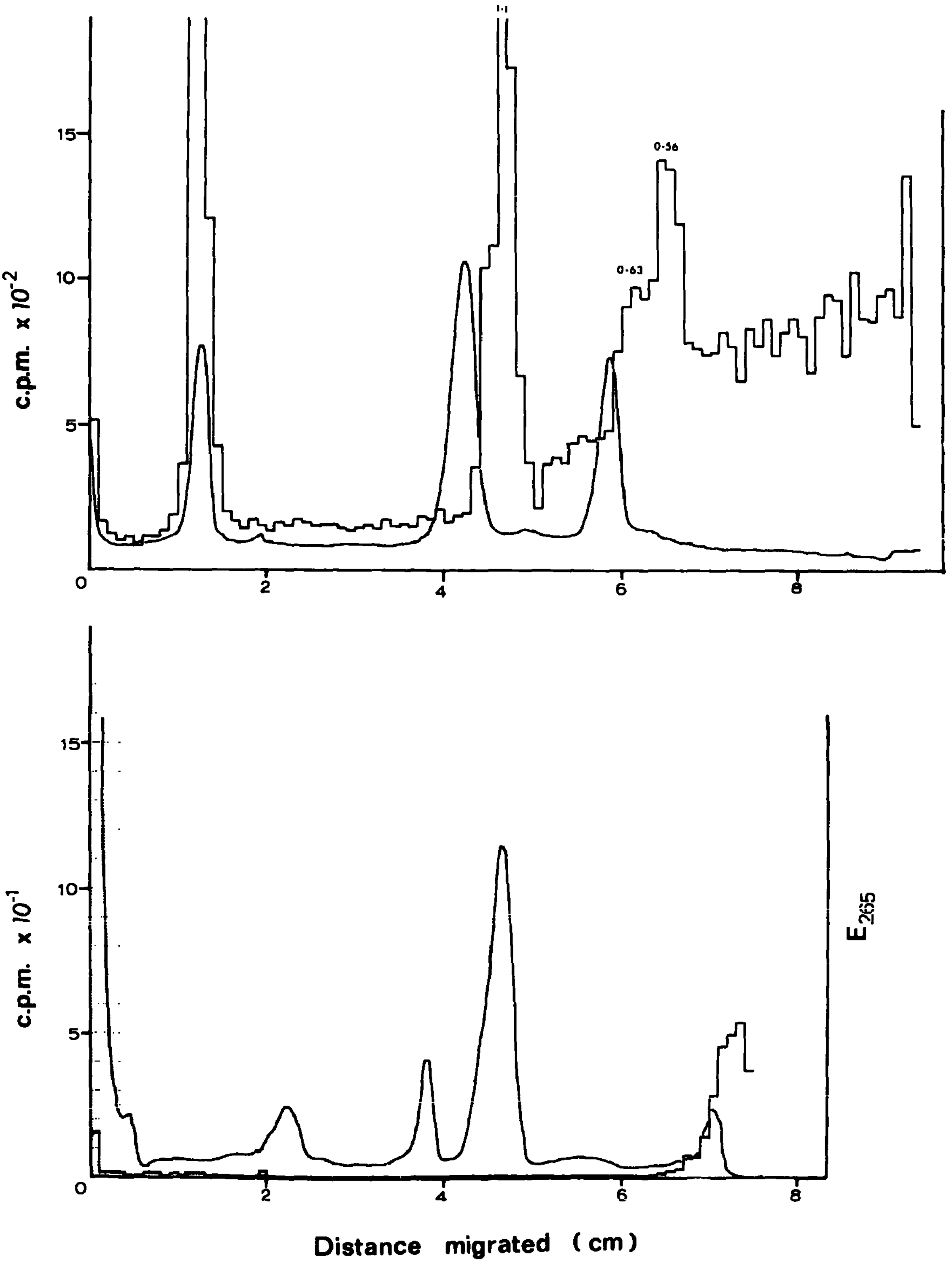

c.p.m. x $10^{-2}$
E$_{265}$
15
10
5
0
2
4
6
8
1·1
0·63
0·56
c.p.m. x $10^{-2}$
E$_{265}$
15
10
5
0
2
4
6
8
Distance migrated (cm)

Fig. 77.

The nucleic acids of root tips of *V. faba*. Seedlings were labelled (as described in the methods, section 10(ii)) with $[^{32}P]$-orthophosphate for 30 min, and incubation was continued in distilled water for 15 min. The root tip was then excised and the nucleic acids extracted (methods section 4(i)). Radioactivity is plotted as a histogram. The numbers above the peaks refer to their MW x $10^6$. Electrophoresed on a 2.2% gel for 4h. Vertical expansion : cam A.

Fig. 78.

The nucleic acids of 50-day old cotyledons, cultured *in vitro* (methods section 11(i)). Radioactivity is plotted as a histogram. Electrophoresed on a 7.5% gel for 4h. Vertical expansion : cam D.


1·1
0·56
0·63
c.p.m. x 10⁻²
15
10
5
0
2
4
6
8
E265
c.p.m. x 10⁻¹
15
10
5
0
2
4
6
8
E265
Distance migrated (cm)

The level of incorporation of $[^{32}P]$-orthophosphate was greater than in the previous samples.

## 9. Incorporation of $[^{32}P]$-orthophosphate into developing seeds

### (i) *In vitro*.

Primary analysis of the samples indicated incorporation of $[^{32}P]$-phosphate into RNA (Table 6). Subsequent analysis of the samples on 7.5% acrylamide gels showed no incorporation into nucleic acid fractions (Fig. 78). In all samples the LMW RNA's having a mobility less than the 5S RNA were detected. There was no apparent degradation of the 25S RNA and the 18S RNA (not shown).

### (ii) *In vivo*.

Analysis of the nucleic acids, extracted from the testa and cotyledons of a 40-day old developing bean, on 2.2% (not shown), 2.6% and 7.5% acrylamide gels (Figs. 79 a and b; 80 a and b), showed that radioactivity was associated with all RNA fractions. The DNA was labelled to a small extent in the cotyledon sample, but virtually no labelling of DNA was detected in the testa.

Primary analysis of the two samples (Table 6) indicated a significantly higher degree of incorporation of $[^{32}P]$-orthophosphate into the testa nuclei acids. Actual incorporation into rRNA was not significantly different between the two samples (compare Figs. 79a and 80a), though incorporation into the 4S RNA was higher in the testa (compare Figs. 79b and 80b).

There was no evidence, in either sample, for the existence of ribosomal precursor RNA, bacterial or chloroplast rRNA (see section 4.(iv)) or non-ribosomal RNA.

## 10. An attempt to identify the messenger RNA coding for the storage protein legumin

Previous results have shown that an active nuclease, present in the

Fig. 79.

40-day old testa nucleic acids. Seeds were labelled *in vivo* with $[^{32}P]$-orthophosphate (methods section, 11(ii)(a)). Radioactivity is plotted as a histogram.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

(b) Electrophoresed on a 7.5% gel for 4h. Vertical expansion : cam D.

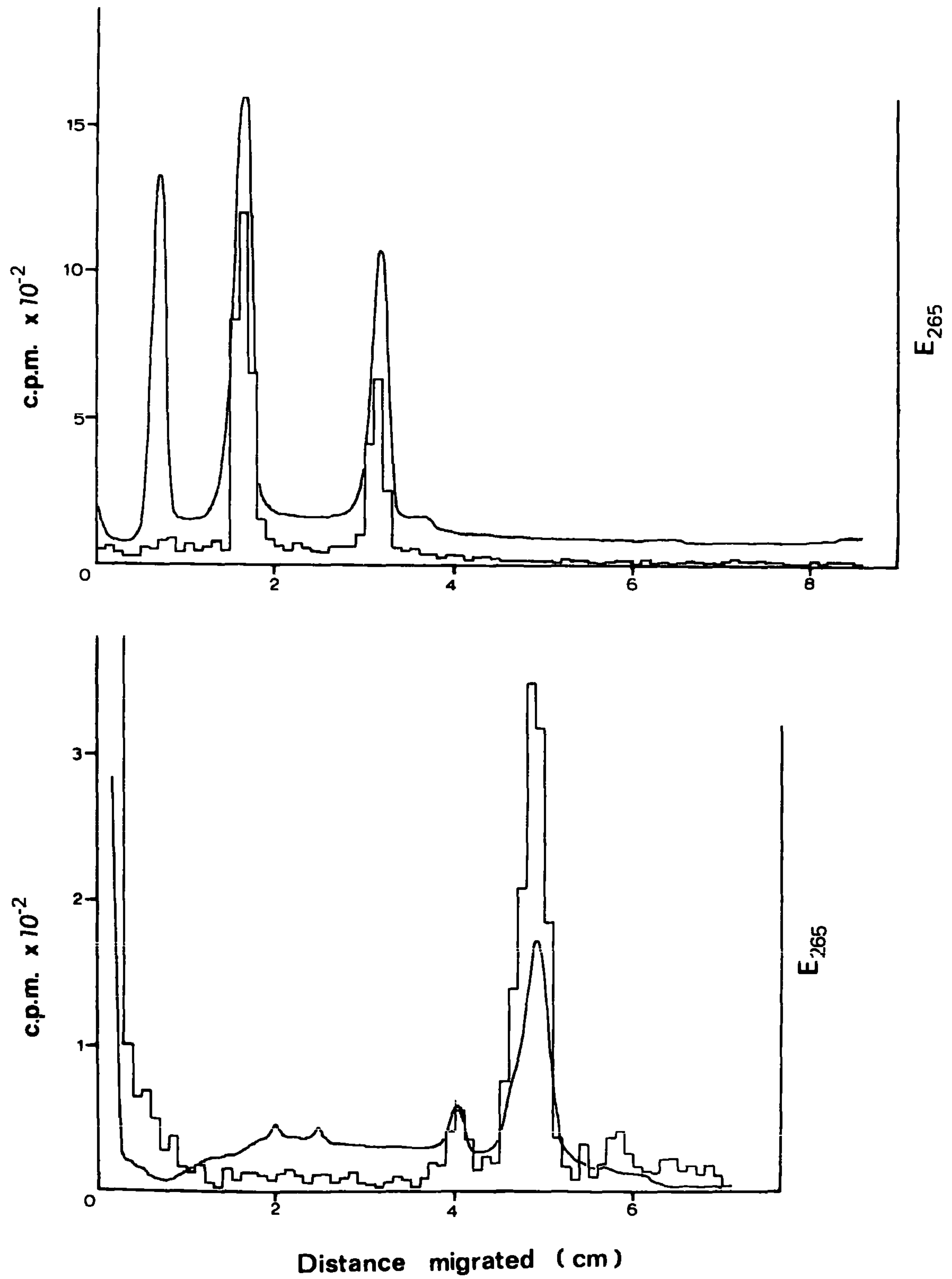
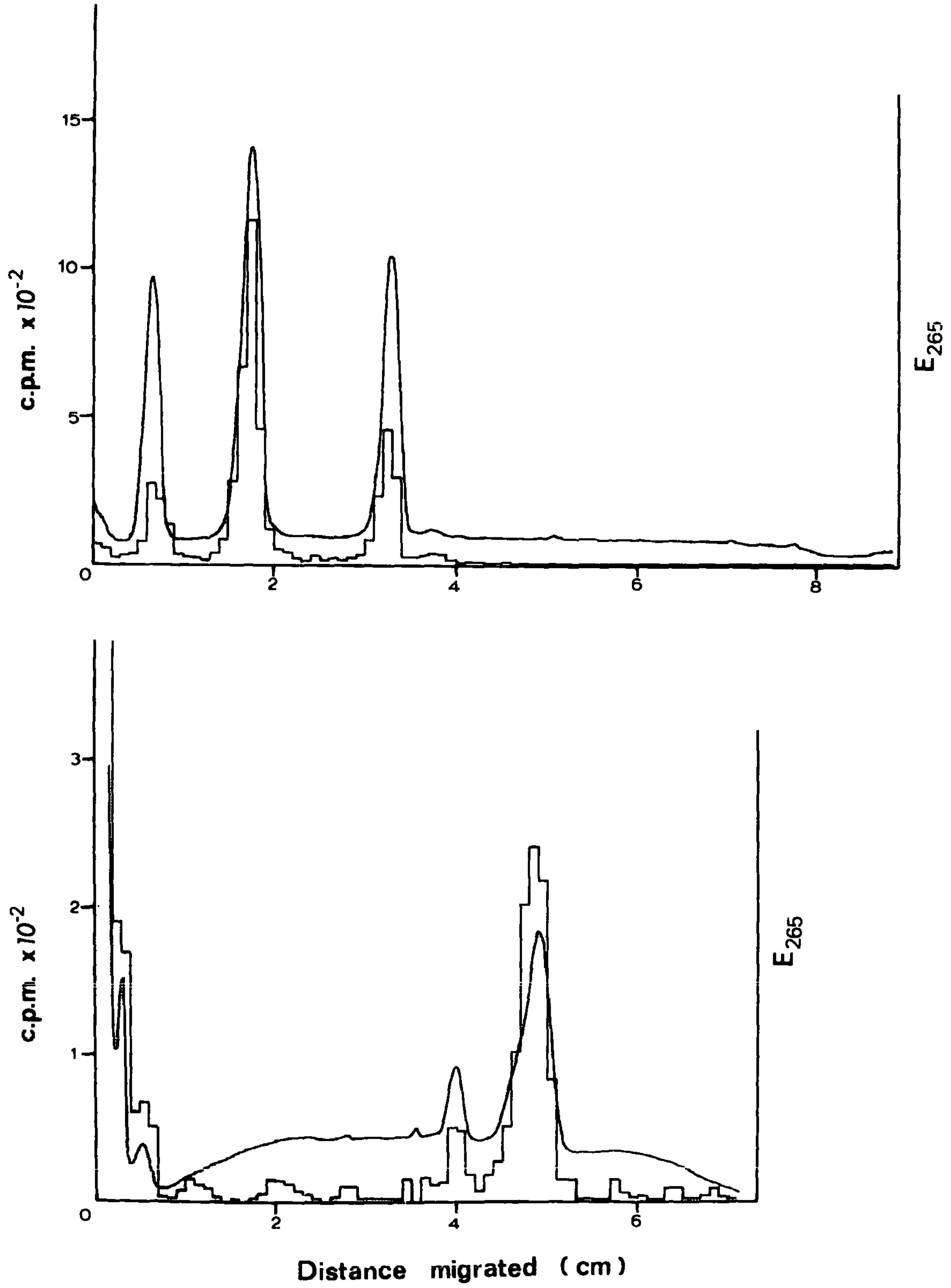

c.p.m. x $10^{-2}$
15
10
5
0
2
4
6
8
$E_{265}$
c.p.m. x $10^{-2}$
3
2
1
0
2
4
6
$E_{265}$
Distance migrated (cm)

Fig. 80.

40-day old cotyledon nucleic acids. Seeds were labelled in vivo with $[^{32}P]$-orthophosphate (methods section, 11(ii)(a)). Radioactivity is plotted as a histogram.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

(b) Electrophoresed on a 7.5% gel for 4h. Vertical expansion : cam D.


c.p.m. x $10^{-2}$
15
10
5
0
2
4
6
8
$E_{265}$
c.p.m. x $10^{-2}$
3
2
1
0
2
4
6
$E_{265}$
Distance migrated ( cm )

developing cotyledons of V. faba has prevented the isolation of undegraded polysomes and their constituent rRNA's. The primary problem was, therefore, the preservation of polysome structure. The much acclaimed RNase inhibitor, DEP, has been shown by Weeks and Marcus (1969) to have a protective effect on polysome structure. This technique and the RNA extraction technique of Oda and Joklick (1967; see also: Gaskill and Kabat, 1971), therefore formed the backbone of the experiments described below.

(i) The analysis of particles extracted in the presence of DEP.

Sucrose density gradient analysis of the ribosomal pellet obtained following the addition of triton x-100 to the post-mitochondrial supernatant (Fig. 81), showed that polysome structure had been preserved to a greater extent than particles extracted in the presence of bentonite (Fig. 59).

Sucrose density gradient analysis of triton x-100 disrupted microsomes (Fig. 82) showed a preponderance of subunits and polysome; the monomeric ribosomes being visible as a shoulder on the large subunit peak.

A comparison of Figs. 81 and 82 indicates that polysome structure is effectively preserved by the presence of DEP and membrance. It is also apparent (compare Figs. 81 and 82 with 59) that DEP causes subunit formation.

The inconvenience of preparing fresh sucrose gradients before each experiment necessitated an attempt to store them by freezing at -70°C (Shore et al., 1969) for later use after thawing at room temperature. Analysis of the gradient slope of freshly prepared gradients, and gradients following freezing and thawing (Fig. 83) showed that following freezing and thawing the gradient slope steepened and changed from convex to sigmoid.

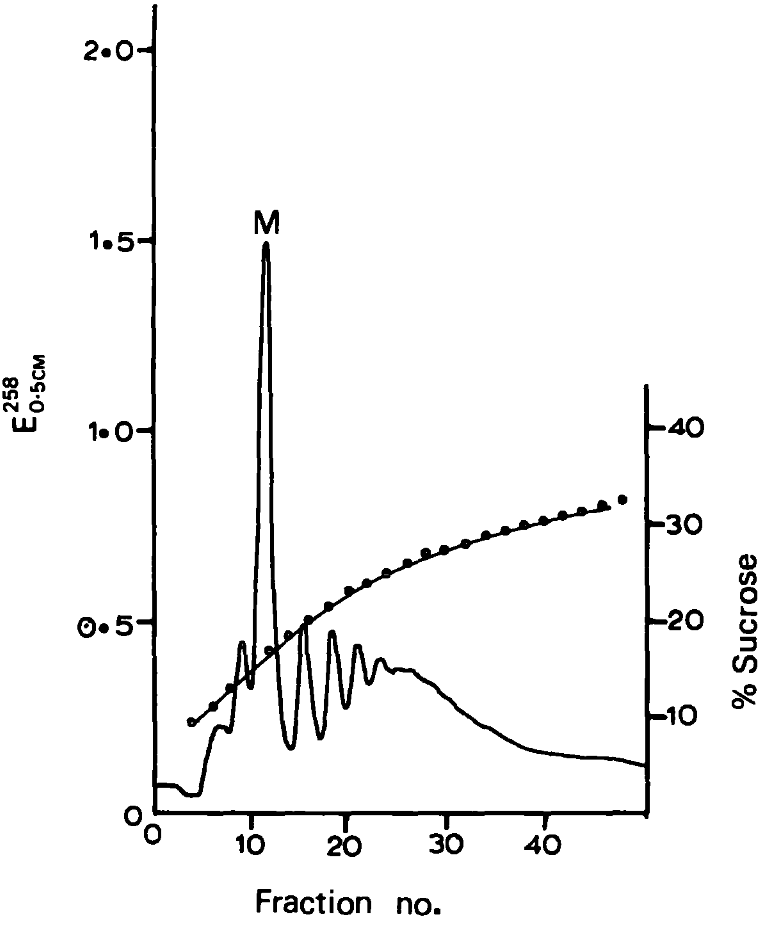
The analysis of triton x-100 disrupted microsomes (extracted in the presence of DEP but with the omission of untitrated-Tris) on frozen and thawed gradients (Fig, 84) showed very poor resolution of the bands, and the use of these gradients was discontinued. It was evident that the

Fig. 81.

Sucrose-gradient sedimentation profile of polysomes from 40-day old cotyledons. The polysomes were prepared in the presence of DEP as described in the methods (section 7(ii)), the post-mitochondrial supernatant being made 4% (v/v) with respect to triton X-100. 1.0 mg of polysomes were layered on to the gradient and centrifuged at 65,000 g av. for 2h at 4°C. The gradient was prepared as described in the legend to Fig. 60, and fractionated as detailed in the legend to Fig. 62.

Fig. 82.

Sucrose-gradient sedimentation profile of triton X-100 disrupted microsomes from 40-day old cotyledons. The microsomes were prepared in the presence of DEP as described in the methods, section 7(ii). 1.0 mg of microsomes were disrupted with triton X-100 and layered on to the gradient. Gradient parameters are given in the legend to Fig. 81.


M
$E^{258}_{0\cdot5\text{CM}}$
2•0
1•5
1•0
0•5
0
0
10
20
30
40
Fraction no.
40
30
20
10
% Sucrose

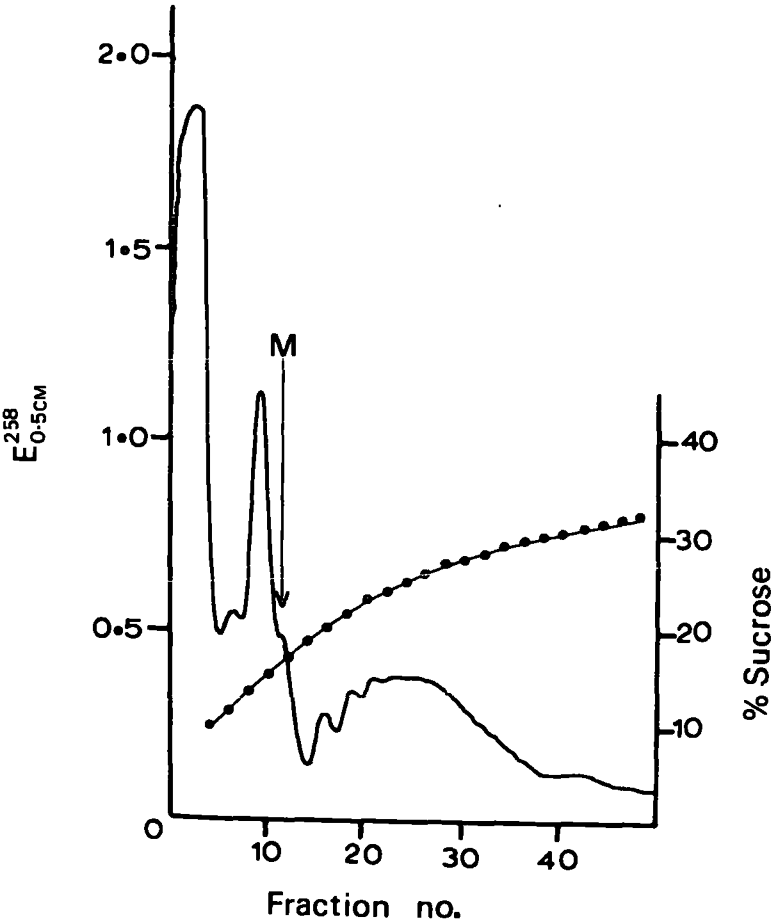

M
$E^{258}_{0\cdot5\text{CM}}$
2•0
1•5
1•0
0•5
0
10
20
30
40
Fraction no.
40
30
20
10
% Sucrose

Fig. 83.

The effect of freezing and thawing convex sucrose gradients. Gradients were prepared as detailed in the legend to Fig. 60.

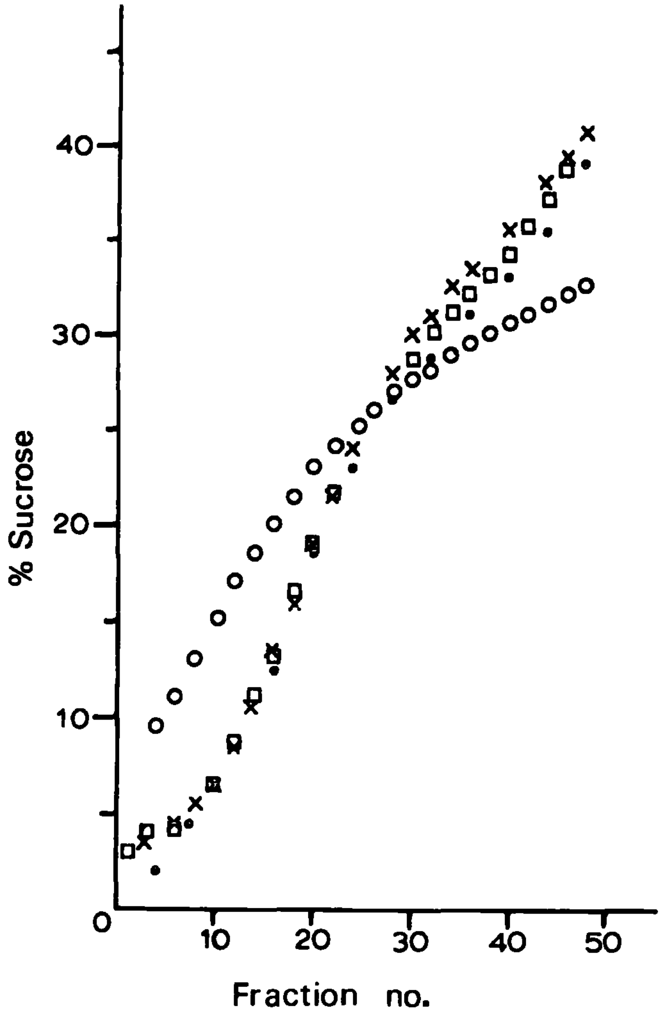
○ : gradient shape immediately after preparation.

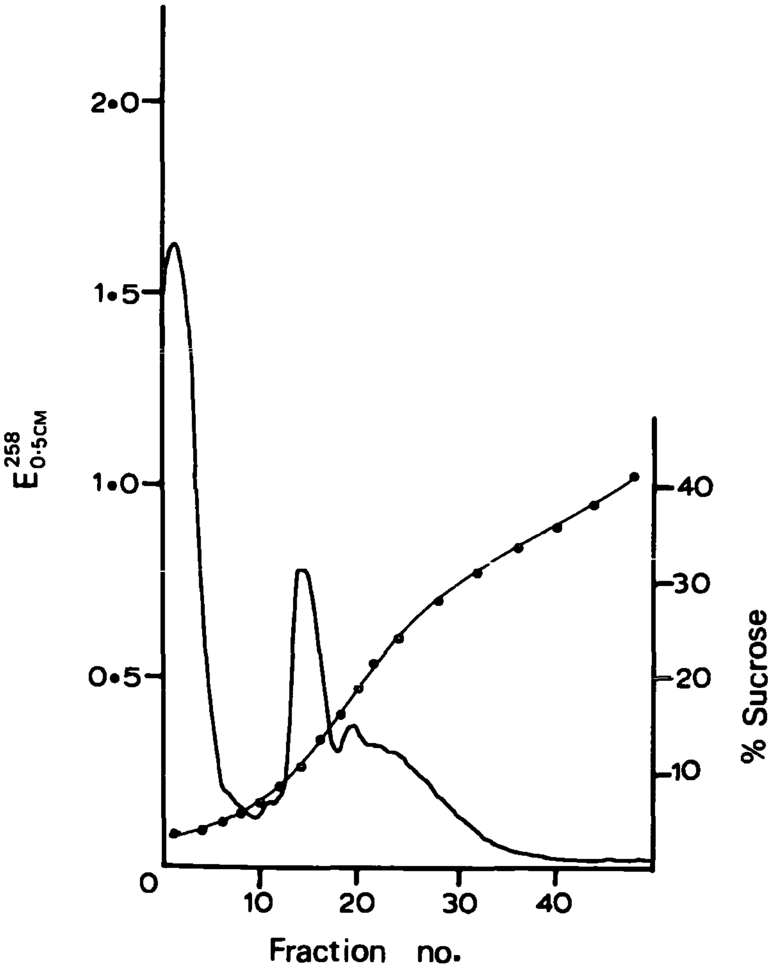
□, ● : gradient shape after freezing and thawing.

× : gradient shape after freezing, thawing and centrifugation; taken from Fig. 84.

Fig. 84.

Sucrose-gradient sedimentation profile of triton X-100 disrupted microsomes from 50-day old cotyledons. The microsomes were prepared in the presence of DEP, but the addition of the untitrated-Tris was omitted. 1.0 mg microsomes were disrupted with triton X-100 and layered on to a frozen and thawed gradient. Gradient parameters are detailed in the legend to Fig. 81.


% Sucrose
40
30
20
10
0
10
20
30
40
50
Fraction no.


$E^{258}_{0\cdot5\,CM}$
2•0
1•5
1•0
0•5
0
10
20
30
40
Fraction no.
40
30
20
10
% Sucrose

omission of untitrated-Tris from the extractant caused the breakdown of polysomes (compare Figs. 82 and 84).

Analysis of the nucleic acids extracted directly from the particles was carried out on 2.6% acrylamide gels. The rRNA's of the ribosomal and microsomal pellets were similar and are represented by one figure (Fig. 85a). Both the 25S and 18S RNA's were degraded. The MW's of the observed breakdown products were 1.02, 0.61, 0.44 and 0.12 x $10^6$ daltons respectively. Melting of the samples at 60°C for 10 min resulted in substantial breakdown of the rRNA's (Fig. 85b). The MW's of the breakdown products are similar to those observed previously (section 6.(v); cf. Fig. 71b).

The nucleic acids of the microsomes (extracted with DEP; untitrated-Tris omitted) showed severe degradation of the 18S RNA component. Melting at 60°C for 10 min resulted in the complete breakdown of the rRNA's (Figs. 86 a and b).

### (ii) An attempt to precipitate legumin synthesising polysomes with antilegumin.

Legumin, kindly supplied by Mr. D. Wright (Department of Botany, University of Durham), was in 0.1M-phosphate, pH 7.0, at a concentration of 1 mg/ml (1.0 mg legumin was precipitated by 1.6 ml antilegumin; Graham, unpublished results).

#### (a) Legumin-antilegumin incubations

The occurrence of the antigen-antibody reaction involves the detection of precipitation at a liquid interphase (RING TEST), following 15 min incubation at 35°C (Graham, personal communication). 0.1 ml (100 μg) legumin overlayering 0.2 ml antilegumin showed a strong reaction following incubation at 35°C for 15 min or at 0°C for 1 h. This reaction was not visibly affected by concentrations of triton x-100 up to 10% (v/v). Mixing of the two phases, following the ring test resulted in the precipitation of the antibody-antigen complex (PRECIPITIN REACTION).

Fig. 85.

Ribosomal pellet RNA. Ribosomes were prepared in the presence of DEP (see Fig. 81) from 40-day old cotyledons; the nucleic acids were extracted as described in the methods (section 4(iv)(b)). The numbers above the peaks refer to their MW x $10^6$.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

(b) RNA heated at 60°C for 10 min, then cooled rapidly. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

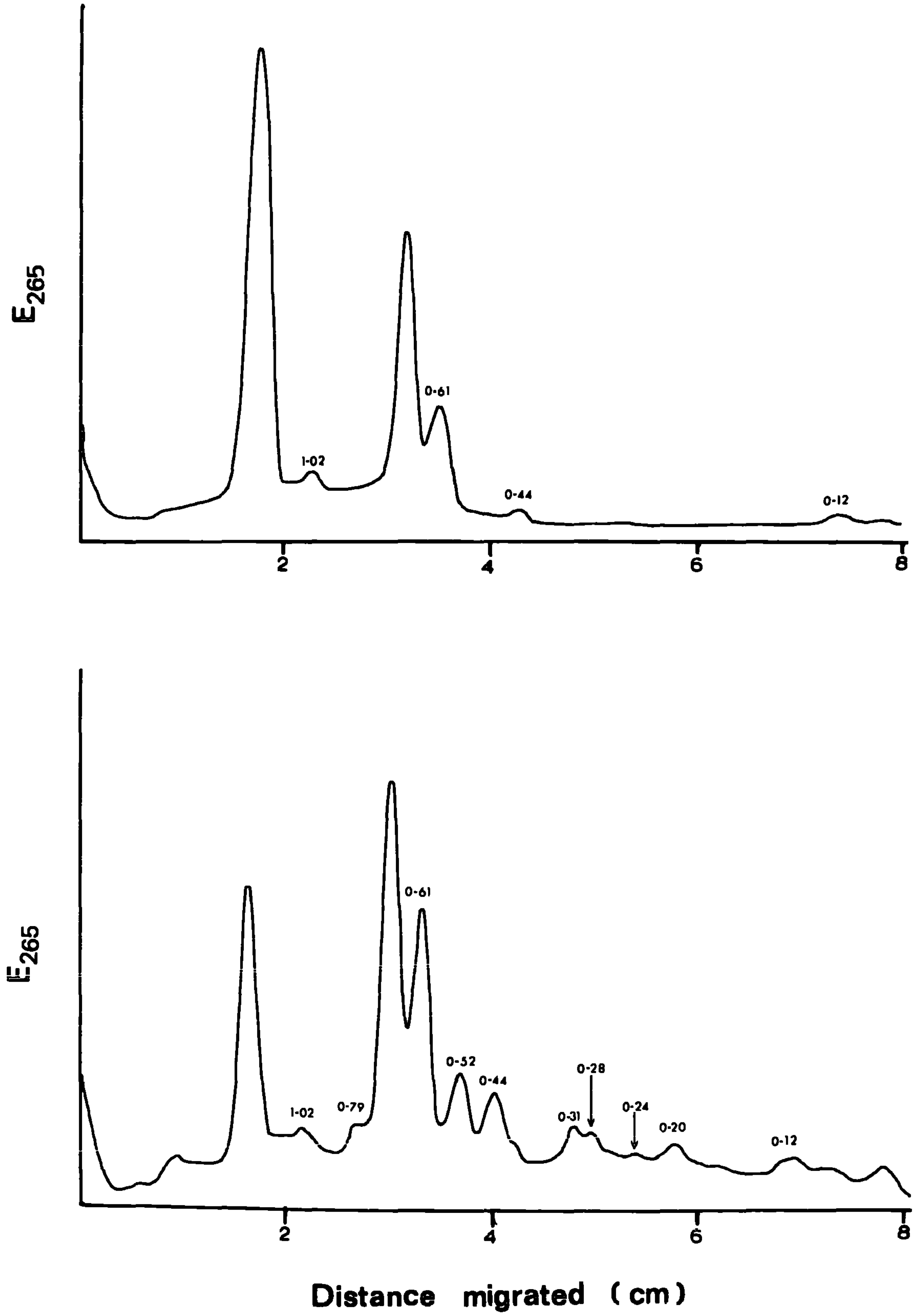
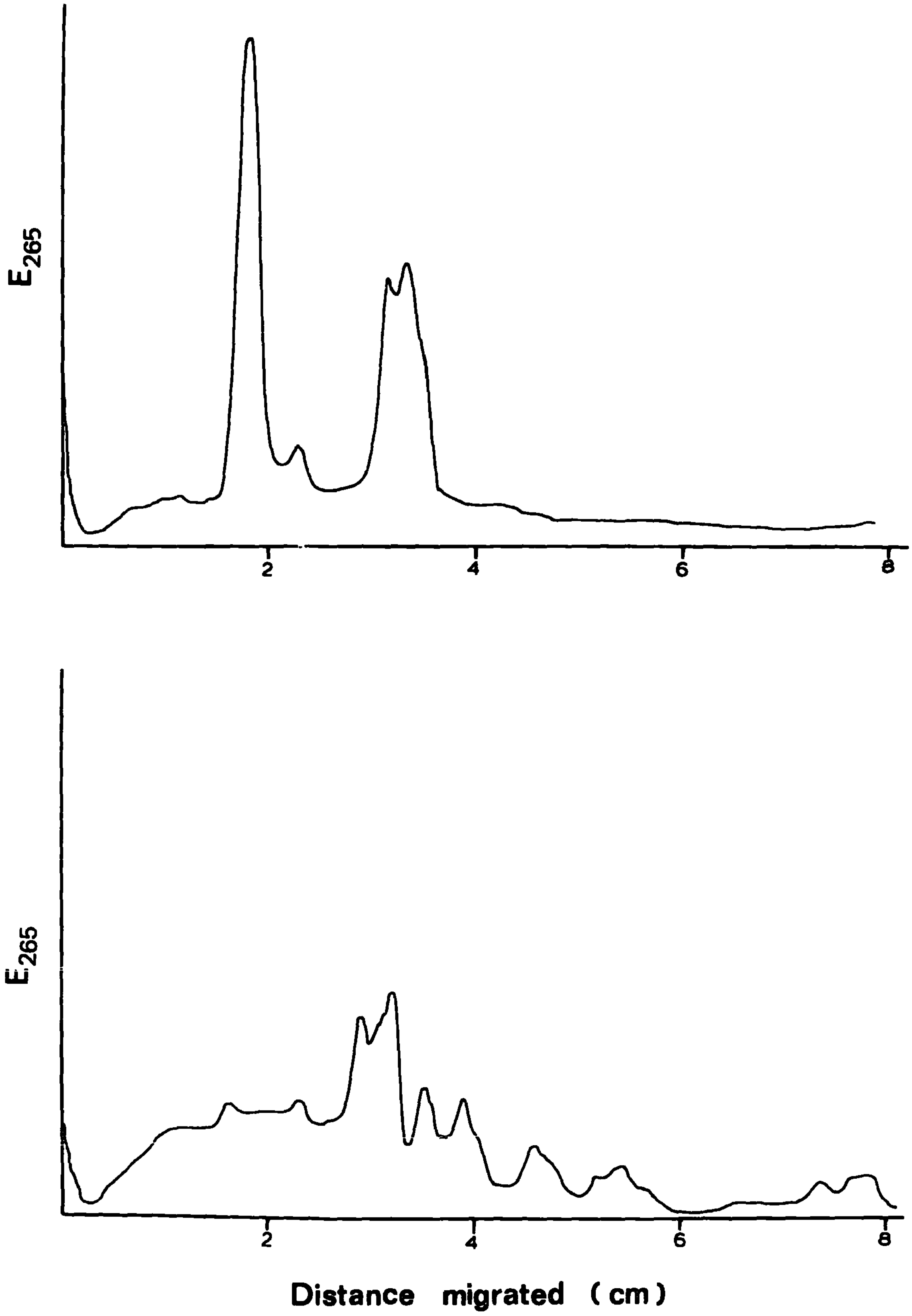

$E_{265}$
1·02
0·61
0·44
0·12
2
4
6
8
$E_{265}$
1·02
0·79
0·61
0·52
0·44
0·31
0·28
0·24
0·20
0·12
2
4
6
8
Distance migrated (cm)

Fig. 86.

Ribosomal pellet RNA. Ribosomes were prepared in the presence of DEP, but the untitrated Tris was omitted, from 50-day old cotyledons (see Fig. 84). The nucleic acids were extracted as described in the methods (section 4(iv)(b)).

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C.

(b) RNA heated at 60°C for 10 min, then cooled rapidly. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C.



$E_{265}$
2
4
6
8
$E_{265}$
2
4
6
8
Distance migrated (cm)

### (b) Microsome-antilegumin incubations

The disruption of microsome preparations with triton x-100, as detailed (Methods, section 7.(ii)) was modified in that the 10% (v/v) triton x-100 was buffered with 50mM-Tris•HCl, 16mM-KCl, 5mM-$MgCl_2$ pH 7.6, in order to maintain the $Mg^{2+}$ concentration in the incubations. Incubations contained:

0.2 ml (2 mg) microsomes
0.4 ml 10% (v/v) triton x-100
0.2 ml antilegumin
0.1 ml (100 µg) legumin
0.1 ml resuspending medium (Methods, section 7.(ii))

Preliminary experiments, using triton x-100 disrupted microsomes (extracted in the presence of DEP), showed a very weak reaction to the ring test. Following mixing of the phases, the precipitin reaction did not occur unless carrier legumin was added.

Microsomes from 40-day old developing cotyledons were incubated with antilegumin, as detailed above, for 1 h at 0°C. The resulting precipitate was pelleted by centrifugation at 1000 g max. for 10 min at 4°C. The resulting supernatant was analysed by sucrose density gradient centrifugation, and showed that the polysomes had been converted to monosomes (Fig. 87). The pellet was subjected to the RNA extraction procedure detailed in the methods (section 3.(iv),(b)) and the nucleic acids obtained were substantially degraded (Fig. 88).

As the most probable source of RNase contamination was the legumin, it was omitted from subsequent incubations. Though no precipitin reaction occurred, the samples were treated in an identical manner, and analysis on sucrose gradients gave a profile similar to Fig. 87.

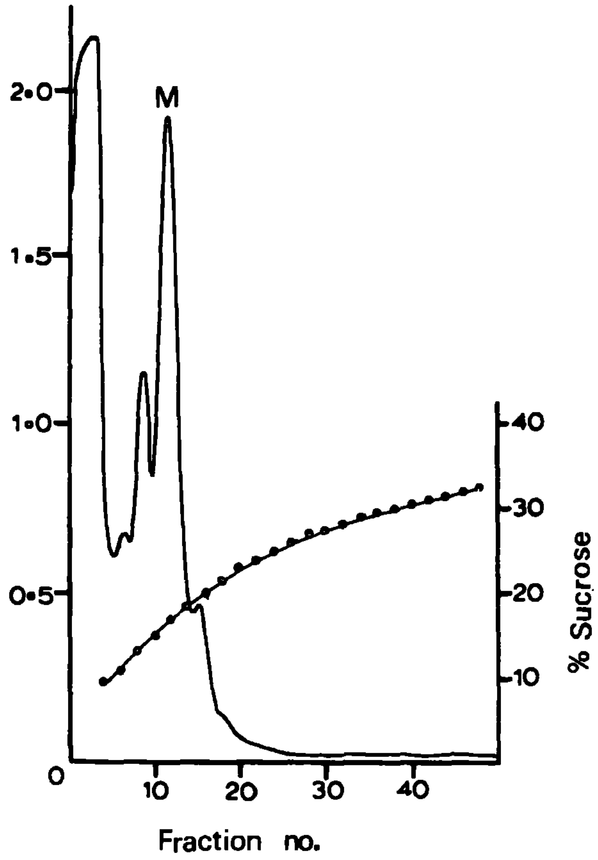
Yarwood (unpublished results) demonstrated that the antilegumin was contaminated with bacteria. Filtration of the antilegumin through a Swinnex bacterial filter (Millipore Ltd.) did not effect the above result.

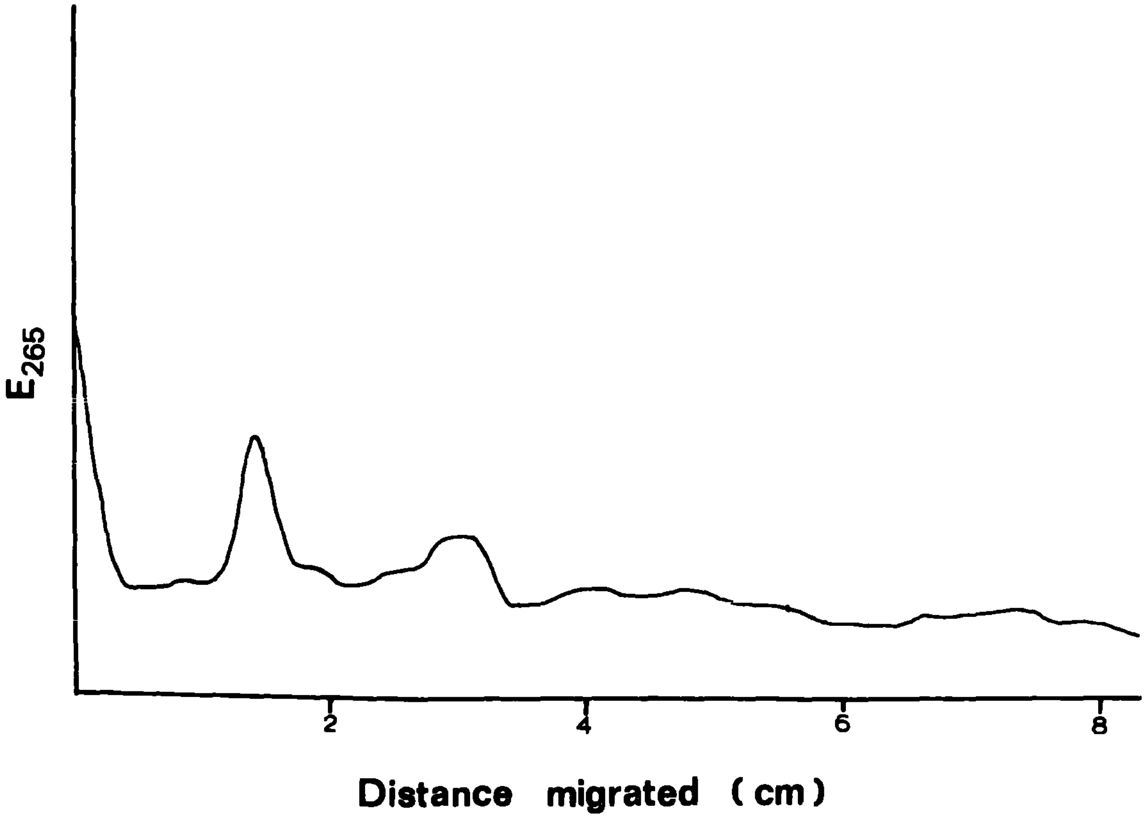
Fig. 87.

Sucrose-gradient sedimentation profile of the supernatant following incubation of triton-disrupted microsomes with antilegumin and legumin, as detailed in the text. The microsomes were prepared in the presence of DEP from 50-day old cotyledons. A volume equivalent to 1.0 mg microsomes was layered on to the gradient. Gradient parameters are detailed in the legend to Fig. 81.

Fig. 88.

The nucleic acids of the antilegumin complex. Triton-disrupted microsomes prepared from 50-day old cotyledons were incubated with antilegumin and legumin, as detailed in the text. The resulting precipitated was extracted as detailed in the methods, section 4(iv)(b). Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam D.


M
2•0
1•5
1•0
0•5
0
10
20
30
40
Fraction no.
40
30
20
10
% Sucrose


E265
2
4
6
8
Distance migrated (cm)

(iii) $[^{32}P]$-Orthophosphate labelled microsomal RNA from 50-day old cotyledons.

50-day old developing seeds were labelled in vivo, as described (Methods, section 11.(ii),(b)). The microsomes were isolated in the presence of DEP and the nucleic acids extracted directly from the microsomal pellet, as described (Methods, section 4.(iv),(b)).

The nucleic acids were analysed on a 2.6% acrylamide gel (Fig. 89a). Less breakdown was evident than had previously been observed (cf. Fig. 85a). Melting of the sample at 60°C for 10 min resulted in the breakdown of the 25S and 18S RNA's (Fig. 89b).

(iv) The analysis of $[^{32}P]$-orthophosphate labelled microsomes from 60-day cotyledons.

60-day old developing seeds were labelled in vivo, as described (Methods, section 11.(ii),(b)). The microsomes were isolated in the presence of DEP and samples treated variously as below:

(1) 2.0 mg triton-disrupted microsomes were incubated for 1 h at 0°C in the presence and absence of antilegumin.

(2) 2.0 mg triton-disrupted microsomes were analysed immediately on a sucrose gradient.

(3) The RNA was extracted from 4 mg of microsomes.

The results obtained from the sucrose gradients are compiled in Figs. 90 and 91. As has been shown previously, antilegumin (RNase) results in the loss of polysomes. This loss is evident by a decrease in the radioactive profile (Fig. 90) as against microsomes not incubated with antilegumin. The incubation period of 1 h at 0°C causes a shift of polysomes to the lighter part of the gradient (compare Figs. 90 and 91).

Analysis on 2.6% acrylamide gels of RNA obtained from (i) total microsomes, (ii) monosomes (Fig. 91) and (iii) polysomes (Fig. 91), showed no significant differences and are represented by one figure (Fig. 92). Melting of the samples at 60°C for 10 min resulted in a similar profile to Fig. 85b.

Fig. 89.

50-day old cotyledon microsomal RNA. Seeds were labelled in vivo with $[^{32}P]$-orthophosphate as detailed in the methods, section 11 (ii)(b). Microsomes were prepared in the presence of DEP and the nucleic acids extracted as detailed in the methods, section 4(iv)(b). The numbers above the peaks refer to their MW x $10^6$.

(a) Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam B.

(b) RNA heated at 60°C for 10 min, then cooled rapidly. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam C.

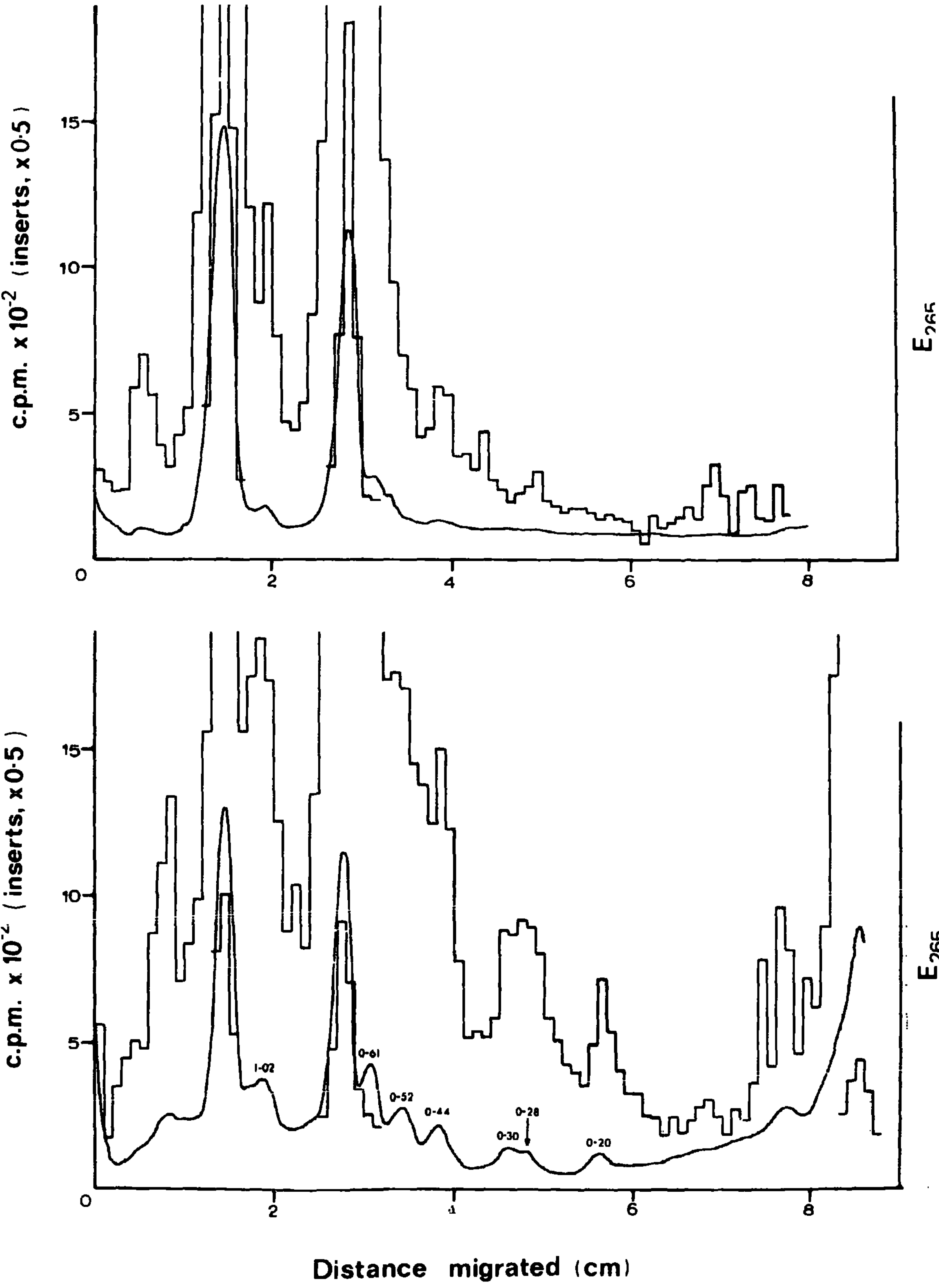

c.p.m. x 10$^{-2}$ (inserts, x 0·5)
15
10
5
0
2
4
6
8
E265
c.p.m. x 10$^{-2}$ (inserts, x 0·5)
15
10
5
0
2
4
6
8
E265
1·02
0·61
0·52
0·44
0·30
0·28
0·20
Distance migrated (cm)

Fig. 90.

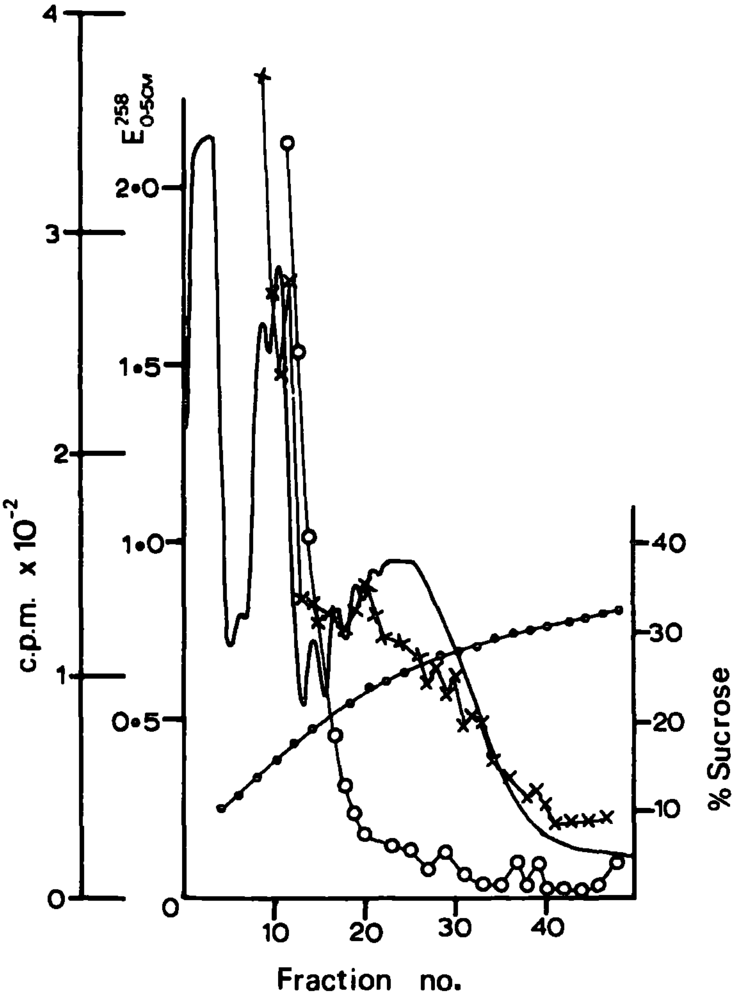
Sucrose-gradient sedimentation profile of triton X-100 disrupted microsomes from 60-day old cotyledons. Seeds were labelled *in vivo* with $[^{32}P]$-orthophosphate as detailed in the methods, section 11(ii)(b). Microsomes were extracted in the presence of DEP and were incubated for 1h at 0°C (± antilegumin) prior to analysis on sucrose gradients. A volume equivalent to 2.0 mg microsomes was layered on to the gradients. Gradient parameters are detailed in the legend to Fig. 81. Radioactivity was determined on 50 µl aliquots dried on to 2 cm filter paper disks.

x-x ; radioactive profile, of microsomes incubated in the absence of antilegumin, corresponds to the optical density profile.

O-O ; radioactive profile, of microsomes incubated in the presence of antilegumin. An optical density profile similar to Fig. 87 was obtained.

Fig. 91.

Sucrose-gradient sedimentation profile of triton X-100 disrupted microsomes from 60-day old cotyledons. Seeds were labelled *in vivo* with $[^{32}P]$-orthophosphate as detailed in the methods. Microsomes were extracted in the presence of DEP and were analysed immediately on a sucrose gradient. 2.0 mg microsomes were layered on to the gradient. Gradient parameters are detailed in the legend to Fig. 81. The shaded areas under the peaks were collected and the RNA extracted as detailed in the methods, section 4(iv)(b).


4
3
2
1
0
c.p.m. x 10-2
E258 0·5CM
2·0
1·5
1·0
0·5
0
40
30
20
10
% Sucrose
10
20
30
40
Fraction no.

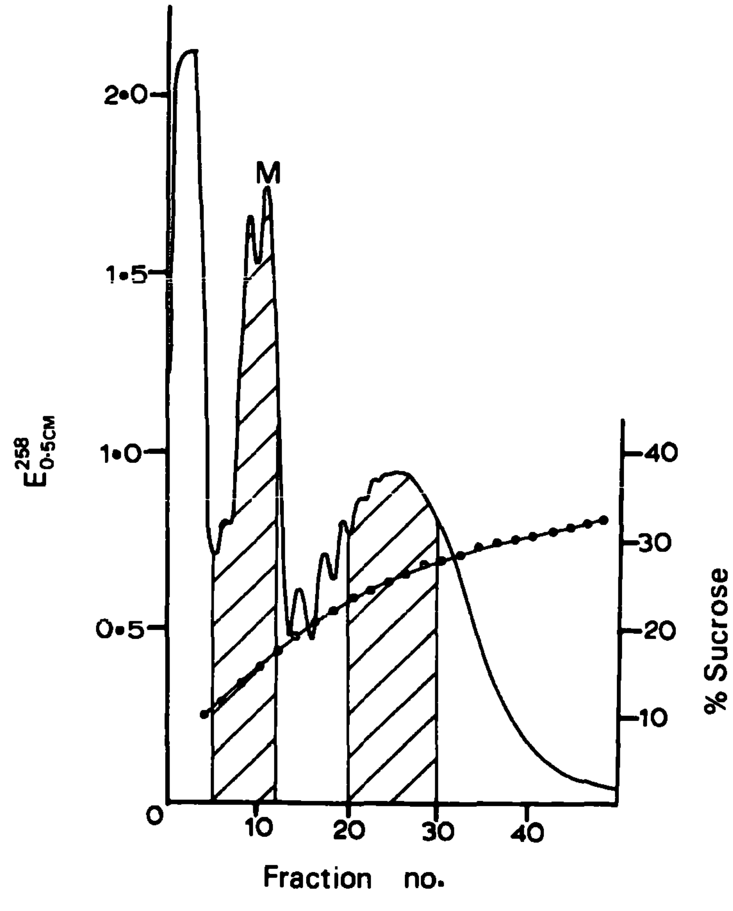
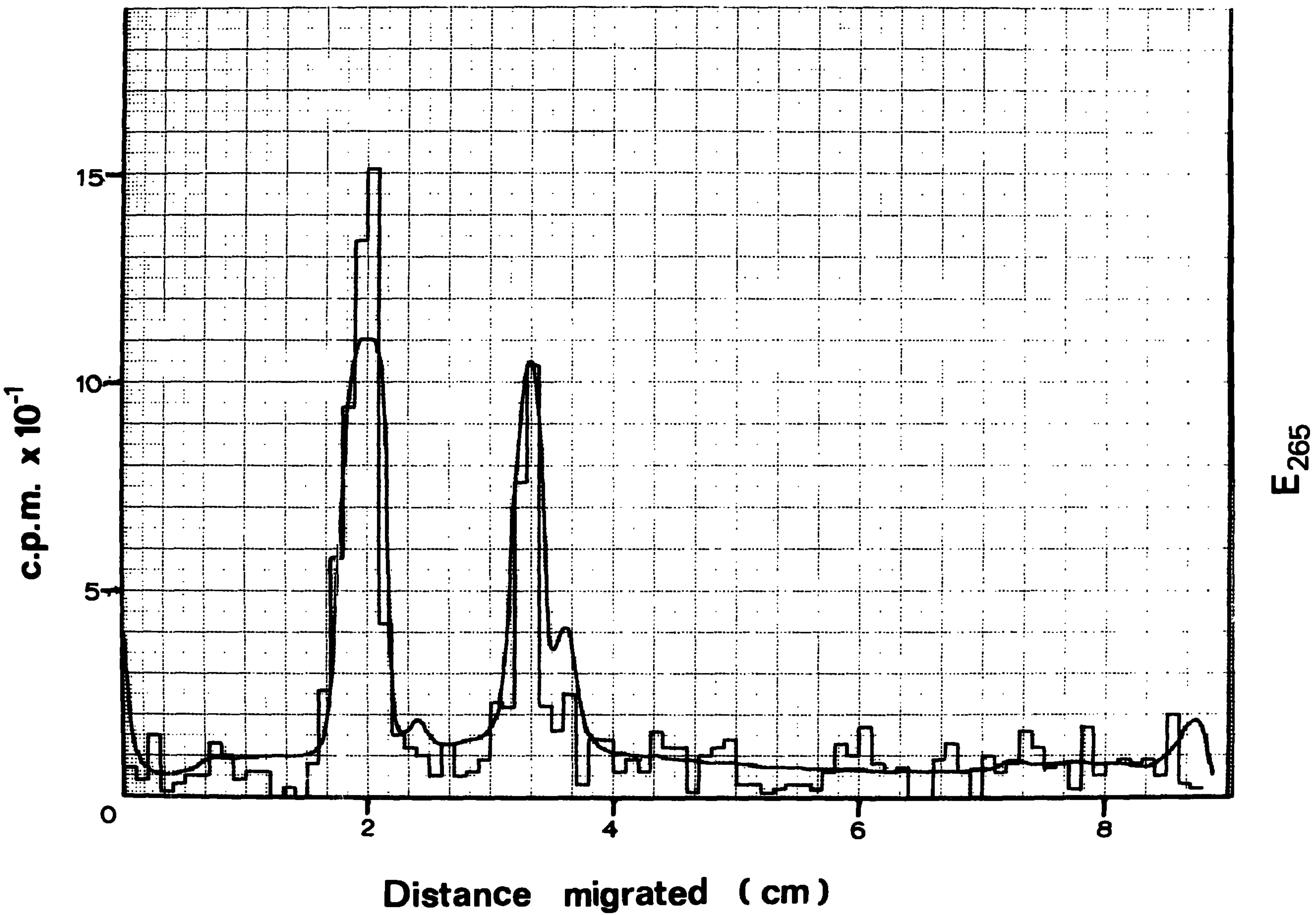

2·0
1·5
1·0
0·5
0
M
E258 0·5CM
40
30
20
10
% Sucrose
10
20
30
40
Fraction no.

Fig. 92.

Polysomal RNA from 60-day old cotyledons. Polysomes were purified and extracted as detailed in Fig. 91. Electrophoresed on a 2.6% gel for 3h. Vertical expansion : cam A. Radioactivity is plotted as a histogram.


c.p.m. x 10$^{-1}$
15
10
5
0
2
4
6
8
Distance migrated (cm)
E$_{265}$

## 11. Spectral characteristics of samples

The spectral characteristics of selected samples prepared by the various techniques are given in Table 7.

## TABLE 7

Spectral characteristics of some selected samples.

| | Method of Preparation | $E_{260}/E_{230}$ | $E_{260}/E_{280}$ | $E_{max.}$ nm. | $E_{min.}$ nm. |
|---|---|---|---|---|---|
| 50-day cotyledon microsomes | 7(ii) | 0.99 | 1.68 | 259 | 239 |
| 50-day cotyledon ribosomes | 7(ii) | 1.35 | 1.55 | 259 | 239 |
| 40-day cotyledon nucleic acids | 4(i) | 2.45 | 2.24 | 257 | 230 |
| 65° RNA | 4(ii) | 1.8 | 1.96 | 258 | 231 |
| Mitochondrial pellet nucleic acids | 4(iii) | 2.14 | 2.25 | 258 | 231 |
| Polysomal RNA | 4(iv)(a) | 2.34 | 2.18 | 257 | 229 |
| Polysomal RNA | 4(iv)(b) | 2.50 | 2.38 | 257 | 228 |
| 4S RNA<br>G75 Fraction III | - | 2.32 | 2.08 | 257 | 229 |

# DISCUSSION

The seed of *V. faba* is characteristic of the Papilionaceae in containing protein as a major component of its nutrient reserves (Danielsson, 1949). The two storage globulins were first identified by Osborne and Campbell (1898), and were termed vicilin and legumin, having MW's of approximately 180,000 and 320,000 (Danielsson, 1949; Bailey and Boulter, 1970).

On the basis of biochemical and cytological studies, Briarty (1967) divided the development of *V. faba* cotyledons into four stages:

I. Mitotic division of the zygote up to the point at which the cellular complement of the embryo approaches that found in the mature seed. Terminates 20 to 25 days after flowering.

II. Increase in fresh weight of the embryo; the embryo swelling to fill the seed cavity. Terminates about 35 days after flowering.

III. The rapid accumulation of starch and protein reserves. Terminates 70 to 100 days after flowering.

IV. The maturation and dehydration of the seed.

The above classification will be referred to in discussing aspects of nucleic acid metabolism of *V. faba* var. Triple White.

The seeds of *V. faba* were obtained from outdoor grown material and therefore were subject to environmental conditions which resulted in a wide variation of the size of seeds of any one age and in the time taken by a seed to reach maturity (Robertson *et al.*, 1962; Wheeler, 1965). Normally the age of a particular seed was determined from its day of flowering and from its size, compared to the average size for that particular age. This method of ageing seeds cannot account for the yearly variation which occurs in the overall maturation period of developing seeds. It was observed that in all previous studies on developing seeds of _V. faba_ (Wheeler, 1965; Briarty, 1967; Payne, 1968), the increase in the average fresh weight of the cotyledons was approximately constant during stage II and stage III of development, though yearly variations in the onset of fresh weight increase occurred. Random samples of seeds, aged by their flowering date, gave the result shown in Fig. 1. This graph allowed the determination of seed age from single cotyledons or cotyledon pairs during the 1969, 1970 and 1971 seasons. Normally good agreement was obtained with outdoor grown material dated by flowering in the 1969 and 1970 seasons, provided that an average size bean was selected. Variation between the flowering age and the weighted age (determined from Fig. 1) was observed in the 1971 season, and in late harvest and winter grown material; in such cases the weighted age was used. Little winter grown material was available for use as difficulties were experienced in obtaining seeds under greenhouse conditions due to the abortion of flowers. As the basic aim of this study was to identify a nucleic acid species directly concerned in information transfer, the majority of the material used for analysis was between 20 and 70 days old.

Rapid RNA synthesis commences in the developing cotyledons about 35 days after flowering (Wheeler, 1965; Payne, 1968), and is evidenced by the appearance of prominent nucleoli and rough ER (Briarty, 1967), and continues up to 60 or 70 days after flowering. The increase in the membrane-bound ribosome fraction and the apparent constancy of the ratio of 25S and 18S RNA to total nucleic acids during the period of RNA synthesis (Payne, 1968), indicates that rRNA synthesis predominates during stage III of development. This was confirmed by the increase in the 25S RNA RNA relative to DNA (Fig. 28) and by in vivo $[^{32}P]$-orthophosphate labelling (Figs. 80, 89 and 92). Relatively little DNA synthesis was detected in 40-day old cotyledons as compared to rRNA synthesis (Fig. 80) and agrees with Payne (1968), who demonstrated a low rate of cell division in cotyledons during stage III of development.

Calculation of the amounts of 25S and 18S RNA's and 5S and 4S RNA's as a percentage of the total RNA gave values of 83% and 17% respectively. These are in agreement with Payne's (1968) values of 87% and 13% for the percentage of high molecular weight RNA and LMW RNA to total RNA respectively, in developing cotyledons. Payne (1968) detected an increase in the LMW RNA fraction, eluted off methylated albumin kieselguhr (MAK) columns, of developing cotyledons between day 50 and day 70. As this fraction consists of 5S RNA, 4S RNA and rRNA breakdown products an increase in any one of these RNA's would effect an apparent increase in the total fraction. The only significant change observed in the LMW RNA's of developing cotyledons was the appearance of the rRNA breakdown products in cotyledons older than 60 days (Figs. 30 and 31). Analysis of the relative amount of 4S RNA to 5S RNA present in the cotyledons at various ages (Fig. 29) gave divergent values and results were subjected to regression analysis in order to determine the best straight line. The correlation coefficient and the t-distribution on the slope obtained were significant ($P > 0.01$ and $< 0.02$). If the values for 30-day old cotyledons were omitted, the regression line would be practically horizontal and therefore little or no significance can be attached to the slope statistics. It is however evident that the 5S and 4S RNA's remaining in approximately constant proportion to each other during development and as it has previously been established that the rRNA content increases during development, then this must also hold true for the 4S RNA fraction. As the 4S RNA fraction is synonymous to tRNA then a situation exists where the tRNA pool increases as the ribosome content of the cell increases. The number of molecules of $_t$RNA per ribosome per cell cannot be calculated with certainty owing to the nature of the data available, but would appear to be somewhere in the region of 6-12 $_t$RNA molecules per ribosome (see Figs. 29 and 80). The number of $_t$RNA molecules bound per ribosome was calculated at 1.5 (Fig. 68) and is in agreement with the values obtained by Wettstein and Noll (1965), and King and Gould (1970).

Analysis of the testa nucleic acids showed a steady decline of rRNA relative to DNA from 30 days to 60 days after flowering (Fig. 32) and a rapid increase in the rRNA breakdown products during this period (Figs. 33, 34 and 35); similar results were obtained by Payne (1968). Following $[^{32}P]$-orthophosphate incorporation into 40-day old seeds and analysis of the nucleic acids of the testa (Fig. 79), it was apparent that rRNA synthesis was continuing even though the relative amount of rRNA was decreasing. This agrees with Wheeler (1965) who showed that the nucleotide content of ageing testa material increases up to day 55 after flowering. Between day 55 and day 80 there was a marked decrease in the nucleotide content of the testa (Wheeler, 1965). This was confirmed by Payne (1968) who demonstrated a very sharp decrease in the rRNA content of the testa during this period. This decrease in the total nucleotide content of the testa probably reflects the translocation of material to the maturing cotyledon (Wheeler and Boulter, 1967). The observed differences in MW of the breakdown products in maturing cotyledons and senescing testa's probably results from a higher concentration of RNase in the testa. These breakdown pdoructs were not observed in mature dehydrated seeds.

The MAK column elution profiles of nucleic acid extracts from 30 to 60-day old cotyledons were atypical in containing a third, high molecular weight RNA fraction (Payne, 1968). A similar fraction constituting 34% of the total high molecular weight RNA was identified in monocotyledon seed nucleic acid extracts by Cherry and Lessman (1967). They suggested that this fraction constituted a store of long-lived mRNA which would direct protein synthesis at the onset of germination. Loening and Ingle (1967) fractionated the RNA's of several seeds of the monocotyledons by polyacrylamide gel electrophoresis and did not detect any unusual RNA fractions. The third high molecular weight RNA fraction of Cherry and Lessman (1967) has therefore arisen from the aggregation of the 25S and 18S RNA's. A similar situation has been found to exist for V. faba cotyledon nucleic acids in that no unusual RNA fractions were found in total nucleic acid preparations (Fig. 27), and therefore the third high molecular weight fraction detected by Payne (1968) constitutes an aggregation artifact. Direct analysis of this fraction by polyacrylamide gel electrophoresis demonstrates the presence of the 25S and 18S RNA's (Payne, 1968; Ingle and Key, 1968). Payne (1968) also identified two minor components of MW $2.2 \times 10^6$ daltons and $1.93 \times 10^6$ daltons. The latter was ascribed to the 25-18S RNA aggregation product. The $2.2 \times 10^6$ daltons component in having a MW significantly different from the 25-25S aggregation component (MW = $2.58 \times 10^6$ daltons) was attributed to either (i) a polycistronic message; (ii) a rRNA - mRNA complex or (iii) a rRNA precursor molecule. Ingle and Key (1968) also identified two minor components of MW $2.5 \times 10^6$ daltons and $2.0 \times 10^6$ daltons, noticeable having MW's similar to the 25S-25S rRNA and the 25S-18S rRNA aggregation components respectively. They concluded that they were not aggregation products as the conditions of gel electrophoresis are not favourable for aggregation, though they made no positive suggestions as to their identity. Although aggregation of RNA molecules is unlikely to occur during electrophoresis, the salt concentration required to elute rRNA from MAK columns does cause aggregation; and if stable aggregates are formed, then they will migrate as an aggregate on acrylamide gels. Such aggregates can only be released into their component molecules by melting the RNA (see Figs. 63

and 64). Two minor components, similar in mobility to those described by Payne (1968) and Ingle and Key (1968) were identified in some preparations of RNA extracted from ribosomes (Fig. 68a). These represent the 25S-25S RNA and 25S-18S RNA aggregation components. Accurate MW data could not be obtained on these components, because the relationship between MW and mobility is non-linear in this region of the gel. The non-linearity arises from a dilution of the acrylamide during overlayering the gel with water during its preparation; this normally results in an under-estimation of the MW and this may explain the results obtained by Payne (1968).

The in vitro labelling of cotyledon slices with $[^{32}P]$-orthophosphate for 30 min followed by a 15 min chase, gave no incorporation into nucleic acids as judged by the distribution of radioactivity on a 7.5% acrylamide gel (Fig. 78). Payne (1968), obtained incorporation of $[^{32}P]$-orthophosphate into nucleic acids following a 3h incubation. The resulting MAK elution profile of radioactivity showed a distinct peak associated with the minor DNA component eluting at a slightly lower salt concentration than the major DNA DNA fraction, and preferential labelling of the third high molecular weight RNA fraction. This distribution of radioactivity has been demonstrated to be typical of plant tissues in general (Key and Ingle, 1964; Ingle et al., 1965; Ingle and Key, 1965; Cherry and Van Huystee, 1965; Cherry et al., 1965; Chroboczek and Cherry, 1966). The analysis of the $[^{32}P]$-orthophosphate labelled rRNA's by sucrose gradient centrifugation demonstrated the associated of radioactivity with the 18S rRNA fraction and with a fraction of lower sedimentation value than the 25S rRNA (Waters and Dure, 1965 and 1966; Ingle et al., 1965). Such a distribution of radioactivity had previously been demonstrated by Glisin and Glisin (1964) in nucleic acids extracted from sea urchins contaminated with bacteria. Lonberg-Holm (1967) using bacterial contaminated seedlings confirmed Glisin and Glsin's (1964) earlier work, and also demonstrated that the rapidly-labelled minor DNA component evident on the MAK column elution profile of earlier workers (see above) was indicative of bacterial contamination. It is concluded that the observed incorporation of $[^{32}P]$-orthophosphate into nucleic acids, following the incubation of cotyledon slices *in vitro* (Payne, 1968), was due to bacteria. In the present study, although no incorporation into any nucleic acid fraction was obtained following the *in vitro* incubation of cotyledon slices, analysis of the nucleic acids showed the presence of LMW RNA's (Fig. 78) which had not been observed in nucleic acid preparations from whole cotyledons (Fig. 80b); though similar LMW RNA's had been detected in mature seeds (Fig. 38b). The ability of cotyledon slices to incorporate $[^{14}C]$-leucine and $[^{35}S]$-sulphate into legumin (Bailey *et al*., 1970) indicates that this system maintains its capacity to synthesise complete globulin molecules, and as globulin synthesis is dependent on rRNA and probably mRNA synthesis, it should be possible to demonstrate RNA synthesis in this system. The apparent inducement of rRNA degradation by slicing the tissue and its inability to incorporate $[^{32}P]$-orthophosphate is anomalous if the results of Bailey *et al*. (1970) are accepted and further experimentation is required before any conclusions can be reached.

Attempts to identify nuclear RNA's either by the hot phenol-SDS extraction procedure or directly from isolated nuclei were not successful. Following the demonstration of the nuclear rRNA precursors in excised and intact roots of pea seedlings (Rogers *et al*., 1970) similar experiments were attempted using the roots of *V. faba*. Initial experiments on excised root tips incubated in $[^{32}P]$-orthophosphate for varying lengths of time resulted in the labelling of bacterial nucleic acids. Further experiments were carried out on carefully selected and sterilised seeds, germinated under sterile conditions, though this apparently enhanced rather than reduced the bacterial flora (compare Figs. 76 and 77). As with cotyledon slices no incorporation into the plant nucleic acid fractions could be detected. It would seem reasonable to assume that in this particular instance, the bacteria in the intercellular spaces or embedded in the cell walls of the roots preferentially used the available phosphate (see Barber, 1966). The bacterial RNA's observed on 2.2% acrylamide gels with the exception of the $0.36 \times 10^6$ daltons component (Fig. 75) are similar to those described by Iwabuchi et al. (1965), Hecht and Woese (1968) and Marrs and Kaplan (1970), though the rate of labelling of the nucleic acids is much slower. The observed labelling of the $0.63 \times 10^6$ and $0.56 \times 10^6$ daltons components is consistent with the $0.63 \times 10^6$ daltons component being a precursor to the $0.56 \times 10^6$ daltons component (see Figs. 75, 76a, 77 and Marrs and Kaplan, 1970). The $0.36 \times 10^6$ daltons component can not be explained as it was only evident in one sample (Fig. 75). Analysis of the LMW RNA's showed that the bacterial 5S and 4S RNA's differed in mobility from V. faba 5S and 4S RNA's (Fig. 76b). This was reproducible in samples contaminated with bacteria whereas the analysis of the in vivo labelled RNA's showed an overlap of the radioactivity and optical density profiles (Figs. 79b and 80b). Recently Payne and Dyer (1971) have confirmed this result in demonstrating a separation of E. coli 5S RNA from V. faba cytoplasmic 5S RNA. They suggested that the V. faba 5S RNA contains 118 nucleotide residues compared to E. coli 5S RNA, which contains 120 nucleotide residues (Brownlee et al., 1968). The observed MW difference between labelled bacterial 5S RNA and the V. faba 5S RNA (Fig. 76b) is approximately 1000 daltons which is in reasonable agreement with the calculations of Payne and Dyer (1971) assuming E. coli 5S RNA to be representative of the bacterial 5S RNA's in general. No attempt was made to identify the contaminating organism(s).

Chloroplast rRNA's were evident in the nucleic acid preparations from leaves of *V. faba* (Fig. 26) and showed a molar excess of the 16S RNA component (0.56 x $10^6$ daltons). This molar excess of the 16S RNA (Loening and Ingle, 1967) or conversely the complete absence of the 23S RNA (Brawerman and Eisenstadt, 1964) has been observed in almost all chloroplast RNA studies, and is due to the lability of the 23 RNA (Ingle, 1968). There was a marked absence of organelle rRNA in nucleic acid preparations from testa and cotyledon tissue (Figs. 27, 80a and 79a) even though they displayed a typical chlorophyll colouration. Utrastructural studies on the developing cotyledon (Briarty, 1967) demonstrated the presence of mitochondria and plastid-like bodies. The latter had a fine structure similar to chloroplasts in that they were bound by a double membrane, contained grana-like lamellae and also, often contained starch grains. Briarty (1967) observed within the matrix of these organelles particles which he concluded to be ribosomes. Similar observations and conclusions had been reached by Swift (1965) using *Beta vulgaris*. It is apparent that, though organelle ribosomes exist in developing cotyledons, they are in an extremely low concentration compared to cytoplasmic ribosomes and therefore their constituent rRNA's cannot be detected by conventional means. The only RNA species identified with the organelles of *V. faba* cotyledons is $_t\mathrm{RNA}_3^{\mathrm{MET}}$ (Yarwood *et al.*, 1971). This $_t$RNA species is capable of being charged and formylated by *V. faba* or *E. coli* enzyme and is indistinguishable from *E. coli* formylmethionine - $_t\mathrm{RNA}_F$ in the *E. coli* cell-free system, suggesting that it functions as the initiator $_t$RNA on the organelle 70S ribosomes (Leis and Keller, 1970; Yarwood *et al.*, 1971).

A minor RNA species with a mobility less than the 5S RNA was first detected in total cotyledon nucleic acid samples (Fig. 33) and later found to be a major species in nucleic acid samples extracted at 65°C (Figs. 37b and 64b). It could also be demonstrated by melting total nucleic acid samples which had been extracted at low temperatures (Fig. 40); this treatment not only caused the release of this RNA but also noticeably reduced the absorbance of the 25S rRNA when compared to the 18S rRNA. Following gradient-purification of the 25S and 18S rRNA's, heat treatment dissociated this LMW RNA species only from the 25S RNA (Figs. 45 and 46). It was therefore similar to the 7S RNA described by Pene et al. (1968) and the 28S associated RNA described by Weinberg and Penman (1968). Its MW and sedimentation coefficient were determined to be 0.52 x $10^5$ daltons and 5.8S respectively using 4S and 5S RNA's as standards. These values are similar to the values obtained for this RNA from pea seedling (Sy and McCarty, 1970). The 5.8S RNA is dissociated from the 25S rRNA at a relatively low temperature (Fig. 41) in agreement with other studies (Sy and McCarty, 1970; King and Gould, 1970; Payne and Dyer, 1971) indicating only 3-6 base pairs involved in the bonding of this molecule to the 25S rRNA (King and Gould, 1970). The 5' terminal analysis of the larger of the two rRNA's from both animal and plant tissues reveals two termini in equimolar amounts (Madison, 1968; Becker and Pollard, 1969). In addition the ratio of absorbance at 260nm of the heat released 5.8S RNA to the 5S rRNA reaches a maximum at 1.35 (Fig. 41) which is very close to the calculated value of 1.37 for equimolar amounts of the two. It would therefore appear that there is one molecule of 5.8S RNA per molecule of 25S rRNA. The occurrence of the 5.8S RNA, as a component of the 80S ribosome, has been demonstrated in a wide range of eukaryotic organisms (Pene et al., 1968; Sy and McCarty, 1970; Payne and Dyer, 1971), and it appears to be absent from the 70S ribosome systems so far analysed (Pene et al., 1968; Payne and Dyer, 1971). The value 5.8S is determined by plotting mobility against sedimentation coefficient, using 4S and 5S RNA's as standards. However, if 3.95S and 4.48S are used, which are the values of the sedimentation coefficients at 20°C corrected for the viscosity and density of the solvent for wheat 4S and 5S RNA's respectively (Soave et al., 1970), then a value of 4.9S is obtained for the '5.8S' RNA of V. faba. This value is similar to the value of 5.0S ± 0.2S, determined by sedimentatio analysis, obtained for rabbit reticulocyte '1' RNA (= 5.8S RNA) by King and Gould (1970). The use of polyacrylamide gel electrophoresis to determine sedimentation coefficients is fallacious in that there is no real relationship between mobility and sedimentation coefficient, and the determination of S values for the '5.8S' RNA from a two point plot (Sy and McCarty, 1970; Payne and Dyer, 1971) is therefore considered to be meaningless.

An attempt to purify the 5.8S RNA, for sedimentation analysis, was made using as a basis the techniques of molar-salt precipitation and/or DEAE-cellulose chromatography, which had previously been used in the purification of the 4S and 5S RNA species. Basically the purification scheme was to isolate the 25S and 18S RNA species free from 4S and 5S RNA's; heat the purified 25S/18S RNA fraction to release the 5.8S RNA and then isolate the 5.8S RNA. The separation of the 5S and 4S RNA's from the 25S and 18S rRNA's was easily achieved either by precipitation of the 25S and 18S rRNA's by molar-salt or by absorption of the 5S and 4S RNA's on to DEAE-cellulose. Both techniques yielded apparently undegraded heavy rRNA free of LMW RNA's (Fig. 17) and subsequent heat treatment released the 5.8S RNA (Fig. 47) and caused the aggregation of the heavy rRNA's which facilitated their subsequent separation by molar-salt precipitation. The molar-salt soluble RNA (5.8S RNA fraction) was found to be contaminated with LMW RNA species (Fig. 48), the principal contaminants having electrophoretic mobilities indistinguishable from 5S rRNA and 4S RNA (tRNA). The precipitated heavy rRNA's, following washing, were contaminated with a LMW RNA which had a mobility identical to the 5.8S RNA (Fig. 50). An attempt to recover this fraction by high-speed centrifugation only resulted in further breakdown of the RNA's (Fig. 51). Similar results were obtained when DEAE-cellulose chromatography was used to prepare the 5.8S RNA fraction (Fig. 52). Again the main contamination arose from RNA's having mobilities identical to 5S and 4S RNA's. The presence of these components in this particular fraction suggested that some binding of the 25S and 18S rRNA's to DEAE-cellulose had occurred. The binding of the heavy rRNA's to DEAE-cellulose was subsequently demonstrated (Fig. 55) and agrees with the earlier work of Stanley and Bock (1965) who described the formation of a non-elutable complex. Attempts to reduce nuclease nicking of the heavy rRNA's, which was considered to be a possible cause of the breakdown, by modifying the extractant produced no significant difference in the results. The fragmentation of the heavy rRNA's into specific LMW fragments was not due to nuclease activity as the rRNA's were apparently intact prior to heat treatment, and following heat treatment the only LMW RNA observed was the 5.8S RNA. It therefore seems possible that molar-salt treatment of heat treated RNA causes a physical fragmentation of the molecule due to stress set up by unnatural folding. However, purely physical degradation would not result in the release of specific fragments and it is therefore necessary to suggest labile regions within the molecule; such probably correspond to single stranded regions. Owing to these difficulties, purification of the 5.8S RNA was discontinued.

Diethylpyrocarbonate (DEP) was first demonstrated to inhibit enzymes by Hullan et al. (1965) and later by Fedorcsak and Ehrenberg (1966). Subsequent studies on its mode of action have demonstrated that it is non-selective against proteins, reacting with free amino groups and primary amines forming N-carbethoxy derivates (Rosen and Fedorcsak, 1966; Muhlrad et al., 1967; Hegyi and Muhlrad, 1968; Glazer, 1970). The ability of DEP to inhibit nucleases was of obvious interest in this study and preliminary experiments based on polyacrylamide gel electrophoresis were designed to compare the actions of LLS and DEP on nucleases. In all incubations containing LLS, both RNase and DNase were inhibited which was the expected result (see Elson, 1958; Spahr and Hollingworth, 1961), owing to its denaturing effect on protein structure. In contrast DEP did not inhibit DNase activity but inhibited RNase activity. Though the DNase concentration in these incubations was known, the RNase concentration was an unknown factor and was assumed to be low compared to the DNase concentration. As the RNase activity was inhibited by DEP in the Koch-Light DNase incubation, but the DNase activity was apparently uninhibited, a comparison can be made to a result of Fedorcsak and Ehrenberg (1966), in which they obtained inhibition of RNase in the presence of 5% albumin. Little information could be obtained from these non-quantitative experiments, but in general the results indicated that DEP did not effectively inhibit DNase. This apparent discrepancy with the results of Fedorcsak and Ehrenberg (1966) prompted a re-investigation of their results using a DNase incubation system. The incubation system was based on uranyl acetate, in which only the lower oligomer digestion products are soluble and therefore seemed reasonable for determining more precisely the extent of the action of DNase on DNA in the presence of DEP. A similar incubation system for assessing DNase activity has been described by Hurst (1958). Following the determination of the initial rate parameters (Figs. 8 and 9), incubations involving DEP showed that inhibition of DNase activity was rapid and non-linear with respect to DEP concentration (Fig. 10). A similar result was obtained when DNase was pre-incubated with DNase (Fig. 12). Both of these results are in agreement with the previous observations of Fedorcsak and Ehrenberg (1966). As these authors used a 10% solution of DEP in ethanol, similar experiments were conducted and no differences were detectable either in the observed inhibition of DNase (Fig. 10) or in the solubility of DEP which normally produced a two phase system when its concentration was greater than 0.5% (v/v). Incubations which contained DEP at a concentration of 5% (10 x saturation level) and in which the enzyme concentration was varied (Fig. 11), showed that as enzyme concentration increased, the observed inhibition of DNase decreased. A similar situation existed with high concentrations of RNase (Table 3). Owing to the limited solubility of DEP, a factor which must govern its ability to react with the components of the soluble phase, it can be reasonably assumed that protein concentration is a critical factor if DEP is to be used as an effective nuclease inhibitor.

In contrast to RNase, DNase activity was affected by Tris buffer, and this effect was additive to the effect of DEP (See Fig. 13 and Table 3). Whether the inhibition was caused by Tris or acetate was not determined and it was assumed that this effect was negligible at the Tris concentration used and did not warrant repeating the experiments using a different buffer system. This inhibition of DNase activity by Tris buffer indicates that prior to general enzyme studies a careful testing of buffer systems is necessary, and this has in the past been overlooked (Fedorcsak and Ehrenberg, 1966; Hurst, 1958).

Recently new methods, based on the use of DEP as a nuclease inhibitor, have been developed for the extraction of undegraded nucleic acids from plant tissues (Solymosy _et_ _al_., 1968; Lazar _et_ _al_., 1969; Solymosy _et_ _al_., 1970). These extraction procedures incorporate SDS into the initial homogenisation medium, but homogenisation is carried out at low temperatures which probably destroys the effectiveness of SDS against nucleases. Analysis of the nucleic acids either by sucrose-gradient or acrylamide gel electrophoresis demonstrated that from older tissues there was a molar excess of 18S rRNA over the 25S rRNA (Lazar _et_ _al_., 1969; Solymosy, 1970). This was attributed to a preferential extraction of the 18S RNA. However, such profiles may result from extensive nuclease attack on the rRNA's during the initial stages of the extraction procedure in which the SDS is effectively removed by the temperature conditions, and RNase is effectively protected against DEP modification, not only by the limited solubility of DEP but also by the protein concentration of the homogenate. This explanation of the results of Lazar _et_ _al_. (1969) and Solymosy _et_ _al_. (1970) is supported by the work of Melera _et_ _al_. (1970), who found that DEP did not effectively protect rRNA from nuclease attack during its extraction from _Physarum_ *polycephalum*. Also a molar excess of 18S RNA over 25S RNA can be generated by heat treating RNase nicked rRNA (Figs. 64a, 71b and 85b). The reaction of DEP with nucleic acids is generally less well understood than its reaction with proteins. Though DEP destroys the infectivity of TMV RNA (Gulyas and Solymosy, 1970), ~~but~~ it does not destroy the transforming capacity of bacterial DNA (Fedorcsak and Turtoczky, 1966) or the amino acid acceptor capacity of human placental tRNA (Abadom and Elson, 1970). A recent report (Wiegers and Hilz, 1971) has demonstrated that DEP degrades nucleic acids. It is concluded that the use of DEP either as a nuclease inhibitor or conversely as an RNA protecting agent can not be relied upon.

Payne (1968) recommended the use of 0.5% sodium deoxycholate for the solubilisation of membrane during the isolation of *V. faba* polyribosomes. Preliminary experiments using *Phaseolus aureus* tissue demonstrated that the ionic detergent sodium deoxycholate at a concentration of 0.2% ~~produced~~ induced the destruction of ribosome units into subunits when compared to the non-ionic detergent triton X-100 at a concentration of 4%. Similar results were obtained using the developing cotyledons of *V. faba*. The destruction of ribosomes by sodium deoxycholate, though not detected by Payne (1968), has previously been recorded by Burka (1967) and Golub and Clegg (1969); and is attributed to the denaturation and loss of ribosomal proteins. As triton X-100 did not appear to affect ribosome integrity it was routinely used to effect the solubilisation of membrane during the isolation of V. *faba* polyribosomes.

Bentonite, which has often been used in the preparation of plant polysomes (Watts and Mathias, 1967; Tester and Dure, 1966), was used in the current study in an attempt to inhibit RNase activity by selective absorption. Tester and Dure (1966) demonstrated that bentonite was capable of absorbing appreciable quantities of ribosomes, thus effectively reducing the yield. Similar results were obtained by Blanton and Barnett (1969), who also showed that bentonite was capable of absorbing rRNA. The ability of bentonite to remove RNase was routinely checked by extracting the nucleic acids from the subcellular fractions, and analysing them by polyacrylamide gel electrophoresis. The amount of rRNA breakdown varied from preparation to preparation (Fig. 67 and 68a), though generally rRNA breakdown increased through the various stages of polyribosome isolation (Figs. 65, 66, 67, 68 and 69). The greatest amount of rRNA destruction was observed following the separation of ribosome units on sucrose gradients (Fig. 69) and indicates an association of RNase with the ribosomal particles. Whether this is due to absorption of RNase during the initial homogenisation of the material or whether the RNase is a ribosomal protein is not known. Hsiao (1968) has obtained a similar association of RNase with Zea mays ribosomes. The amount of rRNA destruction by RNase could be substantially increased if microsomes were incubated for 24h at 0°C prior to extraction and analysis of the nucleic acids (Fig. 70). The appearance of so many minor degradation components necessitated a study of rRNA cleavage as a prerequisite to any attempted identification of non-ribosomal RNA fractions.

Extensive rRNA breakdown was induced by omitting bentonite and substituting sodium deoxycholate for triton X-100 in the preparation of polyribosomes and by isolating the monosome fraction by sucrose density gradient centrifugation (Fig. 60). Analysis of the nucleic acids showed a complete absence of 4S RNA (c.f. triton treated ribosomes Fig. 68b) and four fragments of MW, 1.02, 0.61, 0.44 and 0.31 x $10^6$ daltons. The 0.61 x $10^6$ daltons component, which was the major fragment, had previously been identified, in cotyledons which had been subjected to liquid $N_2$ freezing, as a specific cleavage product of the 0.70 x $10^6$ rRNA. Cleavage of the 0.70 x $10^6$ rRNA, not only results in the 0.61 x $10^6$ component but also a minor fragment of 0.09 x $10^6$ daltons. A similar cleavage of the 0.70 x $10^6$ rRNA has been described by Payne and Loening (1970) in pea root microsomes. The 1.02 x $10^6$ daltons component which is taken to be a cleavage product of the 1.29 x $10^6$ rRNA had previously been detected in roots (Fig. 25), mature cotyledons (Fig. 38a) and in triton treated ribosome preparations (Figs. 68a and 69). Payne and Loening (1970) described a similar component in pea root microsomes and showed that it probably arose from ribosome damaged during the homogenisation of the tissue, and that it was not related to the age of the ribosomes. However, as this component could be detected in total nucleic acids of roots and mature cotyledons it would appear that there exists *in vivo* a small population of ribosomes which are more exposed to RNase attack. The 0.44 and 0.30 x $10^6$ daltons components are comparable to the 0.45 and 0.31 x $10^6$ daltons components of pea root microsomes (Payne and Loening, 1970), though in the latter system a third component of 0.37 x $10^6$ daltons was identified. No fragment corresponding to the latter could be detected from *V. faba* ribosomes and probably results from a difference in ribosome structure. The 0.44 and 0.30 x $10^6$ daltons components appeared to be heterogeneous and were further resolved by melting the RNA. The 0.44 x $10^6$ daltons component had a minor component at 0.42 x $10^6$ daltons and the 0.30 x $10^6$ daltons component had a minor component at 0.28 x $10^6$ daltons (Fig. 89b). Melting of the RNA produced a dramatic change in the optical density profile due to the release of hydrogen bonded fragments. The 0.70 x $10^6$ rRNA became the prominent species being in molar excess over the 1.29 x $10^6$ rRNA. This type of profile could arise in two possible ways; firstly a large number of cleavage points on the 1.29 x $10^6$ rRNA molecule resulting in many small fragments and the relative stability of the 0.70 x $10^6$ rRNA or secondly a highly specific cleavage point in the 1.29 x $10^6$ rRNA which results in a 0.70 x $10^6$ daltons fragment. A small but apparent increase in the 0.70 x $10^6$ daltons species could be detected following heat treatment, by comparing gels (Figs. 71a and 71b; 85a and 85b; see also 43a and 45a) and is indicative of a 0.70 x $10^6$ daltons fragment derived from the 1.29 x $10^6$ rRNA. The cleavage of the heavy cytoplasmic rRNA into a fragment which is equivalent in size to the light cytoplasmic rRNA has been demonstrated in *Tetrahymena pyriformis* (Bostock *et al.*, 1971).

Fragment derivation studies were not undertaken and therefore conclusive evidence for this specific cleavage was not obtained. Similarly the $0.52 \times 10^6$ daltons component which was totally released by melting the RNA (Figs. 71a and 71b) is tentatively identified as a fragment of the $1.29 \times 10^6$ rRNA (see also Figs. 45a and 46a). The remaining minor cleavage products of the $1.29 \times 10^6$ rRNA, which could be identified on a size basis, had MW's of $1.13 \times 10^6$ daltons (Fig. 71b), $0.93 \times 10^6$ daltons (Figs. 45a and 70) and $0.79 \times 10^6$ daltons (Fig. 71b). Other fragments which have been detected, but not so far mentioned, are the $0.65 \times 10^6$ daltons fragment (Fig. 69), the $0.20 \times 10^6$ daltons fragment (Fig. 71b) and the 0.16 and $0.13 \times 10^6$ daltons fragments (Figs. 46a and 71b). Of these only the $0.20 \times 10^6$ daltons fragment was consistently detected in heat treated preparations.

The size distribution of the polyribosomes obtained from developing cotyledons of V. faba (Fig. 59), did not vary significantly with the age of the tissue and were similar to those obtained from other leaf tissues (Pearson, 1969; Pearson and Wareing, 1970). The observed distribution, using bentonite as an RNase protectant, cannot be representative of the in vivo distribution, as bentonite did not effectively protect the rRNA against RNase attack and probably resulted in a considerable loss of polyribosomes. The nuclease inhibitor DEP, has been reported to have a protective effect during the isolation of polyribosomes from wheat embryos (Weeks and Marcus, 1969). Similar experiment, in which DEP replaced bentonite, markedly improved the yield of polyribosomes from developing cotyledons (Fig. 81; compared to Fig. 59). A further improvement was obtained if microsomes were isolated in the presence of DEP, and the membranous material disrupted with triton X-100 immediately prior to sucrose-gradient analysis (Fig. 82). The decrease in polyribosomal breakdown and monosome content observed in microsome preparations probably results from membrane protection of the polyribosomes against the sheer force incurred during the resuspension of the microsomal pellet. Both the polyribosome and microsome profile showed an increased content of subunits over bentonite treated preparations (Fig. 59) and agrees with the previous observations of Weeks and Marcus (1969), and Anderson and Key (1970) that DEP causes ribosomes to dissociate into subunits. Microsomes were shown to be reasonably stable at 0°C for 1 hr, though a slight decrease in the polyribosomal distribution was noted (Figs. 90 and 91), indicating some residual RNase activity. The omission of the untitrated-Tris from the extractant resulted in a poor polyribosomal profile (Fig. 84), and in part this was caused by the gradient which had been subjected to freezing and thawing (see Fig. 83). Analysis of the nucleic acids from the polyribosome and microsome preparations (Fig. 85) gave profiles practically identical to the profiles obtained when rRNA degradation had been induced (see Fig. 71). The amount of rRNA destruction varied from preparation to preparation (compare Figs. 85, 89 and 92), though in all preparations degradation of the $0.70 \times 10^6$ rRNA occurred. This disagrees with Anderson and Key (1970) who showed that DEP inhibited the breakdown of the $0.70 \times 10^6$ rRNA. However, as has been previously discussed, the action of DEP against nucleases is limited by its solubility and the protein concentration; and therefore in tissues with a high RNase content, the RNase will be effectively protected for a short period during which it will cause polyribosome and rRNA degradation. The degree of DEP catalysed hydrolysis of the rRNA cannot be assessed, though omission of the untitrated-Tris from the extractant resulted in a complete breakdown of the rRNA's following heat treatment.

Antilegumin was originally intended to be used with $[^{32}P]$-orthophosphate labelled polyribosome preparations, which had been extracted in the presence of bentonite, in an attempt to precipitate those polysomes engaged in legumin synthesis. The addition of antiserum to such preparations could not possibly have led to an identification of the mRNA(s) for the storage protein legumin, owing to the degraded nature of the polyribosome preparations, though it may have led to a precipitation of those ribosome units which still carried nascent protein chains. That antiserum can cause such a precipitation has been recently demonstrated by Higashi and Kudo (1970). The use of antiserum against microsome preparations which have been extracted in the presence of DEP again could not lead to an identification of the mRNA(s) for the storage protein legumin, as the ribosome units detected in these preparations represent fragments of much larger polyribosomes which are present in vivo (Briarty, 1967) and the possible modification of the antigenic sites on nascent polypeptide would make the precipitin reaction unlikely to occur, however, a partial complex formation could possibly be detected by an increase in the sedimentation rate of the ribosome units through sucrose-gradients, or be induced to precipitate by the addition of carrier protein. Such experiments demonstrated that the antilegumin was contaminated with RNase, resulting in a conversion of the polyribosomes to monosomes (Figs. 87 and 90), and degrading the rRNA (Fig. 88) which was recovered from co-precipitated ribosomes following the addition of carrier protein to the antisera incubations. That non-specific precipitation of ribosomes can occur in the presence of antiserum has been confirmed by Higashi and Kudo (1971).

Analysis of the nucleic acids of $[^{32}P]$-orthphosphate labelled microsomes, extracted in the presence of DEP (Figs. 89, 90, 91 and 92), only demonstrated the presence of radioactive rRNA and its cleavage products. The radioactive components detected with mobilities less than the $1.29 \times 10^6$ rRNA (Fig. 89), could not be equated to non-ribosomal RNA purely on their size and the size of the ribosome units originally present in these preparations and must arise from rRNA aggregation or from contaminating DNA.

The occurrence of large coiled polyribosomes in the cotyledons of V. faba has been demonstrated by Briarty (1967; see also Bonnet and Newcombe, 1965). The absence of such large polyribosomes in cell free extracts results purely from the physical problems involved in cell breakage and the high content of RNase. In tissues such as barley and wheat seedlings, which have a low RNase content, reasonably large polyribosomes can be obtained (Watts and Mathias, 1967; Weeks and Marcus, 1969); though in no plant tissue extracts do polyribosome profiles approach those found from specialised animal tissue extracts (Kuechler and Rich, 1969a and b; Heywood and Nwagwu, 1969; Low *et al*., 1971). The identification of presumptive mRNA's in animal tissue is aided by a low RNase content, and the absence or low rate of rRNA synthesis (Kuechler and Rich, 1969a and b; Heywood and Nwagwu, 1969). The developing cotyledons of *V. faba* which have both a high RNase content and a high rate of rRNA synthesis is therefore not an ideal tissue for mRNA studies.

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# ABBREVIATIONS

With the exception of those listed below, the abbreviations and conventions used throughout this thesis are those recommended by the Biochemical Journal (Biochem. J., 116, 1 (1970)).

| | |
|---|---|
| Diethylpyrocarbonate | DEP |
| Low molecular weight | LMW |
| Lithium lauryl sulphate | LLS* |
| Sodium dodecyl sulphate | SDS |
| Tri-isopropylnaphthalene sulphonate | TNS |
| Molecular weight | MW |

*Lauryl is synonymous with dodecyl.

# AMENDMENT

Throughout this thesis $E^{258}_{0.5cm}$ should be taken to read $E^{254}_{0.5cm}$.

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