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Protein Synthesis and the Viability of Rye Grains

LOSS OF ACTIVITY OF PROTEIN-SYNTHESIZING SYSTEMS *IN VITRO* ASSOCIATED WITH A LOSS OF VIABILITY

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A study was made of the integrity of some components of the protein-synthesizing system from viable and non-viable embryos of rye grains. In comparison with viable-embryo components both post-ribosomal supernatant and ribosomal fractions from non-viable embryos are impaired, for neither will fully support polyphenylalanine synthesis in poly(U)-directed cell-free systems. The lesion in the supernatant lies in components other than the tRNA or the aminoacyl-tRNA synthetase, for these are as functional as those present in the fully active cell-free systems from viable embryos. The ribosomes of embryos of lowered viability show considerable fragmentation and degradation of both 18S and 25S rRNA. This breakdown does not, however, account for the complete lack of polypeptide synthesis in the poly(U)-directed non-viable-embryo system, for if provided with viable-embryo supernatant, non-viable-embryo ribosomes will sustain 60% of the viable-embryo ribosome activity. A lesion in non-viable-embryo supernatant has been located in the binding of the aminoacyl-tRNA to the ribosome. The impaired components in both supernatant and ribosomes in systems *in vitro* may reflect the site of faults in protein synthesis *in vivo* in the early hours of germination. The development of these lesions during grain storage could contribute to senescence and loss of viability in the embryos of rye.

The length of time that seeds retain their ability to germinate varies greatly from species to species. Long-term storage experiments (Darlington, 1951) have shown that some species retain their viability for decades whereas in others it is lost within a few days of seeds being shed.

The biochemical lesions associated with this loss of viability (i.e. capacity to germinate) have attracted much speculation (Crocker & Barton, 1953; Barton, 1961; Roberts, 1972), but precise information is sparse for this particular aspect of cellular senescence.

Before the loss of viability respiratory activity of seeds declines to a low value (Abu-Shakra & Ching, 1967) and ultrastructural studies (Hallam *et al.*, 1972) have shown the double membranes of the mitochondria of the non-viable rye embryos to be ruptured. As non-viable embryos do not incorporate amino acids into proteins during the early hours (0–6h) of germination, the question arises as to whether embryos which are non-viable contain a fully functional protein-synthesizing system which is limited only by an active energy-generating system.

The present paper describes the activity of protein-synthesizing systems *in vitro* from embryos of grain stocks of different percentage viabilities.

Materials and Methods

Chemicals

ATP, GTP, phosphoenolpyruvate, pyruvate kinase, poly(U) and biuret reagent were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. U-^{14}C-labelled amino acid mixture (52mCi/mg-atom of C), [2-^{14}C]thymidine (57mCi/mmol), [U-^{14}C]uridine (482mCi/mmol) and L-[U-^{14}C]phenylalanine (513mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. (AnalaR grade unless stated otherwise).

Grain stocks

Viable stocks of rye grain (*Secale cereale* var. Lovasz patoni) of over 90% viability were obtained from Nunns, Corn and Coal Co. Ltd., Quayside, Woodbridge, Suffolk, U.K., and attested stocks of lower but known viabilities (64%, 48%, 15% and non-viable) were obtained from a storage experiment commenced in 1964 at the National Institute of Agricultural Botany in Cambridge by Mackay &

Flood (1968). Embryos were either dissected by hand or obtained in quantity by the isolation method of Johnston & Stern (1957). Wheat germ was kindly supplied by Mr. D. F. Payne and Mr. R. S. D. Geary of Spillers Ltd., Birkenhead and Cambridge, U.K.

Methods

Measurement of incorporation of ^{14}C-labelled amino acids, thymidine or uridine in vivo into trichloroacetic acid-insoluble material. Dissected embryos were germinated at 25°C on thin layers of 1% agar (Oxoid no. 3) made up in germination medium [2% (w/v) sucrose and 10μg of chloramphenicol/ml]. A small volume of germination medium was added to wet the embryos. After 5h they were washed with fresh germination medium and incubated for a further 1h with a ^{14}C-labelled precursor (2μCi/ml in germination medium). Embryos were then washed three times with a 150-fold excess of unlabelled precursor and homogenized in germination medium containing the same concentration of unlabelled precursor. A portion of the homogenate was pipetted on to a Whatman GF/A glass-fibre filter, air dried and its radioactivity determined as described below.

The remainder of the homogenate was mixed with an equal volume of 10% (w/v) trichloroacetic acid and kept in ice for 30min. The precipitate was collected on a GF/A filter, washed with 10ml of 5% trichloroacetic acid containing 150-fold excess of the unlabelled precursor and then sequentially with 10ml of ethanol, 10ml of ethanol–diethyl ether (3:1, v/v) and 10ml of ether. The filter was air-dried, transferred to scintillant [7g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole per litre of toluene] and radioactivity was determined to a standard error of 5% or less in a Beckman model LS250 liquid-scintillation spectrometer with a counting efficiency of 60% for ^{14}C on GF/A filters.

The trichloroacetic acid precipitate from embryos incubated with ^{14}C-labelled amino acids was heated at 95°C for 15min before filtration, to discharge amino acids bound as aminoacyl-tRNA.

Preparation of tRNA from wheat germ. tRNA was extracted by a modification of the method of Tissieres (1959). Wheat germ (25g) was homogenized in a chilled mortar and pestle with 100ml of 50mM-KCl, 10mM-$MgCl_2$, 5mM-2-mercaptoethanol and 50mM-Tris–HCl buffer, pH7.6 at 20°C (TKM medium). The homogenate was filtered through two layers of muslin, and centrifuged in an MSE Highspeed 18 centrifuge at 30000*g* for 20min at 1°C. After removal of the layer of fat from the surface, the supernatant was centrifuged at 269000*g* ($r_{av.}$ 5.7cm) for 1h in a Beckman 65 fixed-angle rotor at 1°C. The supernatant was centrifuged again at 269000*g* for 2h, and the resulting supernatant was made 100mM with respect to potassium acetate (pH5.0 at 20°C), and sodium dodecyl sulphate was added to give a final concentration of 2% (w/v). This solution was emulsified with an equal volume of phenol saturated with 100mM-potassium acetate (pH5.0 at 20°C) and centrifuged at approx. 2000*g* for 10min at 2°C. The aqueous layer was removed, made 1M with respect to NaCl, and re-emulsified with an equal volume of the phenol solution. After centrifugation, the aqueous layer (which contained the tRNA) was mixed with 2vol. of ethanol and stored overnight at −30°C. The precipitate was collected by centrifugation, dissolved in 0.5M-Tris–HCl buffer (pH8.9 at 37°C) and deacylated as described by Mosteller *et al.* (1967). The solution was dialysed against water for 6h and centrifuged for 30min at 269000*g* ($r_{av.}$ 5.7cm) in a Beckman 65 fixed-angle rotor. The supernatant containing the tRNA was frozen in small batches in liquid N_2 and stored at −30°C. More than 95% of the preparations migrated as 4S RNA in 7.5% polyacrylamide gels (Loening, 1967).

Extraction of ribosomes from embryos. Embryos (1g) were homogenized in a chilled mortar and pestle with 16ml of TKM medium made 0.5M with respect to sucrose. A defatted postmitochondrial (30000*g*) supernatant was obtained as described above under 'Preparation of tRNA'. Ribosomes were pelleted from this at 269000*g* ($r_{av.}$ 5.7cm) in a Beckman 65 fixed-angle rotor for 2h at 1°C, then were washed as described by Marcus & Feeley (1964) by resuspending the pellet in TKM medium–0.5M-sucrose and recentrifuging at 269000*g* ($r_{av.}$ 5.7cm) for 1h at 1°C. The washed ribosome pellet was resuspended in a small volume of TKM medium, and stored in batches in liquid N_2. These are referred to as twice-pelleted ribosomes.

The period of extraction was decreased in some experiments by rinsing the surface of the first 269000*g* pellet three times with TKM medium, resuspending the pellet and storing as described above.

Preparation of a post-ribosomal supernatant from embryos. A modification of the procedure of Marcus & Feeley (1965) was used. The upper third of the 269000*g* supernatant prepared during ribosome extraction was withdrawn and dialysed for three 2-h periods each against 150vol. of fresh TKM medium. The dialysis residue was then centrifuged at 269000*g* ($r_{av.}$ 5.7cm) for 1h at 1°C. The upper two-thirds of this supernatant was withdrawn and portions were rapidly frozen in liquid N_2 and stored at −30°C.

Preparation of a post-ribosomal supernatant from wheat germ. Wheat germ (25g) was homogenized in a 'Sunbeam' bottom-drive blender with 100ml of TKM medium and a post-ribosomal supernatant was prepared as described in the previous section. The supernatant was dialysed overnight against 500vol. of TKM medium at 2°C and then for a further 2h against fresh TKM medium (500vol.), and stored as

described above. Total protein in the fractions was measured both by the Folin method (Lowry *et al.*, 1951) and by the Biuret method (Weichselbaum, 1946) with bovine serum albumin as a standard.

Polyphenylalanine synthesis by ribosomes in vitro. Ribosomes (70–80 μg) were incubated for 60min at 25°C with 5 or 10mM-$MgCl_2$, 1.7mM-2-mercaptoethanol, 25mM-KCl, 1.7mM-ATP, 0.3mM-GTP, 3.3mM-phosphoenolpyruvate, 430 μg of protein of the post-ribosomal supernatant, 110 μg of tRNA, 100 μg of poly(U), 5 μg of pyruvate kinase, 0.5 μCi of [^{14}C]phenylalanine and 42mM-Tris–HCl buffer (pH7.6 at 20°C) in a final volume of 0.7ml. Reactions were terminated by placing the tubes in ice and adding 2ml of 5% (w/v) trichloroacetic acid. Samples were heated at 95°C for 15min to discharge amino acids from aminoacyl-tRNA, cooled, and the precipitate was pelleted by centrifugation at 1000*g*. After resuspension the precipitate was collected on Whatman GF/A filters, washed with 10ml of 5% (w/v) trichloroacetic acid containing 1% (w/v) L-phenylalanine and then sequentially with 10ml of ethanol, 10ml of ethanol–ether (3:1, v/v) and 10ml of ether. Filters were dried and assayed for radioactivity as described above.

Binding assay. Binding was assessed by the method of Nirenberg & Leder (1964). Twice-pelleted ribosomes from embryos of different viability show dependence on added supernatant for enzymic binding. A high-speed supernatant from 64%-viable embryos, treated with *N*-ethylmaleimide (Yarwood *et al.*, 1971) to inactivate the transfer factors in fraction II, was used as a source of transferase I. The reaction mixture contained in 0.2ml, 50mM-Tris–HCl buffer, pH7.2 at 20°C, 70mM-KCl, 4.7mM-Mg^{2+}, 0.1mM-GTP, 28.5pmol of ^{14}C-labelled phenylalanyl-tRNA, 100 μg of poly(U), *N*-ethylmaleimide-treated supernatant (200 μg of protein) and 80 μg of the appropriate twice-pelleted ribosomes. Incubation was at 25°C for 30min.

Extraction of RNA for fractionation in polyacrylamide gels. RNA was extracted from isolated ribosomes or from dry embryos by the method of Parish & Kirby (1966) as described by Loening (1969), sodium tri-isopropylnaphthalenesulphonate and sodium 4-aminosalicylate being used as detergents and a phenol–cresol mixture for deproteinization.

The RNA precipitate obtained was dissolved in 0.5% (w/v) sodium dodecyl sulphate–0.15M-sodium acetate buffer, pH6.0, and precipitated with 2vol. of ethanol to remove contaminating traces of phenol and extraction detergents. The sample was then stored for at least 4h at −20°C.

The 2.6% polyacrylamide gels were prepared as described by Loening (1967, 1969). Electrophoresis was at room temperature in 'E buffer' [36mM-Tris, 30mM-NaH_2PO_4, 1mM-EDTA (disodium salt) and 0.2% (w/v) sodium dodecyl sulphate, pH7.8 (Loening, 1967)]. Gels were scanned at 260nm with a modified Hilger–Gilford spectrophotometer.

In some experiments, to keep pieces of broken RNA together during extraction and fractionation, magnesium acetate at 5mM was included in the extraction medium and a low-salt (2mM-Mg^{2+}) buffer was employed for electrophoresis (Loening, 1969).

The molecular weights of intact rye rRNA were calculated from their mobilities in gels (Loening, 1968) relative to *Escherichia coli* B rRNA (1.08×10^6 and 0.56×10^6). Mean values of $0.70\times10^6\pm0.01\times10^6$ and $1.29\times10^6\pm0.01\times10^6$ (six determinations) were obtained.

Results

Incorporation of ^{14}C-labelled thymidine, uridine or amino acid mixture into embryos

Although each of the ^{14}C-labelled precursors is taken up by imbibed viable and non-viable embryos, neither thymidine nor uridine are incorporated into trichloroacetic acid-insoluble material for the first 6h, suggesting the absence of discernible DNA or RNA synthesis (Table 1). In viable embryos, however, incorporation of ^{14}C-labelled amino acids into trichloroacetic acid-insoluble material starts in the first hour of imbibition (Hallam *et al.*, 1972); no incorporation can be detected in non-viable embryos. Possible reasons for this absence of protein synthesis in non-viable embryos *in vivo* have been investigated by studying the activities of the following individual components in poly(U)-dependent cell-free systems prepared from embryos with a range of different percentage viabilities.

Polyphenylalanine synthesis by mixtures of washed ribosomes and post-ribosomal supernatants from viable and non-viable embryos

A mixture of ribosomes and a supernatant from viable embryos supports a poly(U)-directed polyphenylalanine synthesis, whereas the capacity of the non-viable-embryo components is much lower (Table 2). The activity of the individual non-viable-embryo components was assessed by mixing them with the complementary viable-embryo components. Two sites of lesions in the non-viable-embryo system are then apparent, one in the supernatant and the other in the ribosomes. When non-viable-embryo supernatant is mixed with viable-embryo ribosomes the incorporation of [^{14}C]phenylalanine is only 26% of that obtained with viable-embryo supernatant (Table 2, Expt. 1). When both supernatants are supplemented with a similar post-ribosomal supernatant fraction obtained from wheat germ (Expt. 2) the incorporation capacity of the viable-embryo supernatant remains unaltered but that of the non-

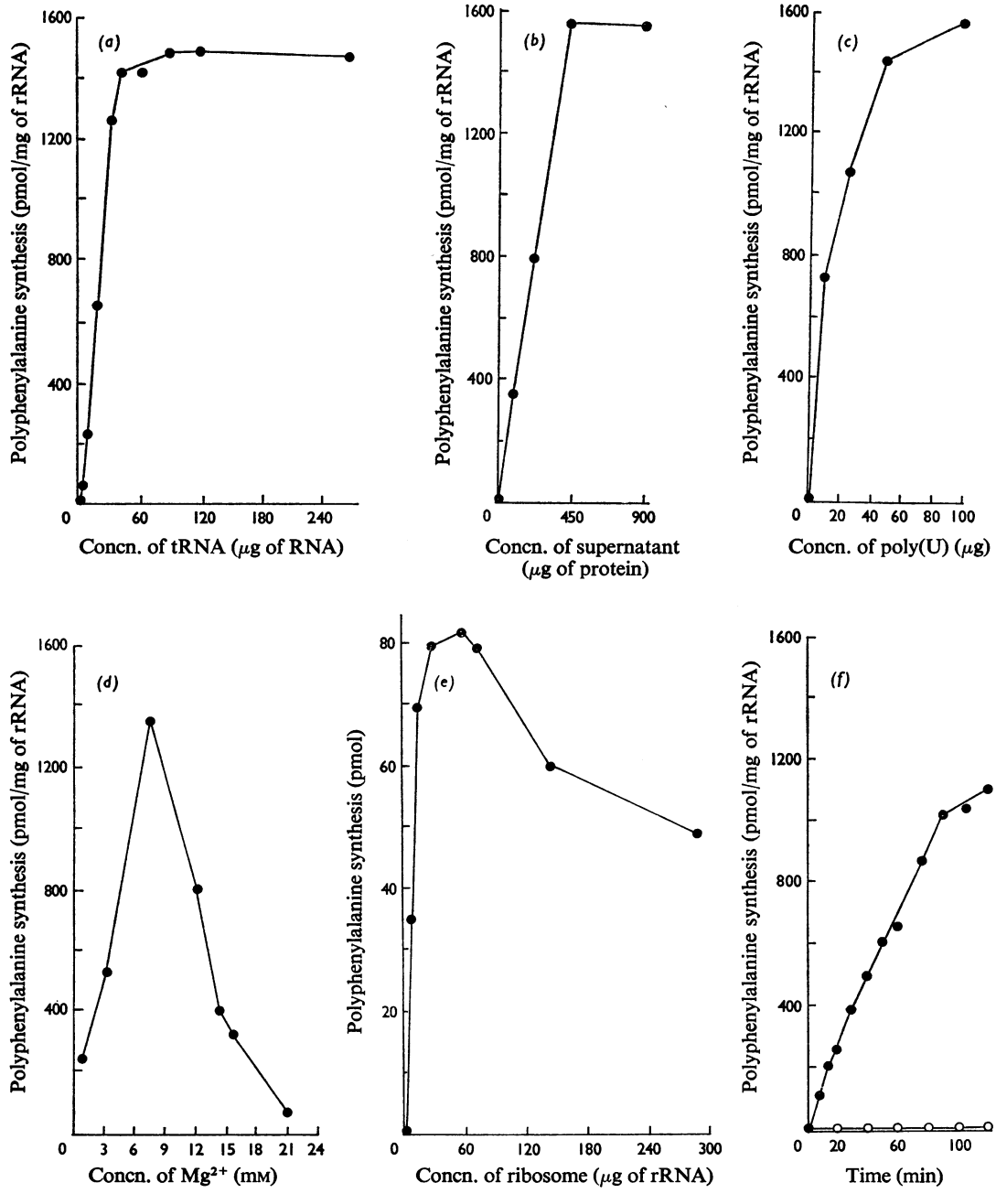

Fig. 1. *Characteristics of polyphenylalanine synthesis in vitro by viable-embryo ribosomes*

(*a*) Effect of tRNA concentration. The reaction mixture containing ribosomes (65 μg), wheat-germ post-ribosomal supernatant (430 μg of protein), poly(U) (100 μg) and 10 mM-Mg^{2+} was incubated for 60 min at 25°C. (*b*) Effect of increasing concentrations of the post-ribosomal supernatant. The reaction mixture containing ribosomes (65 μg), tRNA (100 μg), poly(U) (100 μg) and 10 mM-Mg^{2+} was incubated for 60 min at 25°C. (*c*) Effect of poly(U) concentration. Ribosomes (65 μg) were incubated with post-ribosomal supernatant (430 μg of protein), tRNA (100 μg), poly(U) (100 μg) and 10 mM-Mg^{2+} for 60 min at 25°C. (*d*) Effect of Mg^{2+} concentration. Ribosomes (70 μg) were

Table 1. *Incorporation of ^{14}C-labelled amino acids, thymidine and uridine into isolated embryos*

Embryos were dissected from 5h-imbibed viable and non-viable grain and incubated for 60min at 25°C in germinating medium containing 2μCi of ^{14}C-labelled precursor/ml. To determine the non-specific adsorption background, embryos were mixed with radioactive precursor and immediately washed and processed as described in the Materials and Methods section. These results are representative of three independent experiments. Numbers of embryos used in each experiment are given in parentheses.

^{14}C-labelled precursor		Total uptake into embryos (c.p.m.)	Incorporation into trichloroacetic acid-insoluble material (c.p.m.)
Thymidine			
Viable embryos	(100)	104000	70
Non-viable embryos	(100)	126250	236
Adsorption background	(100)		186
Uridine			
Viable embryos	(50)	75220	171
Non-viable embryos	(50)	54240	94
Adsorption background	(50)		195
Amino acids			
Viable embryos	(100)	158100	19950
Non-viable embryos	(100)	66720	119
Adsorption background	(100)		186

Table 2. *Polyphenylalanine synthesis by mixtures of ribosomes and post-ribosomal supernatants from viable and non-viable embryos*

For Expt. 1, the reaction mixture (0.7ml) contained 200μg of ribosomes, a post-ribosomal supernatant fraction with E_{260}^{1cm} 1.6, poly(U) (100μg), 5mM-Mg^{2+}, 1.7mM-ATP, 0.3mM-GTP and a phosphorylating system buffered as described in the Materials and Methods section, and was incubated at 25°C for 60min. For Expt. 2, a supernatant fraction (430μg of protein) from wheat germ was also present. Each value is the mean of duplicate determinations. Values in parentheses show incorporation expressed as a percentage of that by the complete viable-embryo system.

		Incorporation of [^{14}C]phenylalanine (pmol/mg of rRNA)	
Ribosomes	Supernatant	Expt. 1	Expt. 2
Viable	Viable	69.4 (100%)	70.1 (100%)
Non-viable	Viable	19.1 (27.5%)	5.4 (7.7%)
Viable	Non-viable	18.1 (26%)	55.0 (78.5%)
Non-viable	Non-viable	2.7 (4%)	0.7 (1%)

incubated with reconstituted supernatant and poly(U) (100μg) for 60min at 25°C. (*e*) Effect of ribosome concentration. Ribosomes were incubated with a reconstituted supernatant (post-ribosomal supernatant, 430μg of protein; tRNA, 100μg), poly(U) (100μg) and 10mM-Mg^{2+} for 60min at 25°C. (*f*) Time-course of incorporation with and without poly(U) (100μg) by a reaction mixture containing ribosomes (65μg), reconstituted supernatant and 10mM-Mg^{2+} at 25°C. ○, Without poly(U); ●, with poly(U). All reaction mixtures contained an ATP-generating system buffered as described in the Materials and Methods section. Each value is the mean of duplicate determinations.

viable-embryo supernatant is restored to 78.5% of the activity of the complete viable-embryo system.

The second lesion is associated with the ribosomes. In comparison with viable-embryo ribosomes, the polymerizing capacity of non-viable-embryo ribosomes is much lower (Table 2) and although the activity of viable-embryo ribosomes from different extractions is remarkably constant, that of non-viable-embryo ribosomes varied from 7.7 to 27.5% of the complete viable-embryo system.

Characteristics of protein-synthesizing cell-free systems

The activity of ribosomes extracted from embryos of different viability was assessed by replacing the post-ribosomal supernatant with a reconstituted supernatant containing saturating amounts of tRNA, an aminoacyl-tRNA synthetase fraction from wheat germ, poly(U) and the optimum concentration of Mg^{2+} (Fig. 1). With this reconstituted supernatant the system is saturated with respect to ribosomes at 60–80 μg of rRNA (Fig. 1*e*). The rate of incorporation is linear for 90 min at 25°C (Fig. 1*f*). In the absence of poly(U), incorporation values are close to background. The system (Table 3) is dependent on ATP, an energy-generating system, Mg^{2+}, tRNA, aminoacyl-tRNA synthetase fraction, poly(U) and ribosomes. Incorporation is completely inhibited on the addition of ribonuclease. These dependencies are characteristic of other eukaryotic systems *in vitro* (Allende, 1969; Boulter, 1970), and the measured incorporation can be attributed solely to added ribosomes. Both the characteristics (Fig. 1) and the dependencies (Table 3) of the incorporation system with ribosomes from the embryos of lower viability (64%-, 48%-, 15%-viable and non-viable) are identical with those of ribosomes from viable embryos. When ribosomes are extracted by pelleting once only (Fig. 2*b*) it is only the non-viable-embryo ribosomes that exhibit low incorporating activity. However, when the extraction procedure is lengthened by resuspending the first ribosome pellet and repelleting it (Fig. 2*a*), the activity of the ribosomes from all the embryos of lower viability is decreased. Further ribosomal inactivation therefore occurs during the extended extraction process, particularly in ribosomes of embryos of low viability.

When ribosomes prepared from viable and non-viable embryos were separated in sucrose density gradients, no differences in their sedimentation was detected. However, one aspect of ribosomal inactivation is the slight decrease in the capacity of the ribosomes of different viability to bind [^{14}C]phenylalanyl-tRNA (Table 4).

Integrity of rRNA extracted from embryos and isolated ribosomes

The rRNA extracted from viable embryos was judged to be undegraded after electrophoresis in 2.6% polyacrylamide gels in E buffer by the absence of RNA species other than 25S and 18S RNA (Fig. 3*a*). In contrast, the electrophoretic profile of RNA prepared from non-viable embryos shows several cleavage products (Fig. 3*b*), with mobilities similar to those of fragments of degraded 18S RNA from pea seedlings (Payne & Loening, 1970). Because the ratio of absorbances of 25S to 18S RNA is increased (compare

Table 3. *Dependencies of polyphenylalanine synthesis with a reconstituted post-ribosomal supernatant*

The complete reaction mixture (0.7 ml) contained viable-embryo ribosomes (65 μg), a reconstituted supernatant which contained deacylated wheat-germ tRNA (100 μg) and a wheat-germ supernatant (450 μg of protein), poly(U) (100 μg), 10 mM-Mg^{2+}, 1.7 mM-ATP, 0.3 mM-GTP and a phosphorylating system buffered as described in the Materials and Methods section, and was incubated at 25°C for 60 min. Each value is the mean of duplicate determinations.

	Polyphenylalanine synthesis	
System	(pmol/mg of rRNA)	(% of that with the complete system)
Complete system	868.2	100
–ATP and GTP	110.6	12.7
–ATP, GTP and the phosphorylating system	0.06	0.01
–Mg^{2+}	0.03	0.05
–tRNA	2.4	0.28
–Post-ribosomal supernatant	0.3	0.03
–Poly(U)	3.8	0.43
–Ribosomes	0	0
+Ribonuclease (50 μg)	0.12	0.02

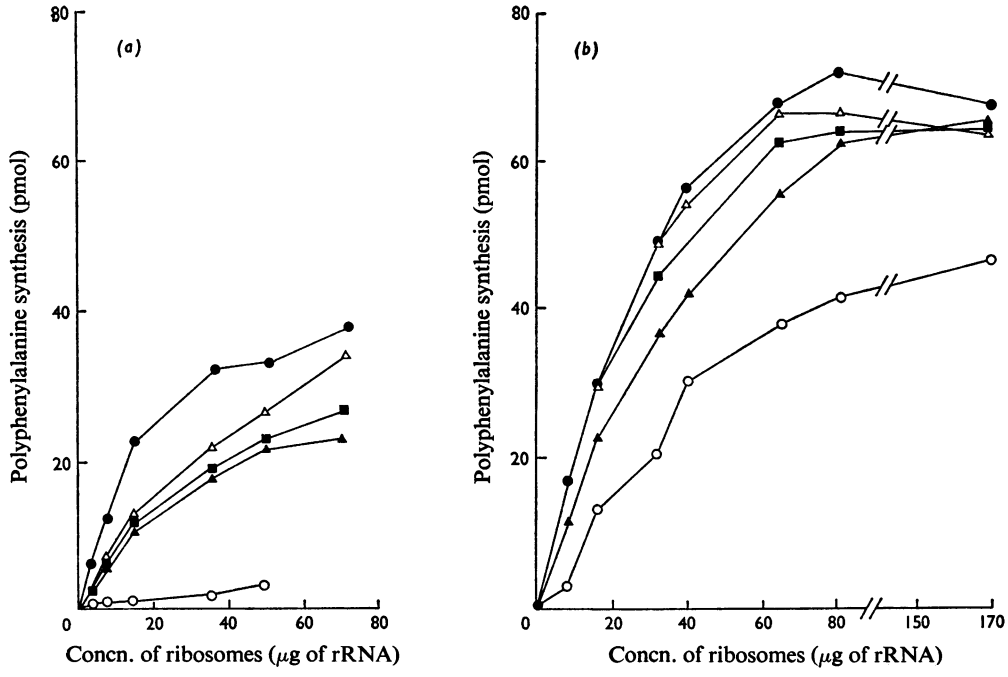

Fig. 2. *Polyphenylalanine synthesis directed by ribosomes extracted from viable* (●), *64%-viable* (△), *48%-viable* (■), *15%-viable* (▲) *and non-viable* (○) *embryos*

The reaction mixture (0.7ml) consisted of ribosomes, reconstituted post-ribosomal supernatant (tRNA, 100μg; synthetase fraction, 430μg of protein), poly(U) (100μg), 10mM-Mg^{2+} and an ATP-generating system buffered as described in the Materials and Methods section. Incubation was for 60min at 25°C. Each value is the mean of duplicate determinations. (*a*) Washed ribosomes (pelleted twice); (*b*) ribosomes pelleted once only.

Figs. 3*a* and 3*b*) most of the cleavage products are probably formed from 18S RNA. Direct extraction of RNA from embryos of intermediate viabilities shows an increasing pattern of 18S degradation, most of which can be accounted for by degradation within the dry grain.

An analysis of the RNA components of isolated ribosomes, as distinct from rRNA extracted direct from the embryo, shows that some further degradation occurs during ribosome preparation (Fig. 4). Whereas RNA degradation of viable-embryo ribosomes is slight, degradation is progressively greater in ribosomes extracted from 64%-, 48%- and 15%-viable and non-viable embryos. The degree of RNA degradation varied somewhat in the different preparations of non-viable-embryo ribosomes (Figs. 4*e* and 5*c*) and this probably reflects small variations in the extraction procedure.

When RNA is cleaved in helical regions, the fragments formed may not dissociate from each other on subsequent fractionation because of hydrogen bonding within the secondary structure (Cox, 1968). The presence of these 'hidden breaks' in the RNA extracted from ribosomes was examined by heating RNA at 70°C for 10min and then rapidly cooling it. The 18S RNA of viable-embryo ribosomes contained only a few 'hidden breaks' as judged by a slight decrease in the 18S RNA peak of the electrophoretic profile (Figs. 5*a* and 5*b*), but 25S RNA contained several breaks. Both 25S and 18S RNA components of non-viable-embryo ribosomes could be separated into many fractions after heating (Figs. 5*c* and 5*d*).

These results suggest that some viable-embryo ribosomes and probably all non-viable-embryo ribosomes contain fragmented 25S RNA which is held together by secondary structure. It is not clear if fragmented 18S RNA (Figs. 4*e* and 5*a*) is similarly held together or if pieces have been lost from the ribosomes. To distinguish between these alternatives, RNA of non-viable-embryo ribosomes was extracted

Table 4. *Capacity of ribosomes from embryos of different viability to bind [^{14}C]phenylalanyl-tRNA*

(*a*) The reaction mixture (0.2ml) contained 50mM-Tris–HCl buffer, pH7.2 at 20°C, 70mM-KCl, 4.7mM-Mg^{2+}, 0.1mM-GTP, 28.25pmol of [^{14}C]phenylalanyl-tRNA, *N*-ethylmaleimide-treated supernatant (200μg of protein) from 64%-viable embryos and 80μg of twice-pelleted ribosomes. This was incubated at 25°C for 30min. From each determination the non-enzymic binding (0.9pmol) has been subtracted. (*b*) Values for post-ribosomal supernatant activity for polyphenylalanine synthesis with and without *N*-ethylmaleimide were obtained as described in Table 3. Each value is the mean of duplicate determinations.

(*a*)

Ribosomes from embryos of viability percentage	Binding of [^{14}C]-phenylalanyl-tRNA (pmol)
95	7.1
64	6.2
15	4.8
0	4.4

(*b*)

Postribosomal supernatant from 95%-viable embryos	Polyphenylalanine synthesis (pmol)
Untreated	28.0
Pretreated with *N*-ethylmaleimide	0.3

and fractionated in the presence of Mg^{2+}. Under these conditions the hydrogen bonds are stabilized by Mg^{2+} and fragments of higher-plant 18S RNA (T. A. Dyer, personal communication) and chloroplast 25S RNA (Ingle *et al.*, 1969) are thereby held together. When RNA of non-viable-embryo ribosomes was separated in the presence of 2mM-Mg^{2+} the 18S RNA fractionated largely as one band at the position expected of this species (Fig. 6; cf. Figs. 4*e* and 5*a*), indicating that pieces of RNA are not lost from the 40S subunit to any appreciable extent. This suggestion is further supported by the absence of RNA components larger than 4S RNA in the post-ribosomal supernatant of ribosome preparations (not shown).

Discussion

During the early hours of germination viable embryos synthesize proteins in the absence of discernible synthesis of DNA and RNA (Table 1). Non-viable embryos, however, synthesize neither proteins nor nucleic acids. According to Marcus & Feeley (1964) hydration of viable embryos results in an activation and translation of a stored mRNA and they reported that this does not occur when non-viable embryos are hydrated. In the present study the ribosomes and supernatant from embryos of different percentage viabilities have been examined in a system *in vitro* with poly(U) as a synthetic message. Although non-viable embryos do not respire [ultrastructural studies of the mitochondria reveal breaks in their double membranes (Hallam *et al.*, 1972)], the lack of an active ATP-generating system may not be the only lesion leading to the loss of protein-synthetic activity. Apart from long-lived mRNA, equally important lesions could be the loss of active supernatant components or loss of ribosomal integrity.

The results with viable embryos show that in the presence of poly(U), polyphenylalanine synthesis is activated by the components extracted from dry embryos, but when poly(U) is added to supernatant and ribosomes from non-viable embryos, negligible quantities of polyphenylalanine are synthesized (Table 2). By mixing ribosomes and supernatants from either viable or non-viable embryos it can be shown that the activity of both components is impaired in the non-viable-embryo material. The absence of protein synthesis in the non-viable embryo cannot therefore be accounted simply to the loss of ATP or an active energy-generating system. Experiments indicate that the loss of supernatant activity is proportional to the loss of viability, so the lesion in the post-ribosomal supernatant could be due to an impairment of tRNA, aminoacyl-tRNA synthetases or transfer enzymes. The addition of deacylated tRNA alone to the mixture of ribosomes from viable or non-viable embryos and their supernatants (as in Table 2) does not modify the percentage differences in activity observed between the supernatants from viable and non-viable embryos. However, the addition of a complete supernatant fraction from wheat germ enhances the activity of the non-viable-embryo supernatant to 78.5% of that of the viable-embryo supernatant. This wheat-germ fraction contains not only tRNA but also aminoacyl-tRNA synthetases and transfer enzymes. Preliminary work suggests that transfer enzyme activity of the non-viable-embryo-supernatant components is impaired.

The second lesion associated with loss of viability occurs in the ribosomes. It may be of secondary importance compared with that of the supernatant as once-pelleted non-viable-embryo ribosomes are up to 60% as active as viable-embryo ribosomes when supplied with viable-embryo supernatant in a poly(U) system (Fig. 2*a*). One aspect of this decreased ribosomal activity is evident in the decline in binding of phenylalanyl-tRNA by ribosomes isolated from embryos of low viability (Table 4).

This ribosome lesion probably results from an

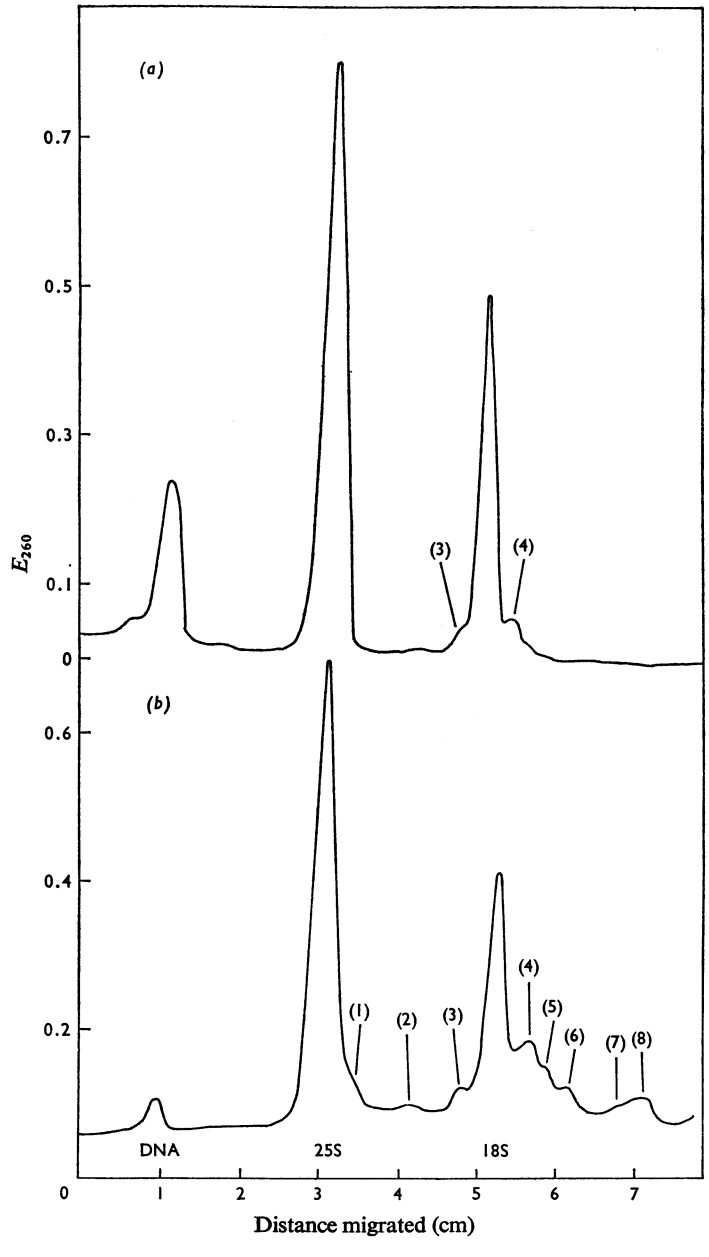

Fig. 3. *Fractionation of the high-molecular-weight nucleic acid components extracted from (a) viable and (b) non-viable embryos*

RNA was subjected to electrophoresis in a 2.6% polyacrylamide gel for 3½ h at 7 V/cm length of gel. The numbers in parentheses above individual peaks refer to specific cleavage products of rRNA. They have molecular weights (relative to the two undegraded rRNA peaks) of: (1) 1.10×10^6 (four determinations); (2) 0.96×10^6 (16 determinations); (3) 0.92×10^6 (seven determinations); (4) 0.86×10^6 (four determinations); (5) 0.79×10^6 (12 determinations); (6) 0.66×10^6 (11 determinations); (7) 0.61×10^6 (six determinations); (8) 0.55×10^6 (eight determinations); (9) 0.45×10^6 (10 determinations) (see Fig. 5).

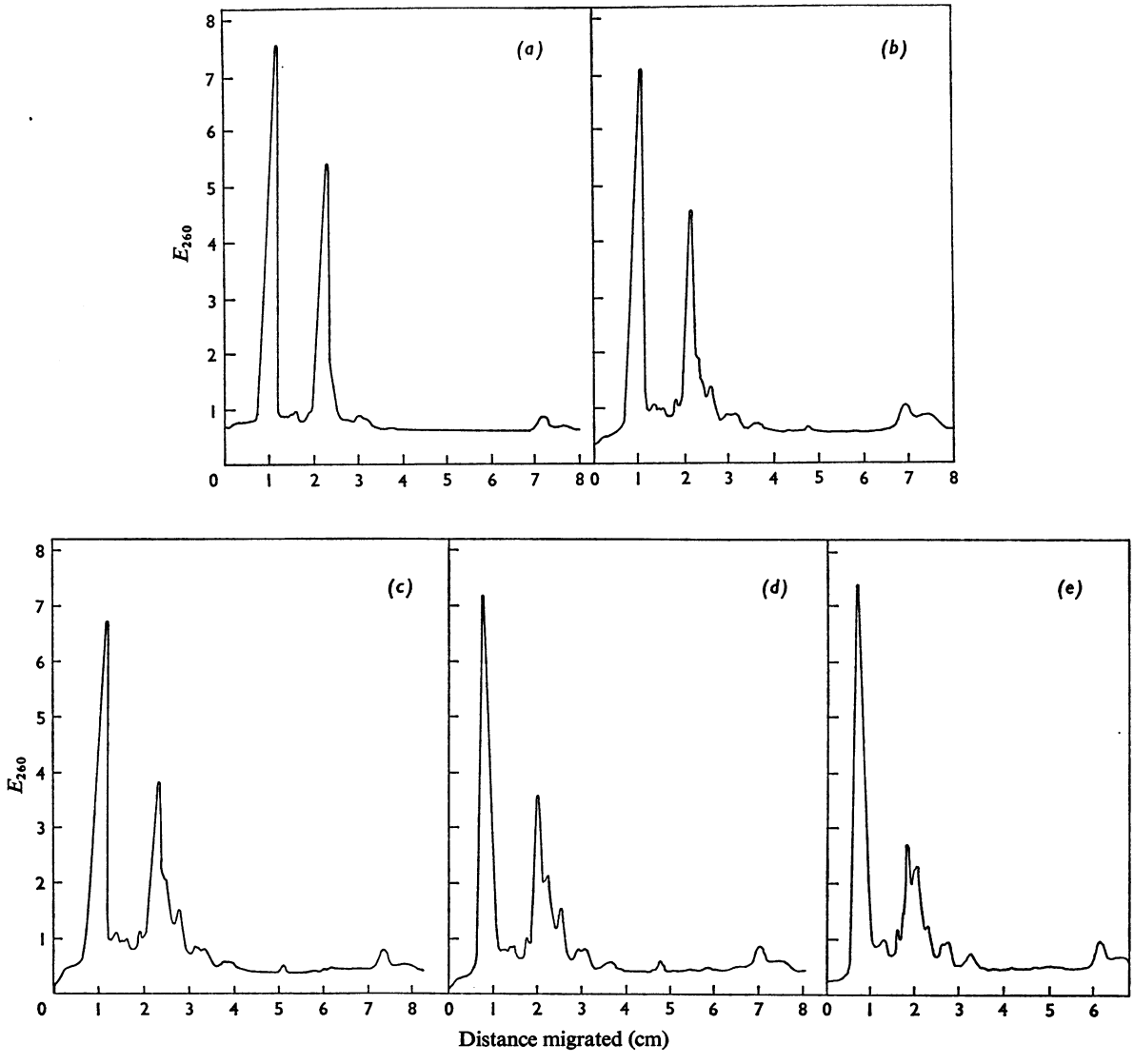

Fig. 4. *Fractionation of the RNA components from (a) viable-embryo ribosomes; (b) 64% -viable-embryo ribosomes; (c) 48% -viable-embryo ribosomes; (d) 15% -viable-embryo ribosomes; (e) non-viable-embryo ribosomes*

RNA was separated by electrophoresis in 2.6% polyacrylamide gels for 2h at 7V/cm length of gel.

alteration in structure or loss of one or more of the rRNA species or ribosomal proteins. The alteration cannot be very great, as ribosomes from viable and non-viable embryos sediment at the same rate in sucrose gradients. Polyacrylamide-gel electrophoresis of RNA extracted from ribosomes clearly shows a decrease in integrity of 25S and 18S rRNA with decreasing viability of grain. The loss of one or more crucial nucleotides at, for instance, the sites of either tRNA or mRNA attachment, would result in a decreased protein-synthetic ability. The impairment of rRNA is therefore a possible cause of the decreased activity of the ribosome. However, animal ribosomes, even after digestion with ribonuclease,

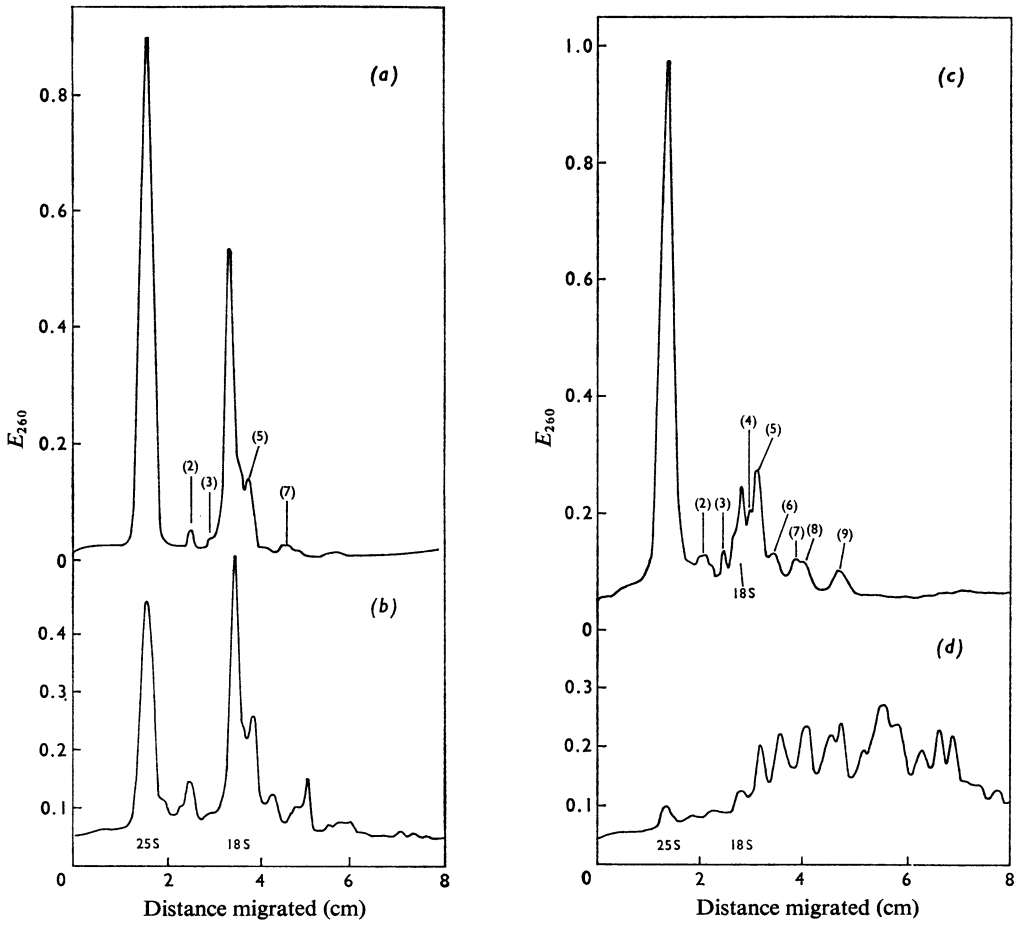

Fig. 5. *Effect of heating RNA from ribosomes from* (*b*) *viable and* (*d*) *non-viable embryos*

Viable- (*a*) and non-viable-embryo (*c*) rRNA controls and heated samples were diluted to 0.1 mg/ml. Samples (*b*) and (*d*) were heated at 70°C for 10 min and then all samples were rapidly cooled by plunging them in liquid N_2. RNA was precipitated by 2 vol. of ethanol and then separated by electrophoresis in 2.6% polyacrylamide gels for 2 h at 7 V/cm length of gel. The molecular wieghts of the numbered fragments are given in the legend of Fig. 3.

largely retain their ability to polymerize amino acids *in vitro* (Huvos *et al.*, 1970; Cahn *et al.*, 1970).

The considerable variation in the activity of extracted non-viable-embryo ribosomes and the integrity of their constituent 18S-rRNA suggests that these preparations contain appreciable ribonuclease activity. This has been confirmed by ribonuclease assays (P. I. Payne, unpublished work). At these concentrations, different ribonucleases preferentially cleave RNA molecules at different positions (Pinder & Gratzer, 1970). However, as the molecular weights of the rRNA breakdown products are similar for all the viability stocks (Fig. 4), it would appear that loss of viability is associated with activation or synthesis of ribonuclease types that are already present in the viable embryo and not with the subsequent production of novel species.

By using stocks of embryos of different percentage viability we have shown that both post-ribosomal supernatant and ribosomes decline in their integrity and activity in protein-synthesizing systems *in vitro*. These impairments may account for the loss of protein-synthetic activity *in vivo* in aged embryos during the early hours of germination, indicating the most

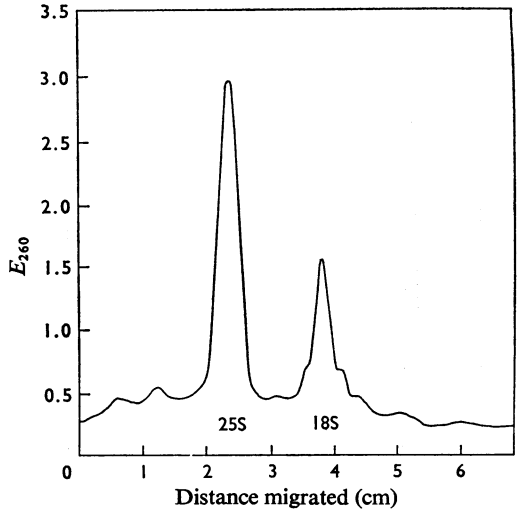

Fig. 6. *Fractionation of rRNA from non-viable embryos in the presence of* Mg^{2+}

RNA, extracted from non-viable-embryo ribosomes in the presence of Mg^{2+}, was separated in a 2.6% polyacrylamide–Mg^{2+} gel for 4h at 7V/cm length of gel.

vulnerable or labile components of the protein-synthesizing system. These lesions could, we suggest, reflect senescent changes that contribute to a loss of embryo viability.

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