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AMBIGUITY IN A SUBCELLULAR PROTEIN SYNTHESIZING SYSTEM FROM SACCHAROMYCES CEREVISIAE

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

The fidelity of a cell-free cytoplasmic protein synthesizing system from the yeast, Saccharomyces cerevisiae, was studied. Polyuridylic acid (poly U)-directed incorporation of phenylalanine and leucine was examined in the presence of environmental factors known to induce ambiguity in bacterial systems. Increasing magnesium, spermine, and spermidine concentrations induced extensive leucine-phenylalanine ambiguity in the yeast system; neomycin and ethanol also induced ambiguity, but to a lower degree; streptomycin and temperature did not induce mistranslation. Magnesium and spermine induced the ambiguous response of the same amino acids, namely, leucine, isoleucine, and serine. Fidelity in the aminoacylation and transfer reactions was examined separately. In the presence of increasing magnesium and spermine concentrations, leucine, isoleucine, and serine were not bound to phenylalanine transfer ribonucleic acid ($tRNA^{phe}$). Conversely, these agents induced the transfer of leucine from tRNA into polypeptide in the presence of poly U and ribosomes. The formal similarity of the findings in the yeast system and the observations reported in Escherichia coli is described. The possible implications are discussed in terms of the evolution of ribosome structure and function.

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INTRODUCTION

The molecular basis of the accuracy of protein synthesis is emerging. The evidence indicates it is more involved than the isolated codon-anticodon pairing. Ambiguous translation of the genetic code, i. e., the response of more than one amino acid to a codon or the response of an amino acid to a nonsense codon, has been shown to occur both in in vitro systems and in the cell. This phenomenon is an invaluable experimental tool for the identification and characterization of the parameters required for accuracy in translation.

Although fidelity has been examined in a variety of bacterial and mammalian extracts, most of what is known of fidelity has been derived from the E. coli system. This communication is the first report of the induction of ambiguity in a crude cell-free system obtained from the eukaryote, Saccharomyces cerevisiae. Whereas comparatively low levels of induced ambiguity are obtained with crude mammalian extracts, reproducibly high levels of ambiguity are obtained in the S. cerevisiae system. Thus,this system is valuable for studying the parameters required for fidelity in a higher organism.

In early experiments designed to decipher the genetic code, it was noted that in addition to phenylalanine, the amino acid leucine also responded to poly U (Matthaei, et al., 1962; Speyer, et al., 1962; Gruneberg-Manago and Bretscher, 1962). This observation,

first made in cell-free systems from E. coli, was also made in protein synthesizing systems derived from mammalian cells (Weinstein and Schechter, 1962; Weinstein, 1963) and a unicellular alga (Sager, Weinstein, and Ashkenazi, 1963). Thus, leucine-phenylalanine ambiguity seemed to be a universal feature of cell-free amino acid incorporating systems.

The first insight into the mechanism of ambiguity came from the studies of Gorini and Kataja (1964) who discovered that some bacterial auxotrophs were able to grow in the absence of their specific growth factors, provided that streptomycin was present in the medium. Their suggestion that streptomycin could influence the accuracy of translation of the code was confirmed in in vitro experiments which showed that streptomycin and other aminoglycoside antibiotics could stimulate misreading of messenger RNA (Davies, Gilbert, and Gorini, 1964).

In vitro ambiguity then emerged as a subject of study. A number of investigations revealed that the fidelity of translation in bacterial subcellular systems was highly sensitive to environmental conditions. In addition to aminoglycoside antibiotics, these factors included elevated magnesium concentrations (Szer and Ochoa, 1964; Friedman and Weinstein, 1964), decreased temperature of incubation (Szer and Ochoa, 1964; Friedman and Weinstein, 1964), polyamines (Friedman and

Weinstein, 1964), and ethanol (So and Davie, 1964). Parameters of the protein synthesizing machinery, tRNA concentration (Gruneberg-Manago and Dondon, 1965; Davies, Gorini, and Davis, 1965; So and Davie, 1965), availability of amino acids (So and Davie, 1965; Szer and Ochoa, 1964), and the chain length of poly U (Sager, 1963) were found to influence the level of ambiguity. Furthermore, ambiguity was observed not only in poly U-stimulated systems, but also with other homopolymers (Davies, Gorini, and Davis, 1965), heteropolymers(Davies, Gilbert, and Gorini, 1964; Friedman and Weinstein, 1964), and messengers of specifically ordered sequences (Davies, Jones, and Khorana, 1966). It was also found that mistranslation in poly U-stimulated bacterial systems was not confined solely to the response of leucine, but certain other amino acids were also incorporated (Davies, Gilbert, and Gorini, 1964; Friedman and Weinstein, 1964; Szer and Ochoa, 1964). A specific group of amino acids responded to each different messenger employed (Friedman and Weinstein, 1964; Davies, Gorini, and Davis, 1965; Davies, Jones, and Khorana, 1966).

In the case of streptomycin, the availability of genetically different strains made it possible to show that the ribosome was the locus of streptomycin-induced ambiguity. Mistranslation was obtained with ribosomes from streptomycin-sensitive cells but not from streptomycin-resistant cells (Davies, Gilbert, and Gorini, 1964).

This finding provided the first evidence that a site on the ribosome contributed to specificity of protein synthesis.

In contrast to the marked sensitivity of bacterial systems to environmentally induced ambiguity, little or no ambiguity was observed in crude amino acid incorporating systems obtained from mammalian cells (Weinstein, Ochoa, and Friedman, 1966; Stavy, 1968; Derr and Scanlon, 1969; Bose, et al., 1969).

The study of ambiguity in the yeast system began with an examination of the effects of environmental conditions known to produce ambiguity in <u>in vitro</u> bacterial protein synthesis. These factors were tested in a poly U-directed protein synthesizing system in which the incorporation of leucine and phenylalanine was measured. This system was chosen because most of the data available from bacterial and mammalian cell-free protein synthesis had been obtained in similar systems. The amino acid specificity of the ambiguous response was determined under conditions found to stimulate high levels of leucine incorporation. Fidelity of translation is a result of specificity in both the charging of amino acids onto their cognate tRNA's, and in the transfer of amino acids from their tRNA's into protein on the messenger-ribosome complex. Therefore, the fidelity of each of these reactions was examined separately under conditions which induced ambiguity in protein synthesis.

MATERIALS AND METHODS

Growth of cells. A starter culture of Saccharomyces cerevisiae ATCC 9767 was grown 20-22 hours in 300 ml of broth containing (in grams per liter) Bacto-Tryptone (Difco), 10.0; Bacto-Yeast Extract (Difco), 5.0; NaCl, 5.0; glucose, 2.0; at pH 7.0. Incubation was at 30 C at 300 revolutions/minute on a Gyrotary Shaker (New Brunswick Scientific). Flasks containing 900 ml of the broth described were seeded with 20 ml of the starter culture, and grown under the same conditions until mid-log phase (approximately 7 hours). The flasks were plunged into ice, and the cells harvested by centrifugation, washed once with buffer (0.005 M magnesium acetate, 0.02 M potassium chloride, 0.01 M tris(hydroxymethyl) aminomethane (TRIS)-HCl (pH 7.8), 0.006 M β-mercaptoethanol), and stored at -20 C.

Preparation of subcellular fractions. Frozen cell pellets were ground in a mortar with twice their wet weight of acid-washed glass beads (0.11 mm diameter), assuming 1 ml of beads weighs 1 gm. A rapid grinding motion was essential to obtaining extracts with high levels of activity. Almost 10 minutes was required to bring a 6 gm pellet to liquid consistency. The broken cells were extracted with a volume of Buffer A [0.005 M magnesium acetate, 0.02 M potassium chloride, 0.01 M TRIS-HCl (pH 7.8), 0.33 mM

dithiothreitol (DTT)] equal to 2 times the wet cell weight. After centrifugation at 30,000 x g for 30 minutes, deoxyribonuclease (EC 3.1.4.5) was added at a concentration of 3 µg per ml. After 5 minutes, the extract was again centrifuged at 30,000 x g for 30 minutes. The supernatant fluid was passed through a column of G-25 Sephadex equilibrated with Buffer A. The peak macromolecular fractions, identified by their yellow opalescence, were pooled and designated the S-30 cell fraction. When S-30 fractions at pH 7.0 and pH 6.5 were required, the column was equilibrated with Buffer A at the respective pH's. The pooled fractions at the different pH levels were adjusted to the same A_{260} before use.

When required, the S-30 fraction was centrifuged at 122,000 x g for 165 minutes, and the top two-thirds of the supernatant fluid was reserved as the S-122 fraction. The ribosome pellet was suspended in Buffer A using a teflon pestle, centrifuged at 10,000 x g for 10 minutes, and the soluble portion adjusted to 500 A_{260} units per ml. All cell fractions were divided into small aliquots, shell frozen in dry ice-acetone, and stored at -72 C. Activity of ribosome suspensions was maintained through at least two cycles of thawing and refreezing and for at least three months of storage.

Preparation of aminoacyl tRNA synthetases. In the preparation of synthetases for use in the aminoacylation of purified $tRNA^{phe}$, endogenous tRNA's were removed from the S-122 cell fraction by a modification of the method of Bergmann (1962). The S-122 fraction (average $A_{280}/A_{260} = 0.7$) was applied to diethylaminoethyl (DEAE) cellulose equilibrated with Buffer I (0.02 M K_2HPO_4-KH_2PO_4 (pH 6.9), 0.001 M magnesium acetate, 0.02 M β-mercaptoethanol 10% glycerol v/v), and the column washed with the same buffer. The material eluting with Buffer II (0.25 M K_2HPO_4-KH_2PO_4 (pH 6.9), 0.001 M magnesium acetate, 0.02 M β-mercaptoethanol, 10% glycerol v/v) was collected. The A_{280} peak fractions were pooled and brought to 70% saturation with ammonium sulfate. The precipitate was solubilized in Buffer A, passed through G-25 Sephadex equilibrated with the same buffer, and the peak A_{280} fractions pooled (A_{280}/A_{260}=1.6). Small aliquots were shell frozen in dry ice-acetone and stored at -72 C.

In the preparation of synthetases for the aminoacylation of unfractionated yeast tRNA, a 35 to 80% ammonium sulfate cut of the S-122 fraction was taken. The precipitate was suspended in Buffer A made 10% (v/v) in glycerol, passed through a G-25 Sephadex column equilibrated with the same buffer, and the A_{280} peak fractions pooled. The preparation was shell frozen in

small aliquots in dry ice-acetone, and stored at -72 C.

Preparation of aminoacyl tRNA's. The reaction mixture contained the following components in a volume of 1.0 ml: 0 006 M magnesium acetate, 0.02 M potassium chloride, 0.01 M TRIS-HCl (pH 7.8), 0.33 mM DTT, 0.0015 M adenosine 5'-triphosphate (ATP), 0.01 M phosphoenolpyruvate, 100 µg of phosphoenolpyruvate kinase (EC 2.7.1.40), 0.0002 M cytidine 5'-triphosphate (CTP), 0.0015 mM ^{14}C L-amino acid (specific activities of phenylalanine and leucine were 455 mc/mmole and 312 mc/mmole,respectively), 0.015 mM each of ^{12}C L-amino acids excluding the radioactive amino acid, 1.6 mg of aminoacyl synthetase protein, and 1 mg of yeast tRNA. The mixture was incubated for 40 minutes at 30 C, and the aminoacyl tRNA's were extracted according to the method of Takeda, Suzuka, and Kaji (1968). The final preparation was suspended in 0.01 M potassium acetate buffer (pH 5.6), stored -72 C, and the tRNA content estimated by assuming that 24 A_{260} units = 1 mg tRNA.

Amino acid incorporation in the standard S-30 system. Components of the standard incubation mixture (given in the legend to Fig. 1) were incubated at 30 C for 35 minutes. The concentration of ^{14}C L-amino acids used in different experiments varied from 244 to 1115 pmoles.

(Specific activities ranged from 112 to 513 mc/mmole). S-30 cell fractions contained 0.51 to 1.4 mg of protein and 0.08 to 0.27 mg of RNA per 0.1 ml. Other modifications in the standard system are indicated in the Table and Fig. legends. Reactions were stopped with 0.7 ml of 8% trichloroacetic acid (TCA) and 3% Bacto-Vitamin-free Casamino Acids (Difco) and heated at 85 C for 30 minutes. Precipitates were collected on Whatman #1 paper or Whatman glass fibre paper (GF/A) and washed with TCA and 70% ethanol. Radioactivity was determined on a Nuclear of Chicago low background gas flow counter at an assumed efficiency of 25%.

When indicated, reactions were carried out in the presence and absence of poly U. "Net poly U stimulation" of a given amino acid refers to the incorporation in the presence of poly U minus that in the absence of poly U.

Aminoacylation of unfractionated yeast tRNA. The basic reaction mixture contained the following components in a volume of 0.2 ml: 0.0095 M magnesium acetate, 0.02 M potassium chloride, 0.01 M TRIS-HCl (pH 7.8), 0.33 mM DTT, 0.001 M ATP, 0.01 M phosphoenolpyruvate, 20 µg of phosphoenolpyruvate kinase (EC 2.7.1.40), 0.0002 M CTP, 300 pmoles of ^{14}C L-phenylalanine (specific activity, 325 mc/mmole), 0.95 mg of S-122 protein, and 0.2 mg of yeast tRNA. Spermine and ^{12}C L-amino acids

(300 or 3000 pmoles of each amino acid in a mixture of ^{12}C L-amino acids excluding phenylalanine) were added as indicated. Incubation was at 30 C for 35 minutes, and was stopped with 1 ml of cold 20% TCA and 5% Bacto-Vitamin-free Casamino Acids. The precipitates were harvested on membrane filters and washed with the same TCA-Casamino Acids mixture followed by 70% ethanol. Samples were counted on a Nuclear of Chicago low background gas flow counter.

Aminoacylation of $tRNA^{phe}$. The basic reaction mixture contained the following components in a volume of 0.1 ml: 0.007 M magnesium acetate, 0.02 M potassium chloride, 0.01 M TRIS-HCl (pH 7.8), 8.9×10^{-5} M DTT, 0.001 M ATP, 0.01 M phosphoenolpyruvate, 10 µg of phosphoenolpyruvate kinase (EC 2.7.1.40), 0.0002 M CTP, 500 pmoles of the indicated ^{14}C L-amino acid (specific activities ranged from 112 to 455 mc/mmole), 0.026 mg of aminoacyl tRNA synthetases, and 2.1 µg (approximately 84 pmoles) of $tRNA^{phe}$. After incubation at 30 C for 35 minutes, the tubes were placed on ice, and 100 µg of carrier yeast RNA and 1 ml of cold 10% TCA were added. The precipitates were collected on membrane filters, washed with 2 ml of cold 10% TCA and 1 ml of cold 70% ethanol, and counted on a Nuclear of Chicago low background gas flow counter.

Transfer of amino acids from aminoacyl tRNA's into protein. The reaction mixture contained the following components in a volume of 0.4 ml: 0.02 M potassium chloride, 0.01 M TRIS-HCl (pH 7.8), 8.3×10^{-5} M DTT, 0.33 mM GTP, ribosomes containing 0.5 mg of protein and 0.4 mg of RNA, and yeast tRNA charged with either ^{14}C L-phenylalanine or ^{14}C L-leucine, and a mixture of ^{12}C amino acids exclusive of the radioactive amino acid, as indicated in the Table legends. Magnesium acetate, spermine, and streptomycin were added as indicated. Reactions were carried out in the presence and absence of 100 µg of poly U, and the net poly U stimulation of a given amino acid was calculated as described for the S-30 system. After incubation at 30 C for the period indicated, the reactions were terminated with 1 ml of 10% TCA and heated at 85 C for 30 minutes. The precipitates were collected on membrane filters and washed with 5% TCA followed by 70% ethanol. In some experiments, the reaction mixture was pipetted onto Whatman #3 filter paper discs (23 mm diam), which were then submerged in 10% TCA, heated at 90 to 100 C for 30 minutes and washed (Mans and Novelli, 1961). Filters were dried and then counted in toluene-based Omnifluor on a Nuclear of Chicago liquid scintillation counter, at 60% and 76% efficiency for samples on Whatman #3 paper and membrane filters, respectively.

Sucrose density gradient. 6.0 $O.D_{260}$ units of an S-30 subcellular fraction were layered on a 5 ml 15-30% (w/v) linear sucrose gradient prepared in Buffer A. Centrifugation was for 70 minutes at 38,000 revolutions/minute in a Spinco SW 50.1 rotor. Fractions were collected, diluted with water, and absorbency at 260 nm was determined.

Biochemical assays. Protein was determined according to Zamenhof (Zamenhof, 1957) and RNA as described by Schneider (Schneider, 1957).

Materials. Deoxyribonuclease (E.C. 3.1.4.5), ribonuclease-free was obtained from Worthington Biochemical Corporation, Freehold, N.J.; G-25 Sephadex, from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; DEAE cellulose, from Bio-Rad Laboratories, Richmond, Calif.; yeast tRNA, ^{14}C and ^{12}C amino acids, from Schwarz Bioresearch, Orangeburg, N.Y.; poly U, from Miles Laboratories, Inc., Kankakee, Ill.; GTP, ATP, and CTP, from P-L Biochemicals, Milwaukee, Wisc.; phosphoenolpyruvate, DTT, spermidine trihydrochloride and spermine tetrahydrochloride, from Calbiochem, Los Angeles, Calif.; phosphoenolpyruvate kinase (EC 2.7.1.40) and $tRNA^{phe}$, from Boehringer Mannheim Corp., New York, N.Y.; Omnifluor, from New England Nuclear, Boston, Mass.; membrane filters

(type B-6), from Schleicher and Schuell, Keene, N.H.; streptomycin sulfate, neomycin sulfate, and cycloheximide, from Sigma Chemical Co., St. Louis, Mo.; and chloramphenicol, from Mann Research Laboratories, New York, N.Y.

RESULTS

I. Establishment of standard conditions for phenylalanine incorporation in the S-30 cell-free protein synthesizing system. Conditions were established for an active poly U-directed system for the incorporation of phenylalanine into protein, employing an extract of Saccharomyces cerevisiae. Amino acid incorporating systems previously developed for E. coli (Nirenberg and Matthaei, 1961) and various species of yeast (Bretthauer, et al., 1963; So and Davie, 1963; Dietz, Reid, and Simpson, 1965) served as guidelines in the determination of the conditions employed in the standard system. This reaction system was developed for use as the control in the study of the effects of a number of variables on fidelity. Therefore, in the selection of conditions consideration had to be given, not only to optima for phenylalanine incorporation, but also to the effect of these optima on the accuracy of translation. The parameters of the reaction which were examined experimentally were time and temperature of incubation, and magnesium and poly U concentration. In the experiments reported here, each of these parameters was varied under the conditions of the standard incubation. Incorporation of phenylalanine given in the results represents polymerization of the amino acid in response to the endogenous messenger as well as to poly U. However, incorporation in the absence of

poly U is low in this system and the highest obtained under all conditions was approximately 3 pmoles. Therefore, the data obtained reflect the effects of the different variables on poly U-directed synthesis.

The kinetics of phenylalanine incorporation in the standard S-30 protein synthesizing system is shown in Fig. 1. A rapid rate of incorporation was obtained for approximately 35 minutes, at which time 94.6 pmoles of phenylalanine were insoluble in hot TCA. Therefore, 35 minutes was chosen as the standard time of incubation.

In a range of magnesium ion concentrations from 0.005 to 0.034 M, the highest level of phenylalanine incorporation (81.2 pmoles) was obtained at 0.009 M magnesium (Table 1). Although a level of 0.009 M magnesium was optimal for phenylalanine incorporation, later studies showed that considerable poly U-directed leucine incorporation (ambiguity) also occurred at this concentration. On the other hand, relatively little ambiguous leucine incorporation was obtained at 0.006 M magnesium. Since the effects of agents other than magnesium itself are more readily detected against a background of low ambiguity, 0.006 M magnesium was selected for use in the standard system.

The effect of varying the temperature of incubation is shown in Table 2. Optimum incorporation of phenylalanine (85.4 pmoles) occurred at 37 C, although considerable activity was obtained throughout the range

Fig. 1. Kinetics of phenylalanine incorporation in the standard S-30 amino acid incorporating system. The incubation mixture contained the following components in a volume of 0.4 ml: 0.006 M magnesium acetate, 0.02 M potassium chloride, 0.01 M TRIS-HCl (pH 7.8), 8.3×10^{-5} M DTT, 0.033 mM guanosine 5'-triphosphate (GTP), 0.635 mM ATP, 3.1 mM phosphoenolpyruvate, 12.5 μg of phosphoenolpyruvate kinase (EC 2.7.1.40), 244 pmoles of ^{14}C L-phenylalanine (specific activity, 513 mc/mole), 12,500 pmoles of each amino acid in a mixture of ^{12}C L-amino acids excluding the radioactive amino acid, 100 μg of poly U, and 0.1 ml of an S-30 subcellular fraction containing 0.87 mg of protein and 0.14 mg of RNA. Incubation was at 30 C for the time indicated. Reactions were stopped and counted as described in Materials and Methods.

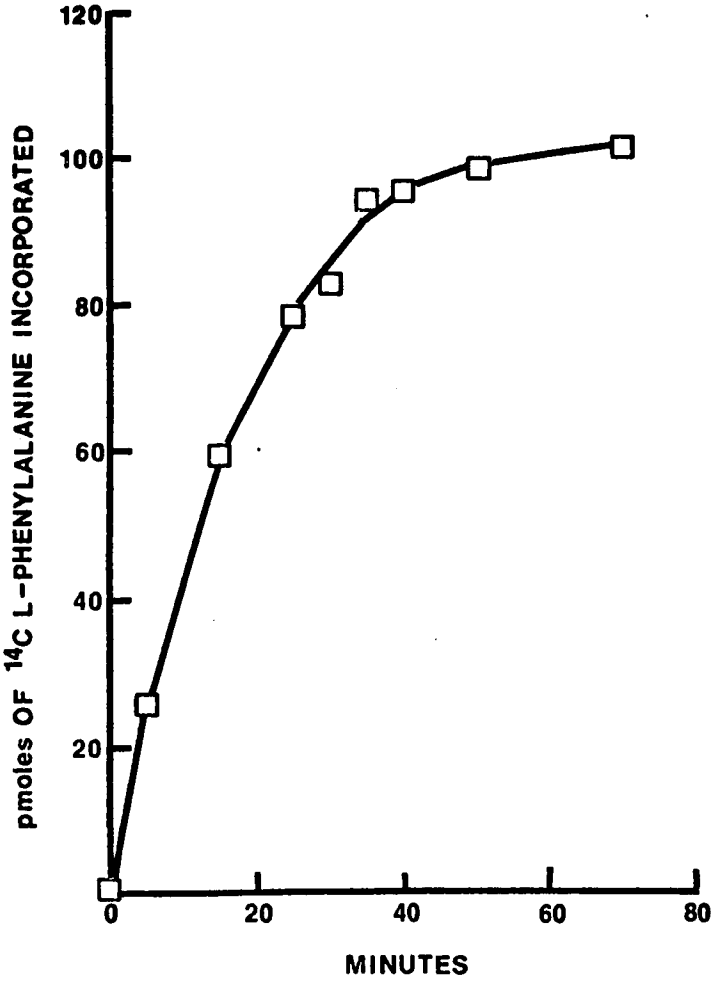
120
100
80
60
40
20
0
20
40
60
80
pmoles OF ^{14}C L-PHENYLALANINE INCORPORATED
MINUTES

FIG. 1

TABLE 1. Effect of magnesium concentration on phenylalanine incorporation in the standard poly U-stimulated S-30 system[a]

Mg (M)	Phe (pmoles)
0.005	36.5
0.009	81.2
0.019	75.9
0.021	62.3
0.029	26.5
0.034	12.3

[a]See legend to Fig. 1 and Materials and Methods. Incubation time was 50 minutes.

TABLE 2. Effect of temperature on phenylalanine incorporation in the standard poly U-stimulated S-30 system[a]

Temp (C)	Phe (pmoles)
12	35.0
15	49.1
20	53.9
30	78.1
37	85.4
42	42.2
45	22.7

[a]See legend to Fig. 1 and Materials and Methods.

tested. The sharp decline in incorporation (42.2 pmoles) noted at 42 C may have been the result of temperature-induced denaturation of one or more components of the system. Compatible with this interpretation, a slight opacity was observed in the reaction mixtures incubated at 42 and 45 C. Since the cells were grown at 30 C and the level of phenylalanine incorporated (78.1 pmoles) at this temperature was close to that obtained (85.4 pmoles) under optimum conditions, 30 C was adopted as the standard temperature of incubation.

In protein synthesizing systems derived from E. coli, an optimum poly U concentration for phenylalanine incorporation is observed and higher levels severely inhibit the reaction (So and Davie, 1964; Szer and Ochoa, 1964). In the yeast system, the highest level of phenylalanine incorporation (108.8 pmoles) was obtained in the presence of 300 µg of poly U (Table 3). Increasing the poly U concentration resulted in moderately decreased incorporating activity. A concentration of 100 µg of poly U, which stimulated considerable incorporation of phenylalanine (66.6 pmoles) was used in the standard system for reasons of economy.

The potassium ion concentration used in the standard system was not derived experimentally. There was evidence that a low monovalent (potassium) to divalent (magnesium) cation ratio improved the structural stability of chloroplast ribosomes from Chlamydomonas (Sager and Hamilton, 1967).

TABLE 3. Effect of poly U concentration on phenylalanine incorporation in the standard poly U-stimulated S-30 system[a]

Poly U (μg)	Phe (pmoles)
20	39.8
50	62.8
100	66.6
200	82.2
300	108.8
500	86.6

[a]See legend to Fig. 1 and Materials and Methods.

The choice of 0.02 M potassium for the standard system was made because this level was lower than the concentration (0.06 M) used in the conventional E. coli system (Nirenberg and Matthaei, 1961), yet within the range (0.007 to 0.06 M) employed in other yeast systems (see references cited above).

In studies with a cell-free system derived from the diploid yeast, Saccharomyces fragilis X Saccharomyces dobzanskii, Bretthauer, et al. (1963) found that phenylalanine incorporation was markedly dependent on the presence of spermine or spermidine. The requirement for spermidine was only partially replaced by magnesium. When the effect of polyamines was examined in the standard yeast system, it was found that spermine and spermidine enhanced phenylalanine incorporation in incubations run at 0.006 M magnesium, a concentration which was sub-optimal for the reaction. This stimulation was not observed consistently when the magnesium concentration was raised to 0.009 M. It appeared, therefore, that polyamines could be replaced by magnesium and were not an essential requirement for protein synthesis in this system. In addition, subsequent studies showed that spermine and spermidine induced ambiguity in the cell-free yeast system. For these reasons, polyamines were not included in the standard system described in this report.

Although the yeast cell contains both cytoplasmic and mitochondrial protein synthesizing systems, it had

been assumed that only the components of the cytoplasmic system were present in the S-30 cell fraction. In the preparation of this fraction, the cell extract was subjected to centrifugation at 30,000 x g, a force known to sediment mitochondria. Nevertheless, an experiment was performed in order to firmly establish that only cytoplasmic protein synthesizing components remained in the S-30 fraction. Chloramphenicol inhibits protein synthesis in the yeast mitochondrial system (Lamb, Clark-Walker, and Linnane, 1968; Scragg, et al., 1971), whereas cycloheximide inhibits protein synthesis in the cytoplasmic system (Lamb, Clark-Walker, and Linnane, 1968). Phenylalanine incorporation in the presence of chloramphenicol or cycloheximide was compared with that obtained in the absence of antibiotics. A concentration of 0.5 mM chloramphenicol inhibits protein synthesis in isolated yeast mitochondria (Lamb, Clark-Walker, and Linnane, 1968). The data presented in Table 4 show that at 0.5 mM chloramphenicol and at 8 times this concentration, phenylalanine incorporation remained essentially the same as in the control (105.7 pmoles). On the other hand, phenylalanine incorporation was inhibited by 97 per cent when cycloheximide was added to the reaction. These results demonstrate conclusively that phenylalanine incorporation in the standard S-30 system occurred on cytoplasmic ribosomes.

TABLE 4. Effect of chloramphenicol and cycloheximide on phenylalanine incorporation in the standard poly U-stimulated S-30 system[a]

Addition	Phe (pmoles)
----	105.7
Chloramphenicol (M x 10^{-3})	
0.5	109.5
4.0	120.6
Cycloheximide (M x 10^{-3})	
0.05	3.4
0.4	3.1

[a]See legend to Fig. 1 and Materials and Methods. Reaction mixtures contained 1.75 x 10^{-4} M spermine.

The ribosome profile of the S-30 cell fraction was obtained on on a linear sucrose gradient and indicated that essentially all ribosomes were present as monomers. As shown in Fig. 2, the $O.D._{260}$ absorbing material sedimented in a single symmetrical peak. Polysomes were probably disrupted by shearing forces incurred during cell breakage.

Thus, standard conditions (see legend to Fig. 1 and Materials and Methods) were established for the poly U-directed incorporation of phenylalanine in a subcellular system obtained from yeast. Protein synthesis in this system was shown to take place on cytoplasmic ribosomes.

II. The effect of various environmental conditions on the fidelity of translation in the standard S-30 cell-free protein synthesizing system. The purpose of these experiments was to examine the effects of several variables on the accuracy of translation in the standard yeast S-30 amino acid incorporating system. The variables chosen for study, all of which have been shown to induce ambiguity in bacterial in vitro protein synthesis, include temperature, magnesium ion concentration, incubation pH, polyamines, ethanol and the aminoglycoside antibiotics, streptomycin and neomycin. These variables were each studied independently and the results obtained were compared with those observed in the standard incubation

Fig. 2. Sucrose density gradient of an S-30 subcellular fraction from yeast (see Materials and Methods).

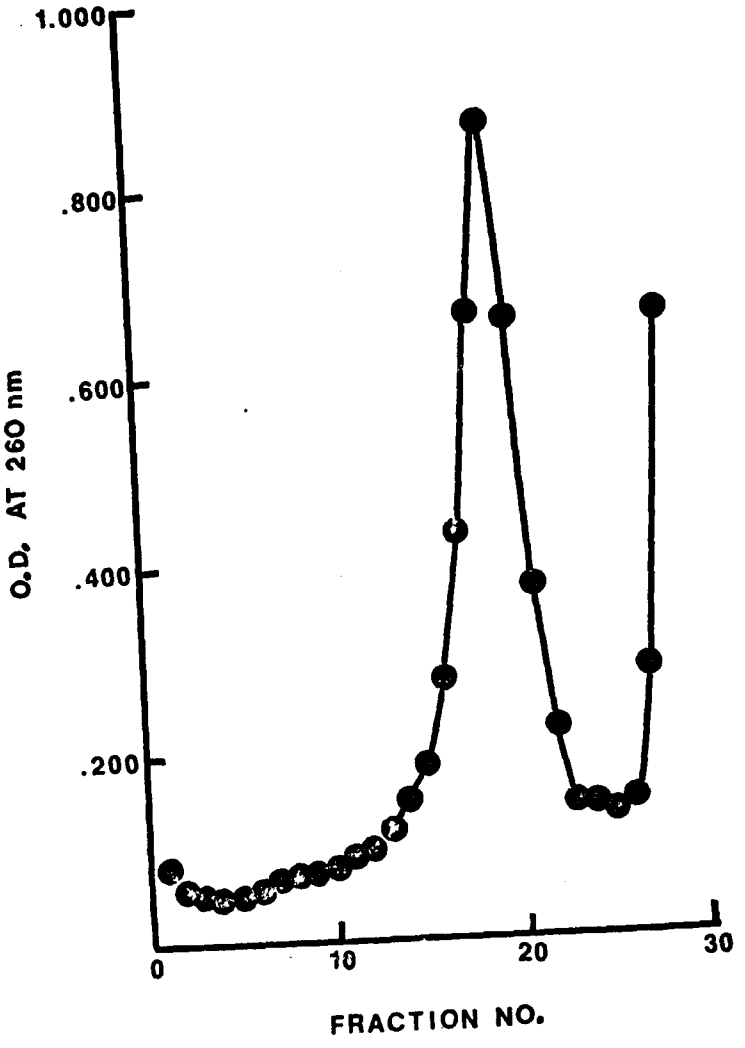

FIG. 2

described in the legend to Fig. 1 and in Materials and Methods. The quantitative basis of comparison was the leucine/phenylalanine ratio as generated under each set of conditions. This ratio was obtained in the following way: Reaction mixtures were incubated under the various in vitro conditions with either ^{14}C L-phenylalanine or ^{14}C L-leucine, in the presence and in the absence of poly U. The amount of amino acid incorporated in the presence of poly U minus that incorporated in its absence represents the "net poly U stimulation." The leucine/phenylalanine ratio is the proportion, net poly U stimulation of leucine incorporation/net poly U stimulation of phenylalanine incorporation. The value of this ratio is low when the fidelity of translation is high.

The results of an experiment in which the standard incubation temperature (30 C) was varied from 12 to 45 C are shown in Table 5 and Fig. 3A. The levels of leucine and phenylalanine incorporation varied with temperature. Optimum poly U-directed phenylalanine incorporation (101.5 pmoles) was obtained at 37 C, while the highest level of poly U-stimulated leucine response (2.6 pmoles) was seen at 30 C. The leucine/phenylalanine ambiguity ratio calculated at 30 C was 0.03, and no significant change in this ratio was observed throughout the range of temperatures tested. Therefore, the data show that varying the temperature of incubation did not perturb the fidelity of the standard system.

TABLE 5. Effect of temperature on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

Temperature (C)	Net poly U stimulation[b] (pmoles)		
	Leu	Phe	Leu/Phe
12	0.8	29.7	0.03
15	1.3	53.6	0.02
20	1.6	67.4	0.02
30	2.6	96.2	0.03
37	1.8	101.5	0.02
42	1.4	79.4	0.02
45	0.2	58.5	0.00

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the presence and absence of poly U.

[b]Incorporation of amino acid in the presence of poly U minus that in the absence of poly U.

Fig. 3. Effect of environmental conditions on net poly U stimulation of ^{14}C L-leucine and ^{14}C L-phenylalanine incorporation in the standard S-30 system (see legend to Fig. 1 and Materials and Methods). Reactions were carried out in the absence and presence of poly U. Graph A, the effect of temperature; B, streptomycin; C, ethanol; D, neomycin; E, magnesium; F, spermidine; G, spermine. (These graphs depict the data presented in Tables 5, 11, 10, 12, 6, 8, and 9, respectively. Symbols: ○·····○, leucine; □·····□, phenylalanine; X——X, leucine/phenylalanine.

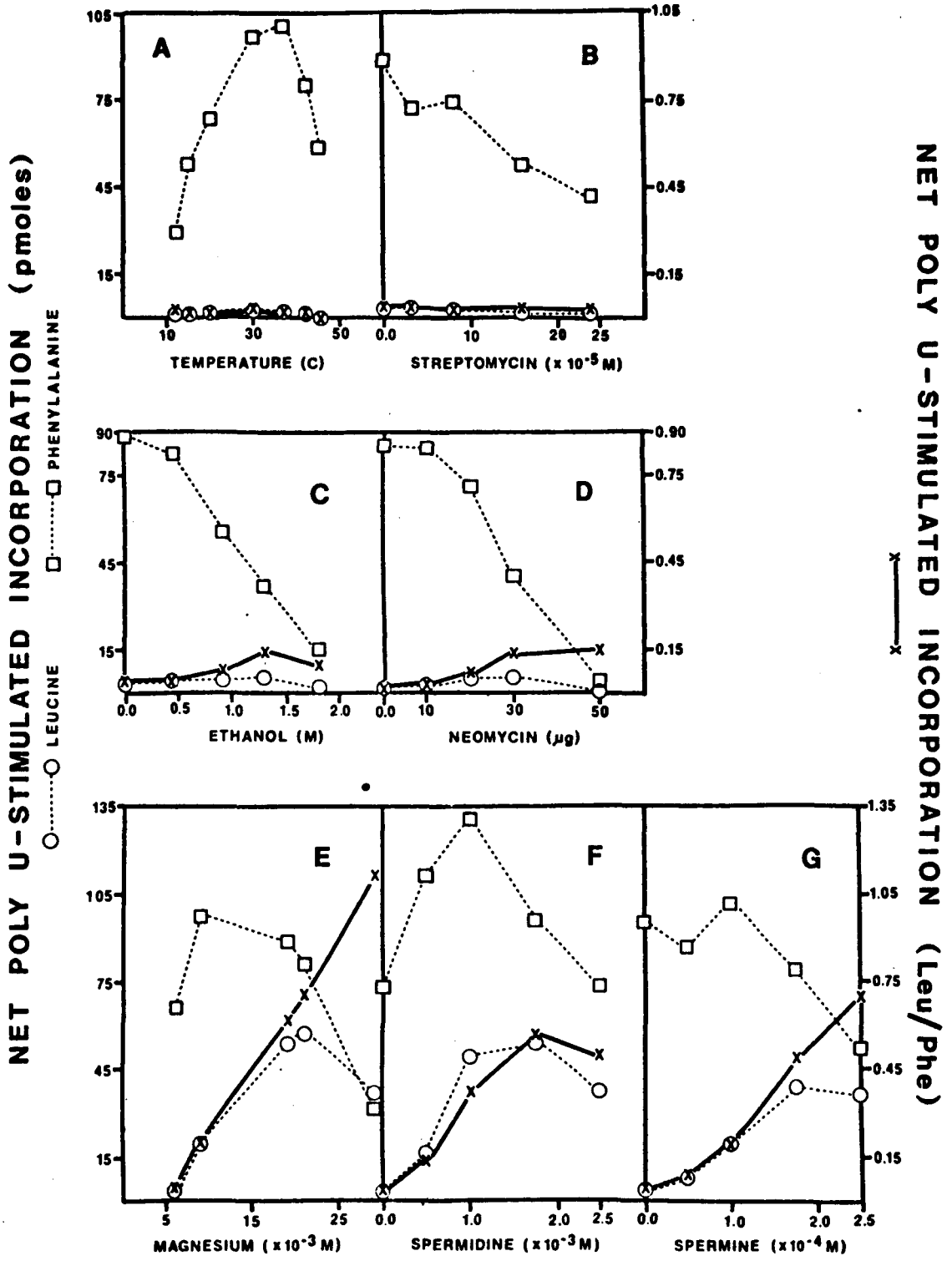
NET POLY U-STIMULATED INCORPORATION (pmoles)
PHENYLALANINE
LEUCINE
NET POLY U-STIMULATED INCORPORATION (Leu/Phe)
A
B
C
D
E
F
G
TEMPERATURE (C)
STREPTOMYCIN (x 10⁻⁵ M)
ETHANOL (M)
NEOMYCIN (μg)
MAGNESIUM (x 10⁻³ M)
SPERMIDINE (x 10⁻³ M)
SPERMINE (x 10⁻⁴ M)

FIG. 3

In contrast with this result, experiments in which the magnesium ion concentration of the standard incubation was varied showed that elevated magnesium concentrations induced a high level of ambiguity. As shown in Table 6 and Fig. 3E, the leucine/phenylalanine ratio obtained at the standard magnesium concentration (0.006 M) was 0.04. Increasing magnesium concentrations resulted in progressively higher values. At 0.029 M magnesium, poly U-directed incorporation of leucine (36.5 pmoles) exceeded that of phenylalanine (32.5 pmoles), and a value of 1.12 for the ambiguity ratio was obtained. This value was 28 times higher than that observed under standard conditions.

Increasing magnesium concentrations (0.006 to 0.021 M) stimulated progressively higher poly U-directed leucine incorporation. The level obtained at 0.021 M magnesium was 20 times greater than that observed in the control. The increased values obtained for the ambiguity ratio in this range of magnesium concentrations were the result of an increase in the numerator (leucine incorporation) and not merely a decrease in the denominator (phenylalanine incorporation). Even at the magnesium concentration (0.009 M) which was optimum for phenylalanine incorporation, both leucine incorporation and the level of ambiguity were higher than the control values.

Lowering the pH of incubation was shown to decrease

TABLE 6. Effect of magnesium concentration on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

	Net poly U stimulation[b] (pmoles)		
Magnesium (M)	Leu	Phe	Leu/Phe
0.006	2.9	65.9	0.04
0.009	19.7	97.8	0.20
0.019	53.5	88.1	0.61
0.021	57.3	81.2	0.71
0.029	36.5	32.5	1.12

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the presence and absence of poly U.

[b]Calculated as indicated in legend to Table 5.

the level of ambiguity obtained in reactions run at an elevated magnesium ion concentration (Table 7). At the pH (7.8) of the standard system, the leucine/phenylalanine ratio was 0.27. This value was reduced to 0.13 and 0.11 in mixtures incubated at pH 7.0 and 6.5, respectively. The lower pH's resulted in diminished incorporation of both amino acids, but the decrease in leucine response was proportionately greater than that of phenylalanine.

Results presented in Tables 8,9 and Figs. 3F,G show that the polyamines, spermidine and spermine, had a pronounced effect on fidelity in the yeast system. The control level of leucine-phenylalanine ambiguity (0.04), increased 14-fold, to a value of 0.57, in the presence of 1.75×10^{-3} M spermidine (Table 8 and Fig. 3F). Similar to the effect observed with magnesium, increasing spermidine concentrations (0.5 to 1.75×10^{-3} M) resulted in increasing leucine incorporation. The amount of leucine responding to poly U at 1.75×10^{-3} M spermidine was 18 times higher than that seen in the control. In addition, leucine ambiguity was expressed even under conditions which were optimum for phenylalanine incorporation (1.00×10^{-3} M spermidine). The elevated value of the ambiguity ratio at this spermidine concentration coincided with increased leucine incorporation. Phenylalanine incorporation was stimulated by low concentrations of spermidine probably because a sub-optimal concentration

TABLE 7. Effect of pH on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

	Net poly U stimulation[b] (pmoles)		
pH	Leu	Phe	Leu/Phe
7.8	25.6	95.3	0.27
7.0	7.4	55.9	0.13
6.5	3.7	33.2	0.11

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out at 0.016 M magnesium, in the presence and absence of poly U.

[b]Calculated as indicated in legend to Table 5.

TABLE 8. Effect of spermidine on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

Spermidine ($M \times 10^{-3}$)	Net poly U stimulation[b] (pmoles) Leu	Phe	Leu/Phe
----	3.0	72.7	0.04
0.50	15.5	111.5	0.14
1.00	50.0	131.3	0.38
1.75	53.7	95.1	0.57
2.50	36.6	73.3	0.50

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the presence and absence of poly U.

[b]Calculated as indicated in legend to Table 5.

TABLE 9. Effect of spermine on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

Spermine ($M \times 10^{-4}$)	Net poly U stimulation[b] (pmoles) Leu	Phe	Leu/Phe
----	3.8	95.5	0.04
0.50	7.7	87.3	0.09
1.00	20.4	102.7	0.20
1.75	39.1	79.7	0.49
2.50	36.0	51.6	0.70

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the presence and absence of poly U.

[b]Calculated as indicated in legend to Table 5.

of magnesium (0.006 M) was used. Others have reported that polyamines could replace magnesium (Takeda, 1969) or have an additive effect (Bretthauer, et al., 1963) in subcellular protein synthesis.

The results presented in Table 9 and Fig. 3G indicate that when the components of the standard system were reacted in 2.5×10^{-4} M spermine, the level of leucine-phenylalanine ambiguity was 0.70, or 18 times that observed in the control. The highest level of leucine incorporation (39.1 pmoles) was obtained at 1.75×10^{-4} M spermine and was 10-fold that found under standard conditions. Similar to the observation with magnesium and spermidine, leucine ambiguity was expressed even when conditions were optimum (1.00×10^{-4} M spermine) for the incorporation of phenylalanine. The addition of a low concentration of spermine stimulated phenylalanine incorporation slightly, presumably on the same basis as the stimulation observed with spermidine.

The kinetics of leucine and phenylalanine incorporation were studied in the absence and presence of spermine. This experiment was a control to determine whether spermine-induced leucine-phenylalanine ambiguity occurred at a time in the course of the reaction when polymerization of phenylalanine was still active. The results of this experiment are presented in Fig. 4. In the absence of spermine, a rapid rate of phenylalanine incorporation

Fig. 4. Kinetics of net poly U stimulation of leucine and phenylalanine incorporation in the standard S-30 system (see legend to Fig. 1 and Materials and Methods). Reactions were carried out in the absence and presence of 3.0×10^{-4} M spermine. 1.2 ml reaction mixtures were incubated with ^{14}C L-phenylalanine or ^{14}C L-leucine, in the absence and presence of poly U. 0.2 ml aliquots were sampled at each time point indicated. Symbols: □, phenylalanine; ○, leucine.

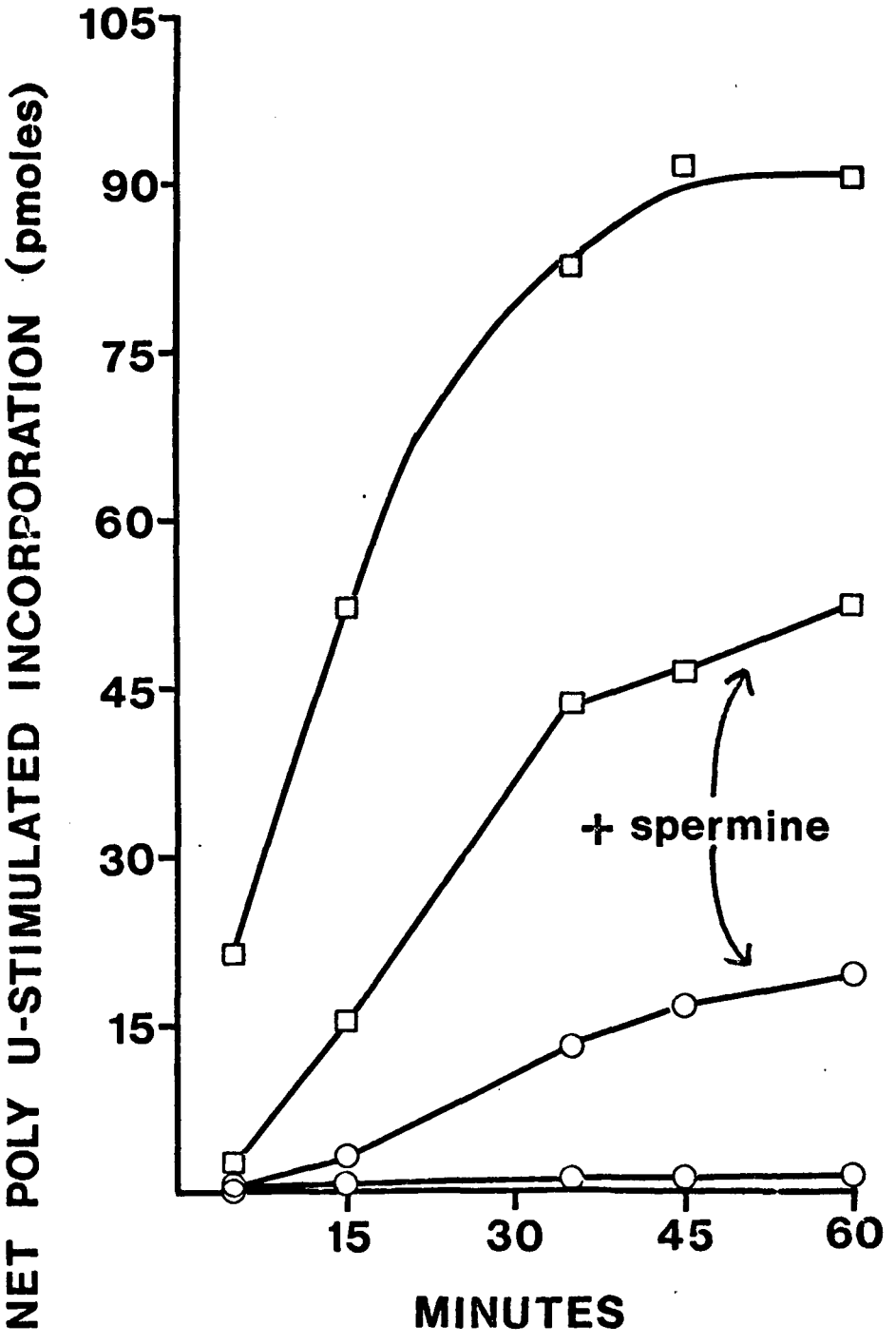
NET POLY U-STIMULATED INCORPORATION (pmoles)
105
90
75
60
45
30
15
+ spermine
15
30
45
60
MINUTES

FIG. 4

was observed for approximately 35 minutes. At this time, 82.6 pmoles of phenylalanine and 1.1 pmoles of leucine were obtained in hot TCA-precipitable material. Although the rate of phenylalanine incorporation was depressed in the presence of spermine, its uptake into protein proceeded at a linear rate for 35 minutes. After 15 minutes of incubation, well within the linear portion of the curve, the value of the leucine/phenylalanine ratio in the presence of spermine was 0.22 as compared to 0.01 in its absence. This observation rules out the possibility that spermine-induced ambiguity was an artifact of a system in which leucine incorporation continued after phenylananine incorporation had ceased.

The results presented in Table 10 and Fig. 3C show that the introduction of ethanol into the system stimulated ambiguity. The index of ambiguity increased 4-fold to 0.14, in the presence of 1.3 M ethanol. At this concentration, phenylalanine incorporation (37.7 pmoles) decreased by a factor of 0.58 while poly U-directed leucine incorporation increased 1.7-fold. Therefore, although ethanol induced ambiguity in the system, its effect was not as marked as that observed with elevated magnesium and polyamine concentrations (see Tables 6,8,9; Figs. 3E,F,G).

The addition of streptomycin had no effect on the

TABLE 10. Effect of ethanol on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

Ethanol (M)	Net poly U stimulation[b] (pmoles) Leu	Phe	Leu/Phe
----	3.2	88.5	0.04
0.45	3.7	83.3	0.04
0.90	4.4	56.3	0.08
1.30	5.3	36.7	0.14
1.80	1.5	15.0	0.10

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the absence and presence of poly U.

[b]Calculated as indicated in legend to Table 5.

accuracy of translation, although both phenylalanine and leucine incorporating activities were reduced in its presence (Table 11 and Fig. 3B). Decreases in leucine incorporation paralleled the decline in phenylalanine response. For example, at 24.0 x 10^{-5} M streptomycin, incorporation of both leucine and phenylalanine were inhibited approximately 50 per cent. Consequently, values for the ambiguity ratio calculated at the various levels of streptomycin tested were essentially the same as that obtained in the control. The concentrations of streptomycin used in this experiment were sufficiently high to depress phenylalanine incorporation. Characteristically, this is the condition under which the highest values for the leucine/phenylalanine ratio are obtained with agents of ambiguity in both the yeast (see Tables 6,8,9,10,12; Figs. 3E,F,G,C,D)and E. coli (So and Davie, 1964; So, Bodley, and Davie, 1964; Sherman and Simpson, 1969) systems.

In contrast to the results obtained with streptomycin, 30 µg of neomycin increased the value of leucine/phenylalanine ratio 7-fold, to 0.14 (Table 12 and Fig. 3D). At this neomycin level, phenylalanine incorporation was approximately one half that of the control value and leucine incorporation was increased by a factor of 3. When the concentration of neomycin was raised to 50 µg,

TABLE 11. Effect of streptomycin on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

Streptomycin (M x 10^{-5})	Net poly U stimulation[b] (pmoles) Leu	Phe	Leu/Phe
----	3.2	88.5	0.04
3.2	3.1	71.8	0.04
8.0	2.4	74.4	0.03
16.0	2.3	55.5	0.04
24.0	1.7	41.9	0.04

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the absence and presence of poly U.

[b]Calculated as indicated in legend to Table 5.

TABLE 12. Effect of neomycin on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system[a]

Neomycin (μg)	Net poly U stimulation[b] (pmoles) Leu	Phe	Leu/Phe
--	1.7	85.1	0.02
10	2.6	84.0	0.03
20	4.9	71.0	0.07
30	5.4	40.0	0.14
50	0.6	3.9	0.15

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the presence and absence of poly U.

[b]Calculated as indicated in legend to Table 5.

incorporation of both phenylalanine and leucine was inhibited markedly, while the value of the ambiguity ratio (0.15) was not increased significantly. Thus, neomycin, like ethanol, induced a comparatively low degree of ambiguity in this system.

Thus, the variables tested in the standard yeast system can be grouped into 3 classes according to their effects on fidelity: Increasing magnesium, spermine, and spermidine concentrations induced markedly high levels of leucine-phenylalanine ambiguity; ethanol and neomycin induced ambiguity, but to a decidedly lower degree; and streptomycin and temperature produced no detectable ambiguity in the system. It was also found that lowering the pH of the reaction mixture reduced the level of ambiguity obtained at an elevated magnesium ion concentration.

III. Amino acid specificity of the ambiguous response to poly U induced by elevated magnesium and spermine concentrations. The possibility that increased levels of magnesium and spermine might also induce incorporation of other amino acids, in addition to leucine, was examined. Ambiguity ratio values were obtained for a series of ^{14}C L-amino acids in poly U-stimulated incubations run at a high magnesium concentration or

with spermine. These values were compared with those observed under standard conditions of amino acid incorporation.

Of the 10 amino acids tested, only leucine, isoleucine, and serine showed significantly increased values of the amino acid/phenylalanine ratio at 0.016 M magnesium (Table 13). At this level of magnesium, the values of the ambiguity ratio for these amino acids, in order, were 0.29, 0.20, and 0.03, respectively, as compared to 0.03, 0.01, and 0.01, at the standard magnesium ion concentration. Furthermore, these elevated levels of ambiguity reflected approximately 9-fold, 32-fold, and 3-fold increases in the poly U-directed responses of leucine, isoleucine, and serine, respectively. The values of the ambiguity ratio calculated for all of the other amino acids tested was 0.02 or less, and no significant differences in the poly U-directed incorporation obtained at the two magnesium concentrations were noted.

The results of an experiment in which the incorporation of phenylalanine and 8 other amino acids was examined in the presence and absence of spermine are presented in Table 14. In this study, the control values of the ambiguity ratio for leucine, isoleucine, and serine, were 0.06, 0.02, and 0.02, respectively.

TABLE 13. Amino acid specificity of magnesium-induced ambiguity in the standard poly U-stimulated S-30 system[a]

Amino Acid	0.006 M Mg		0.016 M Mg	
	Net poly U stimulation[a] (pmoles)	Amino-acid/phe	Net poly U stimulation[a] (pmoles)	Amino acid/phe
Phenylalanine	62.3	----	62.0	----
Leucine	2.0	0.03	18.0	0.29
Isoleucine	0.4	0.01	12.6	0.20
Serine	0.7	0.01	1.9	0.03
Tyrosine	0.2	0.00	0.0	0.00
Valine	0.3	0.01	0.4	0.01
Proline	0.0	0.00	0.0	0.00
Methionine	0.1	0.00	0.0	0.00
Lysine	0.2	0.00	0.0	0.00
Arginine	0.7	0.01	1.0	0.02
Histidine	0.1	0.00	0.0	0.00

[a]See legend to Fig. 1 and Materials and Methods. Reactions were carried out in the presence and absence of poly U.

TABLE 14. Amino acid specificity of spermine-induced ambiguity in the standard poly U-stimulated S-30 system[a]

Amino acid	No spermine		1.75×10^{-4}M spermine	
	Net poly U stimulation[a] (pmoles)	Amino acid /phe	Net poly U stimulation[a] (pmoles)	Amino acid /phe
Phenylalanine	100.8	----	74.2	----
Leucine	6.3	0.06	50.7	0.68
Isoleucine	2.0	0.02	27.5	0.37
Serine	2.1	0.02	3.3	0.04
Tyrosine	0.2	0.00	0.7	0.01
Valine	1.2	0.01	0.5	0.01
Proline	0.8	0.01	0.3	0.00
Methionine	0.1	0.00	0.0	0.00
Lysine	0.7	0.01	0.1	0.00

[a]See legend to Fig. 1 and Materials and Methods.

Reactions were carried out in the presence and absence of poly U.

These values increased to 0.68, 0.37, and 0.04, respectively, in the presence of spermine. These increments in the values of the amino acid /phenylalanine ratio were coincident with approximately 8-fold, 14-fold, and 1.5-fold more poly U-directed incorporation of these amino acids, in the same order. With the possible exception of tyrosine, spermine did not stimulate the incorporation of the other amino acids. Incorporation of tyrosine, however, was extremely low (less than 1.0 pmole), and no conclusion concerning this amino acid was drawn from this experiment. The ambiguity ratio values for amino acids other than leucine, isoleucine, and serine, were in the range of 0.00 to 0.01.

Thus, magnesium and spermine induced the ambiguous incorporation of the same amino acids, leucine, isoleucine, and serine. Furthermore, the effectiveness of both magnesium and spermine in stimulating incorporation was in the order: leucine $\rangle$ isoleucine $\gg$ serine.

IV. <u>Determination of the specific reaction(s) responsible for the ambiguity induced by magnesium and spermine in the S-30 cell-free system</u>. Fidelity of translation in the S-30 amino acid incorporating system requires specific recognition in two reactions: (1) the tRNA aminoacylation reaction, in which a molecule of tRNA is charged with its

amino acid by an aminoacyl tRNA synthetase, and (2) the transfer reaction, in which an amino acid is transferred from tRNA into peptide linkage on a messenger-ribosome complex. The fidelity of the aminoacylation and transfer reactions was examined separately in order to determine the operational level(s) responsible for ambiguity induced by magnesium and spermine in the S-30 system.

A. The effect of elevated spermine and magnesium concentrations on the fidelity of the tRNA aminoacylation reaction. In one approach to this problem, a competition experiment was used to test the possibility that spermine induces the anomalous aminoacylation of $tRNA^{phe}$. The substrate used in this study was unfractionated yeast tRNA in limiting concentration. In outline, yeast tRNA was reacted with ^{14}C L-phenylalanine in the absence and presence of 0.001 M spermine. Under each of these conditions, the effect of increasing concentrations of ^{12}C L-amino acids excluding phenylalanine was examined. In the absence of spermine, the conditions used approximated those commonly employed to charge tRNA's with their specific amino acids. The expected results for the alternatives under consideration were: (1) If anomalous charging of $tRNA^{phe}$ occurs, the addition of ^{12}C amino acids should result in reduced amounts of cold TCA-precipitable phenylalanine. (2) If $tRNA^{phe}$ is charged correctly, the presence of ^{12}C amino acids should have

no effect on the counts obtained.

The results of this experiment are shown in Table 15. In the absence of spermine and ^{12}C amino acids, 87.5 pmoles of phenylalanine were bound to tRNA. The addition of ^{12}C amino acids produced essentially no change in the amount of phenylalanine bound. This lack of competition supports the premise that phenylalanine is bound to its cognate tRNA under these conditions. 0.001 M spermine stimulated the incorporation of phenylalanine and 94.6 pmoles of the amino acid were obtained in cold TCA-precipitable material. When ^{12}C amino acids were added, no significant decrease in this incorporation was observed. ^{12}C amino acids did not compete with phenylalanine, suggesting that spermine did not alter the specificity of recognition involved in the aminoacylation of $tRNA^{phe}$. This study was limited as only two concentrations of ^{12}C amino acids were tested. It is possible that higher levels may have demonstrated competition.

In another approach to the problem, purified $tRNA^{phe}$ was the substrate in charging reactions carried out at increasing concentrations of magnesium and spermine. The reaction mixtures contained only one amino acid at a time (^{14}C L-phenylalanine, ^{14}C L-leucine, ^{14}C L-isoleucine, or ^{14}C L-serine). These amino acids were involved in the ambiguity induced by magnesium and spermine in the S-30

TABLE 15. Effect of spermine and ^{12}C L-amino acids on the aminoacylation of unfractionated yeast tRNA with ^{14}C L-phenylalanine[a]

Conditions		Phenylalanine (pmoles) bound to tRNA
Spermine (M)	^{12}C L-amino acids[b] (pmoles of each amino acid)	
-----	----	87.5
-----	300	89.3
-----	3000	91.1
0.001	----	94.6
0.001	300	94.3
0.001	3000	91.5

[a]See Materials and Methods.

[b]A mixture of ^{12}C L-amino acids excluding phenylalanine.

system. The data presented in Table 16 represents cold TCA-precipitable incorporation corrected for blank values. In reactions run at magnesium concentrations ranging from 0.007 to 0.1 M, $tRNA^{phe}$ accepted 46.8 to 49.1 pmoles of phenylalanine. Only 0.8 pmoles or less of leucine, isoleucine, and serine were bound. Furthermore, the level of each of the non-specific amino acids bound remained constant at increasing magnesium concentrations. These low levels of cold TCA-insoluble leucine, isoleucine, and serine probably reflected the presence of contaminating tRNA's. High levels of leucyl, isoleucyl, and seryl tRNA synthetase activity were demonstrated when unfractionated yeast tRNA was added to the reaction mixture (data not shown).

When the $tRNA^{phe}$ aminoacylation reaction mixture was brought to 2.4×10^{-4} M spermine (Table 16), 45.5, 0.7, 0.2, and 0.8 pmoles of phenylalanine, leucine, isoleucine, and serine, respectively, were bound to tRNA. Acceptance of all four amino acids was diminished at 0.06 M spermine, possibly because of the presence of inactive spermine-tRNA complexes. The observation that the addition of carrier RNA to these reaction mixtures yielded a visible precipitate supports this interpretation. The uppermost levels of magnesium and spermine used in this experiment were 11-fold and 1200-fold, respectively,

TABLE 16. Effect of magnesium and spermine on aminoacylation of $tRNA^{phe}$ with phenylalanine, leucine, isoleucine, and serine[a]

Conditions	Amino acid (pmoles) bound to tRNA[b]			
	Phenyl-alanine	Leucine	Isoleucine	Serine
Mg (M)				
0.007	46.8	0.8	0.3	0.8
0.026	46.9	0.6	0.2	0.7
0.05	46.9	0.8	0.3	0.8
0.1	49.1	0.6	0.1	0.8
Spermine (M)				
2.4×10^{-4}	45.5	0.7	0.2	0.8
0.001	44.9	0.5	0.7	0.8
0.01	44.5	0.5	0.3	0.8
0.06	35.0	0.3	0.0	0.4

[a]See Materials and Methods.

[b]Figures were corrected by subtracting blank values.

higher than concentrations which gave increased values of the leucine/phenylalanine ambiguity ratio in protein synthesis (see Tables 6,9; Figs. 3E,G).

Thus, even in the absence of competing phenylalanine, extremely high magnesium and spermine concentrations did not cause the attachment of leucine, isoleucine, or serine to $tRNA^{phe}$. This finding suggests that the ambiguity induced in the S-30 system by these agents was not a consequence of anomalous charging of this tRNA.

B. <u>The effect of spermine and elevated magnesium concentrations on the fidelity of the transfer reaction</u>. Spermine and elevated magnesium concentrations were shown to induce ambiguity at the transfer level of protein synthesis. In these experiments, yeast tRNA was charged with either ^{14}C L-phenylalanine or ^{14}C L-leucine and a mixture of ^{12}C amino acids excluding the radioactive amino acid. The charging reaction was carried out at a low magnesium concentration in the absence of spermine. This aminoacyl tRNA was subsequently incubated with ribosomes at increasingly high magnesium and spermine levels in transfer reaction mixtures. The leucine/phenylalanine ambiguity ratio was calculated from the data as described for the S-30 system. Since the ribosome preparations used in these studies were unpurified and contained all of the cellular components necessary for the transfer of

amino acids from tRNA into peptide linkage, the ribosome-free supernatant fluid was not used in these reactions.

An alkaline TRIS-HCl buffer system had been used in all of the preceding studies and it was desirable to retain this buffer for the transfer experiments. However, non-enzymatic deacylation of aminoacyl tRNA's in an alkaline TRIS buffer system had been reported (Heredia and Halvorson, 1966). In addition, aminoacyl tRNA synthetases were found to contaminate the ribosome preparation used in this study. Therefore, in order to avoid recharging of tRNA during incubation of the transfer reaction, ATP and its regenerating system were omitted from the reaction mixture. Under these conditions, the transfer of phenylalanine into protein was greatly inhibited. Downey, So and Davie (1965) found that the GTP concentration optimum for the transfer reaction in a yeast system increased 10-fold when the ATP regenerating system (but not ATP) was omitted. Therefore, the level of GTP used in the transfer reaction was increased, and the inhibition of phenylalanine incorporation was partially reversed.

Initial experiments designed to examine fidelity in the transfer reaction in the presence of spermine were run at 0.006 M magnesium. The results of one of these studies, typical of those obtained from several

experiments, are shown in Table 17. The value of the leucine/phenylalanine ratio in the control was 0.08. In the presence of 3.75×10^{-4} M spermine, this value increased to 0.28. An increment in leucine incorporation was barely detectable. Thus, 1.5 pmoles of leucine were transferred in the control, and 1.7 pmoles were transferred in the presence of 1.75×10^{-4} M spermine. In the absence of an increase in poly U-directed leucine incorporation, it can be argued that elevated values of the leucine/phenylalanine ambiguity ratio may result from a preferential inhibition of phenylalanine incorporation. In contrast with the elevated values obtained for the ambiguity ratio with spermine, this value decreased slightly in the presence of 11.2×10^{-5} M streptomycin (Table 17). This concentration of streptomycin inhibited the transfer of phenylalanine by approximately 30 per cent. Thus, the differential effects of spermine and streptomycin on the values of the ambiguity ratio correlated positively with the influence of each agent in the S-30 system (see Tables 9,11; Figs. 3G,B). It appeared that spermine induced ambiguity in the transfer reaction. However, in the absence of an increased poly U-directed transfer of leucine, this conclusion was considered tentative.

The effect of varying the magnesium ion concentration (from 0.004 to 0.019 M) on the transfer reaction was studied.

TABLE 17. Effect of spermine and streptomycin on net poly U-stimulated transfer of leucine and phenylalanine from tRNA into protein at 0.006 M magnesium[a,b]

	Net poly U stimulation (pmoles)		
Addition	Leu	Phe	Leu/Phe
----	1.5	18.9	0.08
Spermine ($M \times 10^{-4}$)			
1.75	1.7	11.8	0.14
3.75	1.2	4.3	0.28
Streptomycin ($M \times 10^{-5}$)			
3.2	1.3	16.8	0.08
11.2	0.7	12.9	0.05

[a]See Materials and Methods. Incubation time was 60 minutes.

[b]All assays contained approximately 120 µg of yeast tRNA charged with either ^{14}C L-leucine or ^{14}C L-phenylalanine. The specific activities of the ^{14}C leucyl tRNA and the ^{14}C phenylalanyl tRNA were 64 and 60 pmoles of ^{14}C amino acid per 100 µg of tRNA, respectively.

As shown in Table 18, the values of the leucine/phenylalanine ratio increased from 0.07 to 0.83. A 5-fold increase in the level of poly U-stimulated leucine transfer (from 0.3 pmoles at 0.004 M magnesium, to 1.5 pmoles at 0.019 M magnesium) was also observed.

The highest level of phenylalanine transferred into protein (4.4 pmoles), was obtained at 0.004 M magnesium (Table 18). Therefore the effect of spermine on the transfer reaction was reexamined at 0.004 M magnesium rather than at the concentration (0.006 M) used in the previous experiments. The results of this study are presented in Table 19. The values of the leucine/phenylalanine ratio increased from 0.08 in the control to 0.70 in the presence of 3.85×10^{-4} M spermine. Furthermore, a 3-fold increase in poly U-directed transfer of leucine (0.3 and 1.0 pmole in the absence and presence of 1.75×10^{-4} M spermine, respectively) was obtained. Thus, a spermine-induced increase in leucine incorporation was demonstrated when the system was run at 0.004 M magnesium. The observation that a high magnesium level can obscure a spermine-induced increase in poly U-directed leucine incorporation has also been reported in a bacterial S-30 system (Friedman and Weinstein, 1964).

Increasing magnesium and spermine concentrations were shown to induce the ambiguous response of leucine to poly U in the transfer reaction. This effect can

TABLE 18. Effect of magnesium concentration on net poly U-stimulated transfer of leucine and phenylalanine from tRNA into protein[a,b]

	Net poly U stimulation (pmoles)		
Magnesium (M)	Leu	Phe	Leu/Phe
0.004	0.3	4.4	0.07
0.006	0.7	3.8	0.18
0.009	1.3	3.2	0.41
0.014	1.6	2.1	0.76
0.019	1.5	1.8	0.83

[a]See Materials and Methods. Incubation time was 50 minutes.

[b]All assays contained approximately 100 μg of yeast tRNA charged with either ^{14}C L-leucine or ^{14}C L-phenylalanine. The specific activities of the ^{14}C leucyl tRNA and the ^{14}C phenylalanyl tRNA were 64 and 24 pmoles of ^{14}C amino acid per 100 μg of tRNA, respectively.

TABLE 19. Effect of spermine on net poly U-stimulated transfer of leucine and phenylalanine from tRNA into protein at 0.004 M magnesium[a,b]

	Net poly U stimulation (pmoles)		
Spermine (M x 10^{-4})	Leu	Phe	Leu/Phe
----	0.3	4.0	0.08
0.88	0.6	3.6	0.17
1.75	1.0	2.9	0.34
2.50	1.0	2.0	0.50
3.85	0.7	1.0	0.70

[a]See Materials and Methods. Incubation time was 50 minutes.

[b]All assays contained the aminoacyl tRNA's described in Table 18.

account for the ambiguity observed in the S-30 system (see Tables 6,9: Figs. 3E,G). Furthermore, the aminoacyl tRNA's used in these experiments were prepared under conditions used to charge amino acids onto their cognate tRNA's. Therefore, it is reasonable to assume that the aminoacyl tRNA wich responded to the UUU codon on the ribosome was leucyl $tRNA^{leu}$, rather than a complex of leucine with a heterologous tRNA.

DISCUSSION

The ribosome was once considered to play a passive role in the reading of the genetic code. However, recent evidence indicates that not only the ribosome (see Discussion below) but also portions of the tRNA molecule outside of the anticodon (Hirsh and Gold 1971) contribute to the fidelity of codon reading. Conventional codon-anticodon interaction, in accord with the rules of the "wobble" hypothesis (Crick, 1966), occurs in specific translation. However, when translation is ambiguous, nonconventional codon-anticodon base-pairing occurs. When the elements of ribosome and tRNA structure and conformation contributing to ambiguity are known, the molecular basis of accurate translation will be better understood.

Ribosomal contribution to codon translation; evidence from the E. coli system. A growing body of evidence from the E. coli system indicates that the decoding site of the message-ribosome complex consists of not only the codon, but also part of the ribosome (Kurland, 1970; Gorini, 1971). The two sources of evidence indicating that a ribosomal site contributes to the reading of the code are investigations of (1) environmentally induced ambiguity, and (2) ribosome mutations which produce ambiguity intrinsically.

Aminoglycoside antibiotics, ethanol, and elevated magnesium concentrations are among the environmental factors which induce ambiguity in the *E. coli* system. The effect of streptomycin has been unequivocally localized to the ribosome. The first experiments to show this (Davies, Gilbert, and Gorini, 1964) used ribosomes and post-ribosomal supernatant fluid from genetically different cells (sensitive and resistant to streptomycin). Homologous and heterologous combinations of these components were employed in amino acid incorporating systems. Streptomycin induced ambiguity with ribosomes obtained from sensitive cells, but had little or no effect with ribosomes from resistant cells. The source of the supernatant fluid was inconsequential. The observation that an external agent acting on the ribosome can alter the specificity of protein synthesis was the first evidence that the conformation of a ribosomal site may be involved in the pairing of tRNA and messenger (Davies, Gilbert, and Gorini, 1964). Furthermore, using the technique of ribosome reconstitution, it was found that when the ribosomal protein P10 was obtained from streptomycin-sensitive (but not from streptomycin-resistant ribosomes), the completed particle was sensitive to the antibiotic (Ozaki, Mizushima, and Nomura, 1969). Thus, P10, a "core" protein of the

30S subunit, was identified as the gene product of the streptomycin locus (*str A*).

Genetic evidence indicates that the determinant of neomycin and kanamycin sensitivity (the *nek* locus) is ribosomal and separate from the *str A* locus (Apirion and Schlessinger, 1968). Neomycin and kanamycin induce ambiguity in extracts from sensitive cells (Davies, Gilbert, and Gorini, 1964), and the degree of misreading induced by kanamycin depends upon whether the source of the ribosomes is sensitive or resistant organisms (Tanaka, et al., 1967).

Evidence for the locus at which magnesium and ethanol exert their influence is less direct than that for antibiotics. A number of environmental agents causing ambiguity did not exert their effects on ribosomes reconstituted without protein P10 (Nomura, et al., 1969). In addition to streptomycin, these agents were neomycin, kanamycin, ethanol, and magnesium. The molecular basis of this observation is obscure, but the evidence suggests that ethanol and magnesium influence the ribosome. However, it is possible that these agents alter the recognition characteristics of aminoacyl tRNA's which can then be accepted by the complete ribosome, but not by the P10-deficient ribosome. Other experiments show that ethanol and magnesium function in the transfer reaction

to give ambiguity, and it is the source of the ribosome (E. coli or reticulocyte) which determines the degree of ambiguity observed (Friedman, Berezny, and Weinstein, 1968). Although the latter result is subject to the same kind of ambivalent interpretation as the experiment with the P10-deficient ribosome, the two findings taken together suggest that the ribosome is the locus of magnesium and ethanol activity.

The same amino acids (leucine, isoleucine, and serine) are involved in the ambiguous response to poly U stimulated by magnesium, streptomycin, neomycin, and kanamycin. Ethanol stimulated leucine and isoleucine ambiguity, but serine was not tested (see Discussion below). Since the antibiotics have been shown to influence the ribosome, the similarity in amino acid response can be taken as further evidence that ethanol and magnesium also exert their effect on this organelle. Experiments which showed that ethanol did not induce anomalous charging of tRNA with leucine and isoleucine (So and Davie, 1965) also lend support to this interpretation.

Thus, streptomycin, neomycin, and kanamycin have been shown to exert their effect on the ribosome in the E. coli system. The evidence strongly suggests that magnesium and ethanol also function at this organelle.

The evidence cited above for a ribosomal contribution to codon translation was derived from studies of environmentally induced ambiguity. However, genetically altered ribosomes can confer ambiguity intrinsically. A mutation, ram ("ribosomal ambiguity"), suppresses all three nonsense codons in the cell (Rosset and Gorini, 1969), and the ribosomes from this mutant give extensive misreading in vitro as seen in the response of isoleucine and serine to poly U. The functional characteristics of the ram ribosome have been correlated with the presence of an altered "core" protein (P4a) in the reconstituted 30S subunit (Zimmerman, Garvin, and Gorini, 1971).

Nature of ambiguity. Observations in the E. coli system made in Gorini's laboratory have lead to his formulation of what may be viewed, in part, as a unified hypothesis explaining both the intrinsic and streptomycin-induced ribosome function in controlling the translation of the genetic code (Gorini, 1971). Gorini's evidence suggests that a low level of ambiguity is intrinsic to the decoding process in wild type E. coli. Thus, "leaky" levels of enzyme are produced with nonsense mutations in a suppressor-negative strain. Intrinsic ambiguity is under ribosomal control since no enzyme is detected in strains bearing mutations at the str A locus.

Furthermore, evidence from *in vitro* studies suggests that intrinsic ambiguity is not random, but only specific amino acid substitutions occur. (Anderson, Gorini, and Breckenridge, 1965; Gorini, 1969).

"Leaky"translation of nonsense codons is more efficient with the *ram* mutation and streptomycin addition (Gorini, 1971). Gorini's interpretation of these and other findings is that *ram* and streptomycin addition do not introduce a new kind of misreading into the system. Rather, the state of the ribosome appears to determine the degree of expression of intrinsic ambiguity. Whereas *str A* mutations to streptomycin resistance restrict its expression, the *ram* mutation and streptomycin addition increase the level of pre-existing intrinsic ambiguity. Thus, the message-ribosome complex must restrict or increase the entry of ambiguous tRNA's on the basis of interaction with a portion of the tRNA molecule outside of the anticodon.

In the context of Gorini's interpretation, then, two separable aspects (qualitative and quantitative) of ambiguity emerge. The qualitative aspect concerns the mechanism by which the ribosome selects the specific tRNA's involved in the ambiguous response. The quantitative aspect concerns the mechanism by which the ribosome relaxes its discrimination against

the entry of these tRNA's.

Nature and function of the putative decoding site on the messenger-ribosome complex in E. coli. When ambiguity is enhanced in in vitro systems, and the ribosomal decoding site is presumably altered, the codon still exerts its influence on the selection of aminoacyl tRNA's. For example, amino acids which respond ambiguously to poly U are those whose codons contain U's (Szer and Ochoa, 1964; Davies, Gorini, and Davis, 1965; Pestka, Marshall, and Nirenberg, 1965). Different messengers restrict the ambiguous response to a specific group of amino acids (Davies, Gorini, and Davis, 1965).

It is clear that the codon triplet must determine the range of allowable responses. It is to be noted, however, that with poly U, not all amino acids (e.g., valine) whose codons contain U's are incorporated. It is proposed, therefore, that the integrated decoding site (codon plus ribosomal contributions) of the messenger-ribosome complex determines the amplitude (amino acid specificity) given to messenger expression. Furthermore, the unmodified ribosomal component must confer fidelity, i. e., the predominance of conventional codon-anticodon base-pairing accompanied by a low level of unconventional codon-anticodon base-pairing with

specific tRNA's (Gorini, 1971). Increased levels of ambiguity are observed when alteration of the ribosomal component increases the affinity of the integrated site for these specific alternate tRNA's.

Some insight into the nature of the ribosomal site which contributes to ambiguity is obtained from genetic studies. The ram gene product (ribosome protein P4a) and the str A gene product (ribosome protein P10) interact to give an altered phenotype. Thus, ambiguity caused by ram is antagonized in vivo and in vitro by mutations at the str A locus (Rosset and Gorini, 1969). Streptomycin also appears to affect this site. Thus, the effects of streptomycin addition and ram are similar and additive. Furthermore, ambiguity caused not only by ram, but also by streptomycin addition, is restricted by mutations at the str A locus (Gorini, 1969).

The E. coli ribosome is a complex organelle made up of 3 RNA's and approximately 50 proteins which are interdependent and interacting. Therefore, although the gene products of the ram and str A loci have been identified, it is not known if these proteins are part of the site directly involved with the admission of aminoacyl tRNA's to the ribosome, or if they interact allosterically to alter the conformation of those components (protein or RNA) which actually comprise the site.

The precise nature of the change in the putative ribosomal decoding site resulting from the ram and str A mutations and the presence of environmental agents causing ambiguity is not known. Sherman and Simpson (1969) presented evidence in support of the hypothesis (Davies, Gilbert, and Gorini, 1964; Davies, Gorini, and Davis, 1965; Pestka, 1966) that streptomycin stimulates ambiguity by inducing a conformational change in the E. coli ribosome. Perhaps the ram and str A mutations, in addition to all agents of ambiguity affecting the ribosome, function by altering the conformation of the putative ribosomal decoding site.

Comparison of findings in yeast, and bacterial and mammalian systems. In order to gain insight into the mechanism of environmentally induced ambiguity observed in the yeast system, a comparison was made with results obtained with bacterial and mammalian extracts. The observations compared were the effects of several environmental factors on the fidelity of poly U-directed protein synthesis, and the spectrum of amino acids incorporated in response to poly U under conditions stimulating ambiguity.

Elevated magnesium concentrations, found to generate high values of the leucine/phenylalanine

ratio in the yeast system, also produce extremely high levels of leucine-phenylalanine ambiguity with bacterial extracts. Thus, ambiguity ratio values of more than 1.00 were obtained with an *in vitro* system from *E. coli* (So, Bodley, and Davie, 1964) and from *Bacillus stearothermophilus* (Friedman and Weinstein, 1964). In contrast, increasing magnesium concentrations did not induce ambiguity in unpurified rat liver and rabbit reticulocyte cell-free systems (Weinstein, Ochoa, and Friedman, 1966) and in rabbit liver extracts (Stavy, 1968). In the rabbit spleen system, a very low leucine/phenylalanine ratio value of 0.01 was observed (Stavy, 1968). Magnesium-induced ambiguity was reported in a rabbit reticulocyte system, but the highest value obtained for the ambiguity ratio was only 0.14 (Bose, et al., 1969). Although Lamfrom and Gruneberg-Manago (1967) obtained a leucine/phenylalanine ratio whose value was 0.39 in a cell-free reticulocyte system, the ribosomes used in these experiments had been treated by incubation at 37 C in relatively high salt (0.2 M KCl).

Lowering the pH of a yeast system run at an elevated magnesium concentration decreased the level of ambiguity observed. This effect has also been reported in *E. coli* extracts (Gruneberg-Manago and Dondon, 1965).

The influence of spermine and spermidine on the yeast cytoplasmic system was similar to that observed with B. stearothermophilus extracts (Friedman and Weinstein, 1964). Values of the leucine/phenylalanine ratio greater than 1.00 were obtained with the thermophile extract. On the other hand, the fidelity of a reticulocyte system was relatively inert to the effect of these polyamines (Weinstein, Ochoa, and Friedman, 1966). An ambiguity level of 0.06 was the highest observed.

Neomycin, but not streptomycin, induced ambiguity in this yeast system. Davies, Gilbert, and Gorini (1964) found that streptomycin and neomycin stimulated the incorporation of leucine and other amino acids in E. coli extracts. Friedman and Weinstein (1964) also made this observation with dihydrostreptomycin in a B. stearothermophilus cell-free system. However, streptomycin failed to induce leucine-phenylalanine ambiguity in a rabbit spleen system (Stavy, 1968), and rat liver systems were only slightly, if at all, affected by streptomycin and neomycin (Weinstein, Ochoa, and Friedman, 1966; Derr and Scanlon, 1969).

In the presence of ethanol, values of the leucine/phenylalanine ratio greater than 1.00 were observed in the E. coli system (So and Davie, 1964). While ethanol also induced ambiguity in the yeast system, the highest

level obtained was only 0.14. In contrast, ethanol induced no ambiguity in rabbit reticulocyte (Weinstein, Friedman, and Ochoa, 1966; Stavy, 1968) and rabbit spleen cell-free systems (Stavy, 1968).

A lowered temperature of incubation increased leucine ambiguity considerably in both E. coli (Szer and Ochoa, 1964) and B. stearothermophilus (Friedman and Weinstein, 1964). On the other hand, variations in temperature did not influence fidelity in an unpurified reticulocyte system (Weinstein, Ochoa, and Friedman, 1966) or in yeast.

Thus, in a poly U-stimulated cytoplasmic extract of yeast, leucine-phenylalanine ambiguity was induced by neomycin, ethanol, spermine, spermidine, and elevated magnesium concentrations. Streptomycin and temperature had no detectable effect. In comparison, the fidelity of bacterial extracts is perturbed by all of these environmental conditions, and the accuracy of crude mammalian systems is relatively unaffected by them. Therefore, a number of the same environmental conditions induce ambiguity in protein synthesizing systems from bacteria and the cytoplasm of yeast.

Further similarities in the coding properties of in vitro prokaryotic and yeast cytoplasmic systems are seen in the amino acid specificity of the ambiguous

response to poly U. Of the amino acids tested in the yeast studies, only leucine, isoleucine, and serine incorporation was stimulated in the presence of spermine and high magnesium concentrations. The amino acid specificity of the ambiguous response in bacterial systems has been determined in the presence of aminoglycoside antibiotics, elevated magnesium concentrations, spermine, ethanol, and low temperature. In the B. stearothermophilus system, as with yeast extracts, both spermine and elevated magnesium concentrations induced the response of leucine, isoleucine, and serine (Friedman and Weinstein, 1964). Furthermore, in both the yeast and thermophile systems, the ambiguity ratio values obtained in the presence of both magnesium and spermine were in the order: leucine/phenylalanine $\rangle$ isoleucine/phenylalanine $\gg$ serine/phenylalanine. Dihydrostreptomycin elicited large increases in leucine, isoleucine, and serine incorporation in B. stearothermophilus extracts, while tyrosine and valine responded to a lesser extent (Friedman and Weinstein, 1964).

In cell-free systems derived from E. coli, the response of leucine, isoleucine, serine, and in some experiments, tyrosine, was stimulated by streptomycin, neomycin, and kanamycin (Davies, Gilbert, and Gorini, 1964; Davies, Gorini, and Davis, 1965). Low

temperature and elevated magnesium concentrations enhanced leucine, isoleucine, serine, and tyrosine incorporation (Szer and Ochoa, 1964). Ethanol stimulated the incorporation of leucine and isoleucine (So and Davie, 1965), but the uptake of serine and tyrosine was not tested.

Thus, the same amino acids, leucine, isoleucine, and serine, are involved in the ambiguous translation of poly U in systems derived from bacteria and from the cytoplasm of yeast. It is also observed that this amino acid specificity is obtained with all of the agents tested: magnesium and spermine in the yeast system, and streptomycin, neomycin, kanamycin, temperature, magnesium, and spermine in bacterial systems. (Ethanol stimulated leucine and isoleucine incorporation in E. coli, but the response of serine was not examined.)

The reaction involved in environmentally induced ambiguity in the yeast system. Environmentally induced ambiguity in yeast was detected in an S-30 amino acid incorporating system. The identification of the specific component(s) of the protein synthesizing machinery affected by these environmental factors is a first step in understanding the molecular mechanism of this phenomenon.

Three alternate models were considered as pathways for the ambiguous incorporation of amino acids in poly U-directed protein synthesis: (1) An error may have occurred solely at the charging level. Phenylalanine tRNA may have been charged with an incorrect amino acid, and subsequently recognized by the poly U-ribosome complex in the transfer reaction. (2) An error may have occurred exclusively at the transfer level. The incorrect amino acid may have been bound to its cognate tRNA which was then recognized ambiguously by the poly U-ribosome complex. (3) Errors may have occurred in tandem at both the charging and transfer levels. The incorrect amino acid may have been bound to a wrong tRNA which was accepted, in turn, through ambiguous recognition on the poly U-ribosome complex. For example, in the case of leucine ambiguity, an anomalously charged tRNA, leucyl $tRNA^{glu}$, may have responded to the UUU codon. It is possible that any one, or a combination of these pathways, was operative to produce the ambiguous incorporation observed in the S-30 system.

Only the first two models were tested in this study, and these were examined solely with reference to the two agents, magnesium and spermine. The third model was considered less probable by reason of its complexity, and was not investigated.

The results obtained in the study of fidelity in the aminoacylation reaction essentially ruled out the first model. On the other hand, magnesium and spermine were shown to induce leucine ambiguity at the level of transfer, a result compatible with the second model. These findings suggest that leucine-phenylalanine ambiguity observed in the S-30 system resulted from the recognition of leucyl $tRNA^{leu}$ by the poly U-ribosome complex. Although transfer level experiments were not carried out with isoleucine and serine, it is probable that these errors also occurred as a result of ambiguous recognition in the transfer reaction.

Similarities between the yeast and bacterial systems; possible implications. The cytoplasmic protein synthesizing system of Saccharomyces cerevisiae, a unicellular eukaryotic microorganism, resembles that of mammalian cells rather than that of bacteria in certain physical and biochemical properties. For example, the molecular weights and sedimentation coefficients of yeast and mammalian ribosomes are similar (Petermann, 1964), and both systems are sensitive to cycloheximide but resistant to chloramphenicol (Lamb, Clark-Walker, and Linnane, 1968; Ennis and Lubin, 1964).

On the other hand, striking similarities are seen between the cytoplasmic protein synthesizing system of yeast and bacterial systems in *in vitro* fidelity studies. A number of the same environmental conditions induce ambiguity in systems obtained from the yeast cytoplasm and from bacteria. In addition, the same amino acids (leucine, isoleucine, and serine) are involved in the ambiguous translation of poly U in both systems. Therefore, elements of the protein synthesizing machinery of the yeast cytoplasm involved in fidelity must bear some special similarity to counterparts in bacterial systems.

In *E. coli*, the ribosome has been identified as the target of aminoglycoside antibiotics, and has been implicated as the locus of magnesium and ethanol activity. In the yeast system, it was found that ambiguity induced by magnesium and spermine occurred in the transfer reaction. Because the observations with yeast so closely parallel those with *E. coli*, it is inviting to extend the *E. coli* findings and assume that the target of magnesium and spermine in the yeast system was the ribosome. Thus, it is tempting to speculate that the putative site on the bacterial ribosome which modulates codon readout has been conserved in highly specific detail through the course of evolution from prokaryotes to the unicellular eukaryote, *Saccharomyces cerevisiae*.

* * * *

During the preparation of this manuscript, Bayliss and Vinopal (1971) reported finding a mutant strain of S. cerevisiae in which streptomycin gave phenotypic suppression of a missense mutation. The streptomycin mutation segregated as a nuclear gene and cytoplasmic ribosomes of this strain were defective in subunit assembly at low temperature. In addition, these ribosomes were sensitive to streptomycin inhibition of protein synthesis in an in vitro system. These results resemble those in the E. coli system, and therefore tend to support the notion that there is a site on the yeast cytoplasmic ribosome which contributes to the fidelity of translation of the genetic code. It would be of interest to determine if streptomycin stimulates misreading with the ribosomes from this strain in an in vitro protein synthesizing system.

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BIBLIOGRAPHY

Anderson, F., L. Gorini, and L. Breckenridge. 1965. Role of ribosomes in streptomycin-activated suppression. Proc. Natl. Acad. Sci. U. S. 54:1076-1083.

Apirion, D., and D. Schlessinger. 1968. Coresistance to neomycin and kanamycin by mutations in an Escherichia coli locus that affects ribosomes. J. Bacteriol. 96:768-776.

Bayliss, F. T., and R. T. Vinopal. 1971. Selection of ribosomal mutants by antibiotic suppression in yeast. Science 174:1339-1341.

Bergmann, F. H. 1962. Bacterial amino acyl RNA synthetases. p. 708 to 718. In S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. V. Academic Press, N. Y.

Bose, K. K., C. L. Woodley, N. K. Chatterjee, and N. K. Gupta. 1969. Poly r-U directed ambiguous amino acid incorporation in the rabbit reticulocyte system. Biochem. Biophys. Res. Commun. 37:179-185.

Bretthauer, R. K., L. Marcus, J. Chaloupka, H. O. Halvorson, and R. M. Bock. 1963. Amino acid incorporation into protein by cell-free extracts of yeast. Biochemistry 2:1079-1084.

Crick, F. H. C. 1966. Codon-anticodon pairing. J. Mol. Biol. 19:548-555.

Davies, J., W. Gilbert, and L. Gorini. 1964. Streptomycin, suppression, and the code. Proc. Natl. Acad. Sci. U. S. 51:883-890.

Davies, J., L. Gorini, and B. D. Davis. 1965. Misreading of RNA codewords induced by aminoglycoside antibiotics. Mol. Pharmacol. 1:93-106.

Davies, J., D. S. Jones, and H. G. Khorana. 1966. A further study of misreading of codons induced by streptomycin and neomycin using ribopolynucleotides containing two nucleotides in alternating sequence as templates. J. Mol. Biol. 18:48-57.

Derr, R. F., and K. S. Scanlon. 1969. Fidelity of genetic code translation in a rat liver system. Biochim. Biophys. Acta 182:253-254.

Dietz, Jr., G. W., B. R. Reid, and M. V. Simpson. 1965. Ribosomes. A study of active and sluggish preparations. Biochemistry 4:2340-2350.

Downey, K. M., A. G. So, and E. W. Davie. 1965. Characterization of the polynucleotide-dependent transfer reaction in protein biosynthesis employing a cell-free system from the yeast Saccharomyces fragilis. Biochemistry 4:1702-1709.

Ennis, H. L., and M. Lubin. 1964. Cycloheximide: aspects of inhibition of protein synthesis in mammalian cells. Science 146:1474-1476.

Friedman, S. M., R. Berezny, and I. B. Weinstein. 1968. Fidelity in protein synthesis. The role of the ribosome. J. Biol. Chem. 243:5044-5048.

Friedman, S. M., and I. B. Weinstein. 1964. Lack of fidelity in the translation of synthetic polyribonucleotides. Proc. Natl. Acad. Sci. U. S. 52:988-996.

Gorini, L. 1969. The contrasting role of str A and ram gene products in ribosomal functioning. Cold Spring Harbor Symp. Quant. Biol. 34:101-109.

Gorini, L. 1971. Ribosomal discrimination of tRNAs. Nature New Biology 234:261-264.

Gorini, L., and E. Kataja. 1964. Phenotypic repair by streptomycin of defective genotypes in E. coli. Proc. Natl. Acad. Sci. U. S. 51:487-493.

Gruneberg-Manago M., and M. S. Bretscher. 1962. Polyribonucleotide-directed protein synthesis using an E. coli cell-free system. Nature 195:283-284.

Gruneberg-Manago, M., and J. Dondon. 1965. Influence of pH and sRNA concentration on coding ambiguities. Biochem. Biophys. Res. Commun. 18:517-522.

Heredia, C. F., and H. O. Halvorson. 1966. Transfer of amino acids from aminoacyl soluble ribonucleic acid to protein by cell-free extracts from yeast. Biochemistry 5:946-952.

Hirsh, D., and L. Gold. 1971. Translation of the UGA triplet in vitro by tryptophan transfer RNA's. J. Mol. Biol. 58:459-468.

Kurland, C. G. 1970. Ribosome structure and function emergent. Science 169:1171-1177.

Lamb, A. J., G. D. Clark-Walker, and A. W. Linnane. 1968. The biogenesis of mitochondria. 4. The differentiation of mitochondrial and cytoplasmic protein synthesizing systems in vitro by antibiotics. Biochim. Biophys. Acta 161:415-427.

Lamfrom, H., and M. Gruneberg-Manago. 1967. Ambiguities of translation of poly U in the rabbit reticulocyte system. Biochem. Biophys. Res. Commun. 27:1-6.

Mans, R. J., and G. D. Novelli. 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. Biophys. 94:48-53.

Matthaei, J. H., O. W. Jones, R. G. Martin, and M. W. Nirenberg. 1962. Characteristics and composition of RNA coding units. Proc. Natl. Acad. Sci. U.S. 48:666-677.

Nirenberg, M., and J. H. Matthaei. 1961. The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. Proc. Natl. Acad. Sci. U.S. 47:1588-1602.

Nomura, M., S. Mizushima, M. Ozaki, P. Traub, and C. V. Lowry. 1969. Structure and function of ribosomes and their molecular components. Cold Spring Harbor Symp. Quant. Biol. 34:49-61.

Ozaki, M., S. Mizushima, and M. Nomura. 1969. Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in Escherichia coli. Nature 222:333-339.

Pestka, S. 1966. Studies on the formation of transfer ribonucleic acid-ribosome complexes. J. Biol. Chem. 241:367-372.

Pestka, S., R. Marshall, and M. Nirenberg. 1965. RNA codewords and protein synthesis, V. Effect of streptomycin on the formation of ribosome-sRNA complexes. Proc. Natl. Acad. Sci. 53:639-646.

Petermann, M. L. 1964. The physical and chemical properties of ribosomes. Elsevier Publishing Co., Amsterdam.

Rosset, R., and L. Gorini. 1969. A ribosomal ambiguity mutation. J. Mol. Biol. 39: 59-112.

Sager, R. 1963. Cold Spring Harbor Symp. Quant. Biol. 28:577.

Sager, R., and M. G. Hamilton. 1967. Cytoplasmic and chloroplast ribosomes of Chlamydomonas: ultracentrifugal characterization. Science 157:709-711.

Sager, R., I. B. Weinstein, and Y. Ashkenazi. 1963. Coding ambiguity in cell-free extracts of Chlamydomonas. 140:304-306.

Schneider, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis. p. 680-684. In S. P. Colowick and N.O. Kaplan (ed.), Methods in enzymology, Vol. V. Academic Press, N.Y.

Scragg, A.H., H. Morimoto, V. Villa, J. Nekhorocheff, and H. O. Halvorson. 1971. Cell-free protein synthesizing system from yeast mitochondria. Science 171:908-910.

Sherman, M. I., and M. V. Simpson. 1969. The role of ribosomal conformation in protein biosynthesis: the streptomycin-ribosome interaction. Proc. Natl. Acad. Sci. U.S. 64:1388-1395.

So, A.G., J. W. Bodley, and E. W. Davie. 1964. The influence of environment on the specificity of polynucleotide-dependent amino acid incorporation into polypeptide. Biochemistry 3:1977-1982.

So, A. G., and E. W. Davie. 1963. The incorporation of amino acids into protein in a cell-free system from yeast. Biochemistry 2:132-136.

So, A. G., and E. W. Davie. 1964. The effects of organic solvents on protein biosynthesis and their influence on the amino acid code. Biochemistry 3:1165-1169.

So, A. G., and E. W. Davie. 1965. Effects of amino acids, s-RNA, and ethanol on coding ambiguity with polyuridylic acid. Biochemistry 4:1973-1979.

Speyer, J. F., P. Lengyel, C. Basilio, and S. Ochoa. 1962. Synthetic polynucleotides and the amino acid code, IV. Proc. Natl. Acad. Sci. U.S. 48:441-448.

Stavy, L. 1968. Miscoding in a cell-free system from spleen. Proc. Natl. Acad. Sci. U.S. 61:347-353.

Szer, W., and S. Ochoa. 1964. Complexing ability and coding properties of synthetic polynucleotides. J. Mol. Biol. 8:823-834.

Takeda, Y. 1969. Polyamines and protein synthesis. I. The effect of polyamines on cell free polyphenylalanine synthesis in E. coli. J. Biochem. Tokyo 66:345-349.

Takeda, Y., I. Suzuka, and A. Kaji. 1968. Comparative studies on specific and nonspecific binding of transfer ribonucleic acid to ribosomes. J. Biol. Chem. 243:1075-1081.

Tanaka, N., K. Sashikata, and H. Umezawa. 1967. Antibiotic-sensitivity of ribosomes from kanamycin-resistant E. coli. J. Antibiot. 20:115-119.

Weinstein, I. B. 1963. Comparative studies on the genetic code. Cold Spring Harbor Symp. Quant. Biol. 28:579-580.

Weinstein, I.B., S. M. Friedman, and M. Ochoa, Jr. 1966. Fidelity during translation of the genetic code. Cold Spring Harbor Symp. Quant. Biol. 31:671-680.

Weinstein, I.B., M. Ochoa, Jr., and S. M. Friedman. 1966. Fidelity in the translation of messenger ribonucleic acids in mammalian subcellular systems. Biochemistry 5:3332-3338.

Weinstein, I.B., and A. N. Schecter. 1962. Polyuridylic acid stimulation of phenylalanine incorporation in animal cell extracts. Proc. Natl. Acad. Sci. U.S. 48:1686-1691.

Zamenhof, S. 1957. Preparation and assay of deoxyribonucleic acid from animal tissue. p. 696 to 704. In S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, Vol. III.

Zimmerman, R. A., R. T. Garvin, and L. Gorini, 1971. Alteration of a 30S ribosomal protein accompanying the *ram* mutation in *Escherichia coli*. Proc. Natl. Acad. Sci. 68:2263-2267.