Amino Acid Incorporation into Protein by Cell-free Extracts of Yeast*

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The characteristics of amino acid incorporation into protein by cell-free extracts from yeast are described. Active extracts are obtained only from unfrozen, exponentially growing cells which are disrupted by sand grinding. Protein synthesis is dependent upon ribosomes, 105,000 × *g* supernatant fraction, nucleoside triphosphates, an energy source, magnesium, and spermidine. Puromycin and ribonuclease inhibit amino acid incorporation, while chloramphenicol and deoxyribonuclease have no effect. Polyuridylic acid stimulates the incorporation of C^{14}-L-phenylalanine and also the transfer of C^{14}-L-phenylalanine from transfer ribonucleic acid into protein. Maximal polyuridylic acid stimulation is dependent upon a critical concentration of yeast-soluble ribonucleic acid.

The *in vitro* incorporation of amino acids into yeast ribosomes was first reported by Webster (1957). However, only within the last year have reports appeared in the literature of active amino acid incorporation by yeast extracts and characterization of the components required. Cooper *et al.* (1962) described a system in which the major incorporating activity resides in the 20,000 × *g* sediment. In another instance, subcellular fractions were obtained from lysed protoplasts (Barnett *et al.*, 1962). More recently, So and Davie (1963) successfully prepared a protein synthesizing system from *Saccharomyces fragilis*.

This report details the preparation and characteristics of an active cell-free amino acid incorporating system obtained from a hybrid yeast. The system requires ribosomes, supernatant fraction, energy yielding system, GTP, a critical concentration of magnesium, and polyamine for activity. Furthermore, the system responds to synthetic polynucleotides and is thus comparable to systems described in bacteria (Nirenberg and Matthaei, 1961; Lengyel *et al.*, 1961) and mammalian cells (Weinstein and Schechter, 1961; Maxwell, 1962). Observations on the stability of the system are presented which may explain some of the difficulties previously encountered in obtaining active extracts.

Materials and Methods

Preparation of Extracts.—A diploid yeast (*Saccharomyces fragilis Saccharomyces dobzanskii*) was used in these experiments. The cultures were grown in a medium consisting of the following components in grams per 100 ml: 0.2 KH_2PO_4; 0.8 $(NH_4)_2SO_4$; 0.05 yeast extract; 0.00163 $MgCl_2$; 2.0 glucose; and trace elements. The cultures were grown at 30° in shaken Erlenmeyer flasks and harvested during exponential growth. Cells were washed twice with cold buffer (0.05 M Tris, *p*H 7.6, 0.005 M magnesium acetate, 0.01 M potassium chloride, 0.01 M mercaptoethanol, and 0.0005 M spermidine) and then disrupted by grinding with twice their weight of acid-washed sand for 5 minutes in a mortar placed in a salt-ice bath at −5°. The mixture was extracted with 2–3 volumes of buffer for 1–2 minutes then centrifuged at 10,000 × *g* for 10 minutes to remove the sand and large debris. The supernatant fluid was further centrifuged twice at 20,000 × *g* for 20 and 30 minutes, respectively. Ribosomes were then sedimented by centrifugation at 105,000 × *g* for 1 hour in the Spinco Model L centrifuge. In some cases, the supernatant fluid was passed through a Sephadex G-25 column equilibrated with buffer to remove low molecular weight components before use. The ribosomal pellet was suspended in buffer and used directly or washed by resedimenting at 105,000 × *g* for 1 hour.

Composition of the Reaction Mixture.—The complete reaction mixture contained the following components in 0.4 ml (in μmoles unless otherwise specified): 24.0 Tris-HCl, *p*H 7.6; 1.40 magnesium acetate; 2.8 KCl; 0.06 each of GTP, UTP, and CTP; 0.20 ATP; 2.50 PEP;[1] 0.24 spermidine; 0.8 mercaptoethanol; 10.0 μg pyruvate kinase; 0.005 C^{14}-L-phenylalanine (50,000 cpm); 0.3–0.6 mg ribosomal protein; and 0.1–0.5 mg supernatant protein. After incubation for 45 minutes at 25°, 0.4 ml of 10% TCA containing 4 mg/ml C^{12}-DL-phenylalanine was added to stop the reaction. The mixture was then heated at 90° for 15 minutes. The precipitates were washed three times with 2 ml of 5% TCA containing 2 mg/ml C^{12}-DL-phenylalanine, and once with 3 ml of ethanol-ether (3:1, v/v). The precipitates were dried, dissolved in concentrated formic acid, and plated on stainless steel planchets for drying and counting. For "preincubation" experiments, reaction mixtures lacking C^{14}-L-phenylalanine, but containing all components at the same final concentration stated above in a volume of 0.2 ml, were incubated for 30 minutes at 25°. Then C^{14}-L-phenylalanine and the other components were added to the same final concentration in 0.4 ml and reincubated for 45 minutes at 25°. Most experiments were carried out in duplicate.

Yeast-sRNA was prepared as described under "preparation of the low molecular weight RNA" by Monier *et al.* (1960) through the precipitation step with potassium acetate and ethanol. This RNA was readily soluble in 1 M NaCl at 0°. Amino acids were deesterified from the sRNA and replaced with C^{14}-L-phenylalanine and the other nineteen common C^{12}-L-amino acids as described by von Ehrenstein and Lipmann (1961). The RNA was precipitated directly from the reaction mixture by addition of 0.1 volume of 20% potassium acetate and 2 volumes of 95% ethanol. The precipitate which formed after standing at −20° for 4 hours was dissolved in water and dialyzed exhaustively against water at 4°. The RNA was repre-

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[1] Abbreviations used in this paper: PEP, phosphoenolpyruvate; sRNA, soluble ribonucleic acid; poly-U, -A, -C, polyuridylic acid, polyadenylic acid, polycytidylic acid, respectively; TCA, trichloroacetic acid.

TABLE I

INCORPORATION OF C^{14}-L-PHENYLALANINE INTO PROTEIN AS INFLUENCED BY THE METHOD OF CELL DISRUPTION

The complete reaction mixture is described under Materials and Methods. After one passage through a French pressure cell at 8000 psi, the extract was centrifuged in the same manner as from the sand-ground cells. The supernatants were passed through a Sephadex G-25 column before use.

Experiment	French Pressure Cell		Sand Grinding		C^{14}-L-Phenylalanine Incorporated[a]
	Ribosomes	Supernatant	Ribosomes	Supernatant	
1	+	+			5.4
2			+	+	28.5
3	+			+	6.7
4		+	+		27.8
5	+	+		+	6.7
6		+	+	+	30.3
7	+	+	+		7.7
8	+		+	+	7.6

[a] Values are $\mu\mu$moles/mg ribosomal protein.

cipitated with ethanol and potassium acetate, washed with 67% ethanol and 100% ethanol successively, and dried *in vacuo*. The specific activity of the C^{14}-L-phenylalanyl-sRNA was 2400 cpm/mg (0.22 mμmole C^{14}-L-phenylalanine/mg sRNA).

Uniformly labeled C^{14}-L-phenylalanine, -valine, and -leucine were obtained from New England Nuclear Corporation and diluted to 9.8 mc/mmole before use. The ATP was purchased from the Sigma Chemical Company and CTP, GTP, and UTP from Pabst Laboratories. Pyruvate kinase and PEP were obtained from the California Corporation for Biochemical Research. Miles Chemical Company supplied the poly-U, -A, and -C. The poly-U had an average sedimentation coefficient of 8. Chloramphenicol was a gift from Parke, Davis and Company. Puromycin was purchased from Nutritional Biochemical Corporation. Crystalline ribonuclease and deoxyribonuclease were obtained from Worthington Biochemical Company.

Protein was determined by the method of Lowry *et al* (1951). All RNA concentrations were calculated by assuming 1 mg/ml in H_2O has an optical density at 260 mμ of 24.

RESULTS

Effect of Methods of Cell Disruption.—The method of cell disruption for the preparation of active cell extracts was critical. Table I compares C^{14}-L-phenylalanine incorporation into protein by fractions prepared from cells disrupted by two different methods. Incorporation by components obtained from a single passage of cells through a pressure cell was 5-fold lower than into extracts derived from grinding cells with sand (expts. 1 and 2). Experiments 3 and 4 show that the ribosomal fraction from pressure-cell disruption is defective; the supernatant fraction from the pressure cell is as effective with ribosomes from the sand-ground cells as is the supernatant from sand-ground cells. These data as well as those of experiments 5 and 6 eliminate the possibility of an inhibitor in the supernatant fraction obtained from pressure cell disruption. Ribosomes from pressure cell derived extracts effect a 70% inhibition of the system containing ribosomes from sand-ground cells and supernatant fraction from either mode of disruption. The nature of the ribosomal defect remains unclear; the sedimentation characteristics of both ribosomal preparations in the analytical ultracentrifuge and on sucrose density gradients are identical.

TABLE II

CHARACTERISTICS OF C^{14}-L-PHENYLALANINE INCORPORATION INTO PROTEIN

The complete reaction is described under Materials and Methods.

Experiment	System	C^{14}-L-Phenylalanine Incorporated[a]
1	Complete	90.0
	−UTP, CTP, GTP, ATP, PEP, pyruvate kinase	4.1
	−ATP, PEP, pyruvate kinase	6.0
	−Spermidine	62.8
2	Complete	53.0
	−CTP, UTP, GTP	28.1
	−CTP, UTP	45.8
3	Complete	53.6
	−Ribosomes	0.0
	−Supernatant	9.7
	+ 5μg Puromycin	11.4
	+ 5 μg Chloramphenicol[b]	50.7
	+ 5 μg Deoxyribonuclease[c]	49.0
	+ 5μg Ribonuclease	2.8

[a] Values are $\mu\mu$moles/mg ribosomal protein. [b] Chloramphenicol had no effect at concentrations up to 125 μg/ml. [c] Deoxyribonuclease had no effect at concentrations up to 125 μg/ml.

Requirements for Amino Acid Incorporation.—Incorporation into hot TCA precipitable protein showed a linear dependence on ribosomes and a partial dependence on the supernatant fraction. The dependence of incorporation upon the various components of the system is presented in Table II. Omission of nucleoside triphosphates and the ATP generating system resulted in the loss of 95% of the activity. In the absence of ATP and the ATP generating system, incorporation was decreased by 90%. Omission of CTP, UTP, and GTP reduced incorporation by 50%, which could be largely replaced by GTP alone. UTP and CTP gave a small but significant stimulation of incorporation and spermidine increased incorporation by over 30%. The effect of spermidine and other polyamines on poly-U stimulated incorporation are discussed below.

Puromycin and ribonuclease inhibited incorporation 80 and 95%, respectively, at a concentration of 12.5 μg/ml. Chloramphenicol and deoxyribonuclease had no effect when used at concentrations up to 125 μg/ml.

The optimum magnesium concentration in the presence of 5×10^{-4} M spermidine was 3.5×10^{-3} M, and in its absence, 6×10^{-3} M (Fig. 1). Spermidine is functioning in some manner other than substituting for magnesium since even at higher magnesium concentrations spermidine stimulated incorporation.

There was no significant change in incorporation upon varying either the potassium ion concentration from 2×10^{-3} M to 6×10^{-2} M, or the *p*H of the incubation mixture from 6.8 to 7.8.

Stimulation of C^{14}-L-phenylalanine incorporation into protein by addition of the nineteen complementary L-amino acids could not be demonstrated in reaction mixtures containing untreated supernatant and unwashed ribosomes. Therefore, endogenous amino acids were removed by passing the supernatant through a Sephadex G-25 column or by dialysis against buffer, and resedimenting the ribosomes in buffer. In either case the addition of 0.002–0.05 μmole of each of the

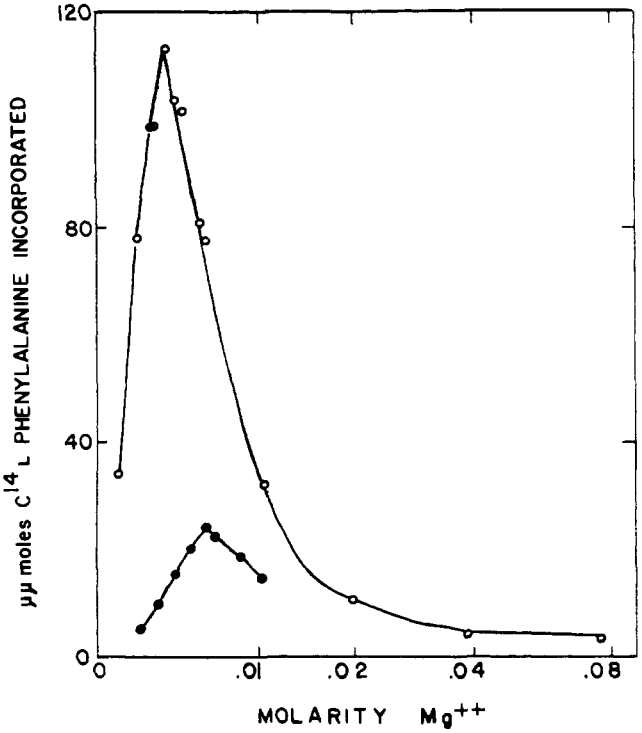

FIG. 1.—The effect of magnesium concentration on the incorporation of C^{14}-L-phenylalanine into protein. The reaction mixture is described under Materials and Methods. Extracts were prepared and incubations were carried out in the presence (O—O) or absence (●—●) of spermidine. Incorporation is given in $\mu\mu$moles/mg ribosomal protein.

nineteen complementary L-amino acids did not stimulate incorporation of C^{14}-L-phenylalanine; at the higher levels (0.05 μmole), varying degrees of inhibition were observed. Other amino acids are incorporated into protein as shown in Table III. The incorporation of C^{14}-L-valine and C^{14}-L-leucine is comparable to that of C^{14}-L-phenylalanine and is dependent on ribosomes, supernatant, and ATP. The failure of the nineteen C^{12}-amino acids to stimulate incorporation may therefore be a problem of rigorous removal of endogenous amino acids from the extracts. Another possible explanation is that amino acids are generated by proteolytic enzymes.

Participation of sRNA.—Previous *in vivo* experiments on the kinetics of $S^{35}O_4^=$ incorporation into yeast revealed that the hot TCA insoluble fraction was labeled at a rate faster than was the hot TCA soluble fraction

TABLE III

CHARACTERISTICS OF C^{14}-L-PHENYLALANINE, C^{14}-L-VALINE, AND C^{14}-L-LEUCINE INCORPORATION INTO PROTEIN

The incubation mixtures were the same as described under Materials and Methods except that C^{14}-L-valine and C^{14}-L-leucine of the same concentration and specific activity as the C^{14}-L-phenylalanine were substituted. The hot TCA precipitates were washed with 5% TCA containing 5 mg/ml casein hydrolyzate. Approximately 0.6 mg ribosomal protein was used.

System	C^{14}-L-Amino Acid Used[a]		
	Phenylalanine	Valine	Leucine
Complete	17.3	9.3	17.5
-Supernatant	5.0	5.9	5.5
-Ribosomes	0.2	0.5	0.2
-ATP, PEP, pyruvate kinase	1.2	0.5	0.2

[a] Values are total $\mu\mu$moles incorporated.

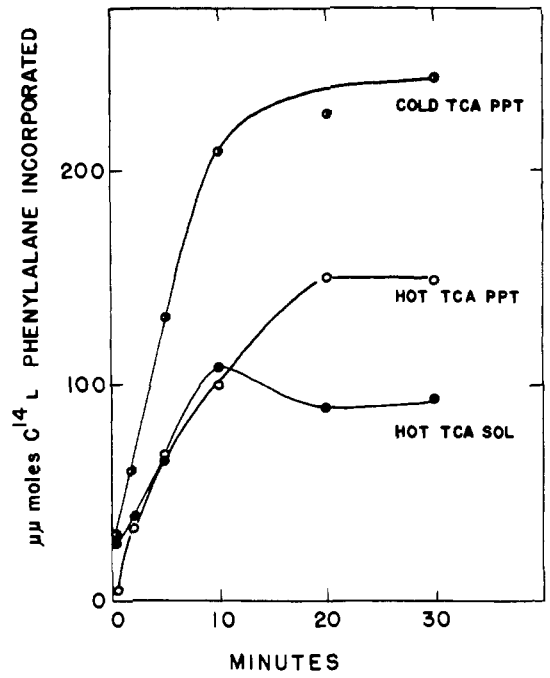

FIG. 2.—The time course of incorporation of C^{14}-L-amino acids into hot and cold TCA precipitable material. The reaction mixture contained thirty times the amount of each component as described under Materials and Methods as well as 0.0125 mmole each of C^{14}-L-leucine, -L-valine and -L-phenylalanine (50,000 cpm each). Duplicate aliquots of 0.8 ml were withdrawn at the times indicated and ice-cold 10% TCA added. One sample of each time interval was heated at 90° for 15 minutes. All samples were washed as described under Materials and Methods with ice-cold 5% TCA. ◑—◑ Cold TCA precipitable counts; O—O hot TCA precipitable counts; ●—● hot TCA soluble counts (difference between cold and hot TCA precipitable counts).

(amino acyl-sRNA) (Young *et al.*, 1961). The kinetics of *in vitro* incorporation of C^{14}-L-valine, C^{14}-L-leucine, and C^{14}-L-phenylalanine into hot and cold TCA precipitable fractions is shown in Figure 2. Incorporation into cold TCA precipitable material proceeded at twice the rate and reached a final level 60% higher than into hot TCA precipitable material. The rate of incorporation into hot TCA soluble components (difference between the curves of cold and hot TCA precipitable fractions, representing amino acyl-sRNA) is equal to or greater than that into protein. Experiments in which the 105,000 × *g* supernatant fraction alone was incubated in the incorporation system revealed that almost all the difference in final incorporation observed in Figure 2 was due to cold TCA precipitable–hot TCA soluble components of the supernatant.

TABLE IV

TRANSFER OF C^{14}-L-PHENYLALANINE FROM C^{14}-L-PHENYLALANYL-sRNA TO RIBOSOMES

The complete reaction mixture was preincubated as described under Materials and Methods, followed by the addition of 0.3 mg C^{14}-L-phenylalanyl-sRNA (0.066 mμmole phenylalanine).

System	C^{14}-L-Phenylalanine Incorporated[a]
Complete	4.4
+100 μg poly-U	18.4
+200 μg poly-U	20.0
+100 μg poly-U + 5 μmole C^{12}-L-phenylalanine	17.6

[a] Values are $\mu\mu$moles/mg ribosomal protein.

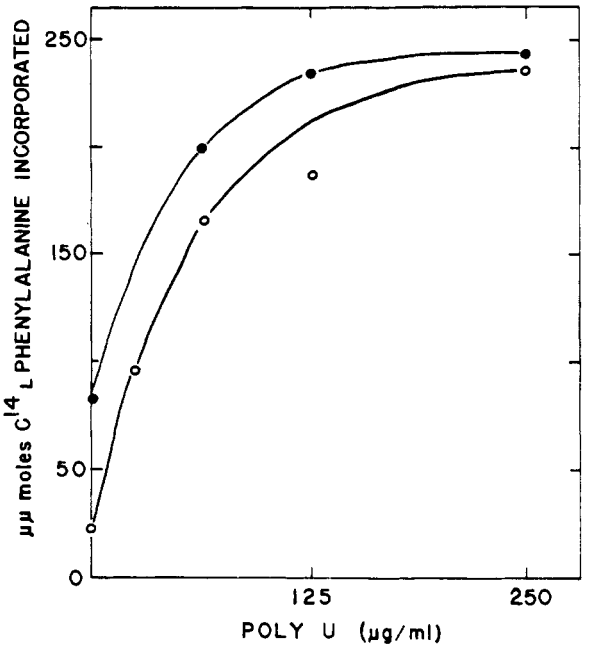

FIG. 3.—Stimulation of C^{14}-L-phenylalanine incorporation into protein by poly-U. The reaction mixtures are described under Materials and Methods. ●—● Not preincubated; O—O preincubated before addition of poly-U and C^{14}-L-phenylalanine. Incorporation is given in μμmoles/mg ribosomal protein.

More direct evidence that transfer RNA functions in this system is that C^{14}-L-phenylalanine is transferred directly from sRNA to protein (Table IV). Addition of poly-U to the yeast system stimulated this transfer as expected if C^{14}-L-phenylalanyl-sRNA were an intermediate in the poly-U directed synthesis of polyphenylalanine. The presence of unlabeled phenylalanine did not lower the incorporation of C^{14}-L-phenylalanine by dilution, thus eliminating the possibility of hydrolysis of the C^{14}-L-phenylalanyl-sRNA to free amino acid before incorporation into protein. These findings are analogous to those previously described by Nirenberg *et al.* (1962) in the *E. coli* cell-free system. The addition of unlabeled phenylalanine to an incorporating system starting with C^{14}-L-phenylalanine does lower incorporation as expected by dilution of the isotope.

Stimulation by Poly-U.—The addition of poly-U to the complete reaction mixture resulted in stimulation of C^{14}-L-phenylalanine into hot TCA precipitable material. The degree of stimulation was dependent on the pretreatment of the ribosomes. Two typical saturation curves of incorporation versus poly-U concentration are shown in Figure 3. The upper curve representing reaction mixtures which received no treatment prior to poly-U addition shows a 3-fold stimulation at saturating poly-U concentrations (approximately 100 μg/ml). The lower curve represents mixtures which were preincubated prior to poly-U and C^{14}-L-phenylalanine addition. Incorporation in the absence of poly-U was reduced about 4-fold, but the final level of poly-U stimulated incorporation reached the same level as with the nonpreincubated samples, resulting in a 12-fold stimulation. Ribosomes washed by resedimentation in buffer or by sedimentation through a 5–25% linear sucrose density gradient in buffer showed the same phenomenon as did preincubation.

No stimulation of C^{14}-L-phenylalanine incorporation into hot TCA precipitable material was afforded by poly-A or poly-C.

Yeast-sRNA enhanced the poly-U stimulated C^{14}-L-phenylalanine incorporation in a preincubated system. With poly-U at a concentration of 125 μg/ml and 3.5×10^{-3} M magnesium, maximal stimulation was obtained with 450 μg/ml of sRNA. Higher concentrations of sRNA inhibited incorporation (90% inhibition at 2.5 mg/ml). As shown in Table V, the addition of either sRNA or poly-U to the complete reaction mixture increased incorporation 3- to 4-fold. However, the addition of both poly-U and sRNA resulted in a 50-fold stimulation, which is consistent with the involvement of sRNA in the transfer of amino acids to ribosomal protein. In a nonpreincubated system sRNA, had no effect on phenylalanine incorporation.

TABLE V

YEAST-SOLUBLE RNA AND POLY-U STIMULATION OF C^{14}-L-PHENYLALANINE INCORPORATION INTO PROTEIN

The complete reaction mixture was preincubated as described under Materials and Methods before the addition of poly-U, yeast-sRNA, and C^{14}-L-phenylalanine.

System	C^{14}-L-Phenylalanine Incorporated[a]
Complete	8.8
+180μg sRNA	25.5
+ 50μg poly-U	39.0
+180μg sRNA + 50μg poly U	430.0

[a] Values are μμmoles/mg ribosomal protein.

Polyamine Stimulation.—The dependence of active incorporation on polyamines is shown in Table VI. In these experiments, cells were washed, disrupted, and centrifuged in buffer lacking spermidine. Spermidine, spermine, or putrescine was then added to the incubation mixture to a final concentration of 5×10^{-4} M, which is maximal for spermidine in the presence of 3.5×10^{-3} M magnesium. Not only is the basal system (minus poly-U) stimulated, but the incorporation of C^{14}-L-phenylalanine in the presence of poly-U is markedly dependent on the presence of either spermidine or spermine. As the magnesium optimum is 6×10^{-3} M in the absence of polyamine (Fig. 1), it is possible that some of the polyamine stimulation observed in Table VI is due to replenishment of the magnesium deficiency.

Effect of Freezing and Storage.—Attempts were made to utilize ribosome and supernatant fractions after freezing and storage at −20°. Freezing the components either in a −20° freezer or in dry ice–ethanol, followed by thawing 1 hour later, did not alter their activity. However, about 80% of the activity was lost if fractions similarly frozen were stored at −20°

TABLE VI

POLYAMINE AND POLY-U STIMULATION OF C^{14}-L-PHENYLALANINE INCORPORATION INTO PROTEIN

The complete system is the same as described under Materials and Methods and contains 180 μg yeast-sRNA; 50 μg poly-U was added where indicated. Cells were disrupted in buffer lacking spermidine.

System	C^{14}-L-Phenylalanine Incorporated[a]	
	Minus Poly-U	With Poly-U
Complete minus spermidine	4.4	7.8
+0.2 μmole spermidine	23.1	228.0
+0.2 μmole spermine	16.7	210.0
+0.2 μmole putrescine	10.0	11.7

[a] Values are μμmoles/mg ribosomal protein.

TABLE VII

INCORPORATION OF C^{14}-L-PHENYLALANINE INTO PROTEIN BY FROZEN AND FRESH COMPONENTS WITH AND WITHOUT POLY-U

To the complete reaction mixture described under Materials and Methods were added 0.2 mg yeast-sRNA and, where indicated, 50 μg of poly-U. Extracts were frozen in dry ice–ethanol and stored at −20° for 5 days.

	Fresh		Frozen		C^{14}-L-Phenylalanine Incorporated[a]	
Experiment	Ribosomes	Supernatant	Ribosomes	Supernatant	Minus Poly-U	With Poly-U
1	+	+			56.5	347.0
2			+	+	9.2	17.4
3	+			+	45.3	254.0
4		+	+		16.8	36.5

[a] Values are $\mu\mu$moles/mg ribosomal protein.

for 5 days to 3 weeks. Freezing the whole cells resulted in the loss of 75% of the activity of fresh cells.

Phenylalanine incorporation with a nonpreincubated system from unfrozen cells varied between extracts from 30–100 $\mu\mu$moles/mg protein. Similar incorporating activity has been reported by So and Davie (1963) for extracts from frozen cells of *S. fragilis;* however we have been unable to confirm these results with the hybrid yeast.

Recombination of frozen and fresh components (Table VII) revealed that the ribosomal fraction was defective after freezing. Frozen supernatant fluid and fresh ribosomes are nearly fully active whereas fresh supernatant fluid and frozen ribosomes have only 30% the activity of the unfrozen control. In the presence of poly-U a similar phenomenon was observed, and therefore the inactivation observed is not entirely due to messenger RNA degradation.

DISCUSSION

Previous attempts in this laboratory to prepare an active cell-free amino acid incorporating system from yeast have been unsuccessful. These difficulties were attributed to the disruption of cells by methods other than sand grinding and in some instances the cells or extracts were frozen prior to use. Using the above preparative methods, one does obtain extracts which weakly incorporate amino acids (1–2 $\mu\mu$moles C^{14}-amino acid incorporated/mg ribosomal protein) (unpublished data). This system has similar requirements to the one described in this paper. Amino acid incorporation is dependent upon ATP and a critical magnesium ion concentration (4×10^{-3} M). However, no stimulation is obtained with spermidine or poly-U, implying that the system is extensively degraded or that amino acid incorporation into protein can proceed by another mechanism.

The data presented in this paper illustrate that active extracts which incorporate 50–100 $\mu\mu$moles of C^{14}-amino acid/mg ribosomal protein are obtained only from unfrozen, exponentially growing cells which are disrupted by sand grinding. The failure of extracts obtained from pressure cell disruption of yeast to actively incorporate amino acids has been traced to the ribosomal fraction, yet a comparison of the sedimentation characteristics of the ribosomes obtained by the two methods reveals no differences. It is possible that "heavy" ribosomal aggregates which have been implicated to be the active species in protein synthesis in *E. coli* (Risebrough *et al.*, 1962) and rabbit reticulocytes (Warner *et al.*, 1962; Warner *et al.*, 1963) are degraded by pressure cell disruption of yeast cells. Experiments in which yeast ribosomes containing synthetic messenger are fractioned by density gradient centrifugations (Spyrides and Lipmann, 1962; Barondes and Nirenberg, 1962), and characterization of the labeled fractions following pulse labeling *in vivo* with $P^{32}O_4^{=}$, are currently in progress in an attempt to define the ribosomal species that are functional in yeast protein synthesis.

The characteristics of the protein synthesizing system reported here (i.e., the requirement for ribosomes, soluble supernatant, ATP, energy generating system, GTP, and magnesium, the participation of amino acyl-sRNA, and inhibition by ribonuclease and puromycin) are similar to systems previously reported for bacteria and mammalian systems. The slight stimulation by UTP and CTP may be due to repairing of the terminal pCpCpA sequence of transfer RNA. So and Davie (1963) demonstrated that inhibition of amino acid incorporation by transfer RNA was eliminated after reconstitution of the terminal pCpCpA sequence. The lack of chloramphenicol and deoxyribonuclease inhibition (So and Davie, 1963) and the failure to observe stimulation by the nineteen complementary amino acids is in agreement with the results of Barnett *et al.* (1962) and So and Davie (1963) for yeast systems. The stimulation by spermidine has not been previously reported for a yeast incorporating system and appears to be a requirement which is not entirely replaced by magnesium as reported for the *Salmonella typhimurum*–incorporating system by Martin and Ames (1962). These authors concluded that polyamines function by converting nonfunctional 70 S ribosomes to functional 100 S particles.

The incorporation of C^{14}-L-phenylalanine by yeast extracts is stimulated by poly-U but not by poly-A or poly-C, thus extending the biological code for phenylalanine to yet another species. Coding experiments with other synthetic polyribonucleotides are necessary to determine the extent of this analogy. Other studies on the stimulation of amino acid incorporation into protein by natural polyribonucleotides from bacterial, yeast, and viral sources as well as by partially degraded synthetic polyribonucleotides will be reported elsewhere.

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Inhibition of Hemoglobin Synthesis by Puromycin*

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The inhibition of hemoglobin synthesis by puromycin was proportional to inhibitor concentration. However, inhibition at low levels of puromycin increased with time. Preincubation studies showed that a small amount of protein synthesis in the presence of puromycin resulted in almost complete inhibition at 2×10^{-5} M concentration of inhibitor. The inhibitor caused the release of labeled protein from the ribosomes, and this release was related to the degree of inhibition. The released material contained incomplete globin chains, as shown by solubility, chromatographic behavior, and *N*-terminal analysis. About 25% of the released material contained chains which were newly started in the cell-free system. Release of incomplete chains took place at 4° in the absence of added enzyme, although this was a slow reaction. The mechanism of puromycin action suggested was that the inhibitor substitutes for the next incoming amino acid at the growing point of the peptide chain (the carboxyl end). This displaces the chain. Further synthesis can occur, but yields only small peptides which are displaced, and are acid soluble.

The inhibition of protein synthesis by puromycin, first reported by Yarmolinsky and de la Haba (1959), has been confirmed in various systems (Nathans and Lipmann, 1961; Morris and Schweet, 1961; Hultin, 1961). More complex effects of puromycin have been reported by Nemeth and de la Haba (1962), Mueller *et al.* (1961), and Rabinovitz and Fisher (1962). A direct effect of puromycin on ribosomes resulting in the release of soluble protein was found by Morris and Schweet (1961), Hultin (1961), Morris *et al.* (1962), Allen and Zamecnik (1962), and Lamborg (1962). Detailed studies of the mechanism of puromycin action on reticulocyte ribosomes are reported here.

EXPERIMENTAL

Materials.—DL-1-C^{14}-leucine was purchased from the California Corporation for Biochemical Research and had a specific activity of 10.3 $\mu c/\mu$mole. Uniformly labeled L-valine, L-arginine, and L-lysine were obtained from the Nuclear-Chicago Corporation and had specific activities of 6.5, 2.5, and 8.3 $\mu c/\mu$mole, respectively. Puromycin hydrochloride was kindly donated by Dr. E. Stokstad of Lederle Laboratories. Rabbit reticulocytes, enzyme fractions, and other components of the cell-free system have been described (Allen and Schweet, 1962). The data have been calculated for C^{14}-amino acid at a specific activity of 7 $\mu c/\mu$mole, which gave 2.3×10^6 cpm per μmole in the thin-window Geiger counter used.

Preparation of Labeled Ribosomes.—Incubations of intact cells were carried out by a modification of the procedure of Borsook *et al.* (1957). For each 2 ml of washed reticulocytes was added 0.33 ml of rabbit plasma, 1.84 ml. of twice-concentrated NKM[1] salt solution, 0.067 ml. of 1 M Tris buffer, *p*H 7.5, 1.0 ml of amino acid mixture less valine, 0.067 ml. of 0.01 M ferrous ammonium sulfate, 0.15 ml of 1×10^{-3} M C^{14}-L-valine, and 1.5 ml of water. The mixture was incubated 15 minutes at 37° and the cells were then centrifuged, resuspended in cold NKM solution, and recentrifuged. Labeled ribosomes were then isolated in the usual manner, except that unlabeled high-speed supernatant (approximately 4 mg protein/mg ribosomal protein) was added to the ribosome suspension after the first sedimentation of the particles in order to dilute any adsorbed or occluded *labeled* hemoglobin.

For the labeling of ribosomes in the cell-free system, the ribosome pellet after the first centrifugation (Allen and Schweet, 1962) was suspended in 0.25 M sucrose to a final concentration of approximately 14 mg of ribosomes per ml (6–7 mg of ribosomal protein) and incubated 10 minutes at 37° in the usual complete system with C^{14}-leucine or other C^{14}-amino acid and other components of the cell-free system (Allen and Schweet, 1962). The reaction was terminated by the addition of 6–10 volumes of cold medium B (0.25 M sucrose, 0.0175 M $KHCO_3$, 0.002 M $MgCl_2$) containing a 50-fold excess of C^{12}-leucine. The labeled ribosomes were then reisolated by centrifugation.

Studies of Released Components.—The release of labeled material from labeled ribosomes either with or

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[1] Abbreviations used in this work: TCA, trichloroacetic acid; chloramine T, sodium *para*-toluenesulfonchloramine; DIFP, diisopropylfluorophosphate; NKM, 0.13 M NaCl, 0.005 M KCl, 0.0075 M $MgCl_2$.