AMINO ACID ANALOGUES AND PROTEIN SYNTHESIS *IN VITRO*

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CHAPTER I

INTRODUCTION

Protein Synthesis

The steps involved in protein synthesis are well documented. These include the uptake of amino acids by the cell (1-4), the activation of the amino acids and formation of amino acyl ribonucleic acid derivatives by enzymes specific for each amino acid (5-14), the transfer of the amino acyl ribonucleic acids to ribosomes (15-20) as directed by mRNA* (21-25), the formation of peptide bonds, and the release of the finished protein from the ribosome (26-30).

Tissieres, Schlessinger and Gros (31), Kameyama and Novelli (32), and Matthaei and Nirenberg (33) reported the inhibition of amino acid incorporation into the protein by DNase in cell-free extracts. The inhibition of protein synthesis by DNase was thought to be due to the

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*The following abbreviations are used: mRNA, messenger ribonucleic acid; sRNA, soluble or acceptor ribonucleic acid; DNA, deoxyribonucleic acid; U, G, A and C, the nucleosides of uracil, guanine, adenine and cytosine respectively; AMP, ADP and ATP, adenosine mono-, di- and triphosphates respectively; poly U, poly uridylic acid; poly UC 5:1, poly uridylic-cytidylic acid 5:1; DNase, deoxyribonuclease; RNase, ribonuclease; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; GTPase, enzyme that cleaves GTP to give GDP and P_i; UL, uniformly labeled; pUpU, 5'-O-phosphoryl-uridylyl-(3'-5')-uridine; UpG, uridylyl-(3'-5')-guanosine (the letter p to the left of the nucleoside indicates a 5'-phosphate, the letter p to the right indicates a 3'-phosphate); EDTA, ethylenediamine tetraacetic acid; Tris, tris (hydroxymethyl) aminomethane; phe-phe, the dipeptide phenylalanylphenylalanine.

destruction of DNA and its resultant inability to function as a template for mRNA synthesis.

Subsequently, Nirenberg and Matthaei (34) observed that synthetic polyribonucleotides could stimulate the incorporation of amino acids into protein-like material in a cell-free extract of Escherichia coli. Poly U was shown to specifically stimulate the incorporation of phenylalanine into a protein having many of the characteristics of authentic poly-L-phenylalanine. This discovery provided a model system by which the steps of protein synthesis could be studied in detail, and also stimulated work on the elucidation of the amino acid code, i.e. the means by which the sequence of bases found in nucleic acids may be translated into a given amino acid sequence in a functional protein.

The stimulation of phenylalanine incorporation into trichloroacetic acid insoluble material by poly U has subsequently been observed in a variety of cell-free systems including rat liver and tumor systems (34, 35) ascites tumor components (36), a cell-free yeast system (37), and a cell-free system obtained from rabbit reticulocytes (38).

Work done principally in the laboratories of Nirenberg (39-41) and Ochoa (42, 43) has led to formulation of base doublets and triplets in mRNA which will act as code words for the incorporation of amino acids. Because the bases of the code word have been established using synthetic polynucleotides with random sequences, only the base composition and not the base sequence is known for the code words. Both investigators propose as code words for phenylalanine triplets containing three uridylic acid residues (UUU) and two uridylic-one cytidylic acid residues (2U1C).

That more than one code word may code for a single amino acid is

known as degeneracy. Incorporation *in vitro* of 17 amino acids by polynucleotides containing a random and wide variation of the four bases suggested a highly degenerate code (40). In addition, leucine acceptor sRNA has been found to have ambiguous coding properties (44). Countercurrent distribution studies of sRNA from *E. coli* have revealed several amino acid acceptor RNA's with multiple peaks (45).

Recently, Leder and Nirenberg (46, 47) studied the effect of trinucleotides on the binding of sRNA to ribosomes. The trinucleotides pUpUpU, pApApA, and pCpCpC directed the binding of phenylalanyl-, lysyl-, and prolyl-sRNA to ribosomes respectively. These results are in agreement with code words proposed from the results of amino acid incorporation studies *in vitro* using polynucleotides as messengers (37, 39). The trinucleotide GpUpU, but not its sequence isomers, UpGpU or UpUpG induced binding of valyl-sRNA to ribosomes, suggesting that an RNA code word for valine is GpUpU.

Using poly U in a cell-free system obtained from *E. coli*, Nirenberg, Matthaei and Jones (48) showed that sRNA was indeed an intermediate in the synthesis of poly phenylalanine. Poly U stimulated the incorporation of radioactivity from ^{14}C-L-phenylalanine-sRNA into trichloroacetic acid insoluble material. The incorporation of label was not diminished by the addition of unlabeled amino acid as was true when ^{14}C-L-phenylalanine was incorporated into protein.

The role of sRNA in coding for amino acid incorporation was further elucidated by Chapeville, Lipmann, von Ehrenstein, Weisblum, Ray and Benzer (49). Cysteinyl-sRNA was converted to alanyl-sRNA which was then incubated in an *E. coli* cell-free system using poly UG (5:1) as the messenger. Normally, poly UG stimulates the incorporation of

cysteine into trichloroacetic acid insoluble material but not alanine (41, 43), but in this case radioactive alanine was incorporated into protein. It was concluded that sRNA functions as an adaptor in specifying the fit of amino acids on a template.

The reactions that take place on the ribosome leading to peptide bond formation and release of the finished protein have been somewhat of an enigma. However, Conway (50) has studied the requirement for monovalent cations in the E. coli amino acid incorporation system. In the poly U dependent reaction, ammonium ion stimulation was superior to that of potassium ion. However, in other amino acid polymerizations, ammonium and potassium ion stimulations were similar in magnitude. A lag period in the polymerization of ^{14}C-L-phenylalanine was noticed which was eliminated by incubation of ribosomes, phenylalanyl-sRNA, poly U and ammonium or potassium ions. The monovalent cations seemed to be involved in binding of amino-acyl sRNA to ribosomes.

Spyrides (51) reported that the binding of ^{14}C-phenylalanyl-sRNA to ribosomes was dependent on poly U and ammonium or potassium ions. Ammonium ion was most effective in this reaction. Although ribosomes were washed repeatedly, and there was no indication of peptide bond formation, the possibility of enzymatic participation in the binding reaction could not be ruled out.

A partial separation of the E. coli amino acyl sRNA transfer factor into two complementary factors was reported by Allende, Monro and Lipmann (52). A ribosome-dependent GTPase activity was found to be associated with the "B" fraction, and GTPase activity was found to closely parallel amino acid incorporation (53).

Arlinghaus, Shaeffer and Schweet (54), using the rabbit reticulocyte

system, have resolved the reaction sequence at the ribosome site into two distinct enzymatic reactions. The first reaction utilized GTP in the binding of ^{14}C-phenylalanyl-sRNA to the poly U-ribosome template. When ribosomes with bound phenylalanyl-sRNA were extracted from the first reaction and incubated in the second reaction phe-phe was formed. Their data indicated that only two phenylalanyl-sRNA molecules could bind to one ribosome at a time, but "when both enzymes and GTP are present, longer polypeptide chains on fewer ribosomes are formed." The mechanism of peptide bond formation was inferred to be an alternating sequence of binding reaction and peptide synthetase reaction.

A study of the interaction of ribosomes with poly U showed that the RNA was bound to 30S, but not to 50S ribosomes (55). The observation that 30s ribosomes treated with formaldehyde bound less poly U than untreated ribosomes prompted the suggestion that binding may be through the phosphate of mRNA and amino groups of ribosomes (46).

In the E. coli-poly U system, which evidently does not release finished peptides into the medium, Gilbert (56) demonstrated that phenylalanyl-sRNA was bound to the 50s ribosome.

Gilbert (56) showed that poly phenylalanine synthesis takes place on polyribosomes formed by interaction of poly U and 70s ribosomes. Polyribosomal structures have been observed in reticulocyte systems (57, 58) and during viral infection of mammalian cell cultures (59). Hardesty, Miller and Schweet (60) have observed a concomitant breakdown of polyribosomes with hemoglobin synthesis in a cell-free reticulocyte system. Kiho and Rich (61) have detected β-galactosidase activity on bacterial polyribosomes after induction of E. coli spheroplasts with methyl-β-thiogalactopyranoside.

A pictorial representation of the path of protein synthesis is seen in Figure 1.

Amino Acid Analogues

Since the report that sulfonamide drugs exerted their effect by competing with p-aminobenzoic acid for an essential enzymatic site, numerous compounds analogous in structure to the natural amino acids have been synthesized and tested as metabolic antagonists (62).

The use of p-fluorophenylalanine as a phenylalanine antagonist has been extensively investigated and reviewed (63). The analogue, when added to a synthetic medium of exponentially growing cells, reduced the rate of growth of E. coli and yeast cells. However, the inhibition was immediately reversed by addition of phenylalanine.

Studies on the assimilation of amino acids in bacteria showed that radioactive p-fluorophenylalanine flowed rapidly into the intracellular amino acid pool and thence into cell protein (63). When similar concentrations of amino acid and analogue were present, phenylalanine was preferentially taken up and incorporated into cell protein. However, when p-fluorophenylalanine concentration in the medium was four times that of phenylalanine, some analogue did pass into the pool. Similar results were obtained with other bacterial cultures.

Certain analogues on gaining entry into a bacterial cell inhibit the synthesis of the natural amino acid (63). The analogues, p-fluorophenylalanine and 2-thiophenealanine, appeared to inhibit the biosynthetic pathway of phenylalanine synthesis. This inhibition would increase the relative concentration of the analogue in the cell, and greatly facilitate incorporation of the analogue into cell protein.

Figure 1

Path of Protein Synthesis

Explanation of Symbols:

AA - 1e = Amino Acid - 1 exterior

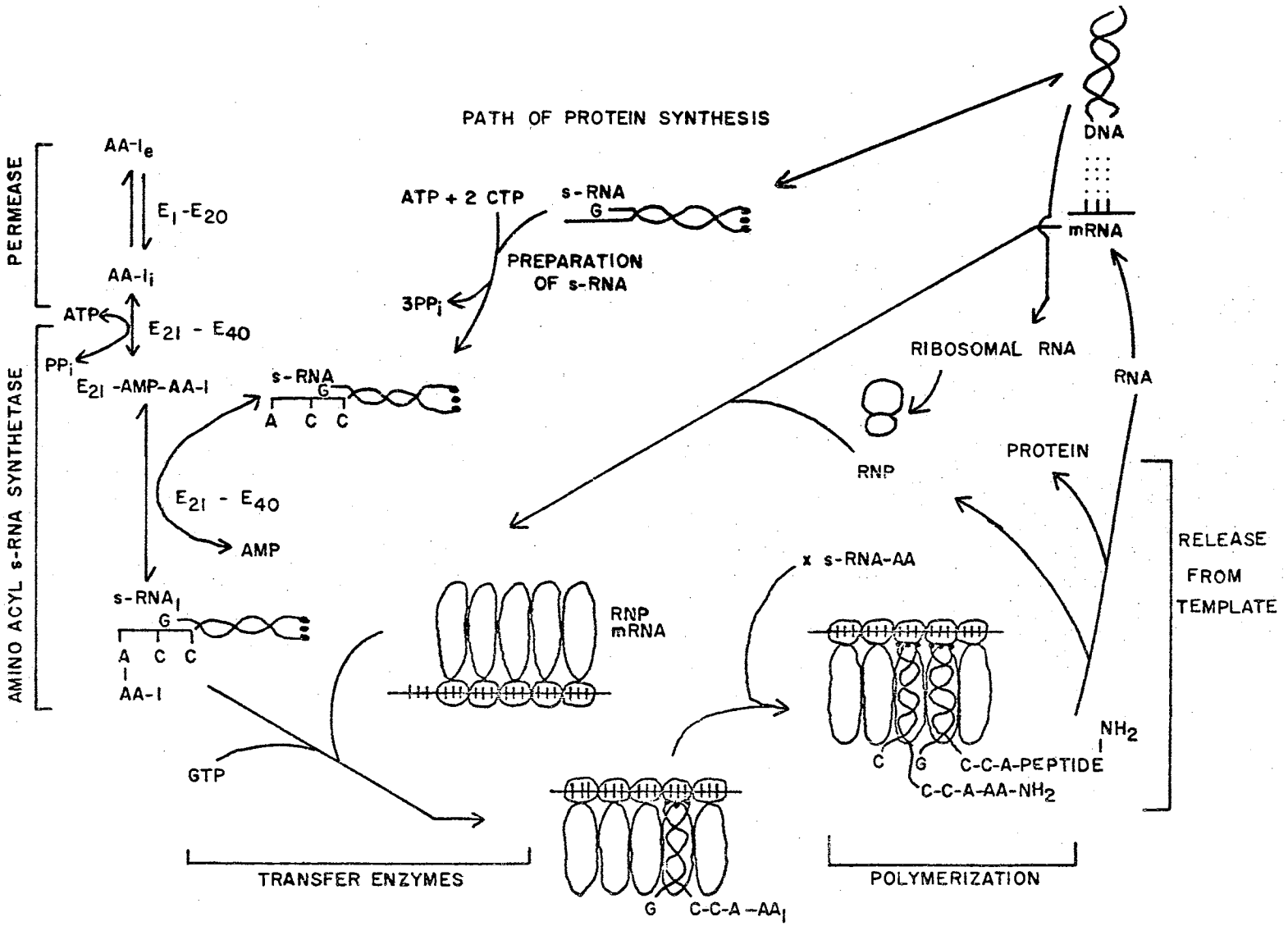
AA - 1i = Amino Acid - 1 interior

E_1 - E_{20} = $Enzyme_1$ - $Enzyme_{20}$

E_{21} - AMP - AA-1 = $Enzyme_{21}$ - Bound Amino Acyl-1-Adenosine Monophosphate

RNP = Ribonucleoprotein Particles

AA - NH_2 - Amino Terminal Amino Acid


PATH OF PROTEIN SYNTHESIS
DNA
mRNA
PERMEASE
AA-I$_e$
E$_1$-E$_{20}$
AA-I$_i$
ATP + 2 CTP
s-RNA
G
PREPARATION OF s-RNA
3PP$_i$
ATP
E$_{21}$ - E$_{40}$
PP$_i$
E$_{21}$ -AMP-AA-I
AMINO ACYL s-RNA SYNTHETASE
s-RNA
G
A C C
E$_{21}$ - E$_{40}$
AMP
RIBOSOMAL RNA
RNA
RNP
PROTEIN
s-RNA$_I$
G
A C C
AA-I
RNP
mRNA
x s-RNA-AA
RELEASE FROM TEMPLATE
GTP
C G C-C-A-PEPTIDE
NH$_2$
C-C-A-AA-NH$_2$
G C-C-A-AA$_I$
TRANSFER ENZYMES
POLYMERIZATION

As measured by ATP-PP_i exchange in an extract from E. coli, p-fluorophenylalanine was activated at only one-tenth the rate of phenylalanine; however, p-fluorophenylalanine could replace the majority of the phenylalanines in E. coli proteins when protein synthesis was one-half that occurring in the absence of the analogue (63). As measured by hydroxamate formation, phenylalanine and 2-thiophenealanine were activated equally, yet 2-thiophenealanine could not sustain protein and RNA synthesis in the absence of phenylalanine.

Using a purified enzyme from E. coli, Conway, Lansford and Shive (64) found that p-fluorophenylalanine was converted to the hydroxamate 95% as effectively as phenylalanine, and 2-thiophenealanine conversion was 58% as effective as the natural amino acid.

Westhead and Boyer (65) observed a 16-25% replacement of phenylalanine by p-fluorophenylalanine in the purified rabbit enzymes aldolase and glyceraldehyde 3-phosphate dehydrogenase. Properties of the p-fluorophenylalanine-containing enzymes were the same as the normal enzymes. Richmond (66) detected a random replacement of phenylalanine by p-fluorophenylalanine in the alkaline phosphatase(s) of E. coli.

Arnstein and Richmond (67) studied the utilization of phenylalanine and p-fluorophenylalanine in protein synthesis in a cell-free rabbit reticulocyte system. In the presence and absence of poly U as added messenger, incorporation of phenylalanine and the analogue were qualitatively the same. Phenylalanine markedly inhibited the activation of the analogue, but after attachment to sRNA no discrimination against the analogue was evident.

Richmond (67) could find no evidence of degeneracy in the code for phenylalanine in the reticulocyte system. It was concluded that

if degeneracy exists, the various phenylalanine acceptor RNA's must have similar specificities for phenylalanine and p-fluorophenylalanine. However, Nirenberg, Jones, Leder, Clark, Sly and Pestka (41) found evidence for the two code words (UUU, 2U1C) for phenylalanine in the E. coli system by comparing the stimulation of phenylalanine incorporation by UC copolymers of varying U content.

Assuming a code word consisting of three letters or bases, the maximum number of code words obtained from the four bases is 4^3 or 64. If the code word is a doublet the maximum number of code words is 4^2 or 16. Although there is evidence to indicate that some code words may be doublets (41), several lines of evidence suggest that three bases are involved in code word recognition (41, 68, 69). The possibility exists that all 64 possible base combinations may code for amino acid incorporation which would mean extensive degeneracy. It is also possible that some base combinations may have other meanings such as start or stop reading. A third possibility is that all possible base combinations may be code words in synthetic mRNA, but in the intact cell base sequence in mRNA and DNA is restricted.

The possibility of extensive degeneracy has been implied as cited previously. Although evidence for phenylalanine code word degeneracy has been presented, existing techniques have not demonstrated degeneracy at the phenylalanine acceptor RNA level as has been shown for other amino acids such as leucine.

The use of amino acid analogues may reveal a difference in specificity in the reactions the amino acid and analogue undergo during protein synthesis. Since p-fluorophenylalanine will compete with or be incorporated in place of phenylalanine into cellular protein, this

analogue has been used to test for phenylalanine acceptor RNA degeneracy.

A comparison of the activation and formation of amino acyl RNA of phenylalanine and p-fluorophenylalanine has been made. Incorporation of the amino acid and analogue from the free amino acid and from the amino acyl RNA into protein as stimulated by poly U and UC copolymers of varying U content has been studied. Preference of one of the phenylalanine acceptor RNA's for p-fluorophenylalanine and subsequent incorporation into protein by one of the code words would indicate degeneracy at the sRNA level.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

The sodium salts of ADP, UDP, GTP and the lithium salt of CDP were obtained from Schwarz Bio Research, Inc. The sodium salt of ATP was obtained from P-L Biochemicals, Inc. The silver-barium salt of phosphoenolpyruvate, pyruvate kinase (2.7.1.40), p-fluorophenylalanine, and p-fluorophenylalanine-1'-^{14}C, Sp. Act. 3.5 mC per mmole, were obtained from California Corporation for Biochemical Research. L-Phenylalanine-UL-^{14}C was obtained from New England Nuclear Corp. Sp. Act. 367 and 33.9 mC per mmole, and DL-phenylalanine-3-^{14}C Sp. Act. 1.1 mC per mmole from Volk Radiochemical Co. DNase (3.1.4.5), Muramidase (3.2.1.17) and dried Micrococcus lysodeikticus cells were obtained from Worthington Biochemical Corp. RNase (2.7.7.16) was obtained from Mann Research Laboratories, bovine serum albumin Fraction V from Nutritional Biochemicals, Inc. and silicic acid from Mallinkrodt Chemical Works. Generous gifts of phenylalanine antagonists are acknowledged as follows: o-aminophenylalanine from T. J. McCord and A. Davis; 2-thiophenealanine, from C. R. Crane; and cis-crotylalanine, 4-aminophenylalanine, 1-cyclopentenealanine, 2-amino-4-ethyl-4-hexanoic acid, p-tolylalanine, 2-pyridylalanine and ethallylglycine from C. G. Skinner. Alumina A305 was a gift from the Aluminum Corporation of America. E. coli B sRNA

was obtained from General Biochemicals and stripped of its amino acids by incubating in 0.5 N Tris HCl pH 8.1 at 35° for 45 minutes followed by dialysis at 4° against two changes of 10 volumes each of glass distilled water for 12 hours.

Methods

Purification of ^{3}H-p-Fluorophenylalanine

Acid-catalyzed tritium exchange on p-fluorophenylalanine was carried out by Volk Radiochemical Co. The labeled p-fluorophenylalanine was purified by ion exchange chromatography (70) and paper chromatography. The solution of radioactive p-fluorophenylalanine was placed on a 1.2 by 2.5 cm column of Dowex 50-X8 (100-200 mesh) which had been washed with 2 N HCl. The column was then washed with 10 ml each of 2 N and 3 N HCl. The radioactive analogue was eluted with 4 N HCl. The fractions containing p-fluorophenylalanine, as determined by measuring absorbance at 264 mμ (A_{264}), were lyophilized to dryness. The residue was dissolved in water and placed on a 1.2 by 3 cm column of Amberlite CG-400-X4 acetate. The analogue was eluted with 0.5 N acetic acid and fractions containing the ninhydrin peak were lyophilized to dryness. The residue was dissolved in water and chromatographed on Whatman 3MM paper utilizing descending technique and butanol-butanone-water (2:2:1) as the developing solvent. This solvent system effected a separation of phenylalanine and p-fluorophenylalanine. The sheet of paper was cut into strips and the radioactive peak located with a Nuclear-Chicago Model 100B paper strip scanner. The radioactive analogue was eluted with water and the solution was again passed through the Dowex 50 and Amberlite CG-400 columns. The final residue was dissolved

in water and adjusted to a concentration of 0.5 µmole per ml using a_M at 264 mµ of 808 M^{-1} cm^{-1}. The use of strong HCl as eluant, though rapid and convenient, did result in some loss of specific radioactivity. The final specific radioactivity was 67.5 mC per mmole.

Purification of Polynucleotide Phosphorylase

Polynucleotide phosphorylase (2.7.7.8) was purified from dried M. lysodeikticus based in part on the procedure of Singer and O'Brien (71, 72). Ten g of dried M. lysodeikticus were dispersed in 500 ml of 0.5% NaCl to which was added 25 mg of muramidase. The suspension was incubated at 37° for 15 minutes. To the viscous solution was added 1 M $MgCl_2$ to 0.01 M, and DNase to 0.5 µg per ml. The solution was incubated an additional 10 minutes at room temperature, and 20 minutes in an ice bath. The suspension was centrifuged at 14,000 x g for 20 minutes at 4° and the supernatant solution decanted (Fraction I). A small sample was dialyzed against cold glass distilled water for assay of enzyme activity.

All further operations were performed at 4°. To Fraction I was added with stirring 29 ml of 0.5% protamine sulfate, pH 5.0 (1.68 ml 0.5% protamine sulfate per 100 mg protein as determined by A_{280}:A_{260} ratio). The solution was stirred for 20 minutes and centrifuged at 14,000 x g for 20 minutes. The supernatant solution was decanted and 16.4 g solid $(NH_4)_2SO_4$ was added with stirring per 100 ml of supernatant solution (30% saturation). After 15 minutes the suspension was centrifuged at 10,000 x g for 15 minutes and the supernatant solution decanted. To the supernatant solution was added with stirring 21.4 g $(NH_4)_2SO_4$ per 100 ml (65% saturation). After 15 minutes the precipitate was collected

by centrifugation. The supernatant solution was discarded and the precipitate dissolved in 40 ml 0.1 M Tris HCl pH 8.1 and dialyzed against 10 volumes of the same buffer (Fraction II).

Fraction II was placed on a column of Sephadex G-75 (4.5 cm x 40 cm) which was equilibrated with 0.01 M Tris pH 8.1, 0.001 M EDTA, and 0.001 M β-mercaptoethanol and eluted with the same buffer. Fractions of 5 ml were collected at a rate of one ml per minute. Fractions containing enzyme activity were pooled (92 ml, Fraction III) and placed on a DEAE-cellulose column (3.6 cm x 10 cm) which was equilibrated with 0.01 M Tris, pH 8.1, 0.001 M EDTA and 0.001 M β-mercaptoethanol (mixer solution). A non-linear gradient elution was employed using 250 ml mixer solution in a 500 ml bottle (mixer flask) and 250 ml mixer solution made 0.5 M in NaCl in a 500 ml separatory funnel (reservoir). Fractions of 5 ml were collected at a rate of 2.5 ml per minute. Fractions containing enzymatic activity were pooled. The dilute protein solution was concentrated by first diluting the solution with 2 volumes of mixer solution and adsorbing the enzyme on a small DEAE-cellulose column (2.0 cm x 2.5 cm) prepared as described above. The enzyme was eluted with mixer solution that was 0.5 M in NaCl. The purified enzyme was dialyzed against the mixer solution to remove NaCl and stored at -20° (Fraction IV).

Assay for the enzyme was carried out in a reaction mixture containing 0.15 M Tris pH 9.0, 0.4 mM EDTA, 0.04 M ADP, 0.01 M $MgCl_2$, and enzyme in a volume of 0.5 ml. Samples of 0.1 ml were pipetted into an equal volume of cold 7% perchloric acid. After 5 minutes at 0° the volume was made to 1.0 ml with 3.5% perchloric acid, centrifuged and 0.5 ml of the supernatant solution removed for determination of P_i by

the method of Fiske and Subbarow (73). Units are expressed as μmole P_i released per mg protein per hour at 35°. In Table I is a resume of the purification procedure.

TABLE I

PURIFICATION OF POLYNUCLEOTIDE PHOSPHORYLASE

Fraction	$A_{280}:A_{260}$	μg Nucleic Acid per mg Protein	Specific Activity	Total KUnits
I	0.65	314	15.1	32.6
II	1.14	33.6	19.1	9.6
III	1.22	15.6	22.0	10.9
IV	1.45	5.3	60.0	12.6

Preparation of Synthetic Polyribonucleotides

Polymerization of the nucleoside diphosphates was carried out in 5-10 ml volumes with the same concentrations of buffer, Mg^{++} and EDTA as in the assay system. Sufficient enzyme was added to complete the reaction in about 2 hours as determined by the release of P_i. When the reaction was complete, the polymers were deproteinized by shaking with 0.25 volume of chloroform and 0.1 volume of isoamyl alcohol (74). After deproteinization, the polynucleotides were precipitated in the cold by addition of 0.1 volume 20% potassium acetate and 2 volumes cold ethanol. The precipitate was collected by centrifugation, dissolved in glass distilled water and dialyzed at 4° against 100 volumes of glass distilled water for 24 hours with 3 changes of water. The polynucleotides were again precipitated with potassium acetate-ethanol and the precipitate

successively washed by centrifugation with cold 75%, 85%, 95% and absolute ethanol, and finally with ether. The washed precipitate was air dried to remove most of the ether, then dried *in vacuo* over KOH pellets and paraffin. The polynucleotides were stored at room temperature in a dessicator containing KOH pellets and paraffin.

Preparation of Cell-Free Extract

E. coli, Crookes strain (obtained from E. Noller, Microbiology Department, Oklahoma State University), was grown in a dextrose-salts medium containing in g per liter: 3 $(NH_4)_2SO_4$; 7.35 K_2HPO_4; 3 KH_2PO_4; 1 NaCl; 0.1 $MgSO_4$; and dextrose as indicated. A 45-50 ml inoculum of 0.5% dextrose-salts was grown for 8-12 hours, and used to inoculate 3 liters of salts medium containing 1.25 mg per ml of dextrose. Following overnight incubation at 37° with aeration, the 3 liters of culture was poured into 7 liters of salts medium containing 50 g dextrose, and incubated at 37° with vigorous aeration. The increase of cell mass was determined by A_{630} measurements in a Bausch and Lomb Spectronic 20 spectrophotometer at 15 minute intervals. When the growth rate reached an increase of 0.2 A_{630} units per hour, and the A_{630} was 0.60 to 0.65, the cells were poured over an excess of cracked ice, harvested in a Sharples centrifuge cooled with a circulating bath kept at 5°, and washed by suspension and centrifugation.

The cell-free extract was prepared in a manner similar to that of Nirenberg and Matthaei (34). The cells were ground with twice their wet weight of alumina for 5 minutes at 5°. All subsequent steps were performed at this temperature. The enzymes were extracted with a volume of buffer containing 0.01 M Tris HCl pH 7.8; 0.02 M KCl; 0.01 M magnesium

acetate and 0.004 M β-mercaptoethanol (standard buffer) equal to twice the weight of cells. The extract was centrifuged at 10,000 x g to remove alumina and debris. DNase was added to the supernatant fluid to make 0.5 µg per ml. The supernatant solution was then centrifuged twice more at 30,000 x g for 15 minutes each. The final supernatant fluid (30S) was incubated for 40 minutes at 35° in a medium containing the following in µmoles per ml: 60 Tris, pH 7.8; 55 KCl; 15 magnesium acetate; 6 mercaptoethanol; 0.075 each of 20 amino acids (glycine, alanine, serine, aspartic acid, asparagine, glutamic acid, glutamine, isoleucine, leucine, cysteine, cystine, histidine, tyrosine, tryptophan, proline, threonine, methionine, phenylalanine, arginine and lysine); 2 ATP; 1 phosphoenolpyruvate, potassium salt; and 15 µg pyruvate kinase. The incubated extract was dialyzed against 60 volumes standard buffer for 6-10 hours and then frozen and stored in liquid nitrogen (Incubated 30S).

Sometimes the 30S extract was dialyzed against standard buffer and centrifuged at 105,000 x g for 2 hours to precipitate the ribosomes. The supernatant solution was aspirated to within 1-2 cm of the precipitate, frozen and stored in liquid nitrogen (105S).

The ribosome precipitate was washed with standard buffer, suspended in 0.01 M Tris, pH 7.2 and 10^{-4} M magnesium acetate and dialyzed overnight against the same buffer. RNase was added to the dialysate to a concentration of 10 µg per ml and incubated for 15 minutes at 20°. The ribosomes were precipitated by centrifugation at 105,000 x g for 2 hours and suspended in 0.02 M Tris, pH 7.2, 0.05 M KCl and 0.02 M magnesium acetate. The ribosomes were washed twice by centrifugation and suspended in the same buffer. The final ribosome suspension was centrifuged at

6000 x g for 15 minutes, and the supernatant solution decanted, frozen in liquid nitrogen and stored at -20°.

Amino Acid Incorporation

The assay medium generally used was a modification of that employed by Conway (50). The assay mixture for the incorporation of the amino acid contained in a volume of 0.25 ml the following in μmoles: 25 Tris HCl pH 7.8; 1 β-mercaptoethanol; 12.5 NH_4Cl or KCl; 10 magnesium acetate; 0.5 ATP; 1 phosphoenolpyruvate; 0.1 GTP; 10 μg pyruvate kinase; 0.2 μC L-phenylalanine-UL-^{14}C (5.4 to 5.9 x 10^{-4} μmoles); or 0.02 μmole DL-p-fluorophenylalanine-^{3}H. Polynucleotide and incubated 30S extract additions are indicated in the legends. Incubation was for 20 minutes at 35°.

For the incorporation of the amino acid from amino acyl sRNA, the sole source of energy was 0.2 μmole of GTP. The reaction mixture contained approximately 3900 counts per minute ^{14}C-phenylalanyl-sRNA or 1800 counts per minute ^{3}H-p-fluorophenylalanyl-sRNA. Additions of polynucleotide and incubated 30S extract are indicated in the legends. Incubation was for 10 minutes at 35°. Details of other reaction mixtures are given in the legends.

A 0.1 ml sample of the assay mixture was pipetted onto a 2.3 cm diameter Whatman 3MM paper disk in a stream of warm air to facilitate absorption of the fluid by the disk and processed according to the O'Neal and Griffin (35) modification of the method of Mans and Novelli (76). The paper disks, numbered and mounted on straight pins, were immersed in cold 10% trichloroacetic acid (5-7 ml per disk) and swirled for at least 10 minutes at 0°. The disks were then washed in 5%

trichloroacetic acid and extracted with hot 5% trichloroacetic acid at 90° for 7 minutes. The disks were then washed twice with ethanol and a final ether wash facilitated drying under a stream of warm air. Radioactivity on the disks was measured with a Model 3003 Packard Tri-Carb liquid scintillation spectrometer in 6 ml of a 100% toluene scintillation fluid containing 4 g per liter of diphenyloxazole and 0.2 g per liter of 1,4-di-(2-(5-phenyloxazoyl) benzene. Counting efficiency was 55-60% for ^{14}C and 4-6% for ^{3}H.

Amino Acyl sRNA Formation

Radioactive phenylalanine or p-fluorophenylalanine was charged to sRNA by a modification of the method of Conway (50). The reaction mixture contained the following components per ml of reaction mixture: 0.4 μmole ATP; 100 μmole Tris HCl pH 7.2; 10 μmole $MgCl_2$; 4 μmole β-mercaptoethanol; 0.75 μC L-phenylalanine; or 0.02 μmole p-fluorophenylalanine-^{3}H; 5 mg sRNA (stripped of its amino acids); and 0.4-1.0 mg dialyzed 105S extract. Incubation was for 10 minutes at 35°. The reaction was terminated by shaking with an equal volume of distilled 90% phenol for 6 minutes. The mixture was centrifuged, the aqueous layer removed and the phenol layer washed with a small volume of water. The aqueous phases were combined and the charged sRNA was precipitated by addition of 0.1 volume of 20% potassium acetate and 2 volumes of cold ethanol. The precipitate was collected by centrifugation, washed with cold 75% ethanol, dissolved in a small volume of glass distilled water and dialyzed at 4° overnight against 2 changes of 100 volumes each of glass distilled water.

Fractionation of Amino Acyl sRNA on a Methylated Albumin Column

Because of the low specific activity of the p-fluorophenylalanyl-sRNA (approximately 500 counts per minute per mg RNA), silicic acid (76) was used as an inert support which gave columns of greater capacity than kieselguhr (77)

Methylated albumin was prepared by the method of Mandell and Hershey (78). Twenty g of albumin were suspended in 2 liters of absolute methyl alcohol, to which was added 32.8 ml of 12 N HCl. The mixture was allowed to stand for 5 days with occasional shaking. The precipitate was collected by centrifugation and washed twice with methyl alcohol and then with ether. Most of the ether was removed by evaporation in air, and the methylated albumin was dried over KOH pellets and paraffin in vacuo. The material was then ground to a powder.

Silicic acid was suspended in water, decanted several times to remove fine particles, washed with 1 N HCl and water and then dried. Solutions containing various concentrations of NaCl buffered at pH 5.4 with 0.02 M sodium acetate were prepared, and will be referred to by NaCl concentration only.

Ten g of silicic acid were suspended in 0.2 M NaCl and stirred briefly with a water solution containing 0.5 g methylated albumin. The slurry was poured into a 1.8 x 18 cm column. The fluid was allowed to drain to the top of the column and the packed column was washed with 50 ml of 0.2 M NaCl. The radioactive amino acyl sRNA was placed on the column and a linear gradient of increasing NaCl concentration was employed. Other details are given in the legends. The A_{260} of each fraction was determined and 0.5 ml of each fraction was put in 10 ml Bray's liquid scintillation solution (79) for radioactivity measurement.

Counting efficiency was 45-50% for ^{14}C and 12-17% for ^{3}H.

In a preparative procedure, 200 mg of E. coli B sRNA and about 20,000 counts per minute of ^{14}C-phenylalanyl-sRNA were placed on a 3.7 x 30 cm column containing 60 g silicic and 2.5 g methylated albumin which was prepared as described above. The column was developed with a linear gradient consisting of 400 ml 0.2 M NaCl in the mixer, and 400 ml 1.2 M NaCl in the reservoir. Fractions of 5 ml were collected at a rate of 4-5 ml per minute. Fractions containing radioactivity were pooled, diluted with 2 volumes water, and the RNA adsorbed from the solution by stirring with ECTEOLA cellulose which had been washed with 1 M HCl and water. The RNA was eluted from the cellulose with 1 M NaCl and the eluate dialyzed at 4° for 36 hours against 3 changes of 60 volumes each of glass distilled water to remove the NaCl. The solution was lyophilized to dryness and the resulting RNA, about 40 mg, was used for charging p-fluorophenylalanine.

CHAPTER III

RESULTS

Analysis of Polynucleotides

The synthetic polyribonucleotides for amino acid incorporation in the E. coli cell-free system were characterized by base analysis and sucrose density gradient centrifugation. The results of the base analysis are seen in Table II. The input ratios of UDP:CDP in the polymerization reaction mixtures were 5:1, 3:1, 1:1 and 1:3. However, higher ratios of UMP to CMP were found in the latter two polymers.

The relative chain length of the polymers as determined by sucrose density gradient centrifugation was found to parallel the amino acid incorporating ability of the polymers under the conditions generally employed for cell-free protein synthesis. Figure 2 shows a comparison of two samples of poly U fractionated by sucrose density gradient centrifugation. The open circles show a relatively high concentration of A_{260} material in regions of greater sucrose density (fractions 10 to 20) and this sample of poly U was quite active, stimulating phenylalanine incorporation up to 40 fold. The poly U represented by the closed circles contained very little material in the more dense region of the gradient, and a very high proportion of the poly U did not sediment appreciably. This polymer did not stimulate phenylalanine incorporation with the assay conditions generally employed.

TABLE II

BASE ANALYSIS OF POLYNUCLEOTIDES

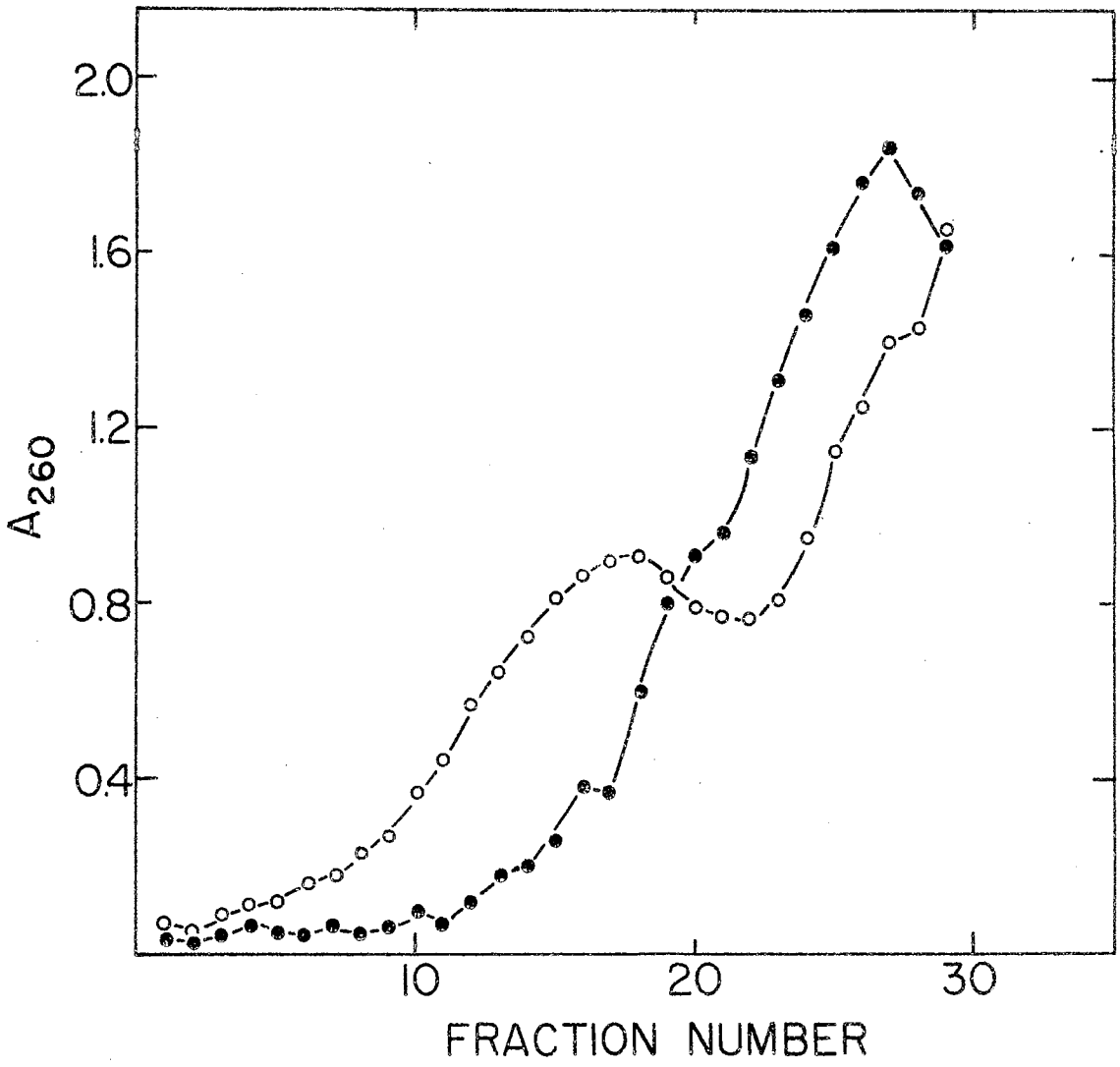
Percent Mononucleotide* U	C	Polynucleotide UC Ratio
81.7	18.3	UC 5:1
76.8	23.2	UC 3:1
67.2	32.8	UC 2:1
35.2	64.8	UC 1:2

*Approximately 500 µg of polynucleotide were hydrolyzed in 0.5 ml of 0.3 N KOH at 37° for 18 hours (80). The solution was then neutralized with 6 N $HClO_4$, and the resulting precipitate of $KClO_4$ removed by centrifugation. The supernatant solution was made 0.05 N in HCl and applied to the top of 1.1 cm x 4.5 cm Dowex 50-X8 (100-200 mesh) column that had been prepared by washing with 3 N HCl, water until neutral, then with 20 ml 0.05 N HCl. The UMP was quantitatively eluted with 6 ml of 0.05 N HCl. Following this GMP was eluted with 7.5 ml of water and AMP and CMP were eluted together with the next 25 ml of water wash. All eluants were made 0.05 N in HCl. Absorbancies of the solutions were determined and the concentrations of the mononucleotides calculated as indicated (80).

Figure 2

Sucrose Density Gradient Fractionation of Poly U Preparations

Relative size of the polymers was determined by sucrose density gradient centrifugation using a modification of the method employed by Matthaei, Jones, Martin and Nirenberg (39). A linear gradient of sucrose concentration from 20% at the bottom to 5% at the top of the tube was employed. The sucrose solutions contained 5×10^{-3} M sodium cacodylate, pH 6.8, and 0.1 M NaCl. Approximately 0.5 mg of the polynucleotide was dissolved in 0.2 ml of the 5% sucrose solution and layered on top of the gradient to give a total volume of 4.4 ml per tube. The tubes were centrifuged in a Spinco SW-39 rotor for 10 hours at 3° using a Spinco Model L centrifuge at 35,000 rpm. Two drop fractions were collected from the bottom of the tube with a #22 hypodermic needle. One ml of water was added to each fraction and A_{260} of the fractions were ○, preparation I; ●, preparation II.


2.0
1.6
1.2
0.8
0.4
A260
10
20
30
FRACTION NUMBER

Poly UC 5:1 was composed almost entirely of rapidly sedimenting material (Figure 3), and this polymer was quite active in stimulating phenylalanine incorporation. Poly UC 3:1, 2:1 and 1:2 gave similar A_{260} profiles as exemplified by poly UC 3:1 in Figure 4. These polymers consisted largely of material that did not sediment appreciably in the density gradient.

Characteristics of Amino Acid Incorporation System

The synthetic polyribonucleotides were used as mRNA in a cell-free system obtained from E. coli. Some of the indications that amino acid incorporation in a cell-free system into trichloroacetic insoluble material is equivalent to protein synthesis are dependence on: exogenous mRNA; ATP and an ATP regenerating system; GTP; ribosomes; enzymes in the ribosome-free supernatant solution; and amino acids (33, 34). The incorporation is also inhibited by RNase and inhibitors of protein synthesis.

In the system employed in this study ribosomes were not separated from the supernatant fluid. DNase was used to destroy DNA and thus prevent any natural mRNA synthesis. An incubation with 20 amino acids, buffer, metal ions and energy as required was used to destroy endogenous mRNA. The characteristics used to show protein synthesis in vitro were: dependence on exogenous mRNA; requirement for ATP, GTP and an ATP regenerating system and inhibition by puromycin. These characteristics are seen in Table III.

Optimum phenylalanine incorporation into trichloracetic acid-insoluble material occurred at 50 µg poly U per ml of reaction mixture and this concentration of polynucleotide was used in most of the

Figure 3

Sucrose Density Gradient Fractionation of Poly UC 5:1

Experimental details are given in the legend of Figure 2.

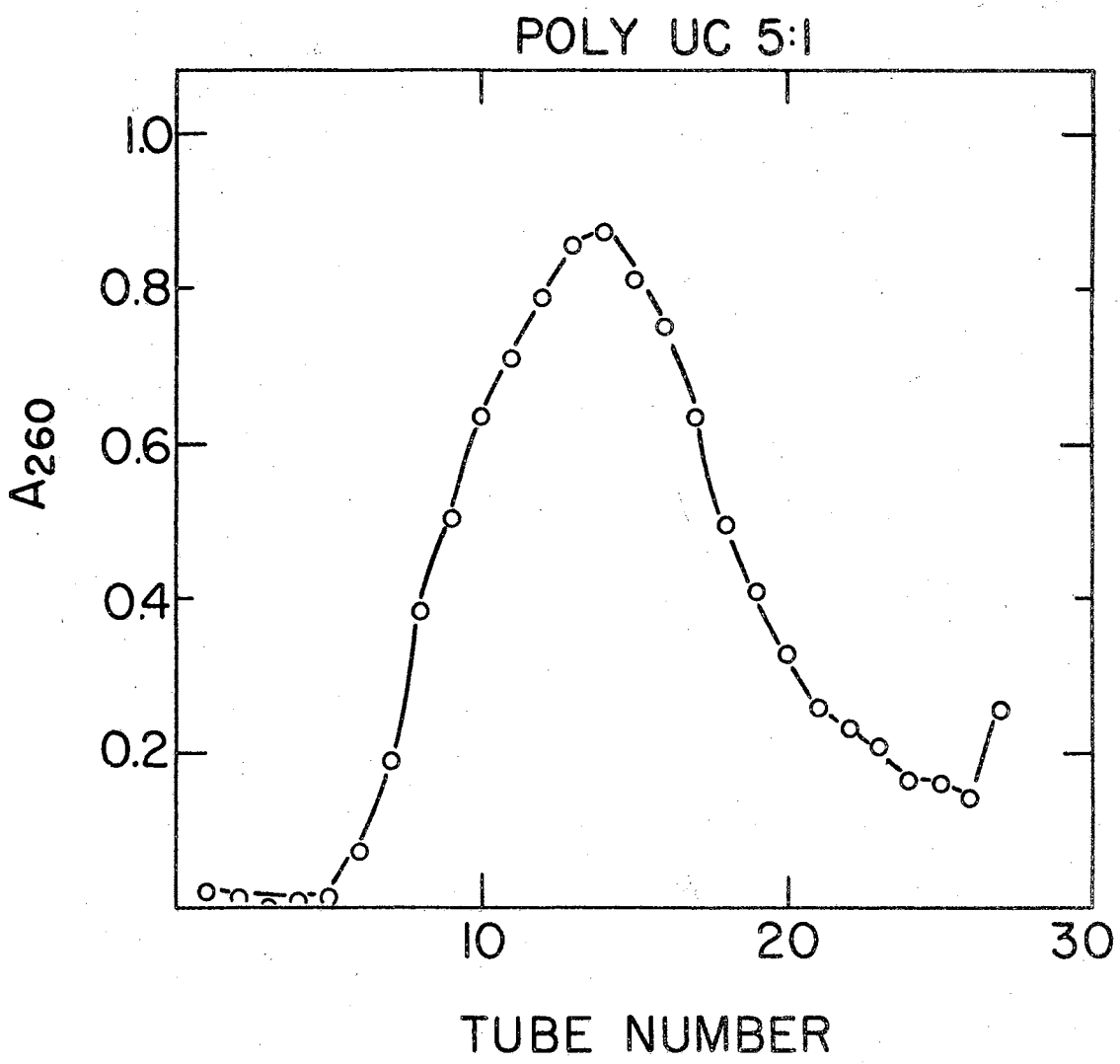

POLY UC 5:1
1.0
0.8
0.6
0.4
0.2
A260
10
20
30
TUBE NUMBER

Figure 4

Sucrose Density Gradient Fractionation of Poly UC 3:1

Experimental details are given in the legend of Figure 2.

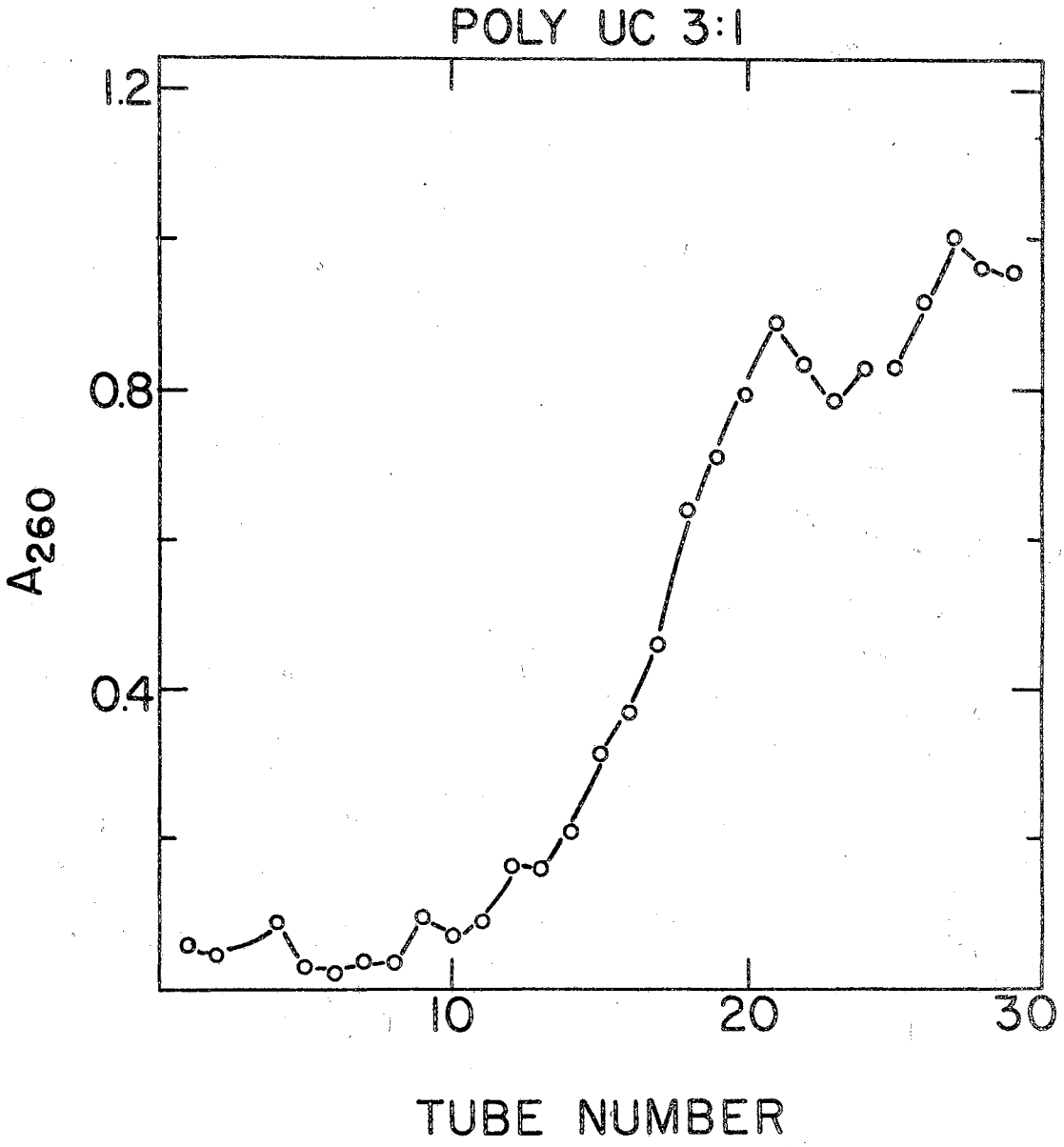

POLY UC 3:1
1.2
0.8
0.4
A260
10
20
30
TUBE NUMBER

TABLE III

CHARACTERISTICS OF THE CELL-FREE AMINO ACID INCORPORATING SYSTEM

Additions	μμmole Phenylalanine-^{14}C Incorporated	
	KCl	NH_4Cl
Complete*	107	152
-Poly U	3.4	2.8
-ATP, GTP and ATP regenerating system	1.3	1.8
+Puromycin, 0.02 μmole	25.9	17.1
	μμmole p-Fluorophenylalanine-^{3}H Incorporated	
Complete*		960
-Poly U		3.6
-ATP, GTP and ATP regenerating system		3.2
+Puromycin, 0.02 μmole		3.4

*The complete system contained the components described previously plus 12.5 μg poly U, 0.75 mg incubated 30S extract, puromycin and 12.5 μmoles monovalent cation as indicated.

experiments. However no inhibition of phenylalanine incorporation was apparent at concentrations of poly U up to 100 µg per ml reaction mixture. The incorporation of phenylalanine was stimulated 30 fold by poly U with KCl as the monovalent cation, and 50 fold with NH_4Cl. With NH_4Cl a 28% to 41% incorporation of the label was routinely observed and NH_4Cl was subsequently used as the monovalent cation. Stimulation of phenylalanine incorporation was dependent on ATP, GTP and an ATP regenerating system. Puromycin, known to be an inhibitor of protein synthesis (81), decreased the poly U stimulation of phenylalanine incorporation by 76% to 89%.

The incorporation of p-fluorophenylalanine in the cell-free system was stimulated 25 fold by poly U and this incorporation was dependent on ATP, GTP and an ATP regenerating system. Puromycin inhibited analogue incorporation by more than 99%.

The reaction mixtures contained 5.45×10^{-4} µmoles of phenylalanine, or 2×10^{-2} µmoles of DL-p-fluorophenylalanine which gave optimum p-fluorophenylalanine incorporation. This explains the greater incorporation of p-fluorophenylalanine.

The incorporation of phenylalanine and p-fluorophenylalanine from sRNA is dependent on poly U (Table IV). The partial dependence of incorporation on GTP may be explained by traces of nucleotides remaining in the crude system. The poly U-stimulated incorporation was inhibited by puromycin. The action of puromycin is to react with the carboxyl end of peptidyl-sRNA ending the sequential extension of the peptide chain (81). Since amino acyl sRNA was present at high concentrations, it is likely that puromycin concentration is not great enough to give optimum inhibition.

TABLE IV

INCORPORATION OF AMINO ACID FROM sRNA

Additions	Counts Per Minute Incorporated	
	Phenylalanine	p-Fluorophenylalanine
Complete*	1770	388
-Poly U	122	68
-GTP	648	177
+Puromycin, 0.015 μmole	300	169

*The reaction mixture contained the components described previously, 0.75 mg incubated 30S extract, 50 μg poly U per ml reaction mixture, puromycin as indicated.

Effect of Analogues on Phenylalanine-^{14}C Incorporation

Many phenylalanine analogues have been synthesized and tested as phenylalanine antagonists. Conway, Lansford and Shive (64, 82) tested a large number of phenylalanine analogues on the growth of E. coli. They reported that p-fluorophenylalanine, 2-thiophenealanine and 2-pyridylalanine were inhibitory to growth of the wild strain of the organism and these analogues were also converted to the hydroxamate by the purified enzyme. These analogues, along with 4-aminophenylalanine inhibited growth of a phenylalanineless mutant at inhibitor to substrate ratios of 10 or less. Of these analogues, p-fluorophenylalanine inhibited phenylalanine conversion to its hydroxamate at an inhibitor to substrate ratio of 10. The other analogues required ratios of 100-200 for such inhibition. The other analogues

(cis-crotylalanine, 1-cyclopentenealanine, 2-amino-4-ethyl-4-hexanoic acid, p-tolylalanine and ethallylglycine) did not inhibit growth of the wild strain of E. coli. Of these latter analogs only 1-cyclopentenealalanine was converted to the hydroxamate by the purified enzyme. The potent inhibition of growth of E. coli by o-aminophenylalanine was reversed competitively by phenylalanine (83).

Of the analogues cited above only p-fluorophenylalanine inhibited the poly U stimulation of phenylalanine incorporation in the cell-free system and 1-cyclopentenealanine was without effect (Table V). All the other analogues caused an increase in the poly U stimulation of phenylalanine incorporation ranging from 50% for 2-amino-4-ethyl-4-hexanoic acid to 108% for p-tolylalanine at the lower concentration used. Increasing the concentration of 2-thiophenealanine, pyridylalanine and ethallyglycine resulted in a further increase in phenylalanine incorporation. One possible interpretation of these results is that the analogues inhibited reactions competing for phenylalanine.

Kinetics of Amino Acid Activation and Incorporation

A significant difference in the kinetics and extent of phenylalanine and p-fluorophenylalanine activation and incorporation may give an indication as to preference for the natural amino acid and phenylalanine code word degeneracy. Therefore a comparison of the kinetics of activation, formation of amino acyl sRNA and incorporation from the amino acid and from amino acyl sRNA into protein has been made.

Hydroxamate Formation

Figure 5 compares the activation of phenylalanine and p-fluoro-

TABLE V

EFFECT OF ANALOGUES ON THE POLY U STIMULATION OF PHENYLALANINE-^{14}C INCORPORATION

Additions in mμmoles		μμmoles Phenylalanine-^{14}C Incorporated	Percent of Control
None*		42.4	100.0
L-Phenylalanine-^{12}C	5	19.6	46.3
DL-p-Fluorophenylalanine	10	29.6	69.8
	20	25.5	60.1
2-Thiophenealanine	10	67.2	158.5
	20	76.6	181.0
2-Aminophenylalanine	10	66.8	157.5
	20	62.4	147.1
cis-Crotylalanine	10	77.6	183.2
	20	54.0	127.3
4-Aminophenylalanine	10	86.0	203.0
	20	67.2	158.6
1-Cyclopentenealanine	10	41.6	98.2
	20	52.8	124.7
2-Amino-4-Ethyl-4-Hexanoic Acid	10	64.0	151.0
	20	47.2	111.3
p-Tolylalanine	10	88.4	208.5
	20	79.2	187.2
2-Pyridylalanine	10	72.8	172.0
	20	76.8	181.5

TABLE V (CONTINUED)

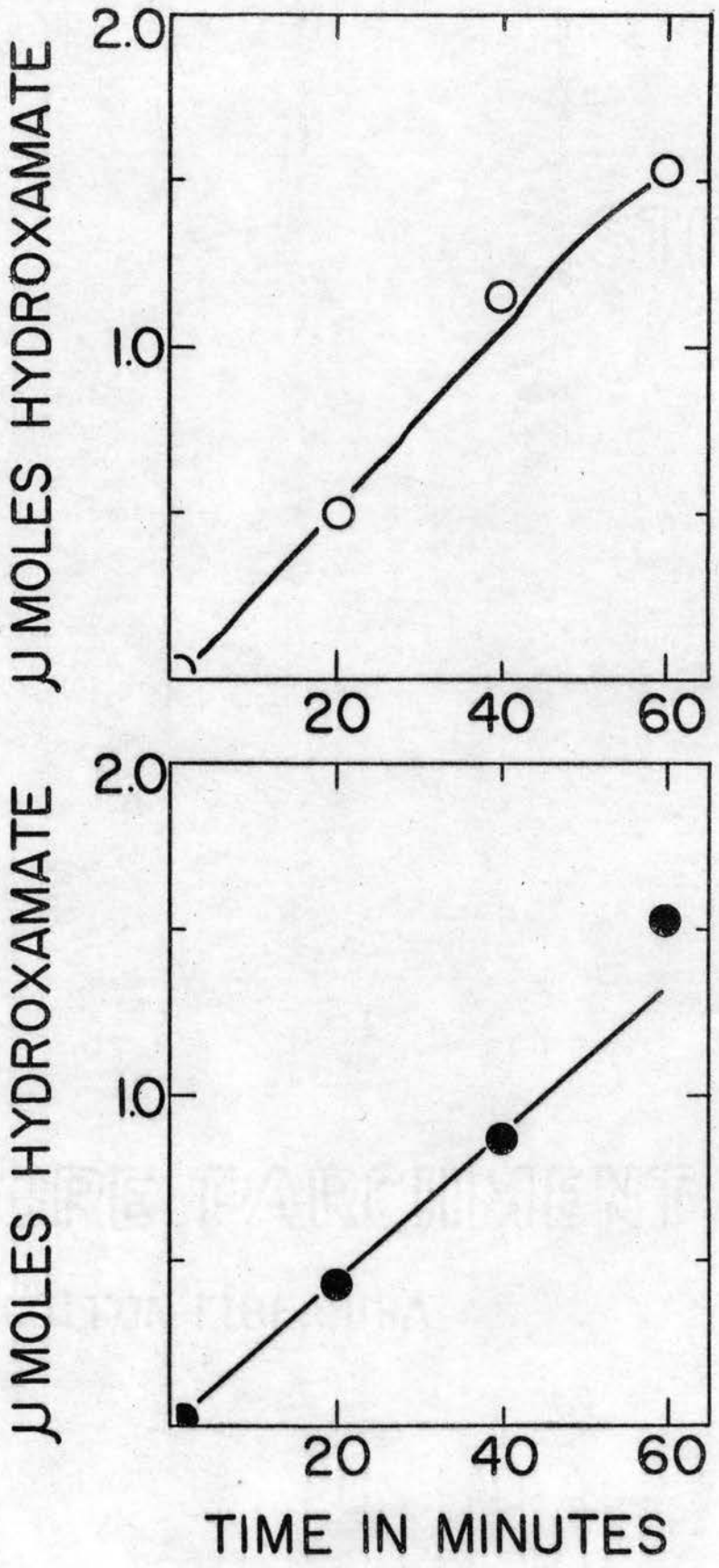
Additions in mμmoles		μμmoles Phenylalanine-^{14}C Incorporated	Percent of Control
Ethallylglycine	10	68.8	162.6
	20	74.8	176.7

*The reaction mixture contained in 0.4 ml the following in μmoles: 40 Tris pH 7.8; 35 KCl; 5 magnesium acetate; 1.6 mercaptoethanol; 0.5 ATP; 1 phosphoenolpyruvate; 1 x 10^{-3} GTP; 10 μg pyruvate kinase; 0.2 μC L-phenylalanine-^{14}C (545 μμmoles); or 0.02 μmole DL-p-fluorophenylalanine-^{3}H; 10 μg poly U; and 2.15 mg 30S extract. Incubation was for 1 hour at 35°. Samples of 0.1 ml were absorbed on paper disks and the disks processed as described previously.

Figure 5

Kinetics of Hydroxamate Formation

The reaction mixture contained in 1.0 ml the following in μmoles: 100 Tris pH 7.2; 10 ATP; 10 $MgCl_2$; 1500 neutralized hydroxylamine hydrochloride; 2 L-phenylalanine; or 4 DL-p-fluorophenylalanine; and 1.5 mg 105S extract. Incubation was for the indicated time at 35°. The reaction was stopped by the addition of 3 ml of a solution containing 10% $FeCl_3$, 10% trichloroacetic acid and 0.67 N HCl (84). The protein precipitate was removed by centrifugation and filtration and A_{500} determined in a Bausch and Lomb Spectronic 20 spectrophotometer against a standard of succinic hydroxamic acid. Upper figure, phenylalanine; lower figure, p-fluorophenylalanine.


2.0
1.0
20
40
60
2.0
1.0
20
40
60
J MOLES HYDROXAMATE
J MOLES HYDROXAMATE
TIME IN MINUTES

phenylalanine as measured by hydroxamate formation. There was little difference in either the rate or amount of activation between phenylalanine and the analogue. This concurs with the report of Conway, Lansford and Shive (64) that a purified enzyme preparation from E. coli activated p-fluorophenylalanine 95% as efficiently as phenylalanine.

Amino Acyl sRNA Formation

A comparison of the formation of phenylalanyl- and p-fluorophenylalanyl-sRNA is seen in Figure 6. Under conditions in which sRNA was limiting and similar concentrations of phenylalanine and fluorophenylalanine were present in the reaction mixtures, the sRNA accepted a greater amount of phenylalanine than p-fluorophenylalanine. This indicates that p-fluorophenylalanine is being charged preferentially to one of the phenylalanine acceptor RNA's. The preparation of phenylalanyl-sRNA was routinely accomplished; however, the charging of sRNA with p-fluorophenylalanine was only sporadically successful. The reason for the difficulty of preparation of p-fluorophenylalanyl-sRNA is not known.

Incorporation of Amino Acid

The kinetics of the incorporation of phenylalanine and p-fluorophenylalanine into protein is observed in Figure 7. The concentration of p-fluorophenylalanine in the reaction mixture was four times that of phenylalanine, which explains the greater incorporation of p-fluorophenylalanine. Since the relative saturation of the amino acid and analogue in the system has not been established, no conclusions can be made as to rate or extent of incorporation.

Figure 6

Kinetics of Amino Acyl sRNA Formation

The assay mixture contained in 0.8 ml the following in μmoles: 80 Tris HCl, pH 7.4; 40 $MgCl_2$; 2 mercaptoethanol; 4 ATP; 0.4 μC phenylalanine-3-^{14}C, Sp. Act. 2.44, or 0.4 μC p-fluorophenylalanine-1'-^{14}C, Sp. Act. 3.5; 0.45 mg 105S extract; and 1.92 mg E. coli B sRNA.

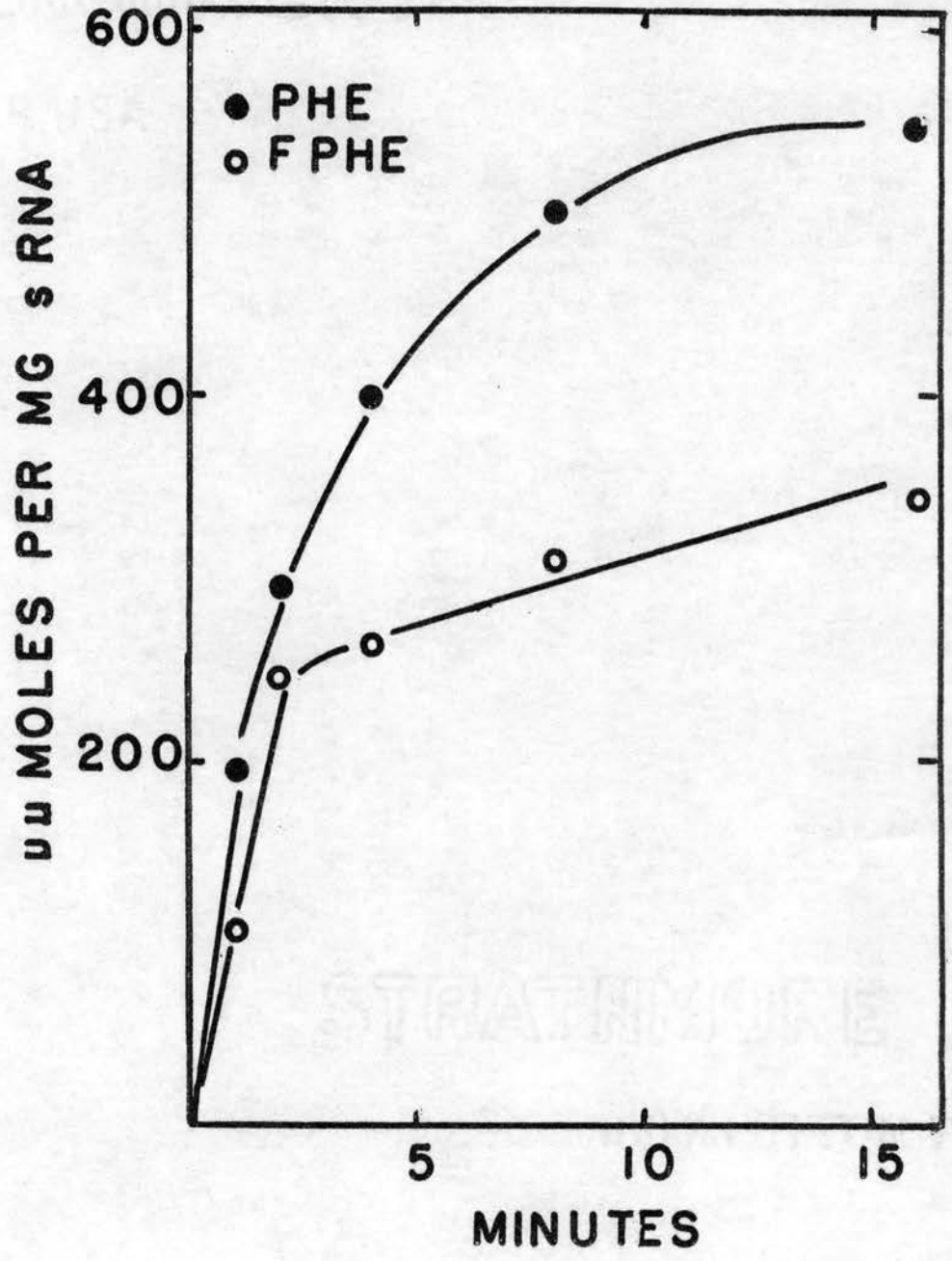
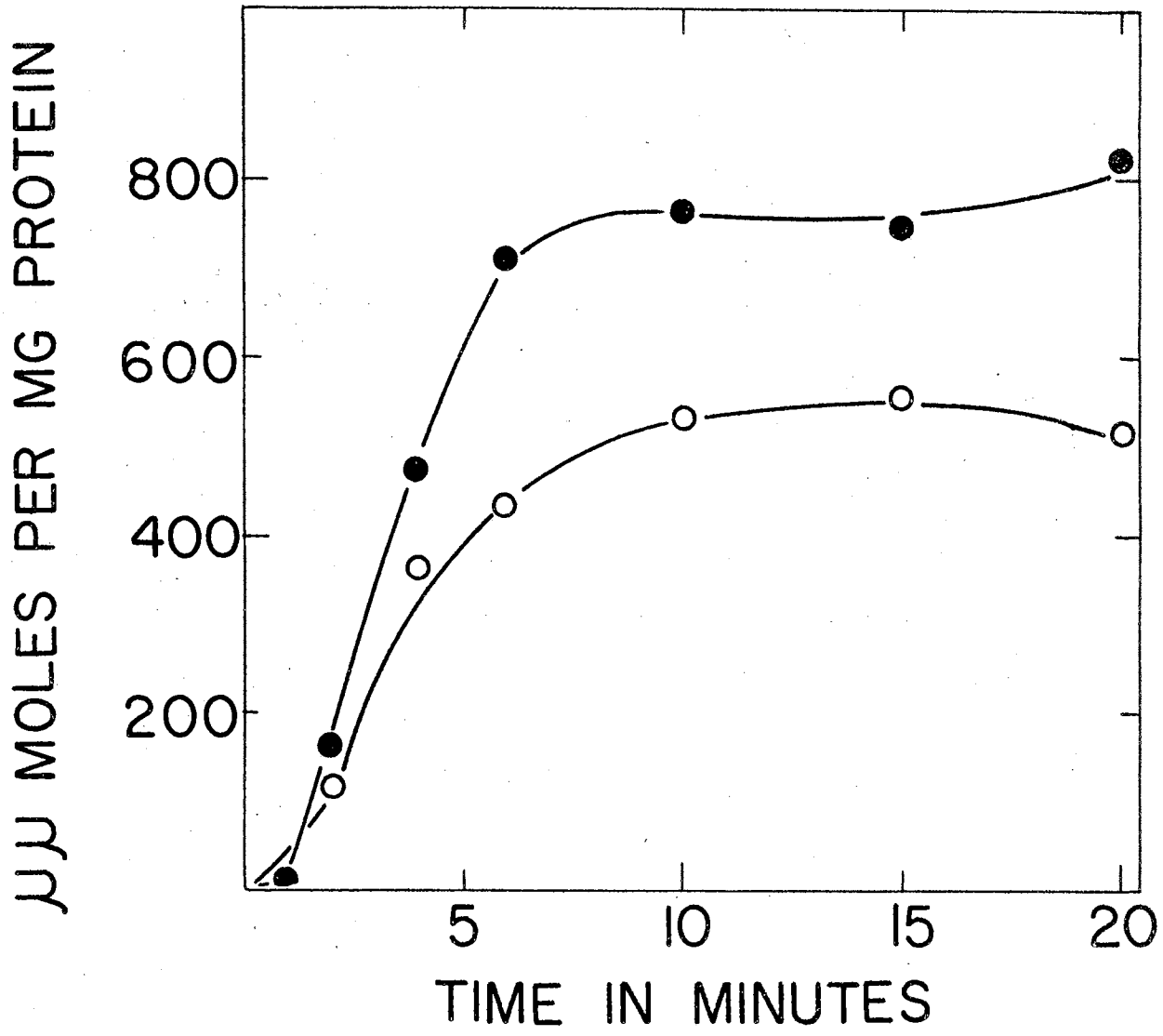

600
400
200
PHE
F PHE
uu MOLES PER MG s RNA
5
10
15
MINUTES

Figure 7

Kinetics of Amino Acid Incorporation

The reaction mixture contained in 1.0 ml the following in μmoles: 100 Tris HCl pH 7.8; 4 mercaptoethanol; 50 NH_4Cl; 10 magnesium acetate; 2 ATP; 4 phosphoenolpyruvate; 1 GTP; 50 μg poly U; 40 μg pyruvate kinase; 0.8 μc phenylalanine-UL-^{14}C (0.0224 μμmole); or 0.08 μmole p-fluorophenylalanine-^{3}H; and 3.0 mg incubated 30S extract. Incubation was at 35°, and 0.1 ml samples were absorbed onto paper disks and processed for radioactivity determination as described. ○, phenylalanine-^{14}C; ●, p-fluorophenylalanine-^{3}H.


JUJ MOLES PER MG PROTEIN
800
600
400
200
5
10
15
20
TIME IN MINUTES

Incorporation from Amino Acyl sRNA

The kinetics of the incorporation of the amino acids from sRNA into protein is shown in Figure 8. Since the amount of ^{3}H-p-fluorophenyl-sRNA in the reaction mixture was much greater than ^{14}C-phenylalanyl-sRNA (173 µµmoles to 32 µµmoles respectively), no comparison as to rate or extent of incorporation is possible. However there is a noticeable lag in the incorporation of p-fluorophenylalanine from sRNA. This indicates that a reaction prior to peptide bond formation is affected by the analogue.

Chromatographic Analysis of Amino Acyl sRNA

Several studies have shown degeneracy at the sRNA level by resolving a given amino acid acceptor RNA into several fractions (44, 45). No evidence for phenylalanine sRNA degeneracy has been obtained from these studies. If one phenylalanine acceptor RNA is preferentially charged by p-fluorophenylalanine, then co-chromatography of ^{14}C-phenylalanyl- and ^{3}H-p-fluorophenylalanyl-sRNA may give sufficient resolution to indicate degeneracy.

The results of chromatography of ^{14}C-phenylalanyl-sRNA on a silicic acid modification of a methylated albumin-kieselguhr column are seen in Figure 9. Chromatography of phenylalanine-3-^{14}C on a comparable column gave an elution profile similar to the first sharp peak noted near Fraction 10. This presumably arises because of the instability of the amino acyl sRNA. This first peak was noted in some but not all preparations of amino acyl sRNA. The relative positions of the A_{260} peak (Fraction 25), and the radioactive peak (Fraction 32), were comparable to that reported by Sueoka and Yamane

Figure 8

Kinetics of Amino Acid Incorporation from sRNA into Protein

The reaction mixtures contained in 0.75 ml the following in μmoles: 75 Tris HCl pH 7.8; 7.5 magnesium acetate; 37.5 NH_4Cl; 3 mercaptoethanol; 0.6 GTP; 50 μg poly U; 1730 counts per minute ^3H-fluorophenylalanyl-sRNA (2.66 mg); or 11420 counts per minute ^{14}C-phenylalanyl-sRNA (1.15 mg); and 2.25 mg incubated 30S extract. Incubation was at 35°, and 0.1 ml samples were absorbed onto paper disks at the indicated time and processed as described for radioactivity determination. ○, phenylalanine-^{14}C; ●, p-fluorophenylalanine-^3H.

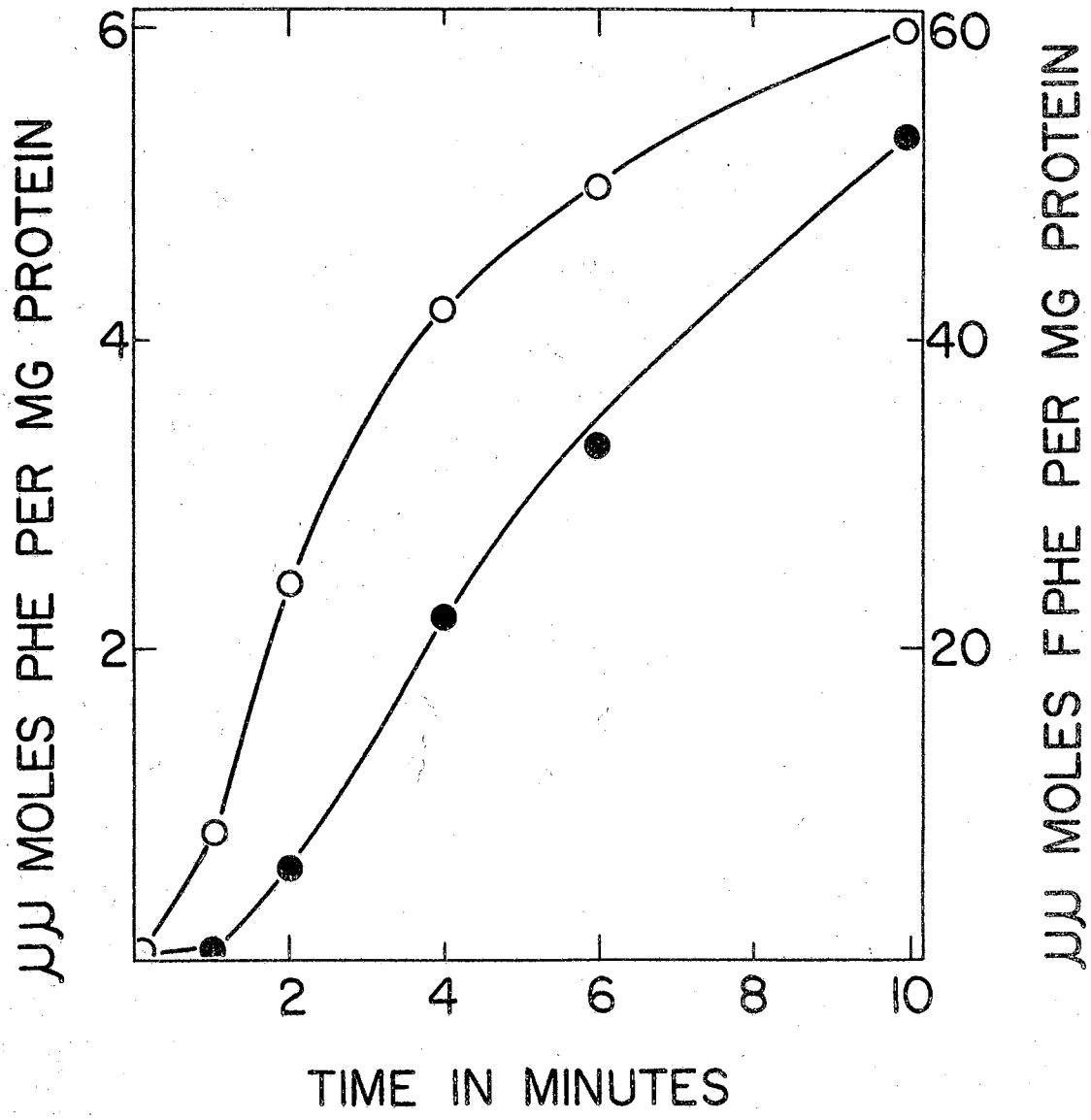
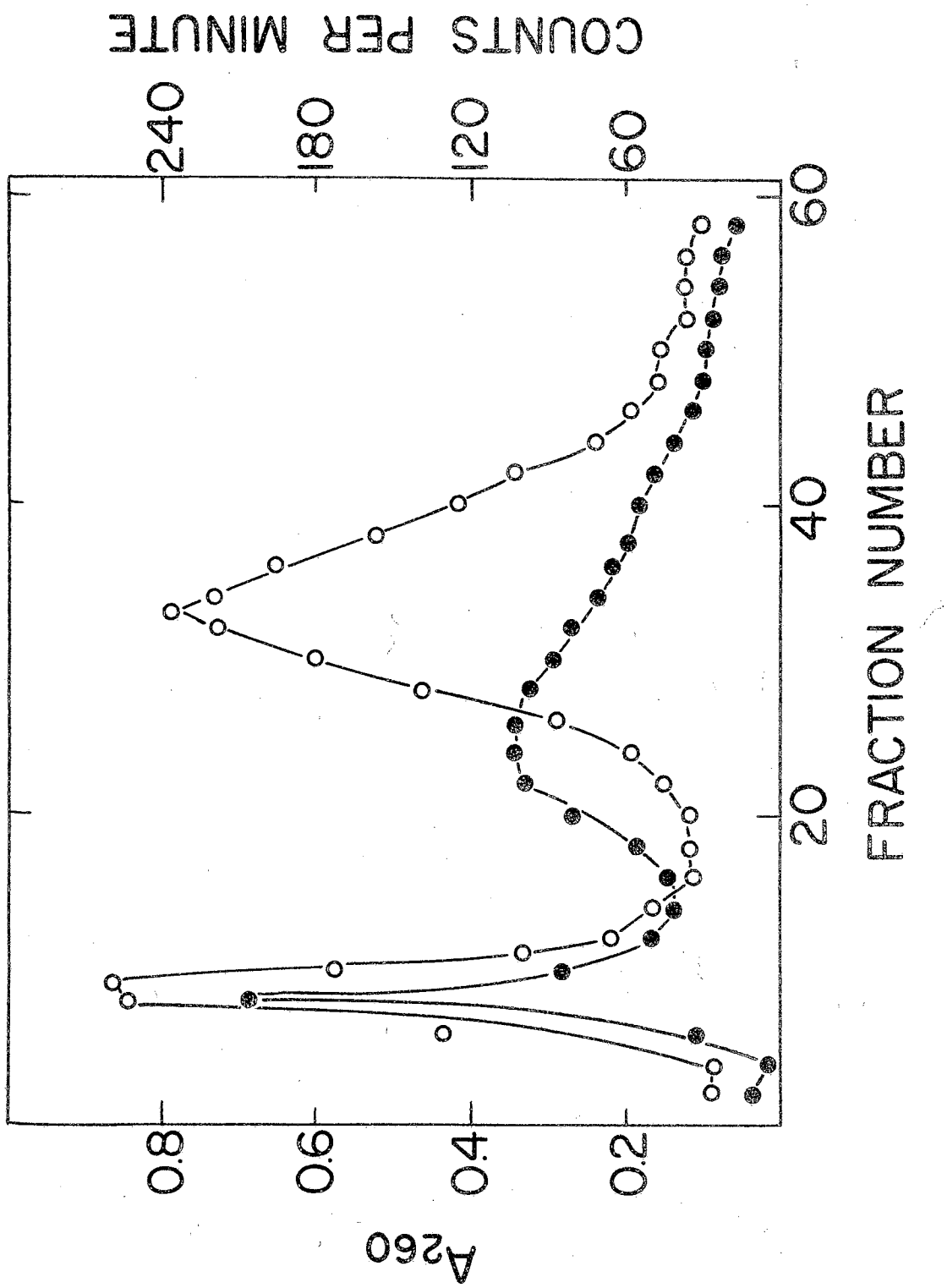

JJJ MOLES PHE PER MG PROTEIN
JJJ MOLES F PHE PER MG PROTEIN
TIME IN MINUTES
6
4
2
60
40
20
2
4
6
8
10

Figure 9

Chromatographic Fractionation of ^{14}C-Phenylalanyl-sRNA

Approximately 20,000 counts per minute (2.2 mg ^{14}C-phenylalanyl-sRNA) were chromatographed on a methylated albumin-silicic acid column. The column was developed with a linear gradient of 100 ml 0.6 M NaCl in the mixer flask (250 ml bottle) and 100 ml 1.2 M NaCl in the reservoir flask (250 ml bottle). ●—●, A_{260}; ○—○, counts per minute.


COUNTS PER MINUTE
240
180
120
60
A260
0.8
0.6
0.4
0.2
20
40
60
FRACTION NUMBER

(77). Similar results were obtained with the chromatography of p-fluorophenylalanyl-sRNA on the methylated albumin column.

In the absence of phenylalanine, poly U has been shown to stimulate the incorporation of leucine into protein (39) in some cell-free systems. This mistake in amino acid coding may be a result of in vitro conditions. As a check on this possibility with phenylalanine at the sRNA level, ^{3}H-phenylalanyl-sRNA, charged in the presence of 19 other amino acids, and ^{14}C-phenylalanyl-sRNA, charged with phenylalanine alone, were mixed and chromatographed on the methylated albumin column. As seen in Figure 10, there is no difference in the ^{14}C and ^{3}H peaks, indicating that in the absence of other amino acids phenylalanine is not charged to any other sRNA.

The possibility exists that one of the phenylalanine acceptor RNA molecules may be preferentially charged with p-fluorophenylalanine. Detection of such a difference would indicate degeneracy at the sRNA level. To test this possibility, sRNA was charged with phenylalanine-^{14}C alone, and with p-fluorophenylalanine-^{3}H alone. The labeled amino acyl sRNA's were mixed and chromatographed on the methylated albumin column. No difference in the shape or relative position of the ^{14}C and ^{3}H peaks is apparent (Figure 11). This experiment gave no indication of phenylalanine degeneracy at the sRNA level.

Binding of Amino Acyl sRNA to Ribosomes

Several investigators (46, 51, 56) have shown that poly U will mediate the binding of ^{14}C-phenylalanyl-sRNA to ribosomes, and this binding can be detected by sedimentation of labeled sRNA in a sucrose density gradient centrifugation. Binding of p-fluorophenylalanyl-sRNA to

Figure 10

Fractionation of ^{3}H-Phenylalanyl- and ^{14}C-Phenylalanyl-sRNA

Approximately 45,000 counts per minute 2,3-^{3}H-phenylalanyl-sRNA (6.22 mg), charged with 19 other amino acids, and approximately 20,000 counts per minute ^{14}C-phenylalanyl-sRNA (2.2 mg), charged with phenylalanine alone, were chromatographed on a methylated albumin-silicic acid column. The column was developed by a linear gradient consisting of 100 ml 0.6 M NaCl in the mixer flask (250 ml bottle) and 100 ml 1.2 M NaCl in the reservoir flask (250 ml bottle). Fractions of 3 ml were collected at a rate of 1 to 1.5 ml per minute. ●—●, ^{14}C; ○—○, ^{3}H.

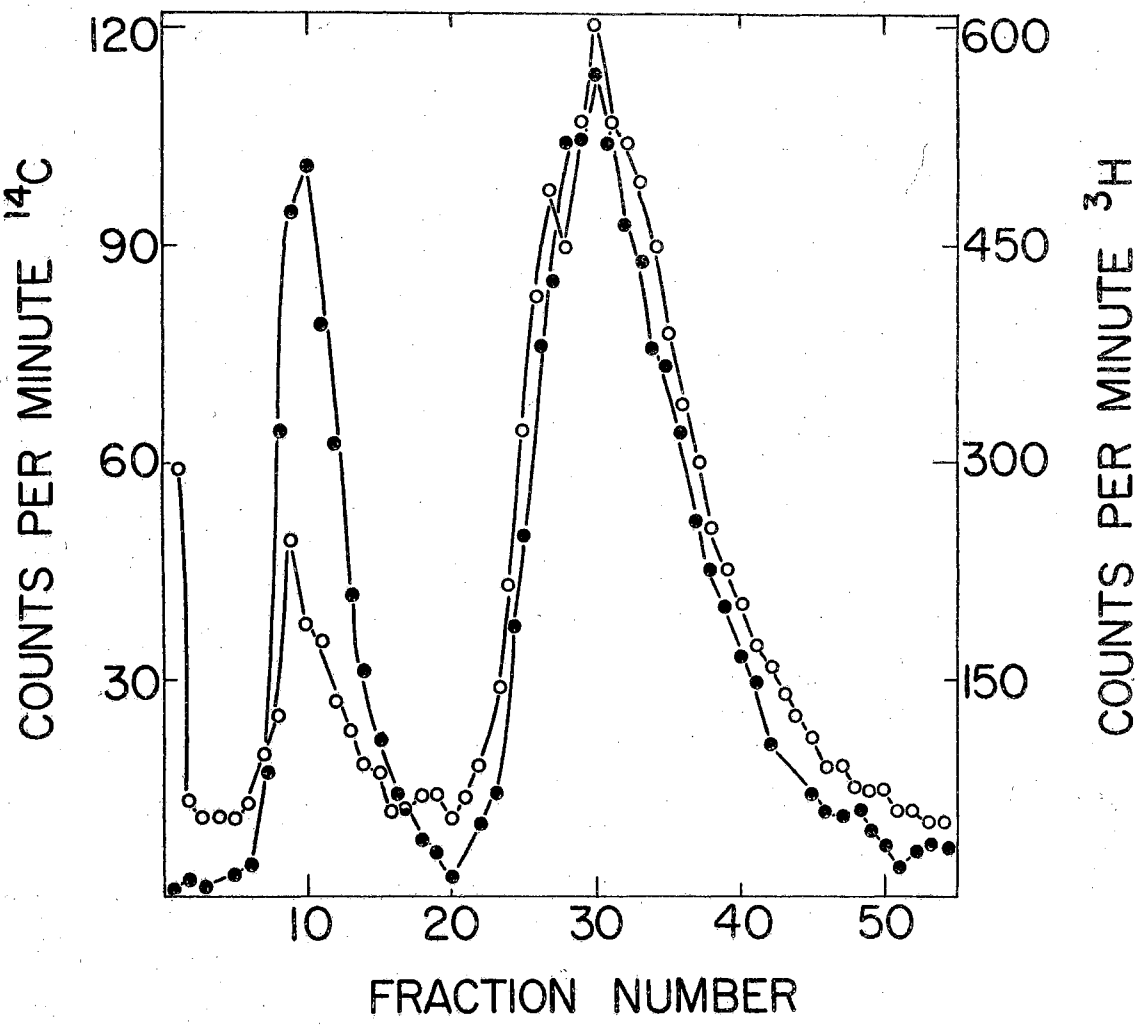
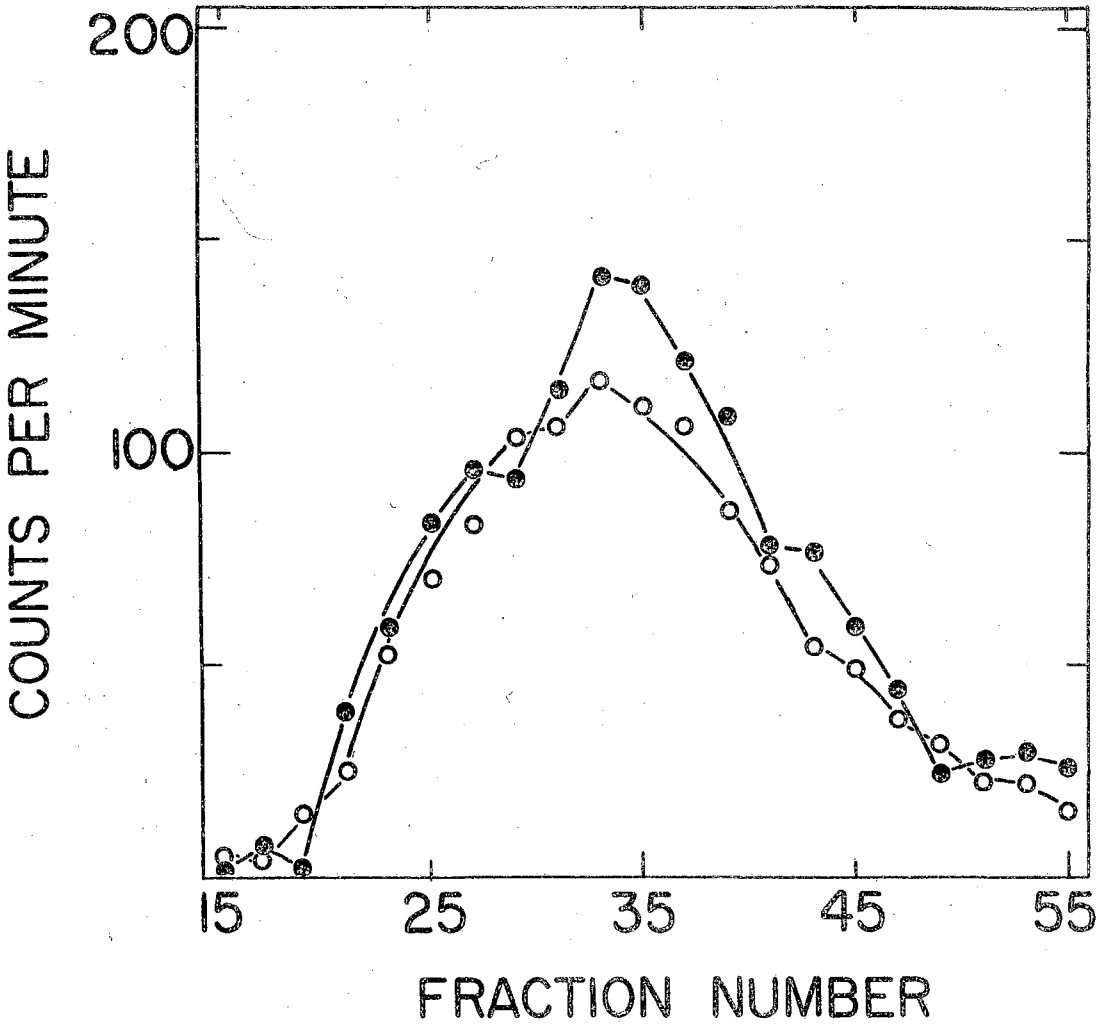

120
90
60
30
600
450
300
150
10
20
30
40
50
COUNTS PER MINUTE ^{14}C
COUNTS PER MINUTE ^{3}H
FRACTION NUMBER

Figure 11

Fractionation of ^{14}C-Phenylalanyl-sRNA and ^{3}H-p-Fluorophenyl-alanyl-sRNA

Approximately 3,300 counts per minute (.36 mg) ^{14}C-phenyl-alanyl-sRNA, and approximately 4,000 counts per minute (7.25 mg) of ^{3}H-fluorophenylalanyl-sRNA were mixed and fractionated on a methylated albumin-silicic acid column. The column was developed by a linear gradient consisting of 35 ml 0.6 M NaCl in the mixer flask, and 35 ml 1.2 M NaCl in the reservoir. One ml fractions were collected at a rate of one ml per minute. ○—○, ^{14}C; ●—●, ^{3}H.


200
100
COUNTS PER MINUTE
15
25
35
45
55
FRACTION NUMBER

to ribosomes in the presence of poly U would be a further confirmation of p-fluorophenylalanine competition with phenylalanine in protein synthesis.

The results of such an experiment are seen in Figure 12. When poly U was present in the reaction mixture radioactivity from ^{14}C-phenylalanyl- or ^{3}H-p-fluorophenylalanyl-sRNA sedimented to the most dense region of the gradient. When poly A was substituted for poly U, or when no polynucleotide was added, the radioactivity was confined to the less dense regions of the gradient.

In all experiments, A_{260} peaks were observed at fractions 14 or 15 and 24 or 25. An additional small A_{260} peak was observed in fractions 1-5 of the tube containing poly U and ^{14}C-phenylalanyl-sRNA. In the p-fluorophenylalanine-sRNA series, no A_{260} peak was evident in fractions containing the sedimented radioactivity, but a small A_{260} peak was evident in fractions 1 and 2 of the tube containing no polynucleotide.

The sedimentation of radioactivity from amino acyl sRNA suggests the poly U-mediated formation of polyribosomes and subsequent binding of amino acyl sRNA which would sediment rapidly in the sucrose gradient.

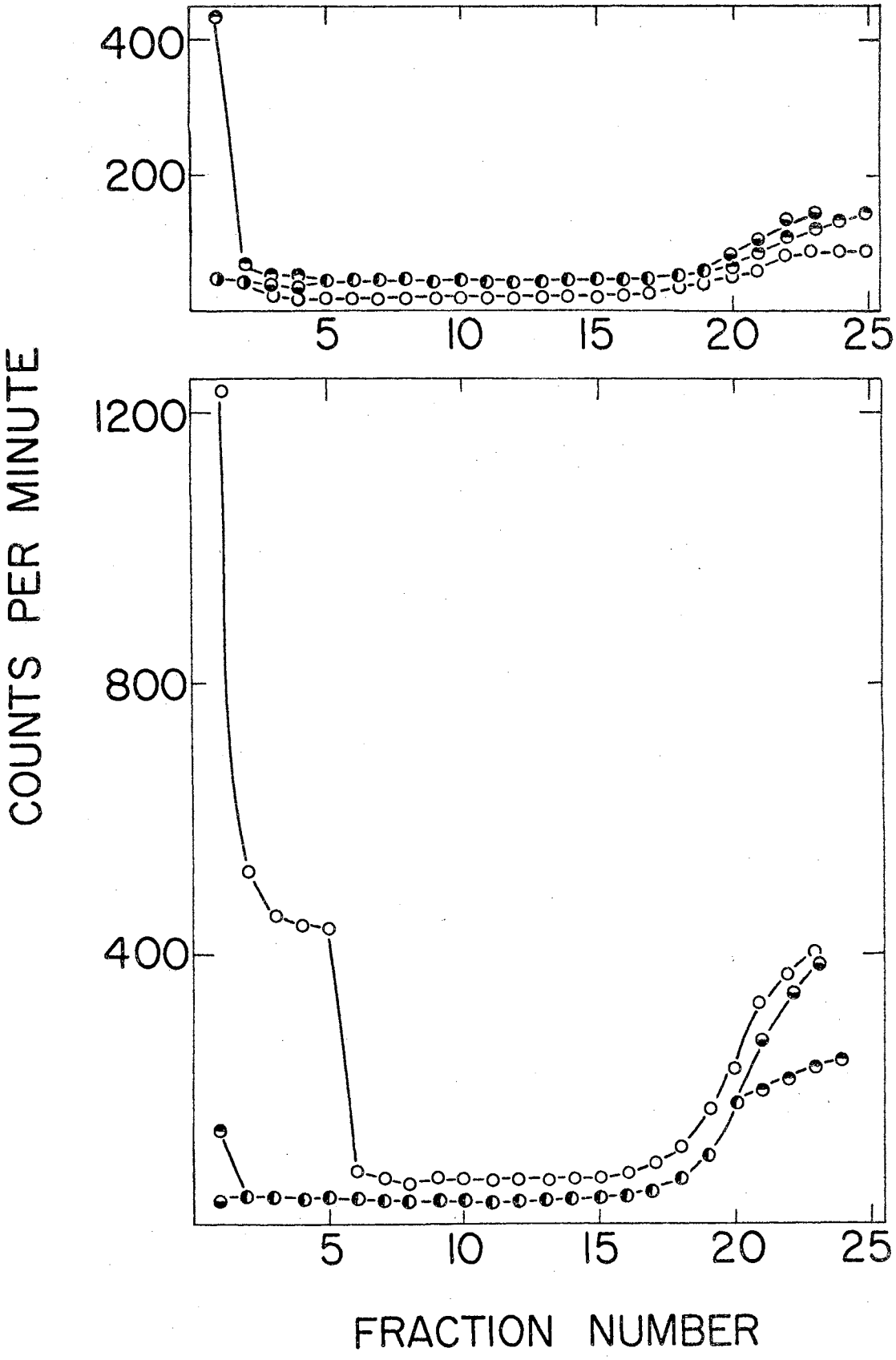
Effect of Polynucleotide Concentration on Amino Acid Incorporation

About 70% of the code words in poly UC 5:1 consists of the code words UUU plus UUC. The incorporation of phenylalanine by UC 5:1 relative to that of poly U averaged 65 to 70%. This is in accord with data already reported (41). However, the incorporation of phenylalanine by poly UC 3:1, 2:1 and 1:2 was much lower than the theoretical code word frequency would indicate. Evidence for additional code word

Figure 12

Binding of Amino Acyl sRNA to Ribosomes

The reaction mixture (46) contained in a volume of 0.3 ml the following: 0.02 M Tris HCl, pH 7.2; 0.02 M magnesium acetate; 0.05 M KCl; 14.18 A_{260} units of RNase-treated ribosomes; 20 ug polynucleotide; and 5010 counts per minute sRNA or 1600 counts per minute ^{3}H-p-fluorophenylalanyl-sRNA. Tubes were kept cold and amino acyl sRNA was added last. Incubation was for 20 minutes at 24°. At the end of the incubation period the tubes were cooled in an ice bath and 0.3 ml of cold 10% sucrose containing the buffer-salts mixture described above were added to the reaction mixture. The mixture was applied to the top of a 5 to 20% linear sucrose gradient containing the buffer-salts mixture to give a total volume of 4 ml. The tubes were centrifuged in a Spinco SW-39 rotor for 1 hour at 3° in a Spinco Model L centrifuge. Two drop fractions were collected from the bottom of the tube, and 0.06 ml of each fraction was added to 10 ml Bray's (77) scintillation solution and counted in a Packard Tri-Carb liquid scintillation spectrometer with a 45 to 50% counting efficiency. To the remainder of each fraction was added 1 ml of water, and the A_{260} determined. Upper figure: ^{3}H-p-fluorophenylalanine-sRNA; ◒—◒, poly U; ◒—◒, poly A; ○—○, no polynucleotide; ◐—◐ and ◑—◑, coincidental points. Lower figure: ^{14}C-phenylalanyl-sRNA;○—○ , poly U; ◒—◒, poly A; ◒—◒ , no polynucleotide; ◐—◐, coincidental points.


400
200
5
10
15
20
25
1200
800
400
5
10
15
20
25
COUNTS PER MINUTE
FRACTION NUMBER

assignments for UC code words was obtained by increasing the polynucleotide concentration used in the amino acid incorporation assays (43). The effect of higher polynucleotide concentrations on phenylalanine and p-fluorophenylalanine incorporation is seen in Tables VI and VII.

Experiment I gives results ordinarily obtained with 50 µg polynucleotide. When the concentrations of poly UC 5:1, 3:1 and 2:1 were doubled (from 80 to 160 µg per ml), phenylalanine and p-fluorophenylalanine incorporation increased by nearly the same factor. Although earlier experiments indicated poly U saturated the system at 50 µg per ml, there was no indication that the system is saturated with respect to poly UC 5:1, 3:1 and 2:1. With poly UC 5:1 at 160 µg per ml, 35% more phenylalanine was incorporated than with poly U at 50 µg per ml.

Increasing polynucleotide concentration from 20 to 50 µg per ml gave comparable results with the stimulation of phenylalanine from sRNA into protein (Table VIII). The stimulation of phenylalanine incorporation relative to that of poly U was increased 9-fold by UC 3:1, and 3-fold by UC 2:1.

Code Word Frequency and Amino Acid Incorporation

If there is no preference of phenylalanine acceptor RNA's for p-fluorophenylalanine, then incorporation of the natural amino acid and analogue by UC copolymers of varying U concentration should be the same. A comparison of theoretical code word frequency and observed amino acid incorporation from amino acyl sRNA is seen in Figure 13. The percent incorporation of phenylalanine by poly UC 5:1 and 3:1 is much greater than p-fluorophenylalanine incorporation. With these two polymers,

TABLE VI

EFFECT OF POLYNUCLEOTIDE CONCENTRATION ON PHENYLALANINE-^{14}C INCORPORATION*

Polynucleotide	µg per ml		µµmoles Incorporated	
	I	II	I	II
Poly U	50		225.8	
Poly UC 5:1	50		142.5	
		80		161.2
		160		309.6
Poly UC 3:1	50		19.2	
		160		43.9
		320		80.5
Poly UC 2:1	50		6.7	
		160		12.7
		320		21.8
Poly UC 1:2	50		1.8	
		160		2.7
		320		3.8

*The reaction mixtures contained the components previously described and 0.75 mg incubated 30S extract and polynucleotide as indicated.

TABLE VII

EFFECT OF POLYNUCLEOTIDE CONCENTRATION ON p-FLUOROPHENYLALANINE-H^3 INCORPORATION*

Polynucleotide	µg per ml		µµmoles Incorporated	
	I	II	I	II
Poly U	50		960	
Poly UC 5:1	50		382	
		80		636
		160		1308
Poly UC 3:1	50		56	
		160		129
		320		221
Poly UC 2:1	50		38	
		160		39.5
		320		64.3
Poly UC 1:2	50		37	
		160		22.0
		320		58.8

*The reaction mixtures contained the components previously described, 0.75 mg incubated 30S extract and polynucleotide as indicated.

TABLE VIII

EFFECT OF POLYNUCLEOTIDE CONCENTRATION ON PHENYLALANINE-^{14}C INCORPORATION FROM sRNA

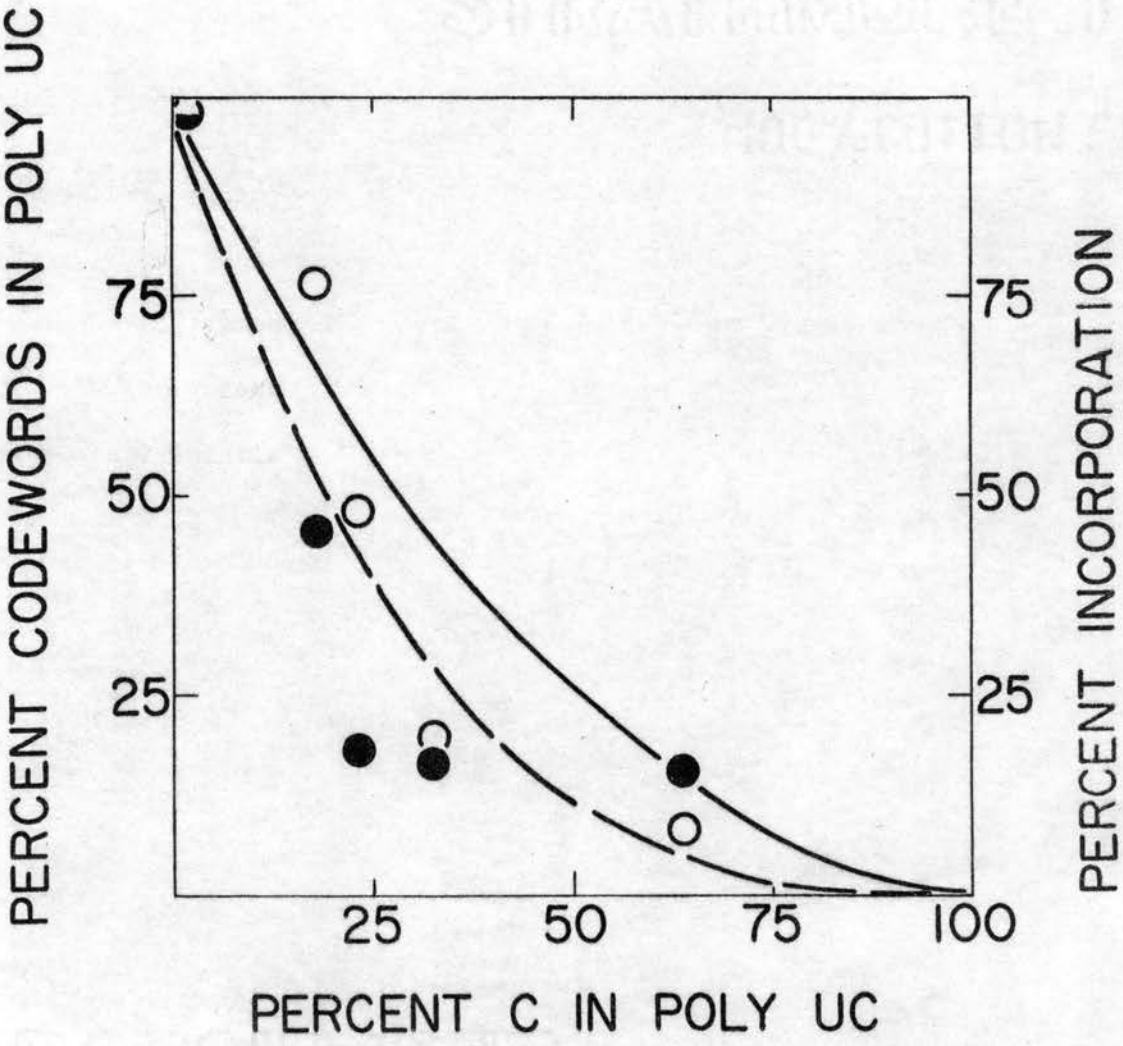
Polynucleotide	Polynucleotide Concentration in µg per ml	
	20*	50**
	Percent Incorporation	
Poly U	100	100
UC 5:1	67	76.8
UC 3:1	5.3	48.3
UC 2:1	5.1	18.9
UC 1:2	4.5	8.6

Reaction mixtures contained the components described previously including 12.5 µmoles NH_4Cl, polynucleotide as indicated above, and 0.375 mg* or 0.75 mg** incubated 30S extract. Incubation was for 10 minutes at 35°.

Figure 13

Comparison of Theoretical Code Word Frequency and Observed Incorporation

The reaction mixtures contained the components previously described for incorporation of amino acids from sRNA, 50 µg polynucleotide per ml and 0.75 mg 30S extract. ——, theoretical frequency of UUU plus UUC or UU; —— ——, theoretical frequency of UUU; ○, phenylalanine incorporation; ●, p-fluorophenylalanine incorporation.


PERCENT CODEWORDS IN POLY UC
PERCENT INCORPORATION
75
50
25
25
50
75
100
PERCENT C IN POLY UC

there is an implication of the two code words UUU plus 2UUC coding for phenylalanine incorporation, and the one code word UUU coding for p-fluorophenylalanine.

CHAPTER IV

DISCUSSION

The tritium exchange labeling of p-fluorophenylalanine gave material of fairly high specific radioactivity (Sp. Act. 67.5). However, the low counting efficiency of ^{3}H on paper disks (4-6%) as compared to ^{14}C (50-60%) partially offset the advantage of higher specific activity than could be obtained with ^{14}C labeled analogue. For detection in terms of counts per minute of incorporated p-fluorophenylalanine-^{3}H, much larger amounts of p-fluorophenylalanine than phenylalanine were necessary in the reaction mixtures. The inequality of substrate concentration made it difficult to draw valid comparisons in some of the kinetic studies of amino acid incorporation into protein.

The equivalence of rates of activation, as determined by hydroxamate formation, and formation of amino acyl sRNA indicated no preference for phenylalanine utilization over that of p-fluorophenylalanine. The kinetic studies of amino acid incorporation were inconclusive both as to rate and extent of incorporation because of the difference in concentration of the amino acid and analogue. Approximately 7% of the phenylalanine, and 3% of the p-fluorophenylalanine in the reaction mixtures was incorporated from the free amino acid. The p-fluorophenylalanine concentration was four times that of phenylalanine; nevertheless the incorporation of phenylalanine was 70% that of p-fluorophenylalanine. There is therefore a question regarding the

relative saturation of the two amino acids. And since poly U is the functional messenger, the incorporation of the amino acid and analogue would be expected to be the same.

However, one indication of degeneracy arises from the extent the amino acids were charged to sRNA. When the ratio of phenylalanine to p-fluorophenylalanine concentration in the reaction mixtures ranged from 3:1 to 1:4, a greater amount of phenylalanine was accepted by the sRNA. This indicates that p-fluorophenylalanine was charged preferentially to only one of the phenylalanine acceptor RNA's.

The kinetic studies of amino acid incorporation from sRNA were also inconclusive because of the difference in concentration of the amino acyl RNA's used. However, the lag in p-fluorophenylalanine incorporation was more pronounced than phenylalanine incorporation. Conway (50) showed that the rate limiting reaction prior to peptide bond formation was the binding of amino acyl sRNA to the ribosomes. It is suggested, therefore, that p-fluorophenylalanine changes the binding characteristics of the sRNA.

Fractionation of the amino acyl sRNA's on a methylated albumin column gave no evidence of degeneracy. However, this does not suggest that degeneracy does not exist. In the light of the other studies cited, the probability is that the column was incapable of resolving the amino acyl sRNA's.

A comparison of code word frequency and observed amino acid incorporation gave evidence of degeneracy. The greater relative incorporation of phenylalanine than p-fluorophenylalanine by poly UC 5:1 and 3:1 indicated that phenylalanine incorporation was coded by UUU plus 2U1C, and p-fluorophenylalanine by UUU. However, the

incorporation of both amino acids with poly UC 3:1, 2:1 and 1:2 was lower than would be indicated by code word frequency in these polymers.

The incorporation of the free amino acids by the same polynucleotides was also performed. With poly UC 5:1 a greater relative incorporation of phenylalanine than p-fluorophenylalanine was again noted. However, the incorporation with poly UC 3:1, 2:1 and 1:2 was too low to be definitive.

The reason for the low incorporation with poly UC 3:1, 2:1 and 1:2 is probably due to the short chain length of these polynucleotides as compared with poly U and UC 5:1. Increasing the concentration of poly UC 3:1, 2:1 and 1:2 increased the incorporation of phenylalanine and p-fluorophenylalanine. But with varying concentrations of the polynucleotides in the reaction mixtures, no comparison of code word frequency and amino acid incorporation with a series of various polynucleotides can be made.

The importance of chain length of the polymers may be seen from the fact that higher concentrations of poly UC 5:1 than ordinarily used gave greater phenylalanine and p-fluorophenylalanine incorporation than poly U. Poly UC 5:1 was composed almost entirely of long chain length material, but only about one-third of the poly U was composed of similar chain length material.

The most satisfactory data would be obtained from the use of a series of polynucleotides that have a similar proportion of long chain length material.

In summary, evidence for degeneracy at the phenylalanine acceptor RNA level stems from two observations. These are: (1) a greater amount of phenylalanine than p-fluorophenylalanine can be charged to sRNA; and

(2) a comparison of code word frequency and observed amino acid incorporation suggests that phenylalanine incorporation is coded by the two code words UUU plus 2U1C and that p-fluorophenylalanine is coded by UUU.

Experiments which may give further evidence of degeneracy are as follows: (1) charging of sRNA with p-fluorophenylalanine followed by charging of the same sRNA with phenylalanine and 19 other amino acids; (2) use of this charged sRNA in a cell-free amino acid incorporation system using tobacco mosaic virus or bacteriophage RNA as messenger. If sRNA can be loaded with the analogue, and further charged with the natural amino acid, then the existence of more than one phenylalanine acceptor RNA would be implied. Incorporation of the natural amino acid and analogue into distinct sites of the protein synthesized in vitro would be additional evidence for degeneracy.

CHAPTER V

SUMMARY

A cell-free system from E. coli was used to study the effect of phenylalanine analogues at various steps of synthetic polynucleotide-mediated protein synthesis. Of the analogues tested and at the levels used, only p-fluorophenylalanine inhibited poly U-mediated phenylalanine incorporation. The kinetics of phenylalanine and p-fluorophenylalanine activation, formation of amino acyl sRNA, and incorporation into protein showed no preferential utilization of phenylalanine over p-fluorophenylalanine. Degeneracy was implied since sRNA accepted a greater amount of phenylalanine than p-fluorophenylalanine. The chromatographic behavior of phenylalanyl- and p-fluorophenyl-sRNA was the same, giving no evidence of degeneracy. A comparison of theoretical code word frequency and observed incorporation of phenylalanine and p-fluorophenylalanine indicated that phenylalanine code words are UUU and 2U1C and the p-fluorophenylalanine code word is UUU.

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