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A POLYURIDYLIC ACID-DIRECTED CELL-FREE SYSTEM FROM 60 DAY-OLD DEVELOPING SEEDS OF *VICIA FABA*

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Abstract-The components and conditions of an active poly U-directed cell-free system from developing beans have been described. Isokinetic sucrose gradients have been used to show that, in the absence of poly U the membrane-bound ribosomal fraction was labelled, whereas in the presence of poly U a monosome fraction was the most active, with a small amount of radioactivity associated with the polysome area.

INTRODUCTION

As YET, no one has described a simple cell-free system from plants capable of the net synthesis of protein detected chemically. There are, however, many reports of cell-free amino acid-incorporating systems isolated from plants,[1–3] and generally there have been two experimental approaches. The first is to isolate ribosomes with endogenous messenger RNA molecules attached, the other to add specific RNA molecules to ribosomal preparations, presumed free of endogenous messenger molecules. The latter method has, in the main, used viral messengers.

Several years ago, as suitable natural plant messengers were not readily available, we decided to develop a polyuridylic acid (poly U) synthetic messenger, directed system from developing seeds of *Vicia faba*. This paper describes the components of the system and the conditions required for optimum activity.

RESULTS

The results in Table 1 show that there was very little endogenous activity in the absence of added messenger, and as with all other thoroughly characterized systems, ribosomes, enzyme fraction, *t*RNA, ATP, GTP, Mg^{2+} and monovalent cation were required for full activity. The time course of the reaction depended on the temperature at which incubations were carried out (Fig. 1); the largest amount of activity was incorporated in incubations at 15". The pH optimum of the process was established as pH 7·8, and there was a very sharp optimum at 9 mM Mg^{2+}.

Characterization of the Components

(1). *tRNA*. Samples of *t*RNA before and after DE52 chromatography were subjected to electrophoresis on 2·6% and 7·5% acrylamide gels.[4] The results given in Table 2 show

[1] R. J. MANS, *Ann. Rev. PI. Physiol.* 18, 127 (1967).
[2] J. E. ALLENDE, in *Techniques in Protein Biosynthesis* (edited by P. N. CAMPBELL and J. R. SARGENT), Vol. 2, Academic Press, London (1969).
[3] D. BOULTER, *Ann. Rev. PI. Physiol. 21, 91 (1970).*
[4] U. E. LOENING, *Biochem. J. 102,251 (1967).*

TABLE 1. CHARACTERISTICS OF POLY U-DIRECTED INCORPORATION OF [^{14}C] PHENYLALANINE

Incubation	$\mu\mu$Mole phenylalanine incorporated per mg *r*RNA
Complete	900
– ribosomes	5
– poly u	50
– enzymes	225
– *t*RNA	180
– ATP*	300
– GTP*	450
– Mg†	5

* A complete dependence on GTP and ATP could be demonstrated if incubation was started without their addition, and then these components added after 15 min, by which time endogenous ATP and GTP had been exhausted. Bacterial counts $< 10^2$/ml.

† Incubation at 25" for 40 min, see Experimental.

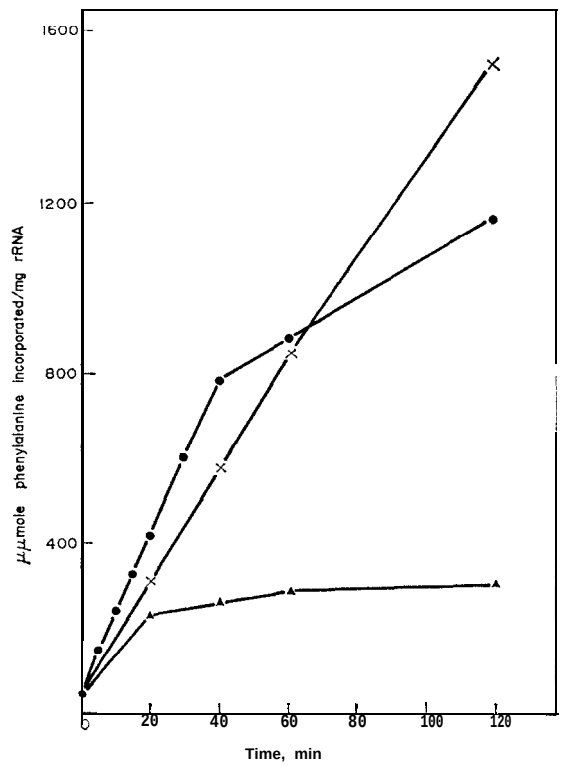

FIG. 1. TIME COURSE OF [^{14}C]PHENYLALANINE INCORPORATION. x, 15"; ●, 25"; ▲, 37".

that 25s and 18s *r*RNA was removed from the crude *t*RNA preparation by chromatography, as was some small molecular weight materials. Some 4s *t*RNA is lost during extraction of *r*RNA with 1 M NaCl, but the molar ratio of 4s/5s RNA remains the same before and after chromatography on DEM.

(2). ***Microsomal preparations.*** The protein: RNA ratio of microsome preparations was determined to be 5·0, indicating the presence of membranes in the preparations.

TABLE 2. PERCENTAGES OF VARIOUS TYPES OF RNA

Sample	4s	5s	25s + 18s
Total RNA prepared by phenol extraction	15	2	83
Crude *t*RNA prepared by method of Mosteller, Culp and Hardesty[11]	61	15	24
RNA preparation after DE52 chromatography	80	20	Trace

RNA separated on Loening gels. Values are % of A/total A at 260 nm.

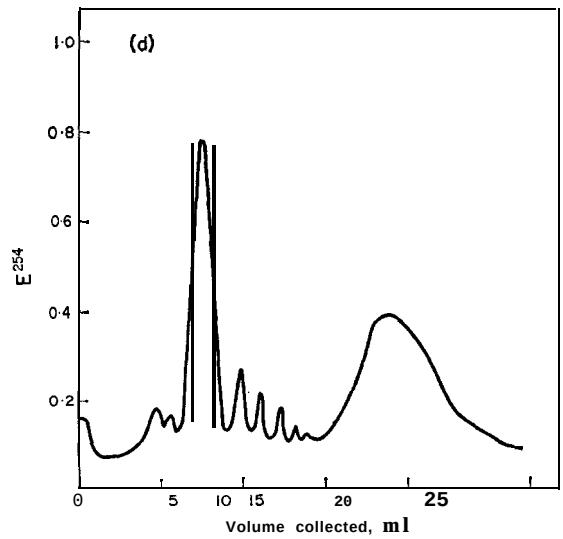

FIG. 2. SUCROSE DENSITY PROFILE OF MICROSOME PREPARATION.

Isokinetic sucrose gradients were used to characterize the preparations further. The results in Fig. 2 show a large broad peak of membrane-bound material which travelled furthest down the gradient, then in order 5 polysome peaks, a large monosome and 2 small sub-unit peaks.

Standard incubation mixtures containing microsomes which had been allowed to incorporate [^{14}C]phenylalanine in the presence and absence of poly U, were analysed on isokinetic sucrose gradients in order to identify the ribosomal fraction most active in phenylalanine incorporation. In contrast to the previous experiment, after incubation the

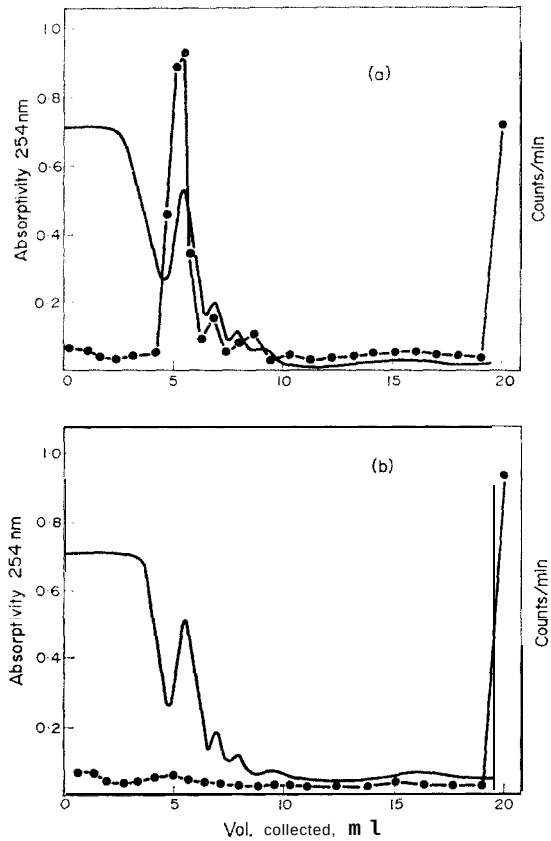

FIG. 3(a and b). [^{14}C]PHENYLALANINE INCORPORATION BY SEPARATED MICROSOME COMPONENTS.
(a) containing poly U, (b) without poly U. ——— optical density. O-----O radioactivity.

membrane-bound material in the preparations sedimented through the gradient and formed a pellet at the bottom of the centrifuge tube; radioactivity was associated with this fraction from incubations both with or without poly U (Figs. 3a and b). Without poly U (Fig. 3b) the membrane-bound fraction was labelled and not the free polysomes, indicating that only the former were sufficiently active in protein synthesis to have detectable radioactivity. In the poly U incubations (Fig. 3a) the monosome fraction was the most active, although a small amount of radioactivity was associated with the polysome area and apparently very little free polyphenylalanine was formed. After incubation the amount of optically active material in the supernatant also increased.

DISCUSSION

Conditions have been determined and extraction methods developed, which have led to the establishment of an active poly U-directed cell-free system from developing beans. This system is more active than most others which have been described.[3]

The true level of incorporation might, in fact, be higher than indicated, due to the presence of endogenous pools of ^{12}C phenylalanine associated with the microsomal preparations. Bean microsomes have in fact been shown to have both loosely and firmly-bound pools of free amino acids.[9] Unpublished work from this laboratory has shown that

some microsomal preparations contain 0·775 mμMole free phenylalanine/mg. If it is assumed that the preparations used in the present work have a pool of phenylalanine of the same order as this, then this would result in a decrease in specific activity of the labelled amino acid of less than 1 per cent.

Moore[5] studying the binding of poly U to 70s ribosomes found that the formation of polysomes depends on the ratio of poly U to ribosomes. When ribosomes were present in molar excess over poly U, polysome complexes were formed, but when poly U was in excess it complexed with single ribosomes. In the present incubations poly U has been added in excess, hence the large peak of activity associated with monosomes in Fig. 3(a). With added poly U radioactivity was also found with free polysomes and membrane-bound material, whereas without poly U only membrane-bound material was radioactive, indicating that protein synthesis directed by endogenous messengers was membrane-bound, whereas poly U associated to some extent with free polysomes.

EXPERIMENTAL

Biological materials. Seeds of ***Vicia faba*** L. var. triple white, were grown in the open. Pods were harvested at 60 days after fertilization of flowers and immediately prior to use; similar-sized seeds were selected.

Chemicals. Chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., except for adenosine monophosphate 2′(3′) mixed isomers; adenosine 5′ triphosphate, Na salt; guanosine 5′ triphosphate, Na salt; creatine phosphokinase; phosphocreatine; polyuridylic acid, K salt, and trizrna base, analytic grade, which were obtained from Sigma Chemical Co. Ltd., London, U.K.; DE52 from Whatman, and radioactive chemicals from The Radiochemical Centre, Amersham, Bucks., U.K.

Buffers and reagents. Extractant A: 0·05 M Tris-HCl, pH 7·6 at 0°; 0·016 M KCl; 0·005 M $MgCl_2$ and 0·45 M sucrose. Solution B 0·1M Tris-HCl, pH 7·6 at 0°; 0·001 M KCl; 0·0001 M $MgCl_2$. Scintillation fluid contained 0·45% (w/v) PPO and 0·01% (w/v) POPOP in toluene. The concentration of magnesium chloride was determined by the method of Mohr, as described in Curnming and Kay.[6]

Protein determination. Protein was determined by the method of Lowry ***et al.***[7]

RNA &termination. RNA was determined by the method of Markham.* Adenosine monophosphate (O-50 μg ml standard solution) was used as the standard, and corrected to RNA content assuming a purine: pyrimidine ratio of 1:0·83 for ***V. faba.***[9] $E^{670}_{1\,cm}$ was measured on a Unicam SP800 spectrophotometer.

Extraction of t**RNA.* t* RNA was prepared from developing bean cotyledons by the method of Mosteller et ***al.,[10] with the modification that 300 g bean cotyledons were homogenized directly in 250 ml. 0·1 M Tris-HCl, pH 7.5, containing 0·003 M-$MgCl_2$ and 0024 M-KCl, plus an equal volume of 90% (w/v) aqueous phenol.

Purification of t**RNA by DEAE-cellulose chromatography.* The crude *t*RNA preparation in 0·05 M Tris-HCl pH 7·6 was adsorbed onto a 1·5 x 15 cm DE52 column, previously equilibrated with 0·05 M Tris buffer. The column was washed through with 0·05 M Tris-HCl pH 7·6 and when the base-line had returned to its original position the *t*RNA was eluted with 1 M NaCl in 0·05 M Tris-HCl pH 7·6. The eluate was adjusted to 0·1 M with respect to KOAc, 2 vol. EtOH added and the *t*RNA precipitated at -20". The *t*RNA was collected by centrifugation at 4000 ***g for 15 min, EtOH decanted and all traces of it removed from the pellet under vacuum. The pellet was redissolved in the minimal vol. 0·01 M KOAc, pH 6·0 and stored in 0·5 ml aliquots at -70".

***Characterization of t**RNA by polyacrylamide gel electrophoresis. t* RNA** preparations were subject to electrophoresis on 2·6% and 7·5% gels using the method of Loening.[4]

Isolation of microsomes. Glassware used in the preparation of microsomes was autoclaved before use. Any apparatus that could not be autoclaved was rinsed out with EtOH and allowed to dry. All solutions were made with sterile H_2O, and precautions were taken throughout to reduce the risk of microbial contamination as much as possible. All procedures were carried out in glassware immersed in crushed ice.

[5] P. B. MOORE, ***J. Mol. Biol. 18, 8 (1966).***
[6] A. C. CUMMING and A. S. KAY, in ***Quantitative Chemical Analysis.*** Gurney-Jackson, London.
[7] 0. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, ***J. Biol. Chem.*** **193.265** (1951).
[8] R. MARKHAM, in ***Modern Methods of Plant Analysis*** (edited by K. PAECH and M. V. TRACEY), **Vol.** IV, Springer-Verlag (1955).
[9] A. YARWOOD, Ph.D. Thesis, University of Liverpool (1968).
[10] R. D. MOSTELLER, W. J. CULP and B. HARDESTY, ***Proc. Nut. Acad. Sci., U.S.*** 57, 1817 (1967).

Pods were surface sterilized with EtOH, the seeds harvested and their testas removed using sterile scalpels and forceps. Approximately 100 g cotyledons were ground in 200 ml of extractant A in an ice-cold mortar with acid-washed sand previously equilibrated with extractant A. The brei was centrifuged at 2000 *g* for 5 min, and the supernatant decanted through 4 layers of sterile gauze to remove some lipid. The pellet was reground in a further 50 ml extractant and centrifuged as before. The supernatant fraction were combined and centrifuged at 20,000 g for 10 min, the supernantant decanted through 4 layers of sterile gauze and then recentrifuged at 105,000 g for 2 hr. The supernatant was decanted through sterile gauze, quickly frozen, and stored at -70". The sides of the centrifuge tubes containing the pellet were carefully wiped clean with tissue, the surface of the pellets rinsed with solution B and the pellets rehomogenized in the same buffer using a loose fitting Teflon homogenizer, at a concentration of 20 mg particles/ml. The concentration of the suspension was determined from the $E^{260}_{1\,cm}$ using a value of 10 absorptivity units for a 1 mg/ml microsome suspension. The particles were stored at -70" until required.

Cell-free amino acid incorporation systems. Thin-walled, narrow bore test tubes were used for all incubations. Incubation mixtures were maintained at 0" in an ice-bath, until the subcellular particles were added, when they were transferred to a constant temperature bath at various temperatures. Incubation tubes were shaken manually every few minutes, and prior to aliquots being removed for assay. The incubation mixture contained the following in μmole/ml incubation: Tris-HCl buffer, pH 7·8 at incubation temperature, 60; $MgCl_2$, 9; KCI, 70; GSH, IO; GTP, 0·2; ATP, 4; creatine phosphate, 10; phosphocreatine kinase, 60 μg; [^{14}C]L-phenylalanine (sp.act. 50 mC/mM), 0.02; each of the other ^{12}C protein amino acids, 0.02; poly U, 0·2 mg; deacylated *t*RNA, 0·2 mg; 0·2 mg microsomes and dialysed 105,000 g supernatant, volume to give maximum incorporation as determined by a concentration curve, usually 0·06 ml.

Measurement of radioactivity. Reactions were stopped by the addition of 5% (w/v) TCA and heated for 15 min at 90". The tubes were cooled to 0" and the insoluble precipitate collected onto a Millipore filter, washed with 5 % (w/v) TCA and dried under an IR lamp. Filters were placed in scintillation vials with 10 ml scintillation fluid and the radioactivity present was measured using a Beckman liquid scintillation counter, model LS-200B. Samples were counted twice and an average taken. Background counts were subtracted. Average counts collected for a complete incubation was 3000 counts/min per Millipore filter.

Sucrose gradients. 15% to 45 % isokinetic gradients were prepared using 6 ml 15 % sucrose and 20 ml 55% sucrose (both containing 10 mM Tris-HCl pH 7.6; 25 mM KCI; 1 mM $MgCl_2$) by the method of Henderson." Gradients were standardized using ribosomes prepared from *E. coli*. Samples from 2·0 ml incubations were layered over the gradient, which was then centrifuged at 95,000 g av. for 3 hr in the 3 x 23 ml rotor of the M.S.E. Superspeed '65' Centrifuge. After centrifugation the E^{254} was measured with an Isco UV analyser, Model 222, attached to a Servoscribe chart recorder, and then the gradient was fractionated and 0·5 ml samples collected in 5 ml 5 % (w/v) TCA containing 0·2 mg bovine serum albumin. The samples were hydrolysed at 90" for 15 min, collected on Millipore filters and radioactivities determined.

Bacterial counts. Aliquots of incubations were checked for bacterial contamination. Serial dilutions were made in quarter strength Ringer's solution and 0·1 ml samples were plated onto Nutrient agar plates. Triplicate plates were made of each dilution. The plates were incubated at 37" for 2 days before the colonies were counted.

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[11] A. R. HENDERSON, ***Anal. Biochem. 27, 315 (1969).***