Fractionation of Transfer Ribonucleic Acid on a Methylated Albumin–Silicic Acid Column. II. Changes in Elution Profiles following Modification of Transfer Ribonucleic Acid*

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ABSTRACT: Uncharged phenylalanine transfer ribonucleic acid and methionine transfer ribonucleic acid are eluted earlier from methylated albumin–silicic acid columns than the aminoacylated species. Four species of phenylalanyl transfer ribonucleic acid are resolved on chromatography of transfer ribonucleic acid extracted from a "relaxed" methionine-requiring mutant of *Escherichia coli* grown in a medium deficient in methionine. These multiple species of phenylalanyl transfer ribonucleic acid were not resolved from each other in the absence of aminoacylation. The terminal 5′-phosphate of the transfer ribonucleic acid does not participate in the chromatographic shift of phenylalanine transfer ribonucleic acid following aminoacylation, since its removal with alkaline phosphatase fails to alter the chromatographic behavior of phenylalanyl transfer ribonucleic acid. Additional evidence is presented suggesting that the terminal 5′-phosphate is sequestered within the molecule. Blocking of the free amino group of phenylalanyl transfer ribonucleic acid or methionyl transfer ribonucleic acid with various aliphatic groups causes consistent retardation on elution from the methylated albumin–silicic acid column as compared with the unblocked species. This retardation is independent of the size of the N-blocking group; *N*-formyl-, *N*-acetyl-, *N*-caproyl-, *N*-palmityl-, *N*-stearyl-, and *N*-acetylalanylphenylalanyl transfer ribonucleic acids emerged from the column at the same position.

Polyacrylamide column chromatography demonstrated that N-blocked aminoacyl transfer ribonucleic acids, such as *N*-acetylphenylalanyl transfer ribonucleic acid and *N*-formylmethionyl transfer ribonucleic acid, are more excluded than their aminoacylated and unacylated counterparts, a finding consistent with a more extended configuration for the N-blocked species. The elution profile of uncharged phenylalanine transfer ribonucleic acid on polyacrylamide columns did not coincide with that found for phenylalanyl transfer ribonucleic acid, the latter eluting latter than the uncharged phenylalanine transfer ribonucleic acid. Similar results were obtained upon chromatography of uncharged methionine transfer ribonucleic acid and methionyl transfer ribonucleic acid. It is suggested that there exist at least three separate configurations for each species of transfer ribonucleic acid: (1) uncharged form, (2) an aminoacylated form, and (3) an N-blocked aminoacyl configuration. These configurations may be required for the specificity of the successive reactions of transfer ribonucleic acid in protein synthesis.

The MASA[1] column of Okamoto and Kawade (1963) has recently been adapted to the resolution of tRNAs from *Escherichia coli* (Littauer *et al.*, 1966; Stern and Littauer, 1968). This column provides a rapid tool for the separation of 100-mg quantities of tRNA. MASA columns are able to separate aminoacylated from uncharged $tRNA^{Phe}$ (Littauer *et al.*, 1966) and are also able to separate Met-tRNA from *N*-formyl-Met-tRNA (Leder and Bursztyn, 1966). The purpose of this communication is to show that these separations are a general phenomenon and appear to depend, in part, upon the configurational state of tRNA. The data presented also support the hypothesis that there are at least three configurational states for each species of tRNA, which can be separated from each other on the MASA column.

Materials and Methods

N-Hydroxysuccinimide esters of formic, acetic, caproic, palmitic, and stearic acids were the generous gifts of Drs. N. de Groot and Y. Lapidot of the Hebrew University Jerusalem and Dr. M. Fridkin of the Weizmann Institute of Science. The hydroxysuccinimide esters

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[1] Abbreviations used: MASA, methylated albumin–silicic acid; MAK, methylated albumin–kieselguhr; Met-tRNA and Phe-tRNA, methionyl and phenylalanyl transfer ribonucleic acid. In this paper "methionine-starved" tRNA refers to the RNA extracted from a "relaxed" methionine-requiring mutant of *E. coli* (A-19 or G-15) grown in a medium deficient in methionine. Methyl-deficient tRNA refers only to those species which contain few or no methyl groups. Methionine-starved tRNA is an approximately equal mixture of methyl-deficient tRNA and fully methylated normal tRNA.

were reacted with *E. coli* Phe-tRNA according to the procedure of de Groot *et al.* (1966). The *N*-acetylalanyl hydroxysuccinimide ester was purchased from the Miles-Yeda Co., Rehovoth, Israel.

Commercial *E. coli* B tRNA and labeled amino acids were purchased from Schwarz BioResearch. Unlabeled amino acids were the products of the California Foundation for Biochemical Research, Membrane filters, MF 50, with a 0.6-μ pore size were obtained from Membranfilter Gesellschaft, Göttingen.

Alkaline phosphatase was purchased from Worthington Biochemical Corp. The enzyme was pretreated with acid-washed powdered glass to remove contaminating ribonuclease activity. Enzyme (0.35 mg) and powdered glass (20 mg, 200 mesh, Fisher Scientific Co.) were stirred together for 1 hr at 4° in 1 ml of 0.1 M Tris (pH 7.0) (L. A. Heppel, 1963, personal communication).

P_i was determined by the method of Chen *et al.* (1956). The assay of phenylalanine acceptance of tRNA on filter paper disks was the procedure of Cherayil and Bock (1965).[2]

E. coli A-19 RC^{rel} RNase I^-met^- was obtained from Dr. S. Spiegelman and *E. coli* G-15 RC^{rel} $met^-his^-biotin^-$ was obtained from Dr. G. Stent. The cultivation of *E. coli* cells under normal and methionine-starved conditions, the isolation of tRNA, the preparation of Phe-tRNA and Met-tRNA, and the preparation of the MASA column were described in previous publications (Littauer *et al.*, 1966; Littauer and Stern, 1967; Stern and Littauer, 1968). Whenever the chromatography of a small amount of labeled aminoacyl-tRNA was required, uncharged carrier tRNA was added to enhance the resolution on the MASA column.

Results

MASA Column Chromatography of Aminoacylated and Uncharged $tRNA^{Phe}$. Previous studies on the chromatographic properties of Phe-tRNA on methylated albumin columns suggested that the chromatographic behavior of uncharged phenylalanine tRNA was radically different from that of tRNA charged with phenylalanine (Littauer *et al.*, 1966). This is confirmed in the present investigation. In the first experiment 18 mg of uncharged normal tRNA was mixed together with 0.17 mg of [^{14}C]Phe-tRNA and the mixture was subjected to MASA column chromatography. The elution profile of the uncharged $tRNA^{Phe}$ was determined by assaying each column fraction for its phenylalanine acceptance capacity, while the acylated tRNA, namely [^{14}C]Phe-tRNA, was followed by removing an aliquot of each fraction and measuring the trichloroacetic acid insoluble radioactivity. Figure 1a demonstrates that the uncharged $tRNA^{Phe}$ precedes the aminoacylated tRNA (Phe-tRNA).

In a control experiment uncharged tRNA was incubated with the reaction mixture used for phenylalanine acylation, with the omission of enzyme and phenylalanine and then treated with phenol and precipitated with ethanol. This treatment did not change the elution position of uncharged $tRNA^{Phe}$.

With some preparations of normal tRNA, preacylated Phe-tRNA emerged from the column as two peaks (Revel and Littauer, 1965; Littauer *et al.*, 1966; Stern and Littauer, 1968). This was only observed with preacylated Phe-tRNA. When uncharged $tRNA^{Phe}$ was fractionated on a MASA column, only one peak of phenylalanine-accepting tRNA was observed, clearly separated from the two peaks of the preacylated Phe-tRNA.

A similar phenomenon was observed with the tRNA extracted from a relaxed methionine-requiring mutant of *E. coli* grown in a medium deficient in methionine. MASA column chromatography of such a methionine-starved tRNA preparation resolved four peaks of the Phe-tRNA (Littauer *et al.*, 1966). The first two peaks correspond to methyl-deficient tRNA, while the last peak corresponds to a fully methylated normal tRNA. Here also only a single peak of uncharged $tRNA^{Phe}$ was observed distinct from the four peaks of preacyltated Phe-tRNA (Figure 1b).

A working hypothesis was adopted that MASA columns resolve tRNA, in part, on the basis of differences in configuration. In addition, it was assumed that there are configurational differences between aminoacylated and unacylated tRNAs.

Alkaline Phosphatase Treatment of tRNA. The change in configuration of the tRNA on aminoacylation may be due to the terminal 5′-phosphate of tRNA, the only primary phosphate moiety in the molecule, interacting with the free amino group of the amino acid to form, or to stabilize, the aminoacyl-tRNA configuration.

To test this hypothesis the terminal 5′-phosphate was removed from tRNA by incubation with alkaline phosphatase at 60° (Figure 2a). The reaction was completed within 2 min and the P_i release was almost equivalent to the number of tRNA molecules present in the reaction mixture. Out of 18.8 mμmoles of tRNA, 20 mμmoles of P_i was released (assuming an average molecular weight of 26,600 for tRNA). Phenylalanine acceptance was not affected by this treatment, confirming the results of Harkness and Hilmoe (1962). The tRNA which had been incubated with alkaline phosphatase was charged with [^{14}C]phenylalanine and mixed together with untreated [^{3}H]Phe-tRNA. This mixture was then chromatographed on a MASA column. Figure 2b demonstrates that after removal of the 5′-terminal phosphate, the behavior of the aminoacylated tRNA on the MASA column remains unchanged and does not revert to the profile of uncharged $tRNA^{Phe}$. It was therefore concluded that the terminal 5′-phosphate of tRNA is not involved in the chromatographic shift of the Phe-tRNA.

It should be noted that alkaline phosphatase is also able to digest the terminal 3′- (or 2′-) phosphate of tRNA ···pCpCp obtained by periodate and cyclohexylamine treatment of tRNA···pCpCpA (Daniel and Littauer, 1965). The digestion of the terminal 3′- (or 2′-) phosphate takes place at 37°, yet from the present experiments it was observed that alkaline phosphatase is unable to remove the terminal 5′-phosphate at that temperature (10 min at 37°). Only by raising the temperature to 60°

[2] This assay was further developed in our laboratory by Mr. E. Canani.

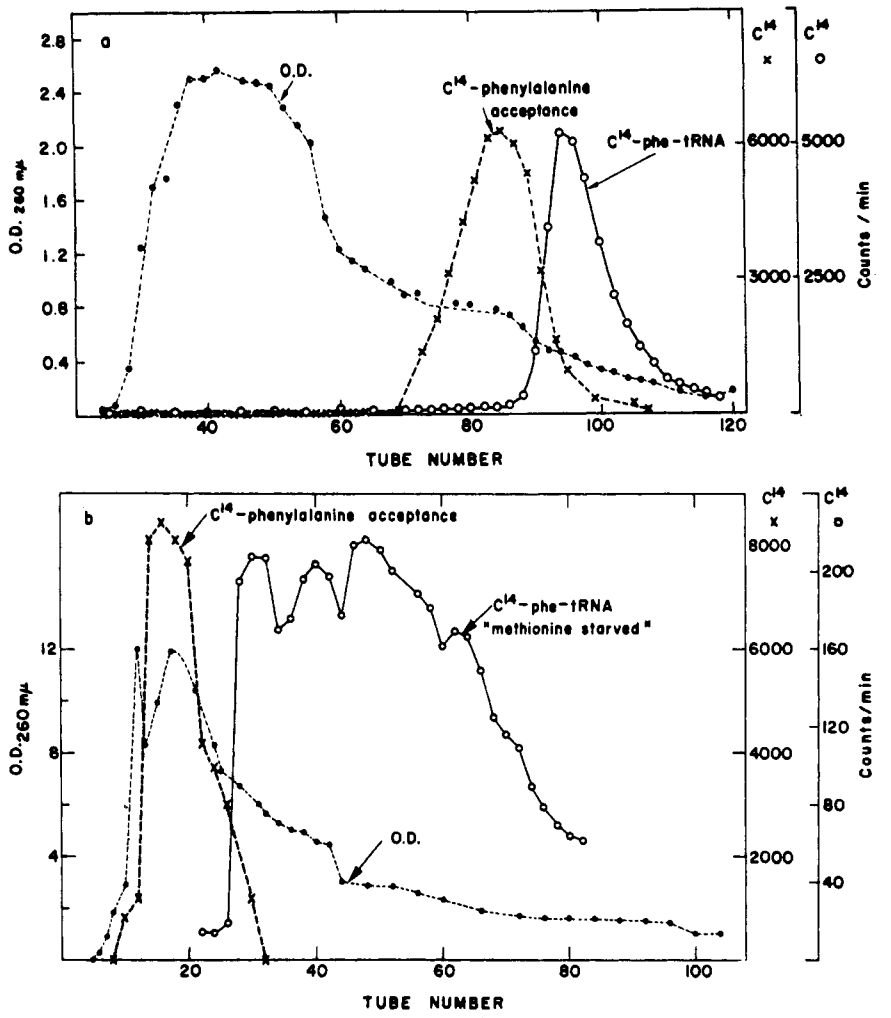

FIGURE 1: MASA column chromatography. (a) Of normal Phe-tRNA and the profile of phenylalanine acceptance of normal uncharged tRNA. Normal tRNA (0.17 mg) from *E. coli* G-15 was acylated with [^{14}C]phenylalanine and mixed with 18 mg of uncharged normal *E. coli* G-15 tRNA together with 5 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.68 M NaCl and placed on a MASA column (2 × 12 cm). The column was washed with 50 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.78 M NaCl. The RNA was eluted at 16° over a period of 24 hr with a linear gradient formed from 200 ml each of 0.05 M sodium acetate buffer (pH 5.5) containing 0.78 and 1.15 M NaCl. Fractions (3.2 ml) (1) were collected. The A_{260} was measured and 3.0-ml aliquots of each fraction were removed for precipitation with trichloroacetic acid and counting of radioactivity (O——O). For the assay of phenylalanine acceptance in the resulting fractions, 0.05 ml of each fraction was added to a phenylalanine tRNA acylation assay mixture containing [^{14}C]phenylalanine and 15 μmoles of Tris-HCl buffer (pH 7.8) in a final volume of 0.1 ml (X- - -X). (b) Of methionine-starved Phe-tRNA and the profile of phenylalanine acceptance of methionine-starved uncharged tRNA. Methionine-starved *E. coli* A-19 tRNA (0.2 mg) was acylated with [^{14}C]phenylalanine and mixed with 50 mg of uncharged methionine-starved *E. coli* A-19 tRNA together with 30 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl and placed on a MASA column. The column was washed with 50 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.8 M NaCl. The chromatographic procedure was the same as that described under part a. Aliquots of each fraction (0.5 ml) were removed for precipitation with trichloroacetic acid and counting of radioactivity (O——O). Phenylalanine acceptance of the column fractions was assayed by adding 0.02 ml of each fraction to a phenylalanine tRNA acylation assay mixture containing [^{14}C]phenylalanine in a final volume of 0.1 ml (X- - -X). Since the reaction mixture for phenylalanine acceptance contained a large excess of unacylated tRNA, the contribution of labeled Phe-tRNA in the assay can be neglected.

was it possible to remove the terminal 5′-phosphate. This observation is consistent with the hypothesis that the 5′ end of the tRNA chain is hydrogen bonded or sequestered within the molecule (*cf.* Armstrong *et al.*, 1966; Herbert and Smith, 1967).

MASA Column Chromatography of N-Blocked Phe-tRNAs. An alternative explanation which might be invoked for the change in the chromatographic behavior of $tRNA^{Phe}$ following aminoacylation is the presence of the free amino group. If so, blocking the free amino group should cause reversion of the profile of Phe-tRNA eluting from the column to that found for the unacylated species. A suitable N-blocking reagent is *N*-hydroxysuccinimide ester of formic acid since it was shown by de Groot *et al.* (1966) that it will react specifically with the NH_2 group of the amino acid without attacking the polynucleotide chain.

N-Formyl-[^{14}C]Phe-tRNA was prepared and applied to a MASA column together with [^{3}H]Phe-tRNA. Figure 3a shows that addition of an *N*-formyl group to the

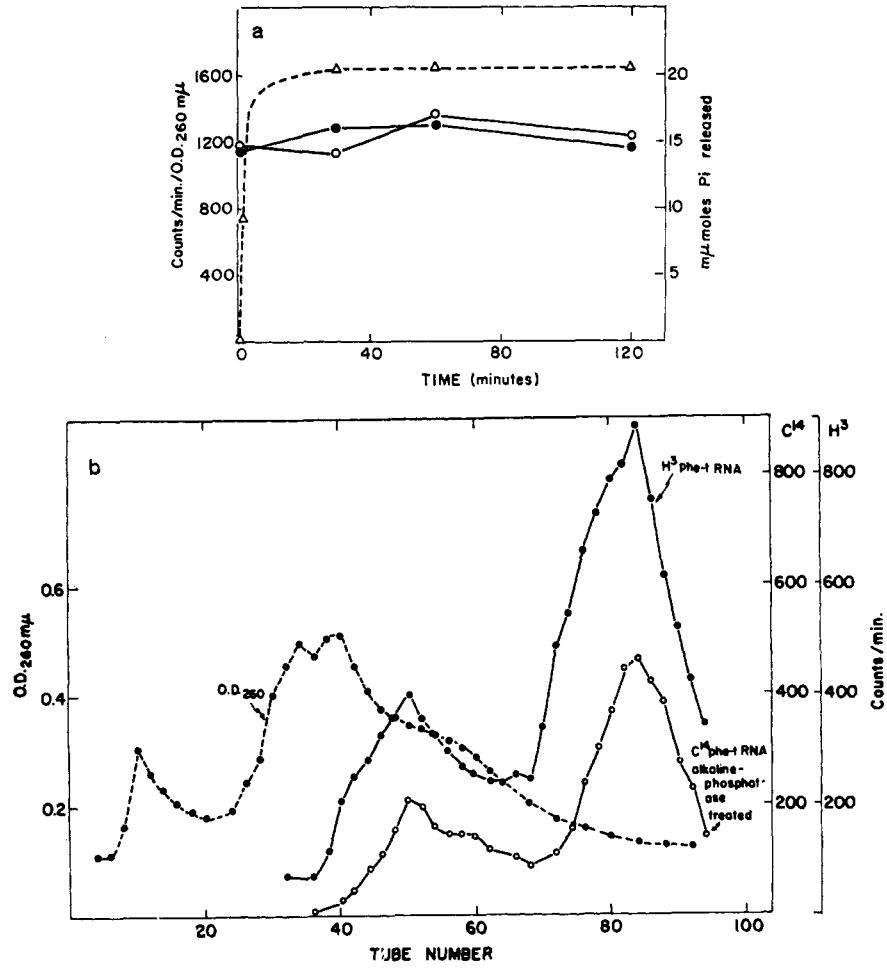

FIGURE 2: Alkaline phosphate studies of tRNA. (a) Time course of alkaline phosphatase digestion of *E. coli* tRNA and phenylalanine acceptor capacity after enzyme digestion. Each point on the graph represents a different tube. The reaction mixture (0.1 ml) contained 0.5 mg of normal *E. coli* A-19 tRNA, 0.1 M Tris-HCl buffer (pH 8.3), and 3.5 μg of bacterial alkaline phosphatase. The suspension was incubated at 60°, and at the indicated time intervals, 0.4 ml of a 0.17% uranyl acetate and 1.7% perchloric acid solution were added to one set of tubes and P_i was determined on the supernatant fluid. The RNA in the duplicate set of tubes was precipitated with 0.4 ml of 80% ethanol containing 0.3 M NaCl. The precipitate was dissolved in 0.1 ml of H_2O and a 0.06-ml aliquot of this solution was added to a phenylalanine tRNA acylation assay mixture. (Δ—Δ) P_i (mμmoles) released per tube containing 18.8 mμmoles of tRNA (assuming an average molecular weight of 26,600); (○—○) phenylalanine acceptance of tRNA incubated in the absence of alkaline phosphatase; (●—●) phenylalanine acceptance of RNA incubated in the presence of alkaline phosphatase. (b) MASA column chromatography of Phe-tRNA pretreated with alkaline phosphatase. Normal tRNA (0.5 mg) from *E. coli* A-19 was treated with alkaline phosphatase as described under part a, then precipitated with ethanol–NaCl, and dissolved in 0.1 ml of H_2O. An aliquot of this solution (0.06 ml) was charged with [^{14}C]phenylalanine, then treated with phenol, precipitated with ethanol–NaCl, and dissolved in 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. A solution of untreated tRNA (0.3 mg/ml) was acylated with [^{3}H]phenylalanine and isolated. The two Phe-tRNA solutions were mixed together with 3.5 mg of uncharged *E. coli* A-19 tRNA in 10 ml of 0.05 M sodium acetate buffer containing 0.4 M NaCl and placed on a MASA column. The column was washed and the chromatography was carried out as described under Figure 1.

Phe-tRNA did not cause the expected reversion in the elution profile of Phe-tRNA. Moreover, *N*-formyl-Phe-tRNA was retarded on the column emerging 16 tubes after the Phe-tRNA.

The effect of the N-blocking group on the elution profile of Phe-tRNA was examined in detail. If retardation of *N*-formyl-Phe-tRNA on the MASA column is due to the additional size contributed by the formyl group, then addition of larger blocking groups should cause even further retardation on the column. A series of *N*-hydroxysuccinimide esters of aliphatic acids were allowed to react with [^{14}C]Phe-tRNA. Each of these *N*-aliphatic acid-[^{14}C]Phe-tRNAs yielded the same elution pattern from the MASA column, including *N*-acetyl-, *N*-caproyl-, *N*-palmityl-, and *N*-stearyl-Phe-tRNA (Figure 3b–e). It was concluded that the chain size made no difference in the elution pattern.

A number of control experiments were performed. [^{3}H]Phe-tRNA was incubated in dimethylformamide in the absence of the N-blocking reagent under conditions identical with the actual N-blocking reactions. The chromatographic profile of this material did not differ from that of untreated [^{14}C]Phe-tRNA, as shown in Figure 3f. Uncharged tRNA was also incubated under the same

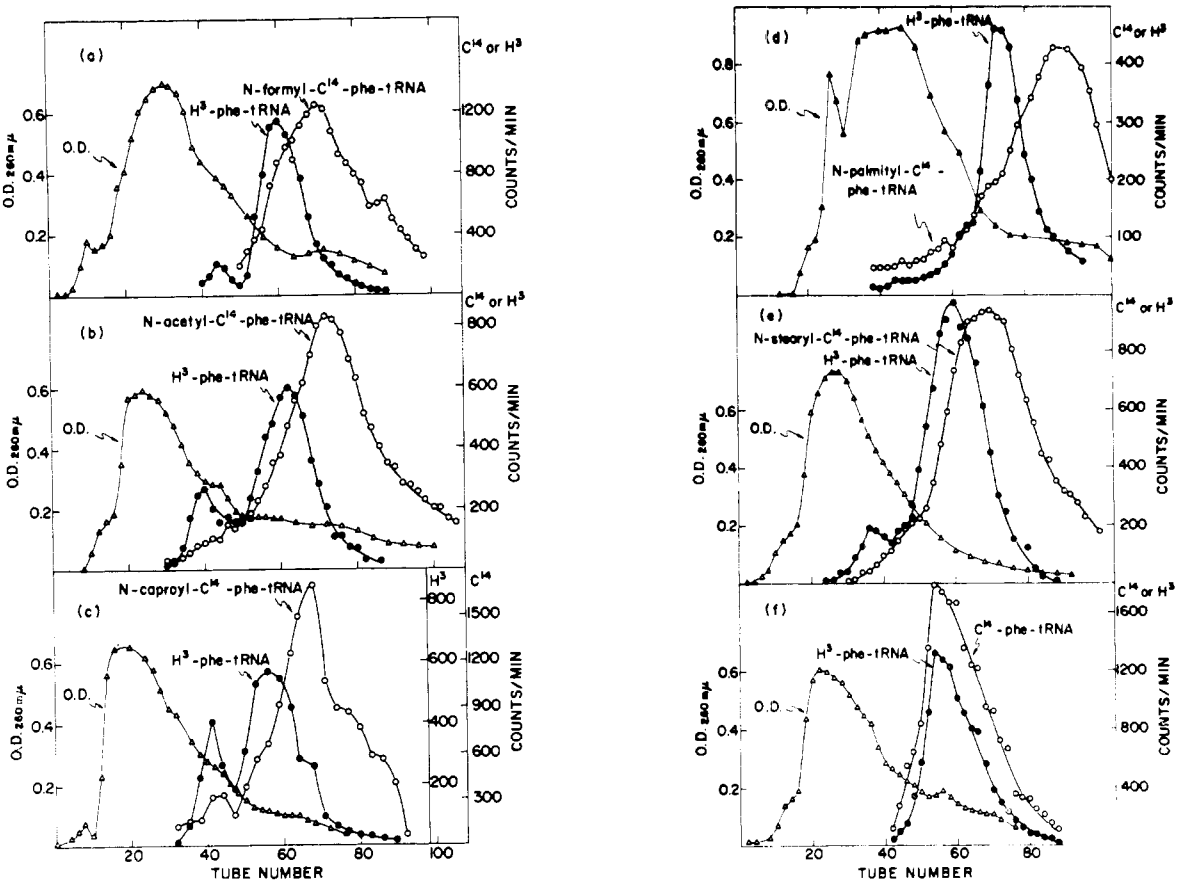

FIGURE 3: MASA column chromatographic profiles of N-blocked Phe-tRNAs. The N-blocked [^{14}C]Phe-tRNAs were prepared by adding 1.9 mg of [^{14}C]Phe-tRNA in 0.4 ml of 0.1 M sodium acetate buffer (pH 5.0) to 1.6 ml of a solution of dimethylformamide, containing 440 μmoles of *N*-acyl hydroxysuccinimide ester. This mixture was stirred vigorously for 15 hr at room temperature and then cooled for 3 hr at −15°. The precipitate was collected by centrifugation, washed twice with 5-ml aliquots of cold dimethylformamide and three times with cold 96% ethanol, and then dissolved with water. An aliquot of this solution (about 30,000 cpm) was added to a solution containing about an equal number of counts per minutes of [^{3}H]Phe-tRNA, together with 4.0 mg of uncharged *E. coli* B tRNA in 10 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl. This was applied to a MASA column. The column was washed and chromatography was carried out as described under Figure 1. In the case of part f, [^{3}H]Phe-tRNA (30,000 cpm) was mixed with 1.9 mg of *E. coli* B tRNA, dissolved in 0.55 ml of 0.1 M sodium acetate buffer (pH 5.0), and stirred overnight with 2.2 ml of dimethylformamide. The RNA was then isolated as described above and placed on a MASA column together with untreated [^{14}C]Phe-tRNA (40,000 cpm) in 10 ml of 0.05 M sodium acetate (pH 5.5) containing 0.4 M NaCl.

conditions in the presence of *N*-acetyl hydroxysuccinimide ester. The tRNA was isolated, acylated with [^{14}C]-phenylalanine, and placed on a MASA column together with untreated [^{3}H]Phe-tRNA. Again no difference in the elution pattern of the two Phe-tRNAs was observed.

The differences in MASA column chromatographic profile between Phe-tRNA and N-blocked Phe-tRNA may also reflect alterations in configuration. One criticism of this theory might be that the additional carbonyl group introduced with the N-blocking group interacts with the column so as to retard the elution of the N-blocked Phe-tRNA. To test this, *N*-acetylalanyl-[^{3}H]-Phe-tRNA was prepared from *N*-acetylalanyl hydroxysuccinimide. The N-blocked dipeptidyl-tRNA was placed on a column together with *N*-acetyl-[^{14}C]Phe-tRNA. If the carbonyl group is affecting the elution pattern, then the introduction of a second carbonyl group should retard the material even more. However, only a slight shift (three tubes) was observed with the introduction of the additional carbonyl (Figure 4). The addition of the first carbonyl caused a shift of 15–20 tubes. It is therefore unlikely that the carbonyl group alone is the basis for the elution pattern change.

Polyacrylamide Gel Chromatography. The changes in configuration of tRNA^{Phe} suggested by differences in MASA column elution may be accompanied by a change in the diameter of the molecule. If so, the change might be reflected in differences in molecular sieve chromatography on polyacrylamide gel columns. This possibility was tested on a column of Bio-Gel P-100. Uncharged tRNA (15 mg) was mixed together with [^{3}H]-Phe-tRNA and *N*-acetyl-[^{14}C]Phe-tRNA. The mixture was placed on the column which was then washed with 0.1 M sodium acetate buffer (pH 4.5). Figure 5 illustrates the results of that chromatography. The *N*-acetyl-[^{14}C]-Phe-tRNA precedes the [^{3}H]Phe-tRNA and the bulk of the extinction profile at 260 mμ. The extinction profile is similar to that reported for the chromatography

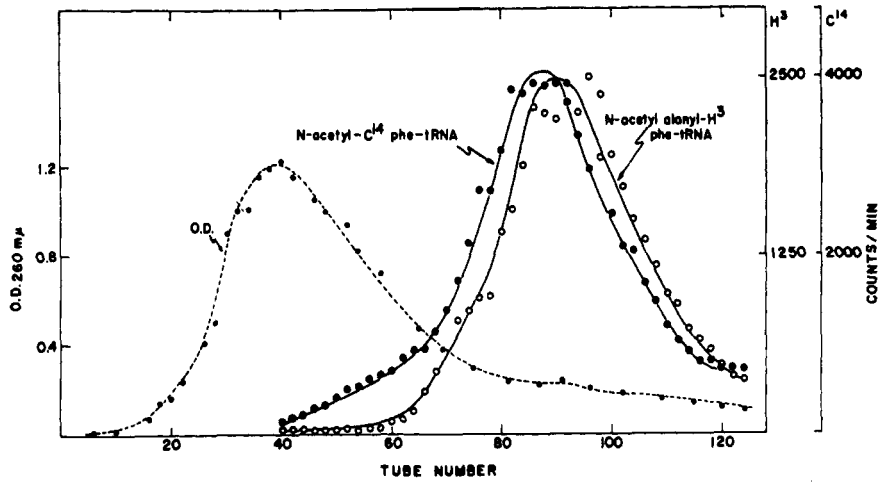

FIGURE 4: MASA column chromatography of *N*-acetylalanyl-Phe-tRNA and *N*-acetyl-Phe-tRNA. *N*-Acetylalanyl-[^{3}H]Phe-tRNA and *N*-acetyl-[^{14}C]Phe-tRNA were prepared from *N*-acetylalanyl hydroxysuccinimide ester and *N*-acetyl hydroxysuccinimide ester as described under Figure 3. Approximately 30,000-cpm aliquots of each N-blocked tRNA were mixed together with 4.0 mg of uncharged *E. coli* B tRNA in 10 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.4 M NaCl and applied to a MASA column. Chromatography was carried out as described under Figure 1.

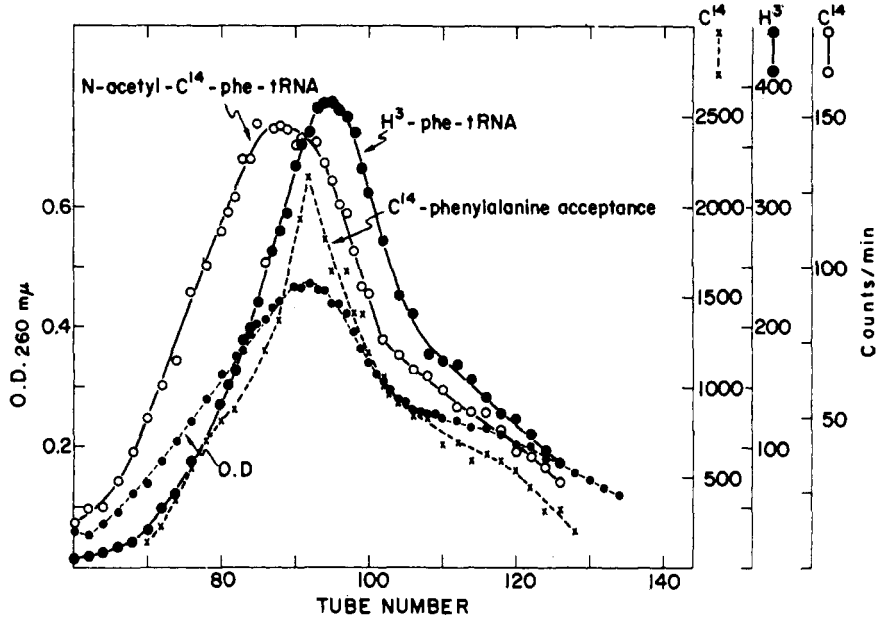

FIGURE 5: Polyacrylamide gel chromatography of *N*-acetyl-Phe-tRNA, Phe-tRNA, and the profile of phenylalanine acceptance of uncharged tRNA. A Bio-Gel P-100 (50–100 mesh) column (1.1 × 100 cm) was equilibrated with 0.1 M sodium acetate buffer (pH 4.5) at room temperature. Normal *E. coli* G-15 tRNA (15 mg) was mixed together with about 10,000 cpm of *N*-acetyl-[^{14}C]-Phe-tRNA and 16,000 of [^{3}H]Phe-tRNA from *E. coli* G-15 prepared as described under Figure 3. The mixture (0.9 ml) was placed on the column, eluted with acetate buffer, and 1.7-ml fractions were collected. Following A_{260} determinations (1:10 dilution), 0.9 ml of each fraction was removed, mixed with 12 ml of Bray's (1960) solution, and radioactivity was measured. Additional aliquots of 0.025 ml were applied to Whatman No. 3MM paper disks and phenylalanine acceptance capacity was assayed by a modified procedure of Cherayil and Bock (1965).

of *E. coli* tRNA on columns of Sephadex G-100 (Schleich and Goldstein, 1964; Röschenthaler and Fromageot, 1965). Figure 5 also illustrates that the elution profile of unacylated tRNA^{Phe} obtained by the assay of [^{14}C]-phenylalanine acceptance does not coincide with that found for Phe-tRNA. The peak of the unacylated tRNA^{Phe} precedes the peak of the preacylated Phe-tRNA.

It may be argued that the *N*-acetyl-[^{14}C]Phe-tRNA is excluded to a greater extent by the polyacrylamide gel because of the slight increase in molecular weight (0.2%) added by the acetyl group. If this is so, then the difference should be more pronounced with a larger N-blocking group. Chromatography was repeated using 15 mg of uncharged tRNA, [^{3}H]Phe-tRNA, and substituting *N*-palmityl-[^{14}C]Phe-tRNA for the *N*-acetyl-[^{14}C]Phe-tRNA which constituted a 1% increase in molecular weight. *N*-palmityl-Phe-tRNA emerged from the col-

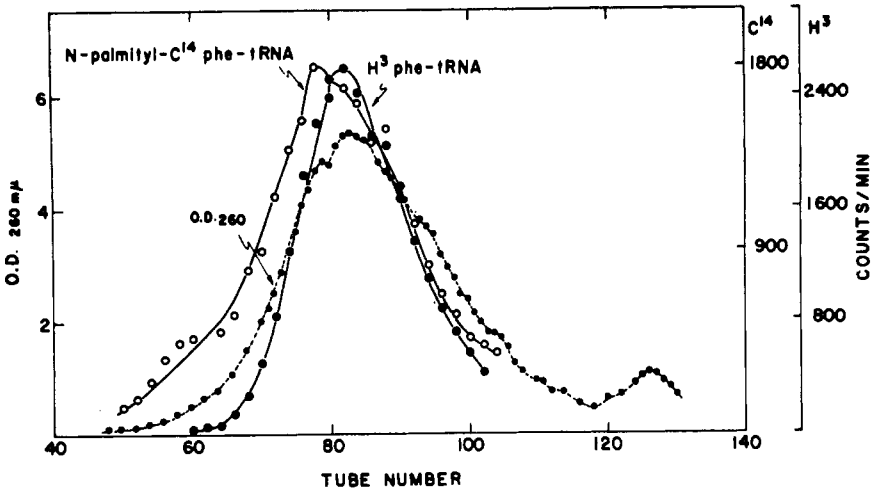

FIGURE 6: Polyacrylamide gel chromatography of *N*-palmityl-Phe-tRNA and Phe-tRNA. A Bio-Gel P-100 column was used as described under Figure 5. *E. coli* B tRNA (15 mg) was mixed together with about 20,000 cpm each of *N*-palmityl-[^{14}C]Phe-tRNA and [^{3}H]Phe-tRNA. The mixture was placed on the column in a volume of 1.5 ml. The RNA was eluted with acetate buffer and 2.0-ml fractions were collected. Following A_{260} determination, 1 ml of each fraction was removed for radioactivity measurement.

umn at the same position as *N*-acetyl-Phe-tRNA (Figure 6). One control was pretreatment of tRNA with *N*-formyl hydroxysuccinimide, prior to charging. This material eluted in the same position as Phe-tRNA. It was therefore concluded that the N-blocked Phe-tRNA derivatives have a more expanded configuration than Phe-tRNA or uncharged tRNA^{Phe}.

Chromatographic Pattern of N-Formyl-Met-tRNA, Met-tRNA, and Uncharged tRNA^{Met}. MASA column chromatography showed that uncharged tRNA^{Met} emerged earlier than Met-tRNA; in addition, there was a clear separation between Met-tRNA and *N*-formyl-Met-tRNA, the latter being more retarded on the column (Figure 7a).

Polyacrylamide column chromatography was then examined with the above derivatives. The chromatographic behavior of the uncharged tRNA^{Met}, Met-tRNA, and formyl-Met-tRNA was similar to that found with tRNA^{Phe} derivatives. The *N*-formyl derivative was more excluded by the column than Met-tRNA (Figure 7b). Thus as with N-blocked Phe-tRNA derivatives, the result of this column suggests a much more expanded configuration for formyl-Met-tRNA than for Met-tRNA or uncharged tRNA^{Met}. Again the elution profile of uncharged tRNA^{Met} did not coincide with that found for Met-tRNA, the latter eluting later than the uncharged tRNA^{Met}.

Discussion

When several species of tRNA exist for a single amino acid, as in the case of methionine-starved phenylalanine tRNAs, no resolution of these species can be detected on MASA columns. Only after amino acid esterification has occurred do several species become resolved one from the other. It is difficult to visualize how the small increase in molecular weight and the net increase of one positive charge from the amino acid could be invoked as the basis of the separation of four different methionine-starved Phe-tRNA peaks since the amino acid is common to all four species. In addition, if the free amino group is the cause of the change in the elution pattern, it would have been expected that blocking the free amino group would cause a reversion to the uncharged position. However, it was observed that the N-blocked derivatives were even further retarded on the column than the Phe-tRNA (Figure 3).

We are therefore led to propose that differences in the chromatographic behavior of charged and uncharged tRNA^{Phe} on MASA columns may reflect a change in configuration. Aminoacylation brings out potential differences between molecules which are not expressed in the absence of the amino acid. The results of the polyacrylamide gel chromatography are also consistent with the concept that aminoacylation changes the configuration of tRNA^{Phe} and tRNA^{Met}. The uncharged tRNA^{Phe} and tRNA^{Met} seem to have a somewhat more expanded configuration since they are excluded earlier from the column than their aminoacylated counterparts (Figures 5 and 7b). Is the difference in configuration between uncharged and aminoacylated tRNA a general phenomenon? Differences in the MASA column elution profile have been observed for tRNA^{Phe}, tRNA^{Met} (Figures 1a,b and 7a), and tRNA^{Lys} (Littauer and Stern, 1967). The work of Sarin and Zamecnik (1965) is in accord with the assumption that a large number of the different tRNA chains change their configuration on aminoacylation. Using optical rotatory dispersion techniques, these authors observed a decrease in the amplitude of cotton effects after acylation of tRNA with a mixture of amino acids. In addition, it was recently observed that certain aminoacyl-tRNAs have a different sedimentation pattern than the corresponding uncharged tRNAs upon sucrose gradient centrifugation (Kaji and Tanaka, 1967).

The results presented in Figure 3 show that blocking of the free amino acid of Phe-tRNA with various aliphatic groups causes a consistent retardation in elution

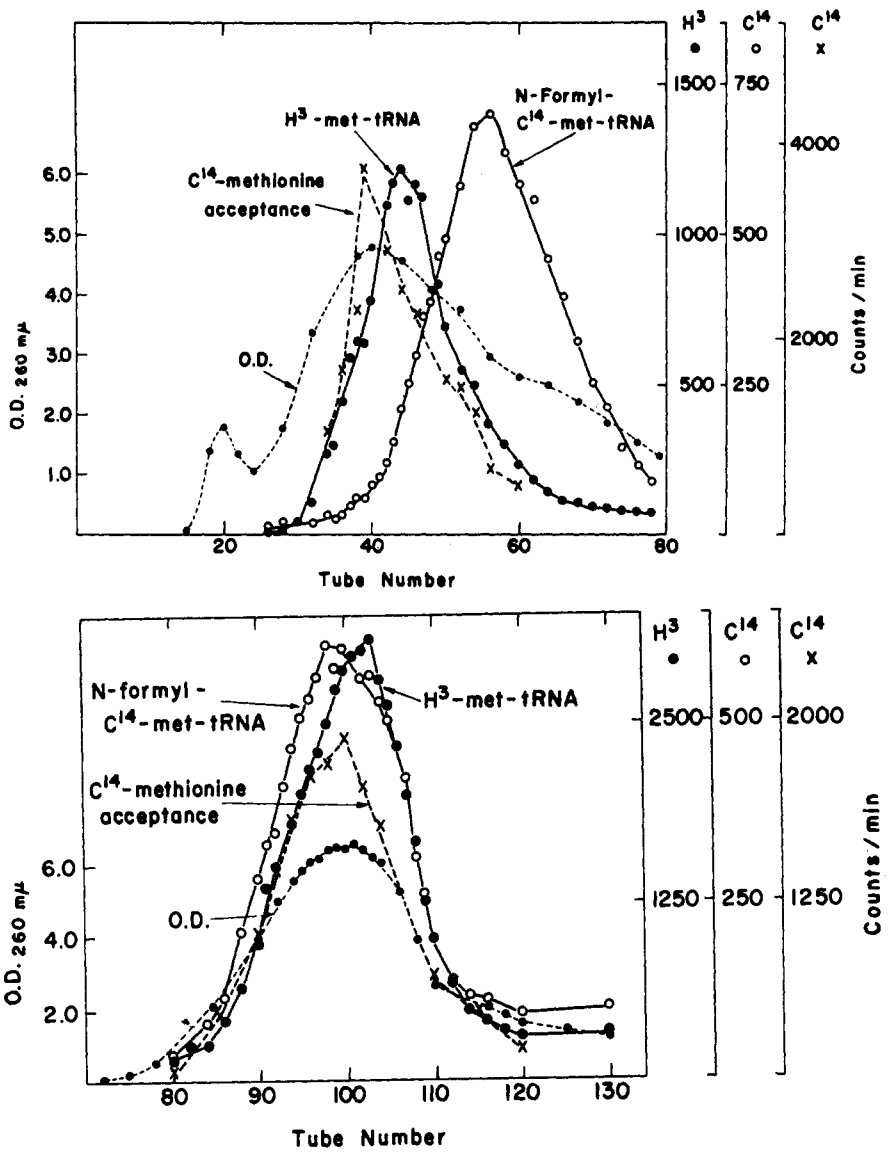

FIGURE 7: Chromatographical studies. (a) MASA column chromatography of *N*-formyl-Met-tRNA, Met-tRNA, and the profile of methionine acceptance of uncharged tRNA. Normal *E. coli* G-15 tRNA was charged with [^{14}C]methionine. The reaction mixture (0.1 ml) contained: 0.6 mg of tRNA, 3.0 μmoles of Tris-HCl buffer (pH 7.4), 0.2 μmole of ATP, 1.5 μmoles of magnesium acetate, 3 mμmoles of [^{14}C]methylmethionine or [^{3}H]methylmethionine (50 mCi/mmole and 3.7 Ci/mmole, respectively), and partially purified methionyl-tRNA synthetase. The reaction mixture was incubated for 30 min at 37° and the Met-tRNA was isolated by phenol extraction and ethanol precipitation. Formylmethionyl-tRNA was then prepared by enzymatic formylation of the [^{14}C]-Met-tRNA (Leder and Bursztyn, 1966). The *N*-formyl-[^{14}C]Met-tRNA (10,000 cpm) was mixed with [^{3}H]Met-tRNA (20,000 cpm) and 25 mg of unacylated normal *E. coli* G-15 tRNA in 10 ml of 0.05 ml of sodium acetate buffer (pH 5.5) containing 0.4 M NaCl, and applied to a MASA column. Aliquots of each fraction (2.0 ml) were removed for precipitation with trichloroacetic acid and measurement of radioactivity. Methionine acceptance of the column fractions was assayed by adding 0.01 ml of each fraction to a methionine tRNA acylation mixture containing [^{14}C]methionine (187 mCi/mmole). (b) Polyacrylamide gel chromatography of *N*-formyl-Met-tRNA, Met-tRNA, and the profile of methionine acceptance of uncharged tRNA. A Bio-Gel P-100 column was used as described under Figure 5. *E. coli* G-15 (15 mg) was mixed together with about 25,000 cpm of [^{3}H]Met-tRNA and 10,000 cpm of *N*-formyl-[^{14}C]Met-tRNA. The mixture (1.0 ml) was placed on the column, eluted with acetate buffer, and 1.8-ml fractions were collected. Following the A_{260} determination, 1.0 of each fraction was removed for measurement of radioactivity. Additional aliquots of 0.01 ml were removed, neutralized with 0.001 ml of 2 M Tris-HCl buffer (pH 8.0), and assayed for methionine acceptance capacity. It should be noted that the *N*-formyl-[^{14}C]Met-tRNA preparation was kept at 0° for more than 10 days before its application to the columns. Under these conditions the more labile [^{14}C]Met-tRNA, that was initially present in this preparation, was completely hydrolyzed.

from the MASA column in comparison with the unblocked Phe-tRNA. This retardation was independent of the size of the N-blocking group. Moreover, N-blocked dipeptidyl-tRNA (Figure 4) emerged from the column very close to *N*-acetyl-Phe-tRNA. Thus, it is unlikely that the aliphatic blocking groups interact with the MASA column. Their effect is quite different from that found when the free amino acid is blocked with an aro-

matic group (*e.g.*, *N*-carbobenzyloxy-Phe-tRNA), as the latter was shown to interact strongly with the column (Stern and Littauer, 1968). It is proposed that an N-blocked Phe-tRNA derivative has a different configuration from Phe-tRNA or uncharged $tRNA^{Phe}$ and is therefore eluted at a higher NaCl concentration from the MASA column. The results of the polyacrylamide column chromatography are consistent with this hypothesis (Figures 5 and 6) and indicate that the N-blocked Phe-tRNA has a more extended configuration than both the Phe-tRNA and the uncharged $tRNA^{Phe}$. The above experiments are also consistent with the results of Kuriki and Kaji (1967) who found that phenylalanyl-tRNA sediments more rapidly than polyphenylalanyl-tRNA on centrifugation through a sucrose gradient.

Is chemical formylation identical with enzymatic formylation? A biological analog of the chemically prepared N-blocked phenylalanyl-tRNAs is found in *N*-formylmethionyl-tRNA, which is an initiating tRNA for protein synthesis of *E. coli* (Marcker and Sanger, 1964). The studies of Leder and Bursztyn (1966) (and confirmed by us in Figure 7a) indicate that *N*-formyl-Met-tRNA is eluted later than Met-tRNA on the MASA column, similar to the relationship between Phe-tRNA and *N*-formyl-Phe-tRNA. The change in structure is therefore not a unique property of the initiator $tRNA^{Met}$ alone, but probably of all N-blocked aminoacyl-tRNAs. However, this methionine tRNA is the only substrate for the formylating enzyme found in *E. coli*. The suggestion of a difference in configuration is strongly supported by the polyacrylamide gel column chromatography of *N*-formyl-Met-tRNA, which also shows a more expanded configuration than Met-tRNA.

The present experiments suggest at least three configurations for each species of tRNA: (1) an uncharged form, (2) a more unique aminoacylated configuration, and (3) an N-blocked aminoacyl configuration which causes the tRNA species to be eluted from the MASA column at a higher NaCl concentration.

It may be postulated that the several configurations proposed for tRNA are required for the successive steps in protein biosynthesis. Configuration 2 may be invoked as an improved substrate for the ribosome binding reactions, though this is not an absolute requirement since uncharged tRNA (configuration 1) can also bind to ribosomes in the presence of mRNA (Kaji and Kaji, 1963). Two or more binding sites have been postulated on the ribosomes (Gilbert, 1963; Warner and Rich, 1964; Traut and Monro, 1964; Watson, 1965; Wettstein and Noll, 1965; Noll, 1966) one aminoacyl-tRNA site for the incoming aminoacyl-tRNA molecule and one polypeptidyl-tRNA site. It may be that the configuration assumed by the *N*-formylaminoacyl-tRNA (configuration 3) makes it suitable for binding to the polypeptidyl-tRNA site. *N*-Acetylalanyl-Phe-tRNA, an analog of polypeptidyl-tRNA, chromatographs in a position close to that of *N*-formyl-Phe-tRNA. It is therefore likely that *N*-formylaminoacyl-tRNA and polypeptidyl-tRNA have analogous if not identical configurations.

The transition of configuration 2 to configuration 3 may be required for the translocation or indeed may cause the movement from the aminoacyl-tRNA binding site to the second ribosomal site, following either formylation of the initiating methionyl-tRNA or peptide-bond formation between polypeptide and the aminoacyl-tRNA.

It has been demonstrated that polypeptidyl-tRNA has a greater binding capacity for the 50S ribosome than aminoacyl-tRNA (Gilbert, 1963; Wettstein and Noll, 1965). This difference in binding ability may also result from a difference in configuration. Finally, release of free tRNA from the ribosome following peptidization of incoming aminoacyl-tRNA may be due to reversion to configuration 1.

From the above results it appears that the entire amino acid, and not only the free amino group, participates in changes in tRNA configuration. It is difficult to visualize how the addition of an amino acid or an N-blocked amino acid to tRNA can cause these alterations in configuration. A plausible explanation is that the terminal pCpCpA sequence of tRNA is involved in establishing the configuration of the uncharged tRNA[3] (Cantor *et al.*, 1966; Cramer, 1967; Doepner *et al.*, 1967; Stulberg and Isham, 1967), and that the addition of an amino acid or its N-blocked derivatives cause the observed changes in the configuration. It is also difficult to define the nature of the structural changes that accompany each of the postulated tRNA configurations; they may arise from changes in the secondary and/or tertiary structure of the polynucleotide chains.

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[3] It is possible that only part of the terminal pCpCpA sequence is required for establishing the configuration of uncharged $tRNA^{Phe}$; the tRNA chains containing only RNA···pCpA sequences, for example, may meet this requirement (Daniel and Littauer, 1965).

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Promitochondria of Anaerobically Grown Yeast. I. Isolation and Biochemical Properties*

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ABSTRACT: *Saccharomyces cerevisiae* cells grown under strict anaerobiosis possess mitochondria-like particles. These particles are tentatively designated as promitochondria. They lack a respiratory chain but still contain oligomycin-sensitive adenosine triphosphatase (F_1), "structural protein," and mitochondrial deoxyribonucleic acid (density 1.685 g/cc), all of which are indistinguishable from their counterparts in aerobic yeast mitochondria. Promitochondria from anaerobically grown cells of the cytoplasmic "petite" mutant exhibit oligomycin-insensitive F_1 and thus differ from the corresponding particles of the wild-type strain. Promitochondria are present in anaerobic yeast cells regardless of whether these are grown in the presence or the absence of Tween 80 and ergosterol. These findings suggest that anaerobic growth of *S. cerevisiae* leads to a dedifferentiation, rather than a complete loss, of the mitochondrial organelles.

Aerobically grown *Saccharomyces cerevisiae* cells possess numerous typical mitochondria (Agar and Douglas, 1957; Vitols *et al.*, 1961) and an active, cyanide-sensitive respiratory chain involving the cytochromes aa_3, b, c_1, and c (Ephrussi and Slonimski, 1950; Slonimski, 1953). In contrast, the anaerobically grown cells are devoid of cyanide-sensitive respiration and the classical cytochrome complement but adaptively regain these characteristics upon aeration (Ephrussi and Slonimski, 1950; Slonimski, 1953; Chin, 1950; Lindenmayer and Estabrook, 1958; Chaix, 1961; Lindenmayer and Smith, 1964).

This oxygen-induced adaptation process obviously represents a promising experimental system for studying the formation of respiratory enzymes. In addition, it may provide a means for investigating the biogenesis of the mitochondrial membranes with which these enzymes are associated. In spite of considerable effort, however, the fate of yeast mitochondria during anaerobic growth and respiratory adaptation has remained a point of continuing discussion. Some years ago, Heyman-Blanchet *et al.* (1959) reported the isolation of "mitochondria" from anaerobically grown yeast cells but presented little evidence to support this claim. On the other hand, Wallace and Linnane (1964) concluded on the basis of

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