Protein Synthesis and Loss of Viability in Rye Embryos

THE LABILITY OF TRANSFERASE ENZYMES DURING SENESCENCE

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Poor germination and eventual loss of viability of rye grains on storage reflects a decreasing ability of embryos to synthesize protein *in vivo*. Cell-free protein-synthesizing systems from low viability embryo stocks exhibit lesions in the soluble components of the post-ribosomal supernatant fractions. tRNA and aminoacyl-tRNA synthetases are not impaired. The activity of enzymes concerned with transfer of phenylalanyl-tRNA from the aminoacyl to the peptidyl site on the ribosome is slightly decreased. The transfer enzymes (transferase I) involved in the binding of phenylalanyl-tRNA to the aminoacyl site show a loss of activity that closely reflects the loss of viability and the decline of protein synthesis of the embryo *in vivo*. The inactivation of labile transferase I components may be a major factor leading to senescence and loss of viability in rye grains.

Viable rye embryos supplied with labelled amino acids in solution show considerable incorporation into trichloroacetic acid-insoluble material within the first hours of imbibition of water (Hallam *et al.*, 1972), but as the viability (i.e. the percentage germination) of the grain stock decreases the rate of this incorporation is decreased (Roberts & Osborne, 1973), and if embryos are non-viable (i.e. do not germinate) no incorporation of amino acids into trichloroacetic acid-insoluble material (Hallam *et al.*, 1973) can be detected. The loss of protein-synthesizing activity can therefore be related to the poorer and slower germination of grains from low-viability stocks. Although the decreased synthesis in these stocks of lower percentage germination could perhaps be attributed partly to an increase in the number of dead embryos in the sample, the decrease in incorporation observed is proportionally greater than the decline in embryo viability. This suggests that some intrinsic factor(s) other than the number of dead embryos are involved. An earlier paper (Roberts *et al.*, 1973) described the lesions that appear in rRNA during loss of viability. The present paper pinpoints lesions in the post-ribosomal supernatant fractions of cell-free protein-synthesizing systems that could contribute to the failure of protein synthesis in aging rye embryos.

Materials and Methods

The stocks of different percentage-viability rye grains (*Secale cereale* L. var. Lovasz patonai, from a 1964 harvest) were a gift from Dr. D. B. Mackay and R. J. Flood of the National Institute of Agricultural Botany, Cambridge, U.K. Grain was obtained in this condition by storage at moisture contents of between 15 and 9% from 1964 to 1969,

and in 1970, when the present work commenced, the stocks had the percentage viabilities (percentage germination) cited here. The embryos used in these experiments were separated from the rest of the grain by the mass-isolation technique of Johnston & Stern (1957). Details of the incorporation of labelled amino acids *in vivo*, the preparation of post-ribosomal deacylated tRNA and the separation and characteristics of the poly(U)-dependent cell-free protein-synthesizing system from these embryos have been described previously (Hallam *et al.*, 1972; Roberts *et al.*, 1973).

Preparation of post-ribosomal supernatant

The post-ribosomal supernatant was obtained by homogenizing 1–3 g of dry embryos in 16 ml of 0.5M-sucrose, 50mM-Tris-HCl, pH 7.7 at 20°C, 50mM-KCl, 10mM-MgCl₂ and 5mM-2-mercaptoethanol. After filtration through muslin and centrifugation at 30000g for 20 min at 1°C in an MSE High-Speed 18 centrifuge, ribosomes were pelleted from the supernatant solution by centrifugation at 269000g for 2 h (*r*_{av} 5.7 cm). The supernatant was then dialysed for three 2 h periods against 150 vol. of fresh extraction buffer. The supernatant was then centrifuged again at 269000g and the top two-thirds of the supernatant were aspirated and stored under liquid N₂ (see Roberts *et al.*, 1973).

Aminoacylation of tRNA *in vitro*

Aminoacylation was studied by a modification of the technique of Mans & Novelli (1961). Deacylated tRNA (100–200 µg) was incubated for 40 min at 25°C with 20mM-KCl, 10mM-MgCl₂, 20mM-Tris-HCl, pH 7.7 at 20°C, 1mM-ATP, 1.5 µCi of ¹⁴C-labelled L-phenylalanine (sp. radioactivity 513 mCi/

mmol) and post-ribosomal supernatant (500 μ g of protein) in a final volume of 0.6 ml. At intervals during the reaction 0.1 ml portions were pipetted on to Whatman filter-paper discs (2.5 cm in diameter), which were supported on pins and dried in a draught of hot air. The dry filters were washed twice for 10 min in 250 ml of trichloroacetic acid (6%, w/v), containing an excess of non-radioactive phenylalanine and rinsed in ethanol, then ethanol-ether (3:1, v/v) and finally ether. The dry discs were counted for radioactivity by liquid-scintillation spectrometry as described by Roberts *et al.* (1973).

Preparation of 14 C-labelled phenylalanyl-tRNA

tRNA containing 14 C-labelled phenylalanyl-tRNA was prepared by a modification of the techniques of Nishizuka *et al.* (1968). Deacylated tRNA from wheat germ (30 mg) was aminoacylated in a 25 ml reaction mixture containing 50 μ Ci of [14 C]phenylalanine (513 mCi/mmol), 2.5 mM-ATP, 10 mM-magnesium acetate and 24 mg of post-ribosomal supernatant protein for 40 min at 25°C. The mixture was placed in ice and adjusted to 0.02 M with potassium acetate, pH 5.0. The solution was adjusted to a final pH of 5 with acetic acid and then made 0.25 M with respect to NaCl and 0.01 M with respect to EDTA. The solution was then applied to a DE-52 DEAE-cellulose column (6 cm $\times$ 2 cm), equilibrated with 0.25 M-NaCl, 10 mM-magnesium acetate and 1 mM-EDTA, pH 5.0, and washed with the buffer until no radioactivity could be detected in the eluate. The elution buffer was then changed to 0.7 M-NaCl, 10 mM-magnesium acetate and 1 mM-EDTA, pH 5.0, and 5 ml fractions were collected. These were assayed for radioactivity in a water-tolerant scintillant [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (7%) in toluene-methanol (1:1, v/v)]. Radioactive fractions were then pooled, adjusted to 0.1 M-potassium acetate, pH 5.0, and precipitated with 2 vol. of ice-cold ethanol and stored overnight at -30°C. The precipitate was pelleted by centrifugation at 2000 g for 10 min and dissolved in 1-2 ml of 10 mM-Tris-HCl, pH 7.6 at 20°C, and 2 mM-magnesium acetate. The insoluble denatured protein was removed by centrifugation in an MSE High-Speed 18 centrifuge at 24000 g for 10 min at 1°C. Elimination of this debris did not decrease the amount of radioactivity in solution. The preparation was frozen in small portions and stored in liquid N₂.

Assay for the transfer of [14 C]phenylalanyl-tRNA into polyphenylalanine on ribosomes

This assay is a modification of those described by Allende (1969), Legocki & Marcus (1970) and Yarwood *et al.* (1971). Twice-pelleted ribosomes (100 μ g) were incubated for 60 min at 25°C with 50 mM-Tris-HCl, pH 7.7 at 20°C, 10 mM-magnesium

acetate, 100 mM-KCl, 50 mM-GTP, 8 mM-creatine phosphate, 16 μ g of creatine kinase, 28.5 pmol of [14 C]phenylalanyl-tRNA (23 200 c.p.m.) and transfer factors or post-ribosomal supernatant as indicated, in a final volume of 0.4 ml. The reactions were terminated by the addition of 2 ml of 5% (w/v) trichloroacetic acid and processed as described by Roberts *et al.* (1973).

Treatment of post-ribosomal supernatants with N-ethylmaleimide

Post-ribosomal supernatants were treated with N-ethylmaleimide to inactivate transferase II enzymes, as described by Yarwood *et al.* (1971). N-Ethylmaleimide (0.1 M, 0.05 ml) was added to 0.2 ml of reaction mixture contained in 50 mM-Tris-HCl, pH 7.7, 50 mM-KCl, 10 mM-MgCl₂ and 0.001 M-GSH at 0°C. After 10 min a further 0.02 ml of 0.05 M-GSH was added to react with the excess of N-ethylmaleimide.

Assay for the enzymic binding of [14 C]phenylalanyl-tRNA to ribosomes

The enzyme-dependent binding of [14 C]phenylalanyl-tRNA to ribosomes was determined by the method of Nirenberg & Leder (1964) as described by Roberts *et al.* (1973).

Results

Incorporation of 14 C-labelled amino acids into embryos in vivo

The incorporation of 14 C-labelled amino acids into trichloroacetic acid-insoluble material by individual embryos from stocks of different viability is shown in Table 1. In addition to the increase in embryos that did not incorporate there was a steep decline in the capacity of those that were still viable to incorporate amino acids during the 60 min incorporation period (Table 1).

Polyphenylalanine synthesis in cell-free systems

Although the poly(U)-dependent cell-free systems *in vitro* derived from non-viable embryos exhibited decreased activity of both the ribosomes and the post-ribosomal supernatant, the ribosomes extracted from 95%-, 64%-, 48%- and 15%-viable embryos were functional and showed very similar activity when they were extracted speedily and pelleted once only (Roberts *et al.*, 1973). Corresponding post-ribosomal supernatants, however, showed large differences in their ability to activate a protein-synthesizing system. The cell-free systems containing washed ribosomes from 95%-viable embryos and post-ribosomal supernatants from dry embryos of different percentage viabilities (Table 2) showed that the capacity of the supernatant to activate the synthesis of polyphenylalanine declined progres-

Table 1. Incorporation of ^{14}C -labelled amino acids into trichloroacetic acid-insoluble material by individual embryos of different percentage viabilities

Embryos were imbibed for 5h at 25°C on thin layers of 1% agar containing 2% (w/v) sucrose and $10\mu\text{g}$ of chlroamphenicol/ml. A small volume of sucrose plus chloramphenicol was added to wet the embryos. They were then rinsed in this medium and incubated for a further 60min in a solution containing $10\mu\text{Ci}$ of ^{14}C -labelled amino acid mixture (sp. radioactivity $45\text{mCi/g-atom of C/ml}$). Each embryo was then washed in a 150-fold excess of unlabelled precursor, homogenized and mixed with an equal volume of 10% trichloroacetic acid. The insoluble precipitate was processed for radioactivity assay as described by Roberts *et al.* (1973). The adsorption background (120c.p.m.) was subtracted.

Viability of embryo	Radioactivity (c.p.m./embryo)				
stocks ...	95%	64%	45%	15%	0%
	8991	6033	921	158	0
	12506	8730	800	236	0
	11622	8380	2280	285	0
	11240	319	0	6	0
	14271	0	0	0	0
Mean ...	11722	5865	1334	171	0

Table 2. Capacity of post-ribosomal supernatants from embryos of different percentage viability to activate polyphenylalanine synthesis utilizing $[^{14}\text{C}]$ phenylalanine

Incubation was at 25°C for 60min in a final volume of 0.6ml containing 5mM-Mg^{2+} , 35mM-KCl , $250\mu\text{g}$ of twice-pelleted ribosomes from 95%-viable embryos, $10\mu\text{g}$ of deacylated tRNA from 95%-viable embryos, $100\mu\text{g}$ of poly(U) and $0.5\mu\text{Ci}$ of $[^{14}\text{C}]$ -phenylalanine.

Source of supernatant (viability of embryos)	Polyphenylalanine synthesized (pmol/mg of rRNA)
64%	34.9
45%	16
15%	9
0%	2.6

sively with the decrease of the embryo viability from which it was obtained. The decrease in supernatant activity was not attributable to inactivation of tRNA, for deacylated tRNA species isolated from 64%-, 45%- and 15%-viable embryos could be aminoacylated with $[^{14}\text{C}]$ phenylalanine (Fig. 1) or ^{14}C -labelled amino acid mixtures (Roberts & Osborne, 1973)

Fig. 1. Aminoacylation of polyphenylalanyl-tRNA in the tRNA mixture isolated from post-ribosomal supernatants of embryos of 64%, 45% and 15% viability

The polyphenylalanyl-tRNA synthetase preparation was from 64%-viable embryos. Incubation was at 25°C in a final volume of 0.6ml containing $35\mu\text{g}$ of deacylated tRNA from embryos of 64% (Δ), 45% ($\bullet$) and 15% ($\circ$) viability, 10mM-Mg^{2+} , 20mM-K^+ , $20\mu\text{M-Tris-HCl}$, pH 7.5 (at 20°C), 1mM-ATP , $1\mu\text{Ci}$ of $[^{14}\text{C}]$ phenylalanine and $450\mu\text{g}$ of supernatant protein from 64%-viable embryos.

to the same extent. In addition, no differences in the activity of the phenylalanyl-tRNA synthetase could be detected in the post-ribosomal supernatants from 64%-, 48%-, 45%-, and 15%-viable embryos as monitored by their rates of formation of phenylalanyl-tRNA (Fig. 2). Because replacement of $[^{14}\text{C}]$ phenylalanine by $[^{14}\text{C}]$ phenylalanyl-tRNA in the complete poly(U)-directed system failed to enhance the synthesis of polyphenylalanine *in vitro* in the presence of the supernatants from lower-viability embryos (Table 3), it was deduced that polypeptide synthesis was limited at some stage coincident with, or after, the binding of aminoacyl-tRNA to the ribosome. Binding of phenylalanyl-tRNA to the ribosomes and its subsequent translocation to the peptidyl site was studied in a series of transfer assays to test the efficiency of the binding (transferase I) and translocase (transferase II) enzymes in the different post-ribosomal supernatants.

Assays for transferase activities in post-ribosomal supernatants

The poly(U)-dependent transferase II assay consisted of washed ribosomes and $[^{14}\text{C}]$ phenylalanyl-tRNA, the soluble transfer enzymes of the post-ribosomal supernatants, GTP, SH groups, K^+ and

Fig. 2. Aminoacylation of polyphenylalanyl-tRNA from 95%-viable embryos by post-ribosomal supernatants from embryos of 64%, 48%, 45% and 15% viability

Conditions were as for Fig. 1, except that all reaction mixtures contained 35 μ g of tRNA from 95%-viable embryos and 450 μ g of supernatant protein from embryos of 64% (Δ), 48% ($\blacksquare$), 45% ($\blacktriangle$) or 15% ($\circ$) viability.

Table 3. Capacity of post-ribosomal supernatants from embryos of different viability to activate polyphenylalanine synthesis utilizing [14 C]phenylalanyl-tRNA

Incubation was at 25°C for 60 min in a final volume of 0.4 ml containing 10 mM-Mg $^{2+}$, 25 mM-K $^{+}$, 100 μ g of poly(U), 70 μ g of ribosomes from 95%-viable embryos, 450 μ g of supernatant protein and 28.5 pmol of [14 C]phenylalanyl-tRNA prepared from 95%-viable embryos.

Source of supernatant (viability of embryos)	Polyphenylalanine synthesized (pmol/mg of rRNA)
64%	305
48%	261
45%	239
15%	156
0%	73

Mg $^{2+}$. It differs from the complete poly(U)-dependent cell-free system in that the K $^{+}$ optimum was shifted from 25 to approx. 100 mM (Fig. 3). Also, the Mg $^{2+}$ concentration was adjusted to 10 mM so that the initial binding of [14 C]phenylalanyl-tRNA to the

Fig. 3. Influence of K $^{+}$ on the transfer of [14 C]phenylalanyl-tRNA to polyphenylalanine

Incubation was at 25°C for 60 min in final volume of 0.4 ml containing 70 μ g of twice-pelleted ribosomes from 95%-viable embryos, 10 mM-Mg $^{2+}$, 50 mM-Tris-HCl, pH 7.7 (at 20°C), 28.5 pmol of [14 C]phenylalanyl-tRNA (23 200 c.p.m.), 50 μ M-GTP, 8 mM-creatine phosphate, 10 μ g of creatine kinase and 450 μ g of transfer enzyme fraction from 95%-viable embryos.

Fig. 4. Kinetics of transfer of [14 C]polyphenylalanyl-tRNA to polyphenylalanine at 100 mM-K $^{+}$

The reaction mixture was as for Fig. 3.

ribosome was non-enzymic and the formation of polyphenylalanine was independent of transferase I enzymes (Allende *et al.*, 1973). The reaction was complete after 60 min at 25°C (Fig. 4). When these

conditions were used, assays of transferase II activity in the different post-ribosomal supernatants showed a slight decline only with loss of viability (Table 4, Expts. I and II); this indicated that this enzyme system does not limit polyphenylalanine synthesis at the lower percentage viabilities.

Treatment of the supernatant with *N*-ethylmaleimide effectively inactivated the transferase II system (Table 4, Expt. III). Post-ribosomal supernatants so treated with *N*-ethylmaleimide do, however, still stimulate the GTP-dependent enzymic binding of [¹⁴C]phenylalanyl-tRNA to washed ribosomes in the transferase I assay of Nirenberg & Leder (1964) even though translocation to the peptidyl site is precluded. In these assays (Tables 5 and 6) the Mg²⁺ concentration is lowered to 4.7mM to decrease the non-enzymatic binding of [¹⁴C]phenylalanyl-tRNA. When *N*-ethylmaleimide-treated supernatants from 64%, 48%, 45%, 15% and 0%-viable embryos were tested in this system for their capacity to bind [¹⁴C]phenylalanyl-tRNA to washed ribosomes from 64%-viable embryos (Table 5a), the activity of the supernatants declined progressively with the loss of viability. When a selected supernatant (from 64%-viable embryos) was tested for its capacity to bind [¹⁴C]phenylalanyl-tRNA to ribosomes from embryos of different viability (Table 5b), only a small decrease in binding to the lower-viability

Table 4. Activity of transferase II enzymes in the post-ribosomal supernatant from embryos of different viability, and inactivation by *N*-ethylmaleimide treatment

Incubation was at 25°C for 60min in a final volume of 0.4ml containing 70μg of twice-pelleted ribosomes from 95%-viable embryos, 10mM-Mg²⁺, 100mM-K⁺, 50μg-GTP, 8mM-creatine phosphate, 10μg of creatine kinase, 28.5pmol of [¹⁴C]polyphenylalanyl-tRNA from 95%-viable embryos and 450μg of post-ribosomal supernatant from embryos of different viability. For *N*-ethylmaleimide treatment see the Materials and Methods section.

Source of supernatant (viability of embryos)	Polyphenylalanine synthesized (pmol/mg of rRNA)		
	Expt. I	Expt. II	Expt. III
95%	272	283	350
64%	—	272	—
48%	214	—	—
45%	—	258	—
15%	245	248	—
0%	141	—	—
95%, + <i>N</i> -ethylmaleimide	—	—	4

Table 5. Activity of GTP-dependent binding enzymes (transferase I) from *N*-ethylmaleimide-treated supernatants

(a) Activity of supernatants from embryos of different percentage viability to bind [¹⁴C]phenylalanyl-tRNA to ribosomes extracted from 64%-viable embryos, and (b) activity of supernatants from 64%-viable embryos to bind [¹⁴C]phenylalanyl-tRNA to ribosomes extracted from embryos of different percentage viabilities. For (a) incubation was at 25°C for 30min in a final volume of 0.2ml containing 80μg of twice-pelleted ribosomes from 64%-viable embryos, 70mM-K⁺, 4.7mM-Mg²⁺, 0.1mM-GTP, 100μg of poly(U), 78.5 pmol of [¹⁴C]polyphenylalanyl-tRNA and 450μg of post-ribosomal supernatant pretreated with 0.025M-*N*-ethylmaleimide. Non-enzymic binding of [¹⁴C]phenylalanyl-tRNA to ribosomes was less than 11pmol/mg of rRNA for all supernatants. For (b) 100μg of ribosomes was used per reaction mixture.

Viability of embryo source	[¹⁴ C]Phenylalanyl-tRNA bound to ribosomes (pmol/mg of rRNA)
(a) Supernatant 64%	59
48%	55
45%	53
15%	20
0%	13
(b) Ribosomes 64%	62
48%	58
15%	48
0%	44

Table 6. GTP-dependent enzymic binding (transferase I-dependent) of [¹⁴C]phenylalanyl-tRNA in systems in which both ribosomes and post-ribosomal supernatants were obtained from embryos of the same viability

Incubation was as described in Table 5; 100μg of ribosomes was used per reaction mixture.

Source of supernatant and ribosomes (viability of embryo)	[¹⁴ C]Phenylalanyl-tRNA bound to ribosomes (pmol/mg of rRNA)
64%	81
48%	79
45%	71
15%	41
0%	5

ribosomes occurred. However, if transferase I-mediated binding is assessed in a homologous system in which both the post-ribosomal supernatants and the ribosomes are derived from embryos of the same viability, the decreased binding can be correlated with the decreasing percentage viability of the parent embryos (Table 6), e.g. the 64%-viable system binds 81 pmol of [^{14}C]phenylalanyl-tRNA, whereas the 0%-viable system binds only 5 pmol.

Discussion

The incorporation of ^{14}C -labelled amino acids into trichloroacetic acid-insoluble material by individual embryos *in vivo* clearly demonstrates that a decrease of synthetic activity occurs well before the embryo becomes non-viable (Table 1). This decrease can be reconciled with the loss of vigour (or senescence) shown by all seeds during storage, and their progressively poorer performance at germination.

The loss in activity of poly(U)-directed cell-free protein-synthesizing systems obtained from embryo stocks of decreasing viability has already been related to a decrease in ribosome activity and a loss of integrity of the rRNA (Roberts *et al.*, 1973). However, another, and possibly more serious, lesion contributing to decreased polyphenylalanine synthesis *in vitro* resides in the post-ribosomal supernatants (Table 2). Since there is no evidence for a lesion in either tRNA or tRNA synthetase (Figs. 1 and 2), and since the replacement of [^{14}C]phenylalanine by [^{14}C]phenylalanyl-tRNA in the poly(U)-dependent system *in vitro* does not stimulate the synthesis of polyphenylalanine by the lower-viability supernatants (Table 3), some failure in the binding or translocation of phenylalanine on the ribosome is implied.

The activity of the translocase or elongation factors (transferase II) assayed at an Mg^{2+} concentration (10 mM) at which non-enzymic binding of [^{14}C]phenylalanyl-tRNA to the ribosome takes place (Table 4, Expts. I and II) show a slight decline only with loss of embryo viability. Post-ribosomal supernatants treated with *N*-ethylmaleimide (Table 4, Expt. III) do not activate the synthesis of polyphenylalanine, yet still activate the GTP-dependent enzymic binding of [^{14}C]phenylalanyl-tRNA to ribosomes, observations which accord with those of Mosteller *et al.* (1966) for reticulocytes and Yarwood *et al.* (1972) for *Vicia faba*. In such assays, the decline in activity of transferase I in the *N*-ethylmaleimide-treated supernatants (Table 5a) was concomitant

with loss in grain viability. The ribosomes (Table 5b) from embryos of different viabilities were, however, active in binding [^{14}C]phenylalanyl-tRNA when provided with an active transferase I system from 64%-viable embryos. When ribosomes and supernatant both from embryos of the same viability were combined in a homologous system (Table 6), a decline in activity was displayed that was similar to that expressed by the individual supernatants (Table 5a).

It is concluded that during dry storage of grain there is an inactivation or denaturation of specific proteins in the embryo. Of the enzymes concerned directly in protein synthesis, transferase I is particularly labile. Indeed, Allende (1969) has reported the instability of extracted transferase I from wheat embryos; this implies that one of the most readily inactivated components of a poly(U)-dependent system synthesizing protein *in vitro* is the binding factor, transferase I. The inactivation of this enzyme may well be a major potential lesion in the loss of viability in cereal grains *in vivo*.

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