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N-Formylmethionyl Transfer Ribonucleic Acid in Mitochondria from *Neurospora**

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ABSTRACT: Although the mechanism for the initiation of protein synthesis in bacteria appears to involve fMet-tRNA, little is known of the initiation process in higher organisms. Since the mitochondria of *Neurospora* contain a protein-synthesizing system with components distinct from those of the cytoplasm, we have examined both mitochondrial and cytoplasmic extracts for fMet-tRNA. A Met-tRNA from the mitochondria can be formylated with an *Escherichia coli* extract, but the corresponding cytoplasmic tRNA cannot be. This mitochondrial fMet-tRNA is chromatographically separable from another mitochondrial Met-tRNA, which cannot be formylated. These are different from the cytoplasmic Met-tRNA and *E. coli* Met-tRNAs. The mitochondrial Met-tRNA synthetase is specific, aminoacylating only the mitochondrial tRNAs and not the cytoplasmic. Similarly, the mitochondrial extracts contain a formylase which reacts only with the mitochondrial fMet-tRNA. Thus, it appears that mitochondrial protein synthesis involves a formylated methionine, presumably as an initiator in analogy to the bacterial system.

The isolation of fMet-tRNA (Marcker and Sanger, 1964; Marcker, 1965) in bacteria and the elucidation of its role as an initiator of protein synthesis (Clark and Marcker, 1966; Adams and Capecchi, 1966) have resulted in a search for a similar system in numerous other organisms. In their early work Marcker and Sanger (1964) reported the presence of *N*-formylmethionine in yeast but not in various mammalian tissues. Similarly, Clark and Marcker (1966) examined mammalian tissues with no success, although Noll (1966) was able to detect the compound in rabbit reticulocytes. Caskey *et al.* (1967) showed that one of two Met-tRNAs from guinea pig liver could be formylated with a preparation from *Escherichia coli*. However, the enzymatic formylation could not be carried out by the liver extract. Recently, Li and Yu (1969) reported both fMet-tRNA and a formylase from Ehrlich ascites cells.

Since mitochondria appear to contain a protein-synthesizing system (*e.g.*, Wintersberger, 1965) distinct from that of the cytoplasm and more bacteria like in nature, a number of investigators have examined organelles for the presence of formylmethionine. Smith and Marcker (1968) showed that fMet-tRNA can be detected in yeast and in rat liver mitochondria, while Galper and Darnell (1969) showed that exclusively the mitochondria of Hela cells contain fMet-tRNA. In addition, the observations of Schwartz *et al.* (1967) suggest that initiation in *Euglena* chloroplasts utilizes *N*-formylmethionine.

Since the mitochondrion of *Neurospora* employs a protein-synthesizing system with components distinct from those of the cytoplasm (Barnett and Brown, 1967; Barnett *et al.*, 1967; Dure *et al.*, 1967), we have examined both mitochondrial and cytoplasmic extracts for fMet-tRNA. We show here that mitochondrial extracts contain a fMet-tRNA and the enzyme that carries out the formylation, whereas cytoplasmic extracts do not.

Materials and Methods

Strains. Neurospora crassa wild-type strain OR23-1A was used. *E. coli* strain A-19 was used in the preparation of bacterial extracts.

Preparation of Mitochondrial and Cytoplasmic Fractions. Hyphas from cultures grown 24–36 hr in aerated flasks of enriched Vogel's (1956) medium were harvested on cheesecloth and washed with cold distilled water. Fractions were prepared as described by Epler (1969) with slight modifications. In a standard preparation of 1500 g (wet weight), each of six 250-g mycelial pads was homogenized with 900 ml of grinding medium (0.005 M EDTA, adjusted to pH 7.5, 8%

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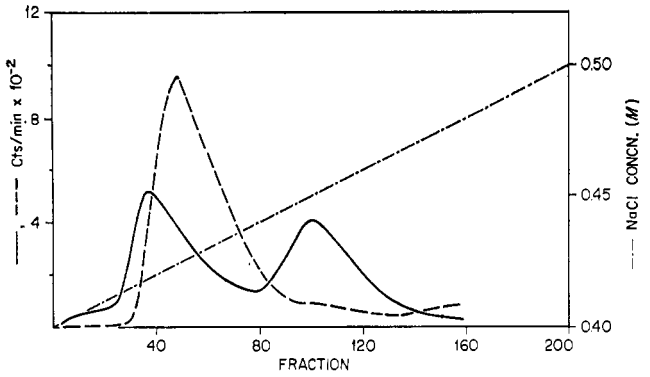

FIGURE 1: Cochromatography (RPC-2) of cytoplasmic and mitochondrial Met-tRNAs from *Neurospora*. The tRNAs and enzymes were prepared from the isolated fractions described in the Methods section. (——) Mitochondrial [^{3}H]Met-tRNA aminoacylated with a partially purified mitochondrial enzyme. (---) Cytoplasmic [^{14}C]Met-tRNA aminoacylated with a cytoplasmic enzyme preparation. All fractions were assayed.

sucrose, 0.15 g of bovine serum albumin or methocel per l., and 0.02 ml of Dow P-2000 polyglycol/l.) plus 2000 g of glass beads in a Gifford-Wood-Eppenbach Colloid Mill (gap setting of 24). After standing, to allow the beads to settle, and a low-speed centrifugation, the mitochondria were pelleted by high-speed centrifugation. The supernatant was retained and used as the cytoplasmic fraction. The mitochondria were resuspended in 100–150 ml and reisolated by zonal centrifugation at 25,000 rpm for 1 hr. The gradient (1200 ml) used was 10–36% sucrose with a 55% sucrose cushion (~500 ml) in the B-XV zonal rotor.

Preparation of tRNA. The tRNA was prepared from cytoplasmic and mitochondrial fractions by phenol extraction (Holley *et al.*, 1963).

Preparation of Enzymes. Cytoplasmic and mitochondrial enzymes were prepared as given in Epler (1969), utilizing high-speed centrifugation, ammonium sulfate precipitation, and DEAE-cellulose column chromatography. The high-speed supernatant (S-100) used as a source of crude transformylase was sampled during the procedure, dialyzed, brought up to 40% glycerol, and frozen until assayed.

The crude enzyme fraction from *E. coli* was prepared by the method of Muench and Berg (1966).

Formation of AA-tRNA. The reactions and assays for AA-tRNA formation were carried out as described by Epler (1969) (see also Table I). For chromatography the AA-tRNA was isolated by phenol extraction from 2.5-ml reaction mixtures. To produce fMet-tRNA the reaction mixture was modified by adding 5,10-[^{14}C]methenyltetrahydrofolate, the formyl donor, together with [^{12}C]methionine. In some cases, ^{12}C donor was used in combination with labeled methionine.

[^{14}C]- and [^{3}H]methionine were obtained from New England Nuclear Co. and Schwarz BioResearch Inc. The [^{14}C]formyl donor was synthesized as described by Shugart *et al.* (1969a).

Chromatography. Aminoacylated tRNAs were dissolved in 0.01 M sodium acetate buffer (pH 4.5), 0.01 M magnesium acetate, 0.01 M β-mercaptoethanol, 0.001 M Na_2EDTA, 0.02% NaN_3, and 0.35 M NaCl. Approximately equal numbers of counts per minute (^{3}H and ^{14}C) from preparations to be compared were combined and cochromatographed by the reversed-phase column (RPC-2) method of Weiss and Kelmers (1967). Jacketed glass columns (240 × 1.0 cm) were used at 23°, and elution utilized a 2-l. linear NaCl gradient (0.40–0.50 M except where noted) at a flow rate of 1.5 ml/min.

TABLE I: Reactions of *E. coli* Enzyme.[a]

tRNA	Methionylation (pmoles/A_{260} per 40 min)	Formylation (pmoles/A_{260} per 40 min)
E. coli crude	57.0	43.0
Mitochondrial *Neurospora*	17.0	4.1
Cytoplasmic *Neurospora*	18.0	0.3

[a] A crude enzyme fraction from *E. coli* was used to methionylate and formylate the following tRNA preparations: *E. coli* crude (General Biochemicals), 1.25 A_{260} units; *Neurospora* mitochondrial, 1.00 A_{260} unit; and *Neurospora* cytoplasmic, 1.05 A_{260} units in a final volume of 0.25 ml. The assays for the measurement of Met-tRNA formation were carried out in reactions which (in addition to enzyme and tRNA) contained per milliliter: 50.0 μmoles of *N*-2-hydroxylethylpiperazine-*N*-2-ethanesulfonic acid (Calbiochem Corp.) buffer (pH 7.5), 0.5 μmole of ATP, 10.0 μmoles of magnesium acetate, 5.0 μmoles of β-mercaptoethanol, 1.0 μCi of [^{14}C]methionine (217 μCi/μmole, New England Nuclear Co.), and 0.1 μmole of each of 19 other [^{12}C]amino acids. In the formylation reaction, 10 nmoles of [^{12}C]methionine and 2.6 μmoles of 5,10-[^{14}C]methenyltetrahydrofolate (56.5 μCi/μmole) were added. A similar amount of ^{12}C donor (Calcium Leucovorin, American Cyanimide) was added to the methionylation mixture. Reactions were carried out at 30° for 40 min; [^{14}C]AA-tRNA was assayed by the filter paper disk method.

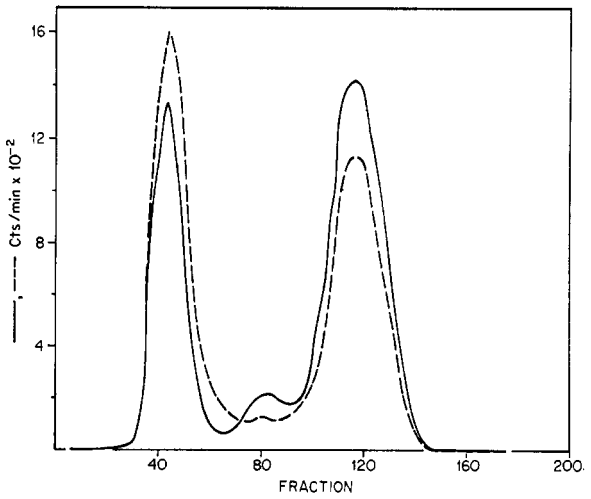

FIGURE 2: Cochromatography of mitochondrial Met-tRNAs from *Neurospora* comparing (——) [^{3}H]Met-tRNA aminoacylated with the mitochondrial enzyme and (---) [^{14}C]Met-tRNA aminoacylated with *E. coli* enzyme.

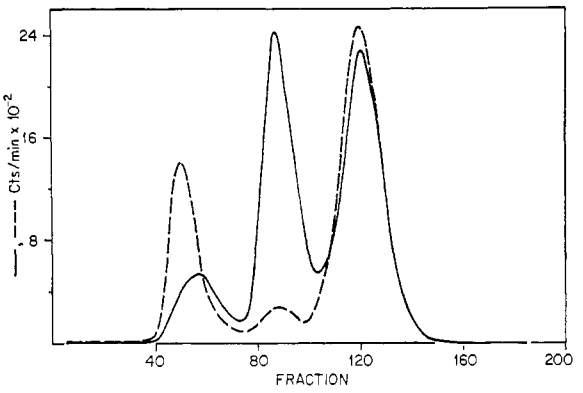

FIGURE 3: Cochromatography of mitochondrial Met-tRNAs acylated and formylated in the presence of the [^{12}C]formyl donor. (---) [^{14}C]Met-tRNA formed by the mitochondrial enzyme. (——) [^{3}H]Met-tRNA formed by the *E. coli* enzyme.

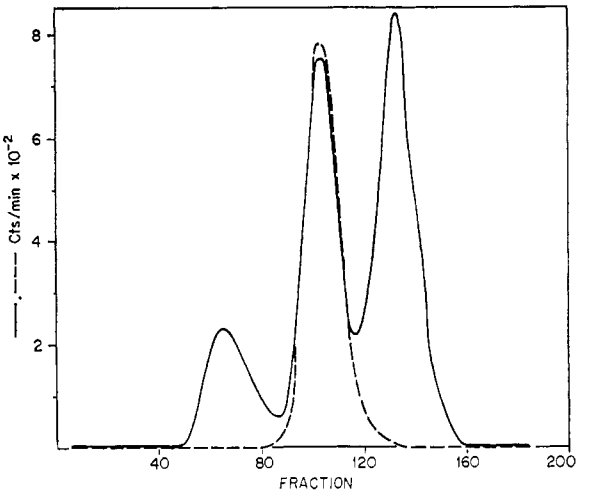

FIGURE 4: Cochromatography of mitochondrial Met-tRNAs acylated and formylated by the *E. coli* crude enzyme preparation. (——) [^{3}H]Met-tRNA formed in the presence of the [^{12}C]formyl donor. (---) [^{14}C]Formyl-Met-tRNA. In order to adjust the peak heights to approximately the same level, the amount of radioactive label of the ^{14}C preparation added to the column was approximately one-third of the ^{3}H preparation.

To each 10-ml fraction, 4 A_{260} units of carrier RNA and 2 ml of 50% trichloroacetic acid were added. The precipitate was collected on type HA Millipore filters (24-mm diameter), washed with 70% ethanol, dried, and counted in a liquid scintillation counter.

Results and Discussion

The fractionation of methionine tRNAs of *E. coli* into two species, one formylatable, the other not, was first reported by Clark and Marcker (1966) using countercurrent distribution. These multiple species of Met-tRNAs can be resolved easily with reversed-phase column chromatographic systems (Kelmers *et al.*, 1965). Furthermore, with their reversed-phase system type 3 (RPC-3), Weiss *et al.* (1968) resolved two fMet-tRNAs and one Met-tRNA from *E. coli*. Exploiting this system in a more analytical fashion, Shugart *et al.* (1969a) observed a shift in the chromatographic position of the major fMet-tRNA resulting from formylation. The same

TABLE II: Reactions of Crude *Neurospora* Supernatants.[a]

tRNA	Methionylation (pmoles/A_{260})	Formylation (pmoles/A_{260})
Mitochondrial supernatant		
E. coli	5.2	2.6
Mitochondrial *Neurospora*	9.5	1.9
Cytoplasmic *Neuropsora*	0.4	0.5
Cytoplasmic supernatant		
E. coli	34.4	0.0
Mitochondrial *Neurospora*	10.1	0.0
Cytoplasmic *Neurospora*	22.2	0.0

[a] Crude S-100's (100,000*g* supernatants) from a standard mitochondrial enzyme preparation (see Epler, 1969) were dialyzed, brought to 40% glycerol, and frozen. These preparations were used to methionylate and formylate the tRNA preparations described in Table I. The reaction mixture also contained 4 μg/ml of polyvinyl sulfate to attempt to inhibit nuclease action. Reactions were carried out at 30° for 5 min with the mitochondrial preparation and 40 min with the cytoplasmic.

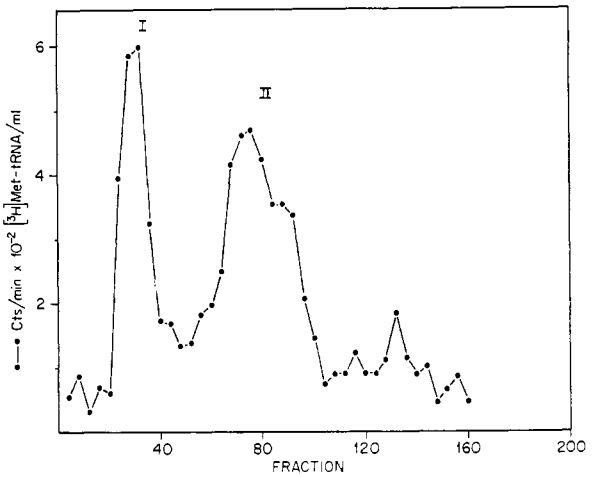

FIGURE 5: Postcharged chromatograph of 5 mg of *Neurospora* mitochondrial tRNA. The tRNA was eluted with a 0.40–0.50 M NaCl gradient and collected in 10-ml fractions. A sample of 0.1 ml from selected fractions (every fourth tube) was assayed for the presence of methionine tRNA with the reaction mixture described by Epler (1969) and Table I, using a crude enzyme fraction from *E. coli* plus the [^{12}C]formyl donor. (——) [^{3}H]Met-tRNA formed in 20 min/ml of fraction. For the rechromatography shown in Figure 6, fractions 24–36 were pooled and noted as I. Fractions 64–96 were pooled and noted as II. Ethanol (2.5 volumes) was added and the precipitate was allowed to stand overnight at 0°. Each pooled fraction was filtered on a Millipore filter, type HA, 47-mm diameter. The filter was dried and the tRNA was eluted with 5 ml of 0.01 M sodium acetate buffer (pH 5).

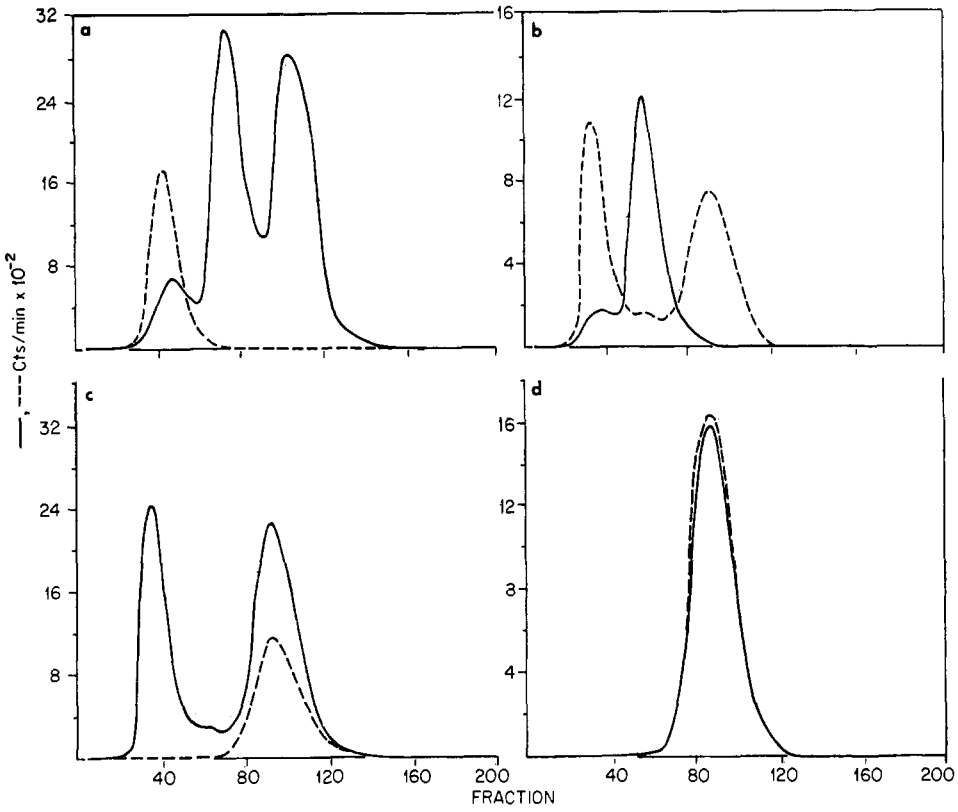

FIGURE 6: Cochromatographs of fractions of Met-tRNA from *Neurospora* acylated with a crude enzyme fraction from *E. coli*. (a) (——) Total mitochondrial [^{3}H]Met-tRNA, formylated with the [^{12}C]formyl donor; (---) [^{14}C]Met-tRNA$_I$ (one-third of ^{3}H cpm), no formyl donor present during aminoacylation. (b) (——) [^{3}H]Met-tRNA$_I$ (one-half of ^{14}C cpm), formylated with the [^{12}C]formyl donor; (---) total [^{14}C]Met-tRNA, no donor present during aminoacylation. (c) (——) total [^{3}H]Met-tRNA; (---) [^{14}]Met-tRNA$_{II}$ (one-third of ^{3}H cpm), no donor present during aminoacylation. (d) (——) [^{3}H]Met-tRNA$_{II}$, donor present during aminoacylation; (---) [^{14}C]Met-tRNA$_{II}$, no donor present during aminoacylation. The counts per minute added to the column were varied as given above to adjust the comparable peaks to approximately equal heights.

authors (1969b) also reported that one of the two fMet-tRNAs previously reported is an artifact that arises from the other during the isolation procedure.

In the *Neurospora* system, which is complicated by the inclusion of both cytoplasmic and mitochondrial components in crude extracts (Brown and Novelli, 1968; Epler, 1969), a number of isoacceptors for methionine also occur. With a reversed-phase column (RPC-2, Weiss and Kelmers, 1967), we have used isolated cytoplasmic and mitochondrial fractions and find one major cytoplasmic species and several chromatographically distinct mitochondrial isoacceptors (Figure 1). Similarly, we find that the *E. coli* enzyme acylates the mitochondrial species and produces the same chromatographic profile (Figure 2).

The crude enzyme preparation from *E. coli* can be used to formylate a part of the methionine tRNAs, showing a high specificity for the mitochondrial component (Table I). Initial attempts to show a formylase activity in partially purified *Neurospora* extracts failed; however, the activity can be detected in the crude high-speed supernatant (S-100) of whole cell *Neurospora* extracts. For example, a dialyzed S-100 from a whole cell homogenate formylated 8.3 pmoles/A_{260} unit of *E. coli* crude tRNA in 10 min. However, cytoplasmic S-100 fractions and partially purified cytoplasmic enzymes showed no activity. Mitochondrial S-100 shows formylation activity with both mitochondrial and *E. coli* tRNAs, but none with cytoplasmic (Table II). In fact, as seen in this table, the mitochondrial Met-tRNA synthetase shows little or no reaction with *Neurospora* cytoplasmic tRNA. Thus, *Neurospora* mitochondria contain both a fMet-tRNA and a formylase.

Figure 3 shows a chromatogram comparing *Neurospora* mitochondrial methionyl-tRNAs acylated with the *E. coli* or the mitochondrial enzyme, respectively. In these experiments, the reactions included the [^{12}C]formyl donor. A third central species is apparent. From Figure 4, it is obvious that the new peak seen is identical with [^{14}C]fMet-tRNA. Thus, the chromatogram is probably analogous to the chromatographic shift (attributable to formylation of the amino acid) seen by Shugart *et al.* (1969a); that is, the central peak is derived from the first peak.

In order to clarify this point, a mitochondrial tRNA preparation (5 mg and discharged) was chromatographed with the RPC-2 column and the fractions "postcharged" with the *E. coli* enzyme (with and without the ^{12}C donor) (see Figure 5). No gross difference in peak position or aminoacylation of the central portion was observed. Fractions I and II were accumulated, concentrated, and aminoacylated with methionine in the presence and absence of the [^{12}C]-formyl donor. From Figure 6a–d, it is clear that the chromatographic properties of fraction I change when converted into fMet-tRNA, while the properties of fraction II are unal-

tered. Thus, the effect is similar to the shift seen by Shugart *et al.* (1969a) but even more pronounced. The remaining portion of the initial peak (fraction I) appears to be refractory to formylation and may represent another Met-tRNA. The methionylation and formylation carried out by the crude preparation from *E. coli* were both taken to completion. However, the possibility that the "refractory" peak is contaminating cytoplasmic tRNA (nonformylatable) cannot be ruled out.

To establish the identity of the amino acid attached to the various *Neurospora* tRNAs, we discharged and chromatographed on paper (Caskey *et al.*, 1967) the appropriate preparations and compared the radioactive amino acid to standard and also to the discharged product of *E. coli* Met-tRNAs. Radioactive peaks were detected at R_F's of 0.22, 0.52, and 0.75, corresponding to methionine sulfoxide, methionine, and *N*-formylmethionine.

More specifically, when the radioactive amino acid, presumably [^{12}C]formyl-[^{14}C]methionine, is stripped from shifted fraction I, the radioactive peak occurs at an R_F of 0.75, *i.e.*, as *N*-formylmethionine. When fraction II is stripped and the amino acid chromatographed, the main component migrates at an R_F of 0.52, *i.e.*, as methionine.

Finally, Figure 7 shows the nonidentity of the mitochondrial fMet- and the *E. coli* fMet-tRNAs. Comparisons to published observations and control chromatograms shown here establish that the first peak of the *E. coli* preparation is the formylated species.

We conclude that the mitochondria of *Neurospora* contain a $tRNA^{fMet}$ and formylase. This $tRNA^{tMet}$ is chromatographically distinct from another mitochondrial $tRNA^{Met}$ that cannot be formulated.

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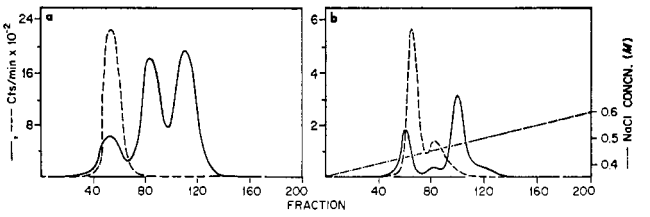

FIGURE 7: Cochromatography of *Neurospora* mitochondrial and *E. coli* Met-tRNAs aminoacylated and formylated with a crude enzyme fraction from *E. coli* (except as noted). (a) (——) Mitochondrial [^{3}H]Met-tRNA, formylated with the [^{12}C]formyl donor; (---) *E. coli* [^{14}C]formyl-Met-tRNA (one-half of ^{3}H cpm added). (b) (——) Mitochondrial [^{3}H]Met-tRNA, charged with the *Neurospora* mitochondrial enzyme, donor present during aminoacylation; (---) *E. coli* [^{14}C]Met-tRNA, formylated with the [^{12}C]formyl donor. In part b, a modified gradient of 0.35–0.60 M NaCl was used.

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