A Study of Transfer Ribonucleic Acid in *Neurospora*. I. The Attachment of Amino Acids and Amino Acid Analogs*

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ABSTRACT: Appropriate conditions have been found for the charging of all 20 amino acids to unfractionated transfer ribonucleic acid from *Neurospora crassa*. A single set of reaction conditions employed for the charging of 14 amino acids is not suitable for charging alanine, glutamic acid, glutamine, glycine, methionine, and serine. The time course of aminoacyl transfer ribonucleic acid formation, with transfer ribonucleic acid limiting, for alanine, glutamic acid, glutamine, and methionine, using standard conditions, reaches a peak in 5–10 min and rapidly declines instead of leveling off. This decline is apparently the result of two factors: (1) instability of the aminoacyl transfer ribonucleic acid in Tris buffer (pH 7.5) and (2) loss of charging capacity of the reaction mixture. By varying the ratio of the concentration of Mg^{2+} to adenosine triphosphate from the standard 20 down to 10 for methionine and alanine, 2 for glutamic acid, and 1 for glutamine and lowering the pH of the reaction to 7.0 for alanine, the loss of charging capacity during the course of the reaction can be prevented. The attachment to transfer ribonucleic acid of glycine and some, but not all, of the other amino acids is inhibited by certain anions, especially phosphate. The transfer ribonucleic acid acceptors for methionine and phenylalanine can be fully charged with the analogs ethionine and *p*-fluorophenylalanine, respectively. In each case the difference in structure of the amino acid and its analog is sufficient to cause a difference in the chromatographic mobility of their respective complexes with identical transfer ribonucleic acid molecules.

We have examined the conditions required for the complete charging of *Neurospora* tRNA. Using sRNA[1] prepared by a simple procedure (described below) and a crude enzyme preparation (Barnett, 1965), we have, by appropriate choice of reaction conditions, measured the specific acceptor activity for all 20 amino acids of protein. For 6 of the 20 amino acids (alanine, glutamic acid, glutamine, glycine, methionine, and serine) the conditions are quite critical. The key variables include ATP, tRNA, and Mg^{2+} concentrations, pH, presence of a sulfhydryl reducing agent, and the kind of anions present. Based on reasonable assumptions about the specific absorbance and average molecular weight of *Neurospora* sRNA it appears that more than 90% of the sRNA molecules in our average preparation has amino acid acceptor activity. A previous report of the specific acceptor activity of *Neurospora* tRNA for 18 of the 20 amino acids using strain 74A (Barnett and Brown, 1967) indicated values much lower than those reported here. The complete charging (>99%) of tRNA from *Escherichia coli* strain A19 has been reported by Matthaei *et al.* (1966). Cantoni and Richards (1966) reported values for the specific acceptor activity of yeast tRNA for 16 amino acids and rat liver tRNA for 15 amino acids.

Ethionine, a methionine analog, and *p*-fluorophenylalanine, a phenylalanine analog, are incorporated into protein by *Neurospora* (Kappy and Metzenberg, 1965; M. Fling, 1968, personal communication). We have investigated the attachment of these analogs to tRNA. While the specific synthetases have less affinity for the analogs than for the amino acids (Novelli, 1967), all of the acceptor activity for these amino acids is available for attachment to the analogs. Cochromatography of aminoacyl-tRNA and analog-tRNA in both cases reveals a shift in chromatographic mobility which may indicate differences in the conformation of the respective complexes.

Experimental Procedures

Materials. Wild-type strain 69-1113a of *Neurospora crassa* was maintained on slants of Horowitz complete medium (Horowitz, 1947). Mycelial cultures of this strain (2-day old), prepared as follows, were used for the extraction of sRNA. A conidial suspension was prepared by adding 10–15-ml sterile-distilled water to a slant (150 × 18 mm) of a conidiating culture. This sus-

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[1] Abbreviations used in this paper: sRNA, RNA which is soluble in 1 M NaCl; $tRNA_x$, tRNA with acceptor activity for amino acid x; x-tRNA, tRNA esterified to x. If more than one chromatographic component exists for any x-tRNA, each is identified by superscript in order of elution, *e.g.*, x-$tRNA^1$. Eth, ethionine; *p*FPhe, *p*-fluorophenylalanine; MAK, kieselguhr coated with methylated albumin; DTT, dithiothreitol–Cleland's reagent.

pension (5–7 drops) was used to inoculate 125-ml erlenmeyer flasks containing 20 ml of "subminimal" medium (a 1:2 dilution of Vogel's medium N (Vogel, 1956) containing 0.5% (w/v) sucrose). The flasks were incubated at 25 ± 1° for 48 hr. The mycelial pads were collected on filter paper in a Buchner funnel in the cold (4°), washed with cold distilled water, pressed dry between paper towels, and stored frozen at −80°.

The procedure for extracting sRNA from *Neurospora* combines aspects of the method of von Ehrenstein and Lipman (1961) for *E. coli* and that of Holley *et al.* (1961) for yeast. The proportions indicated are those used for the extraction of approximately 6 g wet weight of mycelium. The frozen material is ground in a cold mortar with sand. To the ground mycelium 25 ml of water-saturated redistilled phenol and 25 ml of ice-cold buffer (0.001 M Tris-HCl, pH 7.3, containing 0.01 M $Mg(Ac)_2$) are added with vigorous stirring. The homogenate is transferred to a 125-ml screw-cap flask and placed on a rotary shaker for 1 hr at 25°. The mixture is transferred to two centrifuge tubes and centrifuged at 10,000*g* for 30 min. (All centrifugation steps in this procedure were performed in a Sorvall RC-2B at 4° using the SS-34 rotor.) The upper, aqueous phase is removed by pipet and kept at 4°. The lower, phenol layer is reextracted with a small volume of buffer (5 ml/tube) and centrifuged at 10,000*g* for 15 min. The aqueous phase is again removed and added to the first aqueous phase. To the pooled aqueous phases one-tenth volume of 20% (about 2 M) KAc (pH 5.1) is added; RNA is precipitated with two volumes of 100% ethanol at −20°. After 2 hr the precipitate is collected by centrifugation (10,000*g* for 15 min), washed with cold 67% ethanol, and resuspended in 10 ml of 0.5 M Tris-HCl (pH 8.8) containing 0.1 M $Mg(Ac)_2$. The RNA is incubated for 45 min at 37° to remove attached amino acids by saponification and is again precipitated by the addition of one-tenth volume of 20% KAc (pH 5.1) and two volumes of ethanol. The precipitate is left overnight at −20°, collected by centrifugation, and suspended in saline buffer (1.0 M NaCl–0.2 M Tris-HCl, pH 7.3, containing 0.1 M $Mg(Ac)_2$). This operation defines sRNA; high molecular weight RNA is insoluble in 1 M NaCl. Insoluble (ribosomal and other high molecular weight) RNA is pelleted by centrifuging the saline suspension at 48,000*g* for 30 min. The supernatant containing the sRNA is carefully decanted and extracted in a separatory funnel twice with an equal volume of anhydrous ether to remove any remaining phenol. Residual ether is removed by bubbling N_2 though the solution. The RNA is again precipitated with KAc and ethanol for 2 hr as above. The precipitate is washed by suspension and centrifugation twice with 80% ethanol (20 ml), twice with 95% ethanol (20 ml), and dried in a vacuum desiccator over KOH pellets. The dried material is then suspended in 5–10 ml of appropriate buffer and the concentration was measured by the ultraviolet absorption at 260 mμ of a $^1/_{100}$ dilution. The RNA solution, stored at −20°, does not appear to be affected adversely by freezing and thawing.

The ratio of the optical density at 260 mμ to that at 280 mμ of the sRNA prepared in this way is 1.99 ± 0.11. Approximately 20% (by weight) of the dried material is sRNA; the remainder is probably polysaccharide (Chan *et al.*, 1966). The efficiency of extraction of RNA using this procedure is about 25% compared with the amount extractable with hot perchloric acid; 1 mg of sRNA is obtained from each gram (wet weight) of mycelium.

Aminoacyl-tRNA synthetases were obtained from strain 74A because, when grown exponentially, it is virtually free of ribonuclease activity. (We have not investigated whether this is a general property of exponential cultures of *Neurospora* or whether it is peculiar to this strain.) The strain was grown exponentially according to the following modification of the methods of Luck (1963) and Davis and Harold (1962). Large amounts of conidia are produced by growing cultures on 40 ml of solid medium (Vogel's medium + 2% agar) in 250-ml erlenmeyer flasks in constant light. The total yield of conidia from three such flasks is suspended in 50 ml of sterile distilled water, filtered through glass wool, and immediately used to inoculate a 2500-ml low-form culture flask containing 750 ml of Vogel's medium. Several such flasks are incubated at 25° on a reciprocal shaker (approximately 150 excursions/min), and the mycelium is harvested after 18 hr, near the end of the exponential phase of growth.

The synthetase enzymes were prepared from freshly harvested exponential mycelium according to the method developed for *Neurospora* by Barnett (1965) except that the dialyzed preparation was stored as a 50% solution in glycerol at −20°. Such preparations contain enzymatic activity for the charging of all 20 of the amino acids of protein to tRNA.

Dipotassium adenosine 5′-triphosphate (A grade) and trisodium 2-phosphoenolpyruvic acid (A grade) were purchased from Calbiochem. Bovine serum albumin (fraction V) ("Reagent for Microbiological Use") obtained from Armour Pharmaceutical Co. was methylated according to the method of Mandel and Hershey (1960) except that the albumin was methylated for 5 days at 35°, instead of 3 days at room temperature, and the product was stored as a dried powder over KOH in a desiccator. The kieselguhr used as the supporting material of the MAK column is Hyflo Supercel, obtained from Johns Manville. Analytical Reagent grade, liquefied phenol (88%) obtained from Mallinckrodt was redistilled before use and stored as the water-saturated solution in sealed brown bottles at −20°.

Solution A scintillation fluid contains 0.4% (w/v) 2,5-diphenyloxazole obtained from New England Nuclear and 0.01% (w/v) 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] from Packard Instrument Co. in toluene. Samples in aqueous solution were counted in a 2:1 mixture of solution A and commercial grade Triton X-100, a nonionic detergent, supplied by Rohm & Haas (Patterson and Greene, 1965).

Radioactive amino acids (^{14}C labeled with specific activities of 20–100 mCi/mmole or ^{3}H labeled with specific activities of 0.16–27.0 Ci/mmole) were purchased from New England Nuclear, Nuclear-Chicago, Nuclear Supplies, and Schwartz BioResearch. DL-[3-^{14}C]-*p*-Fluorophenylalanine (4.9 mCi/mmole) was purchased from Calbiochem; L-[ethyl-1-^{14}C]ethionine (3.66 mCi/

mmole) and L-[ethyl-1-^{3}H]ethionine (70.0 mCi/mmole) were obtained from New England Nuclear.

Methods. The reaction mixture for the assay of amino acid acceptor activity includes in Tris buffer, generally, sRNA, an amino acid (labeled with ^{3}H or ^{14}C), ATP, $Mg(Ac)_2$, a sulfhydryl reducing reagent, and an enzyme preparation. The proportions of these components are, for some amino acids, quite critical and will be discussed under Results. Assays designed to measure total acceptor activity are performed with tRNA rate limiting and amino acid, ATP, and enzyme in excess. Reactants, without enzyme, are mixed in the cold and then equilibrated at 30°. The reaction is initiated by the addition of enzyme and is completed in 10–20 min. For assay purposes, the formation of aminoacyl-tRNA was measured by the amount of amino acid radioactivity rendered acid insoluble using the filter paper disk procedure of Bollum (1960) as modified by Chambers (1966). The dried disks are counted in 5 ml of solution A scintillation fluid. The control used is an identical reaction mixture without added tRNA; its radioactivity does not exceed 5% of the experimental value. In order to achieve this low background level for two amino acids, tryptophan and cysteine, it was necessary to replace the filter paper disks with glass fiber disks (Whatman GF/A).

To prepare aminoacyl-tRNA for chromatography the reaction is terminated by adding an equal volume of water-saturated phenol and shaking for 7 min at 4°. The mixture is then centrifuged at 5000*g* for 15 min, the aqueous phase is carefully removed with a disposable pipet and the charged tRNA is precipitated by the addition of one-tenth volume of 20% KAc (pH 5.1) and two volumes of cold absolute ethanol. After 2 hr at −20° the precipitate is collected by centrifugation and dissolved in 50 ml of starting column buffer. It is stored in this buffer at −20°.

Chromatography of aminoacyl-tRNA on MAK columns, described first by Sueoka and Yamane (1962), is performed on columns prepared by a modification of the technique of Mandel and Hershey (1960) which is described in detail in the accompanying paper (Shearn and Horowitz, 1969).

Results

Preliminary Experiments. We originally extracted aminoacyl-tRNA synthetases from stationary-phase cultures similar to those used for sRNA extraction. However, such preparations are contaminated with ribonuclease activity. This ribonuclease is somewhat less active with sRNA as substrate than with unfractionated RNA and in this regard is similar to the *Neurospora* endonuclease characterized by Linn and Lehman (1965), which is most active with nucleic acids lacking an ordered structure. In order to circumvent the problems caused by the ribonuclease we tried to charge tRNA *in vivo*. This approach was abandoned when we discovered that aminoacyl-tRNA synthetase preparations from exponential cultures of 74A (Barnett, 1965) are free of interfering ribonuclease.

Standard Reaction Conditions. Two criteria are used to determine the saturation or maximal acceptor activity of tRNA for a particular amino acid. A plot of the amount of aminoacyl-tRNA formed as a function of time should plateau (Figure 1, curve I); and addition of more enzyme, ATP, or amino acid after the plateau is reached should not cause any increase in the amount formed. The interpretation of such evidence is that every tRNA molecule capable of accepting the amino acid has reacted by that time.

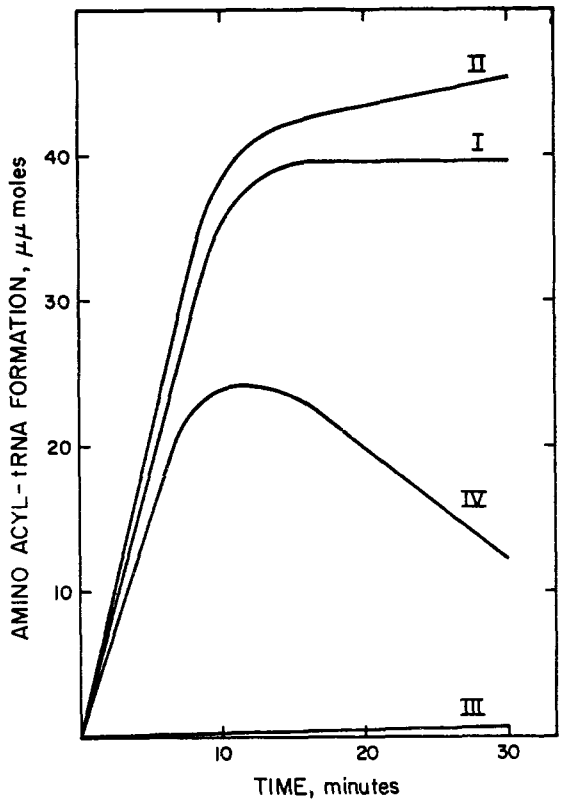

FIGURE 1: Kinetics of charging reaction. Four observed patterns of the amount of aminoacyl-tRNA recovered from the reaction mixture as a function of time. Explanation of each pattern is discussed in the text.

Using a standard reaction mixture (Table I) both criteria were satisfied for the attachment of 14 amino acids to tRNA; the reactions went to completion in 10–20 min at 30°. Quite different kinetics were observed when the same reaction mixture was used to assay the six

TABLE I: Composition of Standard Reaction Mixture.

Component	Amt/ml of Reaction
Tris-HCl, pH 7.3–7.5	100 μmoles
$Mg(Ac)_2$	50 μmoles
ATP	2.5 μmoles
Glutathione (reduced)	5.0 μmoles
[^{14}C]Amino acid	0.02 μmole
sRNA	10 OD_{260} units, ~0.4 mg
Enzyme preparation (in 50% glycerol)	1 mg

TABLE II: Kinetic Parameters of Isolated Serine tRNA Components.[a]

Fraction	Purity (%) I	II	III	IV	K_M (M × 10^7)	V_{max} ($\mu\mu$moles/min)
A	86.4	10.5	3.1		1.70	9.75
B	17.1	75.8	7.1		0.613	13.5
C		18.4	69.1	12.5	<B	20.0
D	1.7	9.6	33.2	55.5	1.22	16.1

[a] Uncharged sRNA was chromatographed on MAK. The eluted fractions were pooled into four groups (A, B, C, and D) based on the expected mobility of the four serine tRNA components. The purity of each pooled fraction was determined by charging it with either [^{14}C]- or [^{3}H]serine and rechromatographing it. In unfractionated tRNA the proportions of the four components are: I = 18.6%, II = 37.3%, III = 34.2%, and IV = 9.9%. Fraction C is believed to have a lower K_m than B because the rate of charging was maximal at the lowest concentration of C tried.

other amino acids: serine, glycine, methionine, glutamic acid, glutamine, and alanine. For serine the result was similar to curve II in Figure 1. Like curve I, the reaction begins with a high initial rate but instead of reaching a plateau after a short time, the reaction continues at a reduced rate. Wettstein (1966) has reported similar kinetics for the attachment of isoleucine to *E. coli* tRNA. No reaction at all occurs with glutamic acid (curve III, Figure 1). The kinetics are quite unusual for the other four amino acids. The amount of aminoacyl-tRNA formed reaches a peak within 5–10 min and then declines (curve IV, Figure 1). Peterkofsky *et al.* (1966b) observed kinetics of this type for valyl-tRNA formation in *E. coli* but the decline began after 90-min reaction.

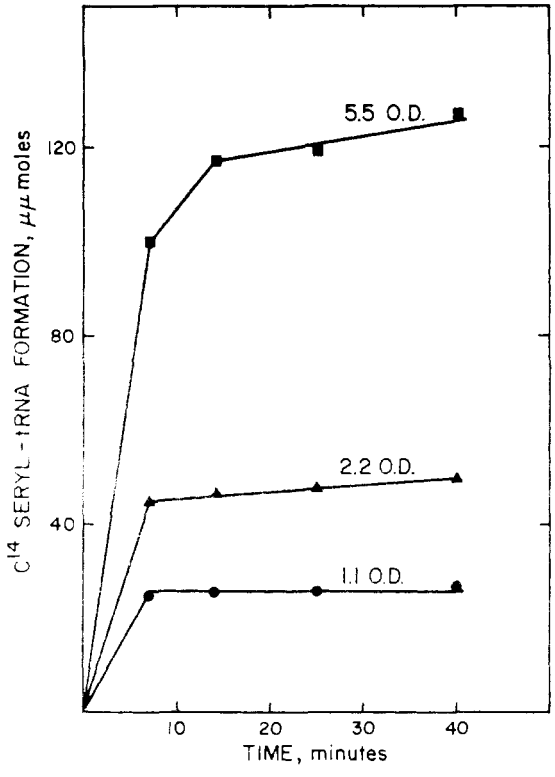

FIGURE 2: Seryl-tRNA formation at different concentrations of tRNA. Attachment of [^{14}C]serine to unfractionated tRNA was assayed at three different tRNA concentrations. Squares, 5.5 OD_{260} units/reaction, triangles, 2.2 units; circles, 1.1 units.

Serine. Saturation kinetics for the attachment of serine to tRNA are observed if the concentration of tRNA in the reaction mixture is lowered by a factor of 5 (Figure 2). This implies that in the original mixture tRNA was in excess. However, the kinetics are not those expected for a reaction with excess substrate. In *Neurospora* we have found four chromatographically separable tRNA components present in different amounts which accept serine. The kinetics observed at high tRNA concentration could be explained if the four components were charged at different rates. This explanation is supported by comparison of the K_M and V_{max} for each of the four partially separated components (Table II).

These data indicate that $tRNA^{I}_{Ser}$ and $tRNA^{IV}_{Ser}$, which are present in unfractionated tRNA as minor components (19 and 10%, respectively), have less affinity for the synthetase enzyme than do the major components. Since the kinetics of charging serine to unfractionated tRNA is the sum of these four reactions proceeding at different rates, the slow rate of reaction at late times may reflect the residual charging of components I and/or IV after II and III are saturated.

Glutamic Acid, Glutamine, Methionine, and Alanine. The high Mg^{2+} concentration in the standard reaction mixture (0.05 M) is responsible for the apparent lack of acceptor activity for glutamic acid. At a lower concentration (0.01 M Mg^{2+}) the initial rate of reaction is maximal, but the kinetics are still not normal; they are now of type IV (Figure 1). The acid-precipitable counts reach a peak and then decline, thus grouping glutamic acid with glutamine, alanine, methionine, and glycine.

The loss of counts suggests either that the RNA molecule is being fragmented or that the aminoacyl bond once formed is unstable. The former explanation was ruled out by extracting the tRNA late in the reaction (after the peak had been reached) and reassaying it. The same peak acceptor activity was observed. The instability of the aminoacyl-tRNA was confirmed for

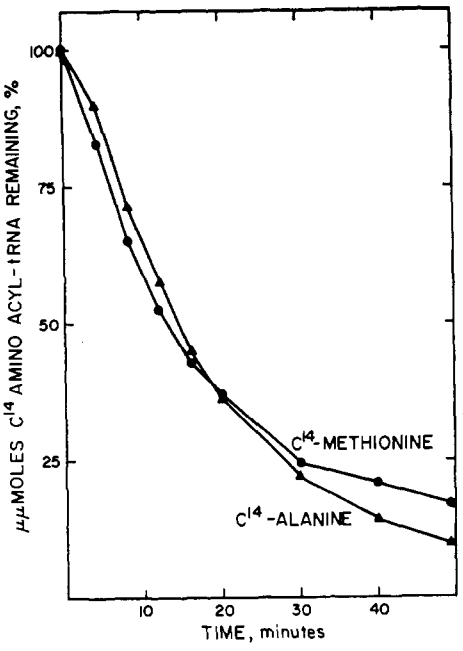

FIGURE 3: Instability of methionyl-tRNA and alanyl-tRNA in buffer. Aminoacyl-tRNA was extracted with phenol from charging mixture after a 10-min reaction at 30°. The ethanol-precipitable material was resuspended in charging buffer and incubated at 30°. The loss of trichloroacetic acid insoluble radioactivity was determined by assaying aliquots at several intervals by the filter paper disk method.

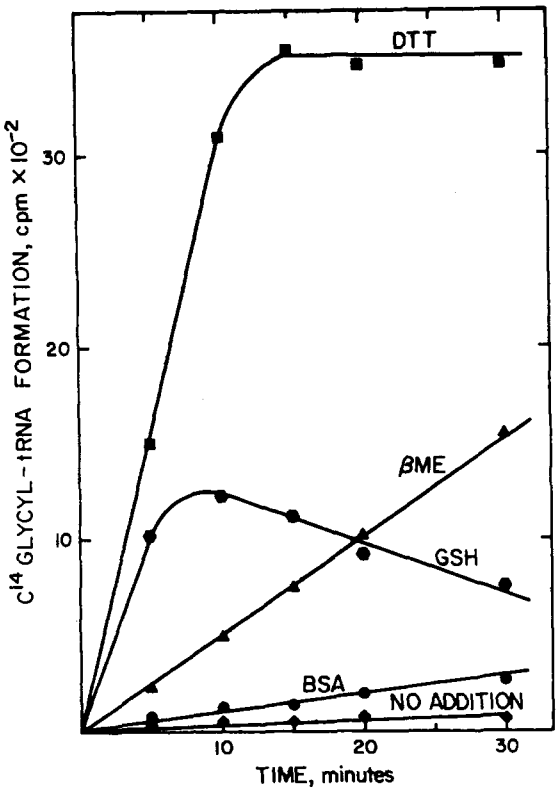

FIGURE 4: The effect of sulfhydryl reducing agents on the attachment of glycine to tRNA. The standard reaction mixture was used to compare the charging of glycine with tRNA in the presence of different additions. Dithiothreitol, β-mercaptoethanol, and glutathione were each used at a final concentration of 5.0 μmoles/ml; 1.9 mg of bovine serum albumin/ml of reaction was used.

alanine and methionine by extracting the peak material and reincubating it in buffer (Figure 3). A loss of trichloroacetic acid precipitable counts was observed.

The instability of the aminoacyl-tRNA is, however, not sufficient to explain the observed type IV kinetics, since as long as all the components required for the formation of the product are present, the reaction should reach a plateau. This contradiction can be resolved by postulating that one of the components is no longer present late in the reaction, but is destroyed in the early part. It was stated above that the tRNA is not destroyed, therefore some other component must be involved.

For the charging reaction with glutamine, glutamic acid, and methionine, the supply of ATP is the critical factor. Maximal initial rate of charging requires a high ATP concentration, 5.0 μmoles/ml. After 10-min reaction, when the aminoacyl-tRNA formation has reached a peak and begun to decline, the addition of another 5.0 μmoles of ATP/ml of reaction results in a burst of charging. Continuous addition of ATP results in a plateau of aminoacyl-tRNA formation. These results indicate that in the initial phase of the incubation, in addition to the amino acid being attached to tRNA, at the expense of an equivalent amount of ATP, the bulk of the excess ATP present (>1000-fold) is hydrolyzed. As the relatively unstable aminoacyl bond is cleaved the amino acid cannot be recharged because the enzyme requires a high concentration of ATP which is no longer present in the mixture. The problem is solved by including in the original mixture a means of regenerating ATP. Only phosphoenolpyruvate is necessary since the synthetase enzyme preparation apparently contains pyruvate kinase activity.

The type IV kinetics observed for the charging of alanine is pH dependent. Normal saturation kinetics are obtained if the reaction mixture is buffered at pH 7.0. Both the initial rate and the extent of the reaction are reduced as the pH is increased. The effect of pH on the

TABLE III: Effect of pH on the Charging of Alanine to tRNA.[a]

pH	Initial Rate (μμmoles/min)	Extent of Reaction (μμmoles)
8.0	1.30	5.22
7.5	3.76	18.9
7.0	8.65	67.2

[a] Three reaction mixtures, differing only in pH, were assayed for aminoacyl-tRNA at several intervals over a 40-min period. The initial rate is defined here as the average rate for the first 2 min. For the pH 8.0 and 7.5 reactions, which never reach a plateau, the extent is the value at the peak.

TABLE IV: Summary of Conditions for Aminoacyl-tRNA Formation in *Neurospora*.[a]

Condition	Ala	Glu	Gln	Gly	Met	Ser	14 Others
tRNA, mg/ml	0.5–1.0					<0.1	0.5–1.0
pH	7.0	7.3–7.5					
$Mg(Ac)_2$, μmole/ml	25	10	5	50			
ATP, μmole/ml	2.5	5.0	5.0	2.5	5.0	2.5	
DTT, μmole/ml	0	5.0					
Phosphoenolpyruvate, μmole/ml	0	20	20	0	20	0	0

[a] All reaction mixtures are in 0.10 M Tris-HCl, have 1 mg/ml of enzyme preparation (in 50% glycerol), and 0.02 μmole/ml of radioactive amino acid.

TABLE V: Specific Acceptor Activity of *Neurospora* tRNA and Its Relationship to Amino Acid Composition.

Amino Acid	Acceptor Act.[a] (μμmoles/OD_{260})	(%)	Amino Acid Composition[b] Protein (%)	Pool (%)	Mycelium (%)
Ala	85.7	6.7	11.0	25.4	13.3
Arg	111.0	8.7	5.6	6.7	5.8
Asn	31.9	2.5	11.5	3.2	10.2
Asp	37.4	2.9			
Cys	16.7	1.3	1.6	4.0	2.0
Glu	33.5	2.6	10.6	14.3	11.2
Gln	66.4	5.2			
Gly	53.8	4.2	9.2	5.0	8.6
His	20.5	1.6	2.6	2.2	2.5
Ile	94.0	7.4	3.2	1.2	2.9
Leu	115.0	9.0	7.8	2.2	6.9
Lys	42.8	3.4	6.9	5.4	6.6
Met	83.9	6.6	1.7	2.2	1.8
Phe	24.6	1.9	3.6	1.0	3.2
Pro	36.2	2.9	3.8	2.5	3.6
Ser	123.0	9.7	7.2	14.8	8.4
Thr	74.3	5.8	5.7	4.7	5.6
Trp	58.7	4.6	0.9	0.1	0.8
Tyr	50.0	3.9	2.5	1.0	2.2
Val	115.0	9.0	4.6	4.0	4.5
Total	1274.4	99.9	100.0	99.9	100.1

[a] Unfractionated tRNA was assayed for specific acceptor activity according to conditions summarized in Table IV.
[b] Data of DeBusk and DeBusk (1967).

initial rate is probably at the level of the activity of the enzyme; however, the decline in the extent of alanyl-tRNA formation may also be affected by an increased rate of deacylation at higher pH values (Table III).

Glycine. Unlike the aminoacyl-tRNA formed with the four other amino acids which display type IV kinetics in the charging reaction, glycyl-tRNA is quite stable. The apparent explanation of the kinetics in this case is rather bizarre. Preliminary experiments indicated the need for a reagent to keep the sulfhydryl groups of the enzyme reduced. However, reduced glutathione proved to be a poor choice. Figure 4 shows the effect of adding different reagents to inhibit the oxidation of sulfhydryl groups. With dithiothreitol, saturation kinetics are observed; less effective agents (such as mercaptoethanol or bovine serum albumin) produce reduced initial rates, compared with DTT, as though the enzyme were limiting. Type IV kinetics are observed only with glutathione.

In the course of experiments on the denaturation of $tRNA_{Gly}$ it was observed that precipitating tRNA with ethanol out of sodium–phosphate buffer results in a

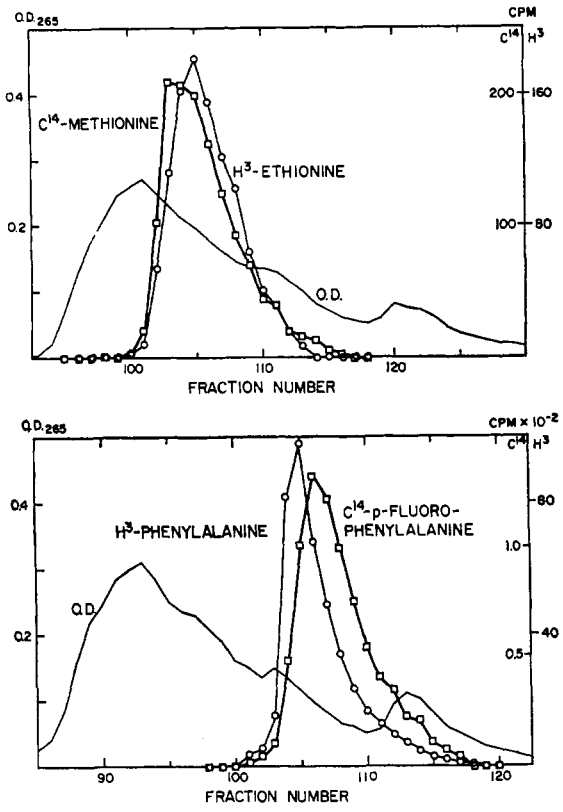

FIGURE 5: Chromatographic profiles of aminoacyl-tRNA *vs.* analog-tRNA. Cochromatography on MAK of [^{14}C]-Met-tRNA with [^{3}H]Eth-tRNA and [^{3}H]Phe-tRNA with [^{14}C]pFPhe-tRNA.

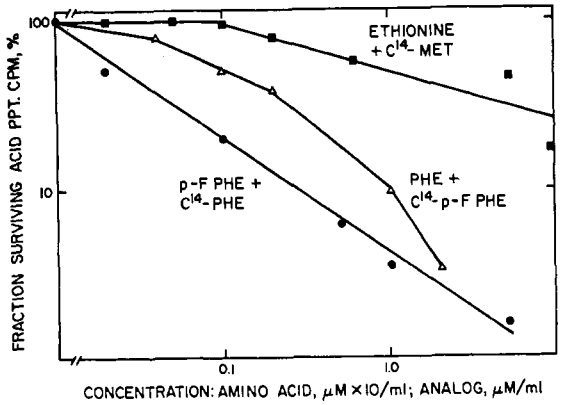

FIGURE 6: Competition experiments between analogs and their respective amino acids. Experiments measuring competition for attachment to tRNA were performed in both directions. A constant amount of labeled amino acid, for example, is charged to tRNA in the presence of an increasing concentration of unlabeled analog. When all of the acceptor sites are occupied the amount of radioactivity is measured. Squares, increasing ethionine concentration, 0.018 μmole/ml of [^{14}C]methionine; circles, increasing pFPhe concentration, 0.002 μmoles/ml of [^{14}C]Phe; triangles, unlabeled Phe in the presence of 1.425 μmoles/ml of [^{14}C]pFPhe. Each value is expressed as the percentage it is of the radioactivity attached in the absence of competing unlabeled material.

substantial loss of glycine acceptor activity. Many other explanations have been ruled out, and it appears that this is a consequence of coprecipitation of some sodium phosphate with the RNA. The salt apparently interferes with the attachment of glycine to tRNA. This phenomenon has been explored further by adding to glycyl-tRNA assay mixtures other salts at varying concentrations. The effect is due to the *anion* and is most pronounced with phosphate; chloride and sulfate are less inhibitory (on a molar basis) and acetate has no effect at all (up to 0.1 M). With each of these anions any of the three monovalent cations, Na^+, K^+, and NH_4^+, give identical results. The effect is fully reversible; neither the enzyme nor the tRNA sustains permanent damage after exposure to 0.1 M phosphate, for example. This anion inhibition has not been examined for all of the amino acids; it does affect alanyl- and aspartyl-tRNA formation but does not affect leucyl-tRNA formation.

Table IV summarizes the sets of optimal conditions determined for assaying the total acceptor activity of *Neurospora* tRNA for the 20 amino acids of protein.

Specific Acceptor Activity. Column 2 in Table V is a summary of the specific acceptor activity of unfractionated *Neurospora* tRNA.

Amino Acid Analogs. In the case of both ethionine and *p*-fluorophenylalanine a considerably higher concentration of analog is required to saturate the synthetase than is required for the respective amino acid (about 100-fold). On the other hand, the specific acceptor activity of unfractionated *Neurospora* tRNA for these analogs is the same as it is for their respective amino acids.

We examined whether in both cases the amino acid and its analog were attached to the same tRNA component(s). Figure 5 shows the results of cochromatographing [^{3}H]Phe-tRNA and [^{14}C]*p*FPhe-tRNA and also [^{14}C]Met-tRNA and [^{3}H]Eth-tRNA. In both cases the analog-tRNA complex elutes reproducibly at a slightly higher salt concentration than does the aminoacyl-tRNA On similar columns [^{14}C]Phe-tRNA and [^{3}H]Phe-tRNA elute in the same place when cochromatographed as do [^{14}C]Met-tRNA and [^{3}H]Met-tRNA.

Three possible explanations of this result are: (1) the analogs are attached to tRNA molecules distinct from those for their cognate amino acids which coincidentally have similar chromatographic mobilities; (2) in the case of Phe and *p*FPhe, *p*FPhe is attached only to the minor, mitochrondrial component of the Phenylalanine tRNA (Barnett and Brown, 1967); and (3) in both cases the amino acid and its analog are attached to the same tRNA molecules but the difference in the structure of the analog is sufficient to cause a shift in chromatographic mobility.

Competition experiments using labeled amino acid and increasing concentrations of unlabeled analog support the third alternative (Figure 6). Competition is detectable when the concentration of analog is five to ten times that of the amino acid. In neither case is there any indication of a plateau (incomplete competition). If the first alternative were correct no competition would be expected, and if the second were correct *p*FPhe would be expected to compete with only 15% of the the Phe acceptors. An objection to this interpretation

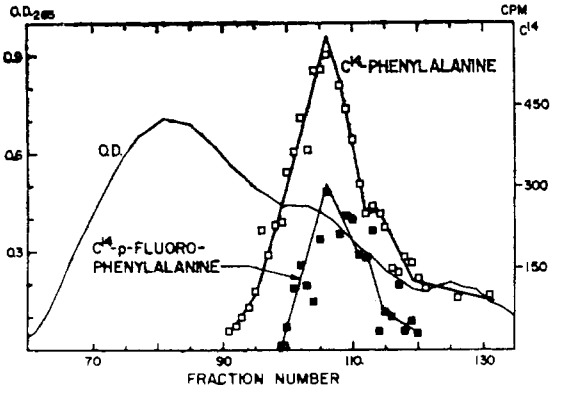

FIGURE 7: Fractionation of Phe and pFPhe acceptor activities. Each fraction from the chromatography on MAK of uncharged tRNA was dialyzed against 0.2 M Tris buffer (pH 7.3) containing 0.1 M $Mg(Ac)_2$ overnight and an aliquot of each was assayed for Phe acceptor activity (open squares). The tRNA from each fraction of interest (99–120) was concentrated by ethanol precipitation, using DNA as carrier, and assayed for pFPhe acceptor activity (solid squares).

of the experiment is that the presence of the analog might noncompetitively inhibit the attachment of the amino acid.

A competition experiment was performed using cold Phe to compete with radioactive *p*FPhe (Figure 6). Complete competition is indicated in this direction as well, with the same reservation as above.

Additional support for the third explanation is provided by the assay of fractionated uncharged tRNA for both Phe and *p*FPhe acceptor activity. If the first or second alternative were correct then the same shift in position should be observed with acceptor activity as is observed with aminoacyl-tRNA. However, the acceptor activity for *p*FPhe is found in the same position as that for Phe (Figure 7).

Discussion

The total acceptor activity, *i.e.*, the sum of the specific acceptor activities for the 20 amino acids, provides an estimate of the amount of tRNA in the sRNA preparation. If one assumes an average molecular weight of 30,000 for *Neurospora* tRNA and an optical density (at 260 mμ) of 24 for a solution containing 1 mg/ml of pure tRNA, then there are 1389 μμmoles of $tRNA/OD_{260}$ unit. If each RNA molecule is capable of accepting 1 amino acid molecule then the maximum acceptor activity of pure tRNA is 1389 μμmoles of amino acid/OD_{260} unit of RNA. The average value for the total acceptor activity of *Neurospora* sRNA which we have measured is 1274 ± 34. Thus about 92% of the RNA soluble in 1.0 M NaCl is tRNA, defined by its amino acid acceptor activity. The remainder of the sRNA is probably composed of ribosomal fragments and/or inactive (denatured) tRNA. The specific acceptor activities reported here average eight to nine times higher than those reported by Barnett and Brown (1967) for *Neurospora* tRNA.

Enzymatic activity for the attachment of all 20 amino acids to unfractionated *Neurospora* tRNA is evident using a single enzyme preparation only when the assays are run under appropriate conditions. Novelli (1967) has reviewed similar situations in other organisms. In this system little activity for glutamine, glutamic acid, and methionine can be detected if the initial ATP concentration is low. Alanyl-tRNA formation is sensitive to alkaline pH, and glycyl-tRNA formation is inhibited by certain anions and requires a sulfhydryl reducing agent.

It seems reasonable then that the failure to detect in any organism all 20 activities in a crude preparation may reflect a failure to find appropriate conditions as well as varying enzyme stability. It is not known whether the same key variables, *i.e.*, pH, and ATP and Mg^{2+} concentrations, play any role in regulating the charging of tRNA *in vivo*.

The fact that type IV kinetics (Figure 4) are observed for glycine only when glutathione is present can be accounted for by assuming that *Neurospora* has an enzyme similar to that of yeast which catalyzes the exchange of free glycine with the glycine residue in glutathione (Snoke and Bloch, 1954). At late times in the charging reaction of glycine to tRNA (after 10 min at 30°) unlabeled glycine released in this way from glutathione dilutes the isotopic glycine remaining in the mixture, and, by exchanging with the labeled glycine attached to tRNA (Yamane and Sueoka, 1964), lowers the specific activity of the attached glycine.

We do not know whether the inhibition of glycyl-tRNA formation by certain anions is due to an effect on the synthetase or on the tRNA molecule. Peterkofsky *et al.* (1966a) have reported that NaCl inhibits the heterologous esterification of *E. coli* tRNA with yeast enzyme. They concluded that the effect was due to modification of the enzyme; however, in that case it appeared that the monovalent cation was the active agent.

Virtually nothing is known about the regulation of tRNA synthesis; no theory has been presented which accounts for the different distributions of acceptor activity in different organisms. A simple hypothesis is that these exists a 1:1 relationship between the distribution of acceptor activity and the amino acid composition of total protein. This naive proposal is probably not the case in *Neurospora*. The distribution of acceptor activity (expressed in per cent) shows no striking relationship to the amino acid composition of total soluble protein, nor to the composition of the free amino acid pools (Table V).

Since the distribution of acceptor activity is not indicative of the average amino acid composition of protein, these parameters may be related by (1) the efficiency of binding of either the amino acids or the tRNA molecules to the aminoacyl-tRNA synthetases or (2) by the concentration of the synthetase enzymes *in vivo*.

We conclude from the experiments reported here on the charging of amino acid analogs to tRNA, that ethionine and *p*-fluorophenylalanine are esterified only to those tRNA molecules which are specific for methionine and phenylalanine, respectively, as might be expected. What was not expected, however, is that the chemical differences between these amino acids and their analogs are sufficient to cause differences in the chromatographic

mobilities of their respective complexes with identical tRNA molecules. These chromatographic differences may be due to slight conformational adjustments of the molecule; if so, they perhaps help to limit the incorporation of these analogs into protein. Formylation of *E. coli* methionyl-$tRNA_{F\text{-}Met}$ shifts its chromatographic mobility and this has been interpreted as the result of a configurational change (Leder and Bursztyn, 1966). We note, with interest, that MAK chromatography has enough resolving power to detect such differences. Study of tRNA molecules attached to modified amino acids may be useful in understanding the conformation of aminoacyl-tRNA which is necessary for amino acid transfer.

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