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MAGNESIUM REPLACEMENT BY POLYAMINES IN HIGHER PLANT CELL-FREE POLYPHENYLALANINE SYNTHESIS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; cell-free polyphenylalanine synthesis; magnesium replacement; polyamines; inorganic cations.

Abstract—The characteristics and specific requirements for the formation of polyphenylalanine from Phe-[^{14}C] in a barley ribosome cell-free incorporation system were detailed. The polyamines spermine, spermidine and putrescine, and the inorganic cations Ca^{2+}, Ba^{2+} and Mn^{2+} demonstrated different capabilities for replacing the Mg^{2+} requirement in the incorporation system. Spermine was extremely efficient in this respect, followed by spermidine; all of the cations tested showed discrete concentration ranges of effectiveness. The data supported the suggestion that, at least to a certain extent, the cation requirement for protein syntheis may be non-specific.

INTRODUCTION

The organic polycations spermine, spermidine and putrescine belong to a class of naturally-occurring aliphatic compounds collectively referred to as polyamines which are widely distributed among both prokaryotes and eukaryotes [1–4]. Various examples of correlative increases in polyamine levels and levels of nucleic acid and protein synthesis in both prokaryotic and eukaryotic tissues have suggested the participation of this class of compounds in the metabolic regulation of macromolecular synthesis [3–6]. Additionally, extensive investigations on Mg^{2+} replacement by the positively-charged polyamines in cell-free polypeptide synthesis systems [7–14] have intimated a possible basis for a causal interrelationship *in vivo* between polyamine regulation and protein synthesis. However, the participation and importance of Mg^{2+} in protein synthesis, especially in the maintenance of ribosome structural and functional integrity has been long established [15–18]. Moreover, as pointed out recently [6], the abundance of both inorganic divalent cations and polyamines in cells leads to difficulties in assessing their relative effectiveness for any given system *in vivo*.

Although the presence of both polyamines [1, 2, 19–21] and the enzymes reponsible for their metabolism [22–26] is well-documented for tissues of higher plants, there has been a paucity of reports on the interactions of these compounds with either *in vivo* or *in vitro* plant protein synthesizing systems. The present investigation has examined the competence of the polyamines spermine, spermidine and putrescine in comparison to that of the inorganic divalent cations Ba^{2+}, Ca^{2+} and Mn^{2+} in replacing the requirement for Mg^{2+} in polyphenylalanine synthesis by a barley ribosome cell-free system.

RESULTS

Characterization of the incorporation system

The experimental results presented in Fig. 1 and Table 1 designate the characteristics and specific requirements for the formation of polyphenylalanine from phenylalanine-[^{14}C] in the barley ribosome cell-free system. This system is functionally a so-called complete system for amino acid incorporation consisting of both aminoacylation and polymerization reactions in contrast to a transfer system which would comprise only the latter steps. Therefore reiterating previous inferences

Table 1. Requirements for Phe-[^{14}C] incorporation by the barley ribosome cell-free system

Component omitted or added	%Incorporation
Complete*	100
− Mg^{2+}	0
− K^{+}	0
− DTT	30
− ATP	<1
− creatine phosphate and creatine phosphokinase	84
− GTP	44
− poly(U)	1
− *t*RNA	<1
− supernatant protein	<1
− ribosomes	<1
+ chloramphenicol (20 μg/ml)	101
(100 μg/ml)	102
+ cycloheximide (20 μg/ml)	30
(100 μg/ml)	12

* Standard reaction mixture as described in the Experimental.

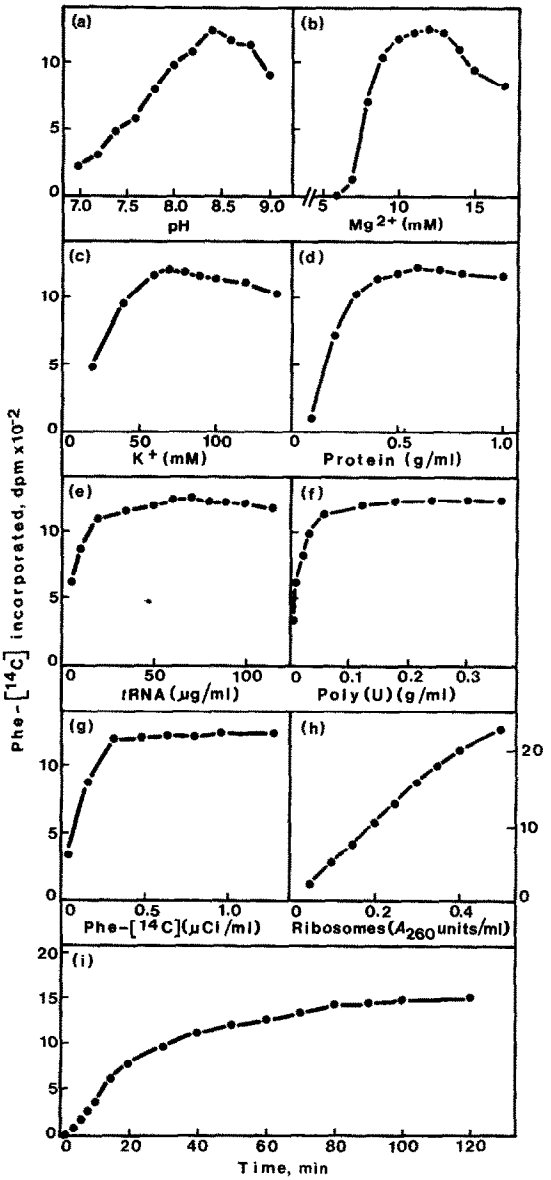

Fig. 1. Characteristics of Phe-[^{14}C] incorporation by the barley ribosome cell-free system. (a) Influence of the pH; (b) effect of the Mg^{2+} concn (as $MgCl_2$); (c) effect of the K^+ concn (as KCl); (d) effect of the dialysed post-ribosomal supernatant concn; (e) effect of the brewer's yeast $tRNA^{Phe}$ concn; (f) effect of the poly (U) concn; (g) effect of the Phe-[^{14}C] concn (sp. act 486 mCi/mmol); (h) effect of the ribosome concn. (i) time course of incorporation. In (a)–(h) the concentrations of all components except for the specific one being considered were as described for the standard reaction mixture in the Experimental and incubation was for 1 hr.

[27], the pH and ionic concentration optima [Fig. 1 (a–c)] for this type of incorporation system may be in essence compromises reflecting the respective optima for the different steps in the overall process of polyphenylalanine synthesis. In this respect, the comparatively high pH optimum [Fig. 1(a)] for the present system containing brewer's yeast *t*RNA, was similar to the higher pH optimum obtained for a bean cell-free incorporation system [27] when the source of *t*RNA was from yeast instead of a higher plant. The indication of a slight lag-phase in phenylalanine incorporation [Fig. 1(i)] has been a characteristic feature of the kinetics of poly(U)-directed polyphenylalanine synthesis systems [27, 28].

Table 1 shows an absolute dependence of the incorporation system on Mg^{2+}, K^+, poly(U), *t*RNA, supernatant protein and ribosomes. GTP is required in polypeptide synthesis for the formation of binding complexes with the proteinaceous elongation factors [29–31] and the partial requirement denoted here may reflect the presence of pre-established levels of such complexes in the dialysed post-ribosomal supernatant. However, the absolute requirement for added *t*RNA suggests that it was not present in the supernatant fraction at levels sufficient to stimulate incorporation. The ability of yeast $tRNA^{Phe}$ to participate successfully in the barley cell-free system was indicative of the reported lack of specificity for heterologous aminoacylation between $tRNA^{Phe}$ and phenylalanine-*t*RNA synthetases from yeast and higher plants [32, 33]. The lack of an inhibitory effect by chloramphenicol attested to the absence of 70S ribosomes of either eukaryotic or prokaryotic origin, while the partial inhibition of incorporation by cycloheximide reflected the differential effect of this inhibitor on plant cell-free protein synthesizing systems [34–36].

Magnesium ion substitution by other cations

The effect of addition of the polyamines spermine, spermidine and putrescine on the activity of the incorporation system at suboptimal Mg^{2+} levels can be seen in Fig. 2. There was a definite stimulation of activity by all 3 polyamines, since at the concentration of Mg^{2+} used (5 mM or less) it alone did not result in detectable activity [see Fig. 1(b)]. The concentration profiles for the polyamines in all cases assumed the characteristic optimum curve of response. With respect to relative effectiveness the order was spermine > spermidine > putrescine, the specific optima for maximal incorporation being 0.3 mM, 2 mM and 16 mM, respectively. This type of graded response was identical to that previously observed for other cell-free incorporation systems [8, 11, 37, 38] although the specific optimal concentrations for stimulation tend to be distinctive for any particular system.

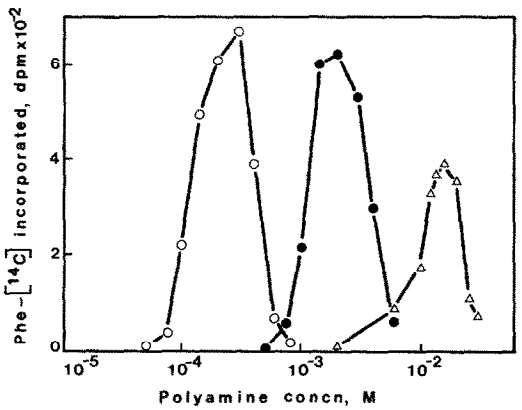

Fig. 2. Effect of polyamine concentration on Phe-[^{14}C] incorporation at sub-optimal Mg^{2+}. (O——O) spermine, at 4 mM Mg^{2+}; (●——●) spermidine, at 4 mM Mg^{2+}; (Δ——Δ) putrescine, at 5 mM Mg^{2+}. All other components of the reaction mixtures were as described in the Experimental.

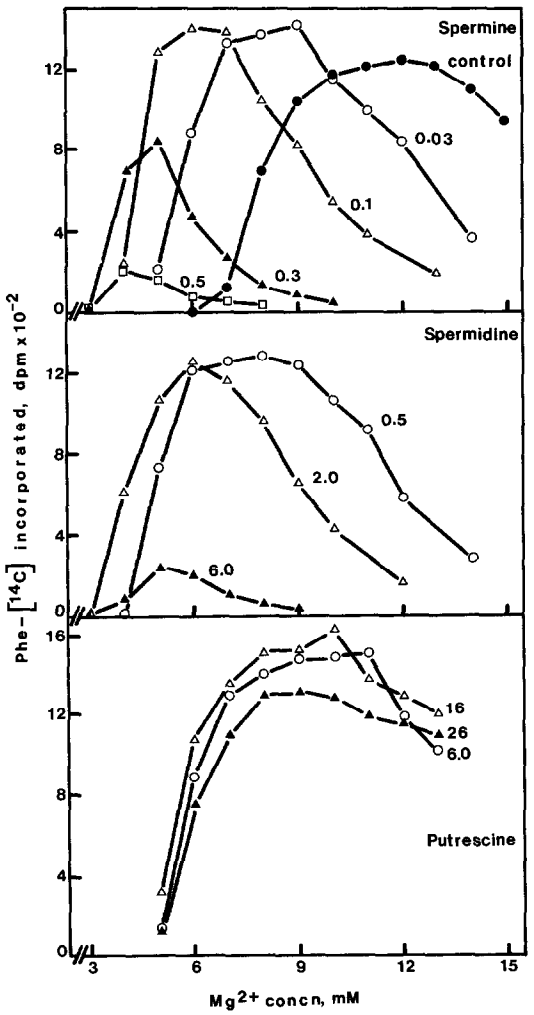

Fig. 3. Effects of polyamines on the optimal concentration of Mg^{2+} in Phe-[^{14}C] incorporation. Control curve is for Mg^{2+} alone; numerical values adjacent to other curves indicate the concentrations (mM) of the respective polyamines in reaction mixtures. All other components of the reaction mixtures were as described in the Experimental.

Using the polyamine optima curves as a guide, the effectiveness of various levels of the polyamines over a range of Mg^{2+} concentrations was tested and the results are shown in Fig. 3. When no polyamine was added to the reaction mixture (control), the optimal concentration of Mg^{2+} for Phe-[^{14}C] incorporation was 12 mM. However, when 0.03 mM or 0.1 mM spermine was added, the optimal concentration of Mg^{2+} was shifted to a lower value, namely 9 mM or 6 mM, respectively; moreover, quantitively there was a slight stimulation of activity at the optimum under these circumstances. With the addition of 0.3 mM or 0.5 mM spermine to the reaction mixture the Mg^{2+} optimum was shifted to 5 mM and 4 mM respectively, but under each of these conditions the level of activity at the optimum was below that when spermine was absent. The results obtained with the various spermine levels tested indicated that as the concentration of spermine was increased within its competent range the degree of shifting of the optimal concentration of Mg^{2+} increased, but the relative increments of shifting became less at the higher spermine levels and there was also reduced incorporation at these levels. When spermidine or putrescine were added to the reaction mixture, results analogous to those for spermine were observed although the effective concentration ranges for each of the polyamines differed. Putrescine was the only polyamine which was required at higher concentrations than the amount of Mg^{2+} it replaced in shifting the Mg^{2+} optimum to lower values. However, in the presence of this polyamine the extent of stimulation of incorporation over the control level was greatest, with 30% stimulation at 16 mM putrescine.

The experimental data shown in Fig. 3 demonstrate what can be considered a sparing effect by polyamines on the Mg^{2+} requirement for polyphenylalanine synthesis, in that comparable levels of synthesis could be achieved at lower Mg^{2+} concentrations in the presence of certain levels of polyamine. This sparing effect in shifting the entire Mg^{2+} optimum curve to lower ranges of concentration in the presence of polyamines has also been reported for other cell-free incorporation systems [8, 11–13, 37]. Additionally, enhancement of incorporation in other such systems ranging from slight stimulation [7–9, 11], as in the present system, to up to twofold stimulation [13] has been observed; in fact, absolute requirements for the presence of polyamines for any

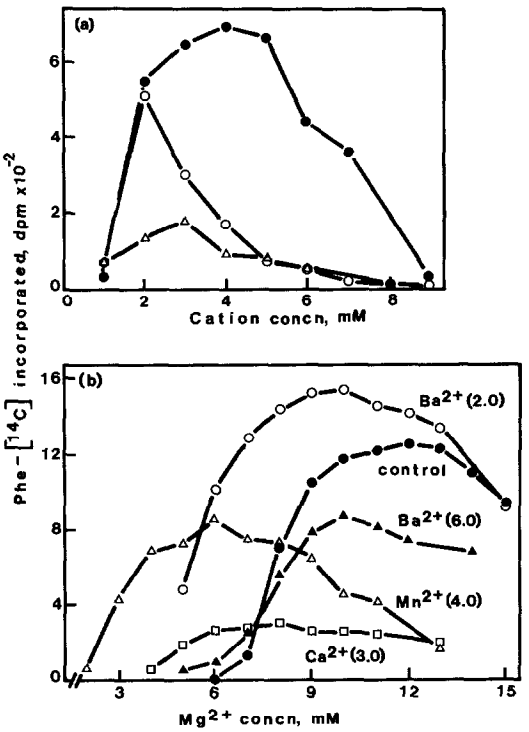

Fig. 4(a). Effect of inorganic cation concentration on Phe-[^{14}C] incorporation at sub-optimal Mg^{2+} (5 mM). (●——●) Mn^{2+} as $MnCl_2$; (○——○) Ba^{2+} as $BaCl_2$; (△——△) Ca^{2+} as $CaCl_2$. All other components of the reaction mixture were as described in the Experimental. (b) Effect of inorganic cations on the optimal concentration of Mg^{2+} in Phe-[^{14}C] incorporation. Control curve is for Mg^{2+} alone; numerical values adjacent to other curves indicate the concentrations (mM) of the inorganic cations specified. All other components of the reaction mixture were as described in the Experimental.

significant levels of incorporation have also been detected [37, 39]. It should be noted from Fig. 3 that even at effective polyamine levels, no activity was evident at concentrations of Mg^{2+} below 3 mM indicating a stringent requirement for the presence of at least some Mg^{2+} in order for the incorporation system to function.

Figure 4 shows the effect of addition of the inorganic divalent cations Mn^{2+}, Ba^{2+} and Ca^{2+} to the incorporation system in experiments paralleling those performed with the polyamines. Firstly, the effects at a fixed suboptimal Mg^{2+} level (5 mM) were observed [Fig. 4(a)]. As had been the case for the polyamines, there was a definite stimulation of activity by all 3 cations with the specific optima for maximal incorporation being 4 mM for Mn^{2+}, 3 mM for Ca^{2+} and 2 mM for Ba^{2+}. Looking at the relative effectiveness of various levels of the inorganic cations over a range of Mg^{2+} concentrations [Fig. 4(b)] there was evidence of a sparing effect by the cations on the Mg^{2+} requirement for polyphenylalanine synthesis. At 2 mM Ba^{2+} the Mg^{2+} optimum was shifted an identical 2 mM to 10 mM and there was a slight stimulation of incorporation (24%) as compared to the control; increasing the Ba^{2+} level to 6 mM did not lead to a further shifting of the Mg^{2+} optimum but it resulted in lower activity over the range of Mg^{2+} concentrations. Mn^{2+} at 4 mM and Ca^{2+} at 3 mM did replace slightly more than equally comparable levels of Mg^{2+} in shifting the Mg^{2+} optimum to 6 mM and 8 mM, respectively; however, in both cases incorporation was reduced in comparison to the control. These results were analogous to an *E. coli* incorporation system [13] where polyphenylalanine synthesis with 6 mM Ca^{2+} plus 7 mM Mg^{2+} was *ca* 30% higher than with 13 mM Mg^{2+} alone, but synthesis in the presence of Mn^{2+} was reduced, even though there was a slightly greater sparing effect on the Mg^{2+} requirement.

Table 2 provides a synopsis of the relative maximal efficiencies of the various cations tested in replacing Mg^{2+} in polyphenylalanine synthesis. It is apparent that 0.03 mM spermine was 100 times more effective on a molar basis than the amount of Mg^{2+} it replaced while giving a slightly higher level of incorporation of Phe-[^{14}C]. In contrast, putrescine was only one-eighth as effective as Mg^{2+} on a similar basis, although a somewhat greater stimulation of incorporation was apparent. Therefore, in this sense of comparison, spermine was up to 800 times more efficient than putrescine in the incorporation system. The inorganic cations tested did not demostrate as wide a disparity in their replacement capabilities but larger differences in incorporation were evident.

DISCUSSION

The observation in this study of the successful replacement by polyamines and inorganic cations of the Mg^{2+} requirement for polyphenylalanine synthesis suggests a certain lack of specificity for the cationic environment in the barley ribosome cell-free system. Moreover, spermine and spermidine were shown to be significantly more efficient than the Mg^{2+} replaced, in that much lower concentrations of these polyamines afforded essentially comparable levels of incorporation. Questions that stem from these observations are: what may be the site and mechanism of action of the polyamines in polypeptide synthesis and what might be the significance of their ability to replace Mg^{2+}? In order to consider these questions it is necessary to take into account the numerous investigations concerned with individual steps involved in the overall process of protein synthesis.

Early studies on isolated ribosomes attributed a critical role to Mg^{2+} in maintaining ribosome structure and in regulating the interactions between ribosomal subunits [15, 16]. Since then, numerous studies have sought to define the possible role played by other cations in the structural and functional integrity of ribosomes. Pea seedling ribosomal subunits, reassociated in the presence of Co^{2+}, were almost as active in amino acid incorporation as Mg^{2+} reassociated subunits, in contrast to Ca^{2+} or Mn^{2+} reassociated subunits which were 50% as active and totally inactive, respectively [18]. In binding studies, Ca^{2+} and Mn^{2+} were found to be freely interchangeable with Mg^{2+} bound to ribosomes [40, 41], although a more detailed investigation revealed that only up to 80% of *E. coli* ribosomal subunit binding sites could be occupied by inorganic cations other than Mg^{2+} without impairment of the phenylalanine polymerizing activity and irreversible structural alterations [42]. Analogous studies with polyamines have likewise indicated that the critical binding requirement for Mg^{2+} could not be satisfied by any concentration of spermidine or putrescine [43, 44]. Although the experimental conditions were not strictly comparable, results from the present investigation also indicated a stringent Mg^{2+} requirement in the presence of polyamines. The hypothesis has been presented that the inability of polyamines to completely displace ribosome-bound

Table 2. Efficiency of the various cations in replacing Mg^{2+} in Phe-[^{14}C] incorporation

Cation	Mg^{2+} replaced in shifting control Mg^{2+} optimum (mM) [a]	Cation level required to replace Mg^{2+} at optimum (mM) [b]	Ratio of Mg^{2+} replaced to cation required [a]/[b]	Incorporation at optimum (% of control)
Spermine	3	0.03	100	114
Spermidine	4	0.5	8	103
Putrescine	2	16	0.125	130
Mg^{2+}	- -	—	1	100
Mn^{2+}	6	4	1.5	68
Ca^{2+}	4	3	1.33	25
Ba^{2+}	2	2	1	124

Mg^{2+} without detrimental effect may be related to the fact that their larger molecular size or charge separation might preclude successful interaction with certain critical portions of the ribosomal RNA [45]. Apart from the direct effects on ribosomal integrity, it has been reported that the Mg^{2+} requirement for the binding of poly(U) to ribosomes, a critical step in polyphenylalanine synthesis, could be completely satisfied by other inorganic cations at equivalent concentration or by polyamines at lower concentrations [46, 47]; also, the binding of aminoacyl-*t*RNA to ribosomes could be stimulated by polyamines in association with Mg^{2+} [13, 48].

Perhaps the major emphasis in recent years on the mode of action of polyamines in polypeptide synthesis has been placed on their ability to replace Mg^{2+} in the aminoacylation reaction [49–51]. This capability has been primarily attributed to the stabilization of *t*RNA conformation rather than to the activation of aminoacyl-*t*RNA synthetases [52–54], although some evidence exists for the latter mechanism [55]. An intriguing aspect of polyamine involvement in aminoacylation *in vitro* has been the discovery of a differential ability to replace Mg^{2+} depending on the specific amino acids involved [56, 57]. This behaviour has intimated that an important role might be played by polyamines as intracellular regulators of aminoacyl–*t*RNA formation in *in vivo* protein synthesis. Certainly the multiplicity of both *t*RNAs and aminoacyl–tRNA synthetases in cells has led to the implicit understanding that cell and tissue differentiation may be regulated by translational control of protein synthesis through the aminoacylation reaction [58–60], and a correspondingly important role could be visualized for polyamines in this regulation,

Nonetheless, one issue which remains to be elucidated is that if magnesium, which is present in all living cells, can adequately fulfil the cationic requirement in aminoacylation or in the various other steps of protein synthesis, why invoke alternative interactions involving other cations? One might speculate as have others [3] that the evolution of organisms may have embodied viable alternatives of differing specificity to deal with environmental irregularities. Moreover, the opportunity for self-regulation of the intracellular cationic environment by polyamine turnover could afford an added focus for the control of growth and development.

EXPERIMENTAL

Biological material. Excised embryo-ends of unimbibed barley seeds (*Hordeum vulgare* cv Gateway) were used as a source of ribosomes and supernatant factors; the portion of the seed utilized comprised *ca* 30% of its total wt.

Buffers and reagents. All buffer solns were made up in sterile deionized-H_2O. Buffer A: 50 mM Tricine [*N*-Tris-(hydroxymethyl)glycine], pH 7.8 (20°); 5 mM $MgCl_2$; 40 mM KCl; 5 mM 2-mercaptoethanol. Buffer B: 10 mM Tricine, pH 7.8 (20°); 5 mM $MgCl_2$; 5 mM 2-mercaptoethanol. Scintillation fluid contained 0.4% PPO and 0.01% POPOP in scintillation-grade toluene.

Preparation of ribosomes. Immediately prior to the isolation procedure, 20 g of seed material as specified above was ground in a Wiley mill fitted with a 20-mesh sieve. Henceforth, all operations and centrifugations were at 4°. The granular powder was added to 80 ml buffer A and this prepn was then homogenized for 20 sec at $\frac{1}{2}$ max. speed with a Polytron homogenizer. The resulting homogenate was filtered through a vegetable juicer lined with a single layer of Miracloth. Then 20% Triton X-100 in buffer A was added to the filtrate at a concn of 2 ml per 10 ml filtrate, followed by stirring for 10 min. This mixture was centrifuged at 9000 *g* (r_{av} 8 cm) for 15 min and the resulting supernatant was filtered through one thickness of Miracloth in order to eliminate the lipid layer at the surface: a further centrifugation at 36000 *g* for 40 min was performed, also followed by the Miracloth filtration step. The final supernatant was layered over 10 ml of M RNase-free sucrose (Schwartz/Mann, Orangeburg, New York) in buffer B and centrifuged at 214000 *g* (r_{av} 6.3 cm) for 165 min to pellet the ribosomes. Ribosomal pellets were gently resuspended in 20% (w/v) glycerol in Buffer B using a cotton-tipped swab, and then centrifuged at 26000 *g* (r_{av} 8 cm) for 15 min to remove aggregated material. After determination of the ribosomal concn by measurement of *A* at 260 nm, aliquots at a concn of 25 A_{260} units per ml were placed in glass ampoules which were subsequently sealed and stored in liquid N_2. Sucrose gradient analysis of such ribosomal prepns showed the presence of only 80S monomeric ribosomes.

Preparation of post-ribosomal supernatant factors. The initial steps of the procedure were identical to those described for the preparation of ribosomes with the following modifications: the extraction buffer (Buffer A) contained 0.5 M sucrose, the Triton X-100 step was omitted and the M sucrose pad was not used in the 214000 *g* centrifugation. After the high-speed centrifugation, the upper two-thirds of the supernatant was carefully recovered and aliquots were then dialysed for 18 hr at 4° against 500 vols of Buffer A, followed by a further dialysis against a fresh 500 vols for 3 hr. The retentates were then combined and centrifuged at 26000 *g* for 15 min to eliminate a ppt. which had appeared during dialysis. Protein estimation was performed on the resulting supernatant [61] using BSA fraction V as a standard; aliquots were then sealed in ampoules and stored in liquid N_2 at a concn of 5.4 mg/ml.

Assay system for polyphenylalanine synthesis. All components (excluding ribosomes and dialysed high-speed supernatant, described previously) were made up individually as solns in sterile deionized-H_2O and stored frozen at −20° in sealed vials until required for expts. Preparation of reaction mixtures and incubations were performed in a positive-pressure filtered ventilation hood. Components in the standard reaction mixture were at the following concns in a final vol. of 0.5 ml: 40 mM Tris–HCl, pH 8.4 (20°); 12 mM $MgCl_2$; 70 mM KCl; 9 mM DTT; 0.5 mM GTP; 1 mM ATP; 8 mM creatine phosphate; 20 μg/ml creatine phosphokinase; 0.7 μCi/ml L-phenylalanine-[U-^{14}C] (sp. act. 486 mCi/mmol); 240 μg/ml poly (U); 60 μg/ml brewer's yeast $tRNA^{Phe}$ (Sigma Chemical Co., St. Louis, Missouri); 0.2 A_{260} units/ml ribosomes; 600 μg/ml dialysed post-ribosomal supernatant protein. In expts where polyamines were used, these were added as neutralized solns of their respective HCl salts. After incubation for 1 hr at 30°, 10 μl aliquots were collected on Whatman 3 MM filter paper discs (21 mm diam) and hot TCA-insoluble radioactivity was measured [62, 63]. Data in figures and tables represent the average of at least two independent expts with 3 sample replicates per expt.

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