Decline in Ribonucleic Acid and Protein Synthesis with Loss of Viability during the Early Hours of Imbibition of Rye (*Secale cereale* L.) Embryos

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A decline in protein synthesis and slow germination accompanies loss of viability in embryos of rye (*Secale cereale* L.) grains. Associated with this, incorporation of precursors into all the major classes of RNA is decreased and the processing of precursor rRNA to 25S and 18S RNA is retarded. Embryos that just reach 0% viability still synthesize some low-molecular-weight non-nucleolar material, although they do not synthesize protein. It is suggested that early-synthesized RNA could play a major part in determining the extent of protein synthesis at early germination, and thereby regulate the rate at which germination can proceed.

The loss of viability in seeds in the dry state is a progressive event expressed at germination by impaired vigour of the embryos and slow establishment of the seedling (Roberts, 1972). In biochemical terms this loss of vigour appears as decreased mitochondrial activity (Abu-Shakra & Ching, 1967), lowered activity of respiratory enzymes (Lakon, 1949) and a lower ATP production (Ching, 1973). Such effects can be related to ultrastructural changes (Berjak & Villiers, 1970, 1972; Hallam *et al.*, 1972*a,b*), which indicate degeneration of a variety of membrane classes within the cells. Although these alterations are clearly contributory factors to the loss of vigour in stored seeds, a major lesion is the progressive failure of the protein-synthesizing system. In rye (*Secale cereale* L.) the rate of protein synthesis in the first few hours of germination is closely correlated with embryo performance, and can be recognized as an index of embryo vigour (Roberts & Osborne, 1973*a*). Although the extracted ribosomes sediment at the same density in a sucrose gradient, both 25S and 18S rRNA exhibit progressive fragmentation as embryos lose viability (Roberts *et al.*, 1973). Cleavage of rRNA has been noted also in embryo axes of non-viable peas (Bray & Chow, 1976*b*). The inactivation of the GTP-dependent transferase I (binding aminoacyl-tRNA to the ribosomes) closely parallels the impairment of protein synthesis in embryos from rye stocks of diminishing viabilities (Roberts & Osborne, 1973*b*) and occurs also after aging of pea seeds (Bray & Chow, 1976*a*) and wheat embryos (Dell'Aquila *et al.*, 1976).

To date it is still an open question whether the early protein synthesis is coded for solely by long-lived mRNA that is stored in the dry seed and activated when the embryo imbibes water (Dure & Waters,

1965; Weeks & Marcus, 1971) or whether the early protein synthesis depends on the production of newly synthesized mRNA molecules (Dobrzanska *et al.*, 1973), or utilizes both old and new RNA, including mRNA. In rye, protein synthesis has been detected within 15 min after imbibition in 95%-viable high-vigour embryo stocks (Sen *et al.*, 1975); nuclear RNA synthesis has been demonstrated by radioautography after 10 min (Osborne *et al.*, 1974), and the presence of newly synthesized high-molecular-weight heterodisperse RNA has been ascertained in embryos after 20 min of imbibition (Sen *et al.*, 1975). The work with rye has now been extended to low-vigour embryos, and the present paper describes a study of early RNA synthesis for a range of percentage viabilities from 100 to 0%, and relates the findings to the extent of early protein-synthetic activity that the embryos display.

Experimental

Materials

Rye grain (*Secale cereale* L., var. Lovaszpatonai) of 100 or 99% viability (1973–1974 harvests) was obtained from Nunns Corn and Coal Ltd., Quayside, Woodbridge, Suffolk, U.K. Attested stocks of lower viabilities (from a single 1964 harvest) were a gift from Dr. D. B. MacKay and Mr. R. J. Flood of the National Institute of Agricultural Botany, Cambridge, U.K., and were from a storage experiment in which grain had been held at moisture contents from 9–14% from 1964 to 1969. Embryos were separated from the rest of the grain by the mass-isolation technique of Johnston & Stern (1957) and stored at 4°C over CaCl₂. The viability of stored embryos declines slowly over many months, and as a routine stocks were checked for percentage germination (percentage viability) at yearly or twice-yearly intervals. 0%-viable embryos were those from the 1964 stock that

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were 15% viable in 1970 and had just reached 0% viability in 1975. The embryos designated 'non-viable' (NV) were also from the 1964 stock, and showed 0% germination in 1969; they had therefore been non-viable for at least 6 years.

Radiochemicals

G-¹⁴C-labelled amino acid mixture (57mCi/mg-atom of C), [U-¹⁴C]uridine (405 or 487mCi/mmol) and [5-³H]uridine (24Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Germination. Entire embryos were germinated at 25°C in Petri dishes on thin layers of 1% agar (Oxoid no. 3; Oxoid Ltd., Basingstoke, Hants., U.K.) made up in a germination medium containing 2% (w/v) sucrose and chloramphenicol (10 µg/ml) (GM medium). Individual embryos were weighed dry and again after 48 h, and the percentage increase in fresh weight of germinated and non-germinated embryos was then calculated (see Table 1).

Incubation with precursors. The procedures for incubating individual embryos at 25°C in 5 µl drops of ¹⁴C-labelled precursors (groups of five embryos in 25 µl) and for measuring the total uptake and incorporation of the precursors into trichloroacetic acid-insoluble material under sterile conditions *in vivo* was described previously (Roberts *et al.*, 1973; Roberts & Osborne, 1973a,b; Sen *et al.*, 1975).

Radioautography. Similar incubation methods using ³H- and ¹⁴C-labelled precursors were used to label embryos of the different viabilities for radioautography. At the end of the incubation period (0–2 or 1–3 h), embryos were rinsed in a 150-fold excess of unlabelled precursor. For microtome preparation embryos were transferred to Carnoy's fixative (ethanol/chloroform/acetic acid, 6:3:1, by vol.), and passed through an ascending concentration series of ethanol and benzene, and finally embedded in wax. Serial sections (4 µm thick) were transferred to dry slides previously coated with gelatin. For squash preparations, root tips were fixed in acetic acid/ethanol (1:1, v/v), then squashed in 45% (v/v) acetic acid and the material was affixed to the slides by freezing over solid CO₂ for 20 min.

To distinguish background graining due to non-specific adsorption from incorporation of radioactive uridine into RNA, sample slides were selected after dewaxing, and treated with pancreatic ribonuclease A (Worthington Biochemical Corp., bought from Cambrian Chemicals, Croydon, Surrey, U.K.) at 100 µg/ml in 1 mM-EDTA (disodium salt)/10 mM-Tris/HCl, pH 7.4 (at 20°C), for 60 min at 37°C. Control slides were treated similarly with buffer alone. Treatment of sample slides with 1 M-HCl at 37°C for 2 h removed all grains. For distinguishing

background graining from amino acid incorporations into proteins, sample slides were incubated for 60 min at 33°C with type IV bacterial proteinase [Sigma (London) Chemical Co., London S.W.6, U.K.] at 200 µg/ml in 10 mM-magnesium acetate/50 mM-Tris/HCl, pH 7.8 at 20°C; other slides were exposed to buffer alone. After rinsing with water, the HCl- or enzyme-treated and untreated slides were covered with AR10 stripping film (Kodak Ltd., Hemel Hempstead, Herts., U.K.), dried and exposed for 1–4 weeks in darkness at 4°C. Slides were developed in Formula D19 or ID19 solution (Kodak) at 19°C for 4 min and fixed for 10 min Metafix or Amfix (Kodak). Sections were then stained with pyronine/Methyl Green and the number of grains was determined under dark-field illumination in a Leitz microscope fitted with a photomultiplier and automated grain counter. The appropriate comparisons were made with background slides.

Polyacrylamide-gel electrophoresis of nucleic acids. At least 30 embryos from the different viability stocks were incubated for 0–1 or 0–3 h in [¹⁴C]uridine. The RNA was extracted by the method of Parish & Kirby (1966) and Loening (1969), and fractionated by polyacrylamide-gel electrophoresis as described in detail in Sen *et al.* (1975). Gels were scanned at 260 nm with a modified Hilger-Gilford spectrophotometer, frozen with solid CO₂ and cut into 1 mm slices. Each slice was dried, placed in 10 ml of scintillant (Sen *et al.*, 1975) and counted for radioactivity (to an s.e. of 1%) in a Beckman liquid-scintillation spectrometer with a ¹⁴C counting efficiency of 80–90%.

Results

The progressive nature of loss of viability can be seen from the ability of the embryo to imbibe water (Table 1). Both germinating and non-germinating embryos take up water more slowly over a period of 48 h the lower the viability of the stock from which they are obtained. The non-germinating embryos are of particular interest; only those that were known as non-viable for 6 years (NV) failed to take up more than 16% of their weight in water, or to increase in size.

Incorporation of radioactive precursors into trichloroacetic acid-insoluble material

Measurements of the incorporation of ¹⁴C-labelled amino acids into trichloroacetic acid-insoluble material by individual embryos of different viabilities demonstrate their declining ability to synthesize protein in the early hours of germination (Fig. 1). The decline is greatest between 100 and 86% viability, and the shape of the curve shows that the values essentially reflect loss of embryo vigour and are not

EXPLANATION OF PLATE 1

Radioautographs of incorporation of [³H]uridine (1 mCi/ml) between 1 and 3 h of imbibition into the cortical region of the root tip
Median microtome sections of whole embryo were prepared (4 μ m thick) from (a) 0%-viable embryos and (b) 100%-viable embryos. Radioautographs of incorporation of ¹⁴C-labelled amino acids (10 μ Ci/ml) into the root tip during 0-2 h of imbibition were obtained from squash preparations of the root tip from (c) 0%-viable embryos and (d) 100%-viable embryos. For further details, see the Experimental section. The mean nuclear diameter was 10 μ m.

Table 1. *Increases in fresh weight after 48 h of imbibition by germinating and non-germinating embryos from stocks of different percentage viability*

Individually weighed embryos were set out in Petri dishes on agar made up in GM medium (see the text). They were moistened with a small volume of GM medium and incubated at 25°C in darkness. Further small volumes of GM medium were added at intervals when the solution was imbibed. Each embryo was blotted and reweighed after 48 h. Results are means for 100 embryos. NV, non-viable.

Viability (%) ...	Increase in fresh weight (%)				
	86	53	15	0	NV
Embryos that germinated	830	833	550	—	—
Embryos that failed to germinate	567	367	159	71	16

Fig. 1. *Incorporation of ^{14}C -labelled amino acids ($5\ \mu\text{Ci}/\text{ml}$ in GM medium) into trichloroacetic acid-insoluble material by embryos from stocks of different percentage viability during incubation in precursor for the first 2 h of imbibition*

For details of the method, see the text. Error bars represent s.d. ($P = 0.05$).

directly attributable to the numbers of non-viable embryos within the sample, or to changes in the uptake of precursor with loss of viability [Fig. 1 (inset)].

Radioautographic assays

RNA synthesis also declines steeply in the early stages of loss of viability, and is shown by the more sensitive method of radioautography (Fig. 2). Although they do not germinate, the 0%-viable embryos show clear nuclear graining (Plate 1a)

resulting from incorporation of [^3H]uridine (one-tenth of the values for 100%-viable embryos; Fig. 2). These embryos do not, however, incorporate any ^{14}C -labelled amino acids into acid-insoluble material above background (Fig. 1), nor do they show graining from incorporation of ^{14}C -labelled amino acids by radioautography (Plate 1c); 100%-viable embryos show heavy cytoplasmic graining (Plate 1d). Embryos that had been non-viable for at least 6 years (NV) incorporated neither RNA nor protein precursors at any time (Figs. 1 and 2).

Incorporation into nucleic acids fractionated by gel electrophoresis

Extraction of total nucleic acid from embryos incubated in [^{14}C]uridine for the first hour of imbibition and subsequent fractionation in either 2.2 or 7.5% (w/v) polyacrylamide gels show that all major classes of RNA are synthesized in 100%- and 95%-viable embryos (Figs. 3a, 3b, 3e and 3f), but demonstrably less radioactivity is incorporated into the 31S, 25S, 18S, 5S and 4S RNA in the 95%-viable embryos. Synthesis of 31S and 4S RNA only (Figs. 3c and 3g) can be shown for the 86%-viable embryos after 1 h, but the incorporation by embryos of viabilities of 53% (Figs. 3d, 3h) or lower (results not shown) is so small over this period that it is below the limit of detectability by the technique with the specific radioactivity of uridine used. An extended incorporation (0–3 h) to compare the high-molecular-weight products synthesized by 99%- and 90%-viable embryo stocks as separated on 2.2% (w/v) polyacrylamide gels is shown in Figs. 4(a) and 4(b) respectively. These embryos were from the stock

Fig. 2. *Mean number of silver grains per nucleus in median longitudinal sections of embryos of different percentage-viability stocks after incubation in GM medium for the first hour of imbibition followed by transfer to [^3H]uridine ($1\ \text{mCi}$ in GM medium) for a further 2 h*

At least 200 nuclei were observed per treatment. Backgrounds due to small amounts of silver graining remaining after ribonuclease incubation of sample slides of each treatment have been subtracted (see the text). Error bars represent s.d. ($P = 0.05$).

Fig. 3. Fractionation on polyacrylamide gels of nucleic acids extracted from embryos of stocks of 100% (a, e), 95% (b, f), 86% (c, g) and 53% (d, h) viabilities incubated in [^{14}C]uridine (700 $\mu\text{Ci}/\text{ml}$ in GM medium) for the first hour of imbibition. In (a), (b), (c) and (d), 60 μg of RNA was separated on 2.2% (w/v) gels, and in (e), (f), (g) and (h), 120 μg was separated on 7.5% gels. Molecular weights of the RNA fractions relative to *Escherichia coli* rRNA markers are: 31S, 2.3×10^6 ; 25S, 1.3×10^6 ; 18S, 0.7×10^6 .

which on storage became 95%- and 86%-viable respectively, and were later used for the experiments shown in Fig. 3. Although all the major RNA species are present in embryos of both viabilities (Fig. 4), the total incorporation in the 90%-viable embryos is less than one-tenth of that in the 99%-viable, and comparisons of the relative proportions of 31S to 25S and 31S to 22S to 18S RNA indicate a relatively slower rate of rRNA processing by the lower-viability embryos.

Discussion

As embryos lose the ability to germinate they also lose the capacity to incorporate precursors into

protein (Fig. 1) and into RNA (Fig. 2). The decreased incorporation can be related to the slower rate of germination of embryos (e.g. loss of vigour) from low-viability stocks. A measure of the extent to which imbibition and growth is impaired is seen from the increases in fresh weight of different-viability embryos 48 h after imbibition (Table 1). Whereas the physical uptake of water over the first 60 min of imbibition is only slightly less in non-viable as compared with viable embryos (Hallam *et al.*, 1972b), the subsequent phase of active water uptake is decreased at the lower viabilities. The decrease in uptake of precursor by low-viability embryos during the first 2 h of imbibition [Fig. 1 (inset)] does not, however, account for the decreased incorporation of

Fig. 4. Fractionation on 2.2% (w/v) polyacrylamide gels of 60 μ g of nucleic acids extracted from (a) 99%-viable embryos and (b) 90%-viable embryos imbibed with [14 C]uridine (700 μ Ci/ml in GM medium) for 0–3 h. For further details, see the legend to Fig. 3.

precursor. The low rate of protein synthesis is attributable rather to the accumulation of cellular lesions that develop with impaired viability, some of which have been shown to be associated with the components of mRNA translation.

If early protein synthesis were coded for solely by long-lived mRNA in the dry seeds, then the present results could be interpreted as indicating that either (a) the efficiency of the translation system rather than long-lived mRNA determines the rate of protein synthesis at germination, or (b) long-lived mRNA in the embryos does limit the rate of early protein synthesis, in which case mRNA molecules must represent some of the relatively less stable macromolecules in the seed. A further possibility, (c), is that the extent of early protein synthesis could be largely determined by the amount of newly-synthesized RNA at germination. In a previous study with 99%-viable embryos (Sen *et al.*, 1975) we distinguished, by polyacrylamide-gel electrophoresis, the following order of detection for RNA transcription of the genome on imbibition; heterogeneous (ribonuclease-sensitive) 4S and 5S RNA by 20 min, 31S, 25S and a 22S RNA by 40 min, and 18S RNA by 60 min. Radioautographic studies (Fig. 2) show that the incorporation of [3 H]uridine into nuclear RNA decreases in all cells of an embryo as viability is lost. Interestingly, however, incorporation is still clearly discernible from grain counts in 0%-viable embryos, although they do not synthesize protein (Plate 1). By using extraction procedures for RNA and protein, a similar obser-

vation was made by Barker & Bray (1972) and Bray & Dasgupta (1976) for non-viable pea seeds obtained by accelerated-aging procedures. Whether or not such incorporation represents a synthesis of intact RNA of low molecular weight or fragments of high-molecular-weight transcripts is not known.

Comparison of gel scans at 1 h (Figs. 3a, 3b, 3c and 3d) and 3 h (Figs. 4a and 4b) indicates that the decrease in the incorporation of radioactive precursor into all fractions of high-molecular-weight RNA is steeper than would be predicted from the grain counting of radioautographs of these viabilities (Fig. 2). A comparison of Figs. 3(a) and 3(b) with 4(a) and 4(b) also indicates a lower rate of conversion of 31S precursor rRNA into the 25S and 18S products as viability is lost. If the processing of any newly synthesized mRNA is similarly delayed, then the decline in early protein synthesis with loss of viability could as reasonably be attributed to a failure of early RNA synthesis as to some loss of integrity in long-lived RNA or malfunction in the translation process.

Although one-half of a 53%-viable stock of embryos will eventually germinate by 24 h, no synthesis of high- or low-molecular-weight RNA is evident from the polyacrylamide-gel separations for the first hour of imbibition. From grain counting, however, incorporation into a nuclear component is still half that determined in 100%-viable embryos (Fig. 2), implying a considerable synthesis of low-molecular-weight oligonucleotides. At 1–3 h, nuclear graining in the higher-viability embryos is mostly concentrated

over the nucleolus (Plate 1b), whereas that in the 0%-viable embryos is distributed throughout the nucleus (Plate 1a). This suggests that most of the grains in the 0%-viable embryos represent a population of small oligonucleotides associated with the chromatin rather than completed high-molecular-weight rRNA molecules associated with the nucleolus.

The reason for the impairment of RNA synthesis with loss of viability is not yet understood, but could stem from a number of causes. The low amount of RNA detected in polyacrylamide gels despite the presence of RNA-sensitive products in radioautographs of nuclei (Fig. 2 and Plate 1) suggests the possibility of an impaired DNA template. By comparison of the amount of DNA that could be recovered by 'spooling' (Marmur, 1961), Roberts & Osborne (1973a) showed that, in embryos of decreasing viability, DNA was increasingly cleaved into low-molecular-weight fragments without loss of total DNA, and that the lower values were related to an increased activity of deoxyribonuclease. The number of chromosomal breaks and aberrations that occur at division in the cells of individuals germinating from old seeds (Roberts, 1972) lends credence to such ideas. By using techniques that do not fragment DNA during extraction, the cleavage of high-molecular-weight DNA into lower-molecular-weight fragments in non-viable rye embryos *in vivo* has been established (K. E. S. Cheah & D. J. Osborne, unpublished work).

Villiers (1974) and Berjak & Villiers (1972) have proposed that cellular repair processes, including those for DNA, may occur as an early event in germination, or during periods of imbibition in dormant seeds (Villiers & Edgcombe, 1975). However, in embryos of 100%-viable rye, detectable incorporation of [³H]thymidine into DNA does not occur until 4–5 h after imbibition, and is delayed until after 7–8 h in 90%-viable embryos (Sen & Osborne, 1974; Sen, 1975). Although any incorporation due to DNA repair might well be too small to detect, the possibility of an early DNA-repair process cannot be excluded. Indeed, a requirement for DNA repair could explain the delay in the start of DNA replication with loss of vigour and the lag in the synthesis of high-molecular-weight RNA species. Failure of DNA repair could be a contributing factor in the slow establishment of seedlings from low-vigour embryos, and indicates also that energy coupling is not the only limitation to germination.

The present results show that in the early hours of germination, incorporation of precursors into all the major classes of RNA is impaired, and that the processing of precursor rRNA to 25S and 18S RNA is retarded, as rye embryos lose viability. Whether the failure of early RNA synthesis is the major cause of the decline in early protein synthesis is unresolved, but since the viable embryos from low-viability

stocks do germinate and eventually, albeit slowly, establish as seedlings, early synthesized mRNA rather than long-lived mRNA could code their early protein synthesis.

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References

- Abu-Shakra, S. S. & Ching, T. M. (1967) *Crop Sci.* **7**, 115–118
- Barker, G. R. & Bray, C. M. (1972) *Symp. Biol. Hung.* **13**, 61–68
- Berjak, P. & Villiers, T. A. (1970) *New Phytol.* **69**, 919–938
- Berjak, P. & Villiers, T. A. (1972) *New Phytol.* **71**, 135–144
- Bray, C. M. & Chow, T.-Y. (1976a) *Biochim. Biophys. Acta* **442**, 1–13
- Bray, C. M. & Chow, T.-Y. (1976b) *Biochim. Biophys. Acta* **442**, 14–23
- Bray, C. M. & Dasgupta, J. (1976) *Planta* **132**, 103–108
- Ching, T. M. (1973) *Seed Sci. Technol.* **1**, 73–88
- Dell'Aquila, A., Zocchi, G., Lanzani, G. A. & De Leo, P. (1976) *Phytochemistry* **15**, 1607–1610
- Dobrzanska, M., Tomaszewski, M., Grzelczak, Z., Rejman, E. & Buchowicz, J. (1973) *Nature (London)* **244**, 507–509
- Dure, L. S. & Waters, L. C. (1965) *Science* **147**, 410–412
- Hallam, N. D., Roberts, B. E. & Osborne, D. J. (1972a) *Planta* **105**, 293–309
- Hallam, N. D., Roberts, B. E. & Osborne, D. J. (1972b) *Planta* **110**, 279–290
- Johnston, F. B. & Stern, H. (1957) *Nature (London)* **179**, 160–161
- Lakon, G. (1949) *Plant Physiol.* **24**, 389–394
- Loening, U. E. (1969) *Biochem. J.* **113**, 131–138
- Marmur, J. (1961) *J. Mol. Biol.* **3**, 208–218
- Osborne, D. J., Roberts, B. E., Payne, P. I. & Sen, S. (1974) in *Mechanisms of Regulation of Plant Growth* (Bielecki, R. L. & Ferguson, A. R., eds.), bulletin 12, pp. 805–812, The Royal Society of New Zealand, Wellington
- Parish, J. H. & Kirby, K. S. (1966) *Biochim. Biophys. Acta* **129**, 554–562
- Roberts, B. E. & Osborne, D. J. (1973a) *Proc. Easter Sch. Agric. Sci. Univ. Nottingham 19th*, 1972, 99–114
- Roberts, B. E. & Osborne, D. J. (1973b) *Biochem. J.* **135**, 275–286
- Roberts, B. E., Payne, P. I. & Osborne, D. J. (1973) *Biochem. J.* **135**, 275–286
- Roberts, E. H. (1972) in *The Viability of Seeds* (Roberts, E. H., ed.), pp. 253–306, Chapman and Hall, London
- Sen, S. (1975) Ph.D. Thesis, University of Cambridge
- Sen, S. & Osborne, D. J. (1974) *J. Exp. Bot.* **25**, 1010–1019
- Sen, S., Payne, P. I. & Osborne, D. J. (1975) *Biochem. J.* **148**, 381–387
- Villiers, T. A. (1974) *Plant Physiol.* **53**, 875–878
- Villiers, T. A. & Edgcombe, D. J. (1975) *Seed Sci. Technol.* **3**, 761–774
- Weeks, D. P. & Marcus, A. (1971) *Biochim. Biophys. Acta* **232**, 671–684