The Mitochondrial and Cytoplasmic Transfer Ribonucleic Acids of *Neurospora crassa**

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ABSTRACT: The transfer ribonucleic acids of the fungus *Neurospora crassa* have been isolated and characterized. Countercurrent distribution and reversed-phase column chromatography were used to separate the individual species of acceptor transfer ribonucleic acids. In transfer ribonucleic acid prepared from whole-cell extracts, multiple forms of glutamic acid, glutamine, glycine, leucine, lysine, methionine, serine, and phenylalanine transfer ribonucleic acids were easily separated by countercurrent distribution. However, the mitochondria of *Neurospora* contain transfer ribonucleic acids and aminoacyl transfer ribonucleic acid synthetases that are included in whole-cell extracts along with the cytoplasmic components.

In a few cases, countercurrent distribution of isolated mitrochondrial transfer ribonucleic acids indicated which of the multiple forms observed in whole-cell extracts were due to mitochondrial transfer ribonucleic acid. For further resolution, reversed-phase chromatography was used to show *distinct* and *separable* species of mitochondrial and cytoplasmic transfer ribonucleic acids of 15 amino acids.

Countercurrent distribution has been a highly successful method for purifying individual tRNAs (*e.g.*, Holley *et al.*, 1965). Application of this technique to the tRNAs from yeast, *Escherichia coli*, and rat liver has aided in bringing our knowledge of the role of tRNAs in protein synthesis to its present state. This report surveys the behavior of the tRNAs of the fungus *Neurospora crassa* in the system of Holley (Holley *et al.*, 1963). Multiple forms of tRNAs (isoacceptors) are easily separated, but the occurrence of isoaccepting species in *Neurospora* is complicated by the observation that the mitochondria of this organism contain tRNAs and aminoacyl-tRNA synthetases in addition to those found in the cytoplasm. Our initial work (Barnett and Brown, 1967; Barnett *et al.*, 1967) has shown that the mitochondrial tRNAs are distinct from those of the cytoplasm in acylation specificities. In addition, it appears that the mitochondria of *Neurospora* lack certain tRNAs which respond to specific codons and are present in the cytoplasm of the fungus and in bacterial systems (Epler and Barnett, 1967).

In order to identify the organelle tRNAs and to compare them with their cytoplasmic counterparts, a separate distribution of mitochondrial tRNAs was carried out. The results from this distribution and from the high-resolution reversed-phase chromatographic system support the hypothesis of a complete and unique set of tRNAs in a separate mitochondrial protein-synthesizing system. These observations corroborate the work of Barnett and Brown (1967) and Barnett *et al.* (1967). The results from the chromatographic separations agree with the preliminary report of Brown and Novelli (1968) on *Neurospora* and with the work of Buck and Nass (1968) on rat liver. Thus it appears that the partition and chromatographic properties of mitochondrial tRNAs are in general different from those of the cytoplasmic tRNAs.

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Materials and Methods

Strains. N. crassa wild-type strain OR23-1a was used.

Preparation of Mitochondrial and Cytoplasmic Fractions. Hyphae 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. Mitochondria were prepared by the method of Hall and Greenawalt (1964), followed by zonal centrifugation as described in Barnett and Brown (1967). 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% sucrose, 0.15 g of bovine serum albumin or methyl cell 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, the nuclei and debris were removed from the homogenate by low-speed centrifugation (1500*g* for 12 min), and the mitochondria were pelleted by centrifugation at 8000*g* for 30 min. The post-mitochondrial supernatant was recentrifuged at 30,000*g* for 30 min to remove residual mitochondria and then used as the cytoplasmic fraction. The pelleted mitochondria were resuspended and reisolated by zonal centrifugation. The gradient (1000 ml) used was 10–30% sucrose with a 55% sucrose cushion (500 ml) in the B-IV zonal rotor. The resuspended sample (100–150 ml) was inserted and centrifuged at 40,000 rpm for 1 hr.

Preparation of tRNA and Countercurrent Distribution. The tRNA was prepared from cytoplasmic and mitochondrial fractions by phenol extraction (Holley *et al.*, 1963). Countercurrent distribution was accomplished by Holley's methods; recovery of the fractions was as described by Barnett and Epler (1966).

Preparation of Enzymes. A. CYTOPLASMIC. After centrifugation at 78,000*g* for 2 hr, 500 ml of the supernatant of the cytoplasmic fraction was adjusted to 0.2 M potassium phosphate buffer (pH 7.5) and 0.01 M β-mercaptoethanol and passed over a 4 × 24 cm DEAE-cellulose column (equilibrated

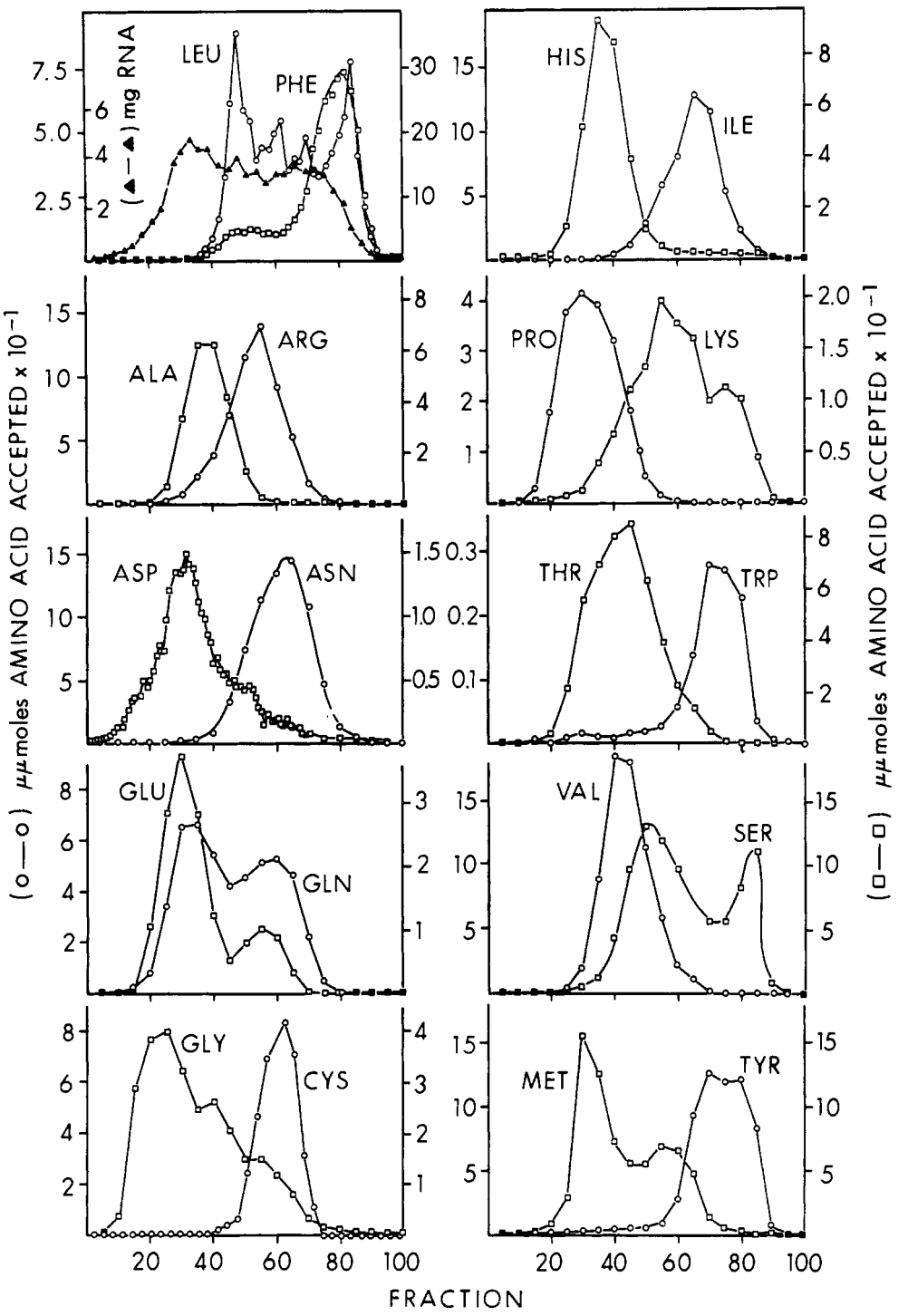


FIGURE 1: 100-Transfer countercurrent distribution of 230 mg of *Neurospora* (whole-cell) tRNA. Distribution of tRNA in milligrams (▲——▲) is shown in fractionation of $tRNA^{Phe}$. The fractionations shown here were subsequently verified with distributions of 146 and 257 mg of *Neurospora* whole-cell tRNA. Charging of the fractionated whole-cell tRNA from the distribution is shown for each amino acid. Samples (0.1 ml) of selected tubes were assayed with an unfractionated *Neurospora* aminoacyl-tRNA synthetase preparation as described in Materials and Methods. (O——O or □——□) Aminoacyl-tRNA formation. The distributions of $tRNA^{Asp}$, $tRNA^{Asn}$, and $tRNA^{Phe}$ are from Barnett and Epler (1966); the figure of $tRNA^{Leu}$ is from Epler and Barnett (1967).

with the same buffer). The eluted protein was precipitated by the addition of $(NH_4)_2SO_4$ crystals to saturation. After being stored overnight in the cold, the precipitate was collected by centrifugation at 10,000*g* for 30 min. The pellet was resuspended in 0.01 M *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid buffer (pH 7.5), 0.1 M KCl, 0.01 M β-mercaptoethanol, and 20% glycerol and then passed over a Sephadex G-25 column that had been equilibrated with the same buffer. Aliquots of the protein were brought to 40% glycerol, frozen, and stored at −20°. An unfractionated whole-cell synthetase was prepared by the same method, except that the hyphae were initially suspended in the phosphate buffer and disrupted in a Gaulen press.

B. MITOCHONDRIAL. After zonal centrifugation the mitochondrial fractions (120–160 ml) were diluted to one-half the sucrose concentration and centrifuged at 30,000*g* for 30 min. The pellet was suspended in 0.2 M potassium phosphate buffer (pH 7.5) containing 0.01 M β-mercaptoethanol and disrupted in a Branson Sonifier (Model S125) at maximum output for five 1-min periods in approximately 50 ml. The homogenate was then treated in the same way as the cytoplasmic fraction, except that the $(NH_4)_2SO_4$ precipitate was adjusted to approximately 3.0 M and centrifuged at 78,000*g* for 1 hr.

The mitochondrial protein precipitate was passed over Sephadex G-25 in 0.01 M potassium phosphate buffer (pH 8.0), 0.01 M β-mercaptoethanol, 20% glycerol, and 0.001 M KCl and applied to a 1 × 24 cm DE23 column. After the bulk of nuclease activity was removed by washing with the above buffer, the remainder of the protein was eluted with buffer containing 0.3 M KCl. The resulting fraction was then adjusted to 0.15 M KCl and 40% glycerol.

Assay of Aminoacyl-tRNA. The assays for the measurement of aminoacyl-tRNA formation were carried out in reactions which (in addition to enzyme and tRNA) contained per milliliter: 50.0 μmoles of Tris-HCl buffer or *N*-2-hydroxyethylpiperazine-*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, 0.5 or 1.0 μCi of [^{14}C]amino acid, and 0.1 μmole of each of 19 other [^{12}C]amino acids. Reactions were performed at 30° for 15 or 20 min in a final volume of 0.25 ml. [^{14}C]Aminoacyl-tRNA was assayed by the filter paper disk method (Bollum, 1966).

All [^{14}C]amino acids (and [^{35}S]cysteine), except [^{14}C]asparagine (Nuclear-Chicago) and [^{14}C]glutamine (Calbiochem), were obtained from New England Nuclear Co. [^{3}H]Glutamic acid, [^{3}H]glycine, [^{3}H]histidine, [^{3}H]proline, [^{3}H]tryptophan, and [^{3}H]tyrosine were obtained from New England Nuclear Co. All others were obtained from Schwartz BioResearch Inc.

Charged preparations for chromatography were prepared from reactions of 2.5 ml and [^{14}C]aminoacyl-tRNA isolated by phenol extraction or by the method of Waters (given in Yang and Novelli, 1968).

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 disodium EDTA, 0.02% NaN_3, and 0.35 M NaCl. Approximately equal numbers of counts per minute (^{3}H and ^{14}C) from cytoplasmic and mitochondrial preparations were combined and cochromatographed by the reversed-phase column method of Weiss and Kelmers (1967). Jacketed glass columns (240 × 1.0 cm) were used at 23°, and elution was with a 2-l., linear sodium chloride gradient at a flow rate of 1.4 ml/min. Fractions (10 ml) were received in a refrigerated collector; 4 A_{260} units of carrier RNA and 2 ml of 50% trichloroacetic acid were added to each tube. The precipitate was collected on type HA Millipore filters, washed with 70% ethanol, dried, and counted in a Packard liquid-scintillation counter.

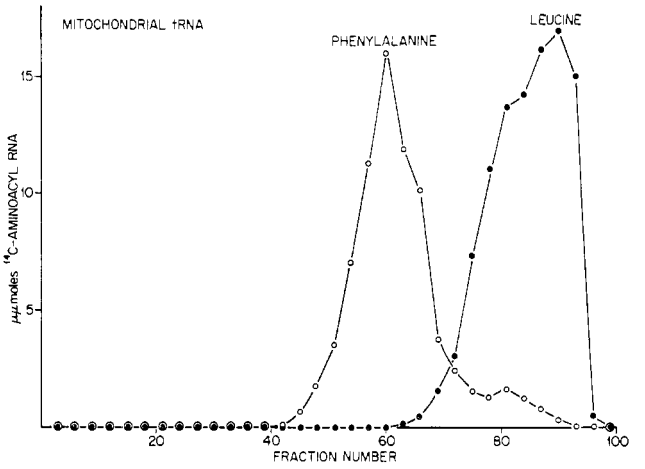


FIGURE 2: Amino acid charging of fractionated *Neurospora* mitochondrial tRNA. Samples were assayed as in Figure 1, except that a mitochondrial synthetase preparation was used. (—●—) Leucyl-tRNA^{Leu} formation; (—○—) phenylalanyl-tRNA^{Phe} formation. The fractionated RNA was obtained from the 100-transfer countercurrent distribution of 46 mg of mitochondrial tRNA.

In cases in which minor peaks were suspect, labels were reversed to further eliminate the prospect of contaminating amino acids. Reisolation and rechromatography of isolated peaks were carried out in an attempt to rule out column artifacts and/or degradation.

Results and Discussion

The results of assaying the fractions obtained by countercurrent distribution with an enzyme prepared from whole-cell extracts are summarized in Figure 1. In parallel with the results from many other organisms, the occurrence of multiple forms or isoaccepting species for a single amino acid appears to be the general case. In *Neurospora*, however, the picture is more complex, since whole-cell extracts also contain tRNAs and synthetases of mitochondrial origin. For example, mitochondrial leucine tRNA (assayed with the specific mitochondrial enzyme) is present as a single peak associated with tRNA$_{II}^{Leu}$ (Epler and Barnett, 1967). Furthermore, cochromatography of cytoplasmic and mitochondrial species by use of a reversed-phase system (Kelmers *et al.*, 1965) has resolved differences in the cases investigated (Brown and Novelli, 1968).

Distribution of tRNA from isolated *Neurospora* mitochondria and its assay with a mitochondrial enzyme produces the profiles of tRNA^{Phe} and tRNA^{Leu} shown in Figure 2. Here the position of the mitochondrial component among multiple peaks is clarified. Since further identification of the *Neurospora* cytoplasmic and mitochondrial species can be obtained with the reversed-phase systems, a detailed study of the chromatographic properties of fungal tRNAs was carried out.

Figure 3 illustrates the chromatographic separations obtained with reversed-phase chromatography. Cochromato-

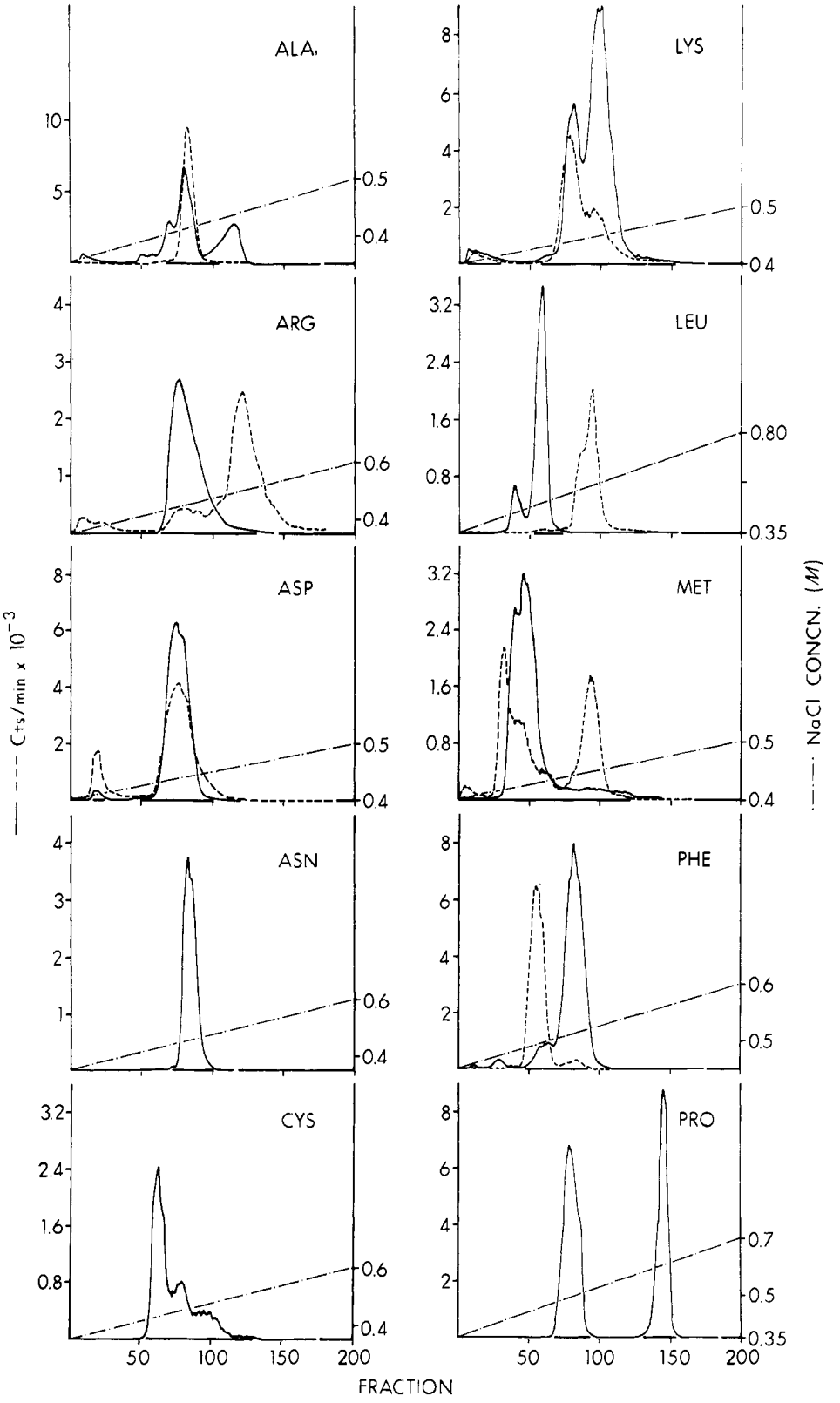
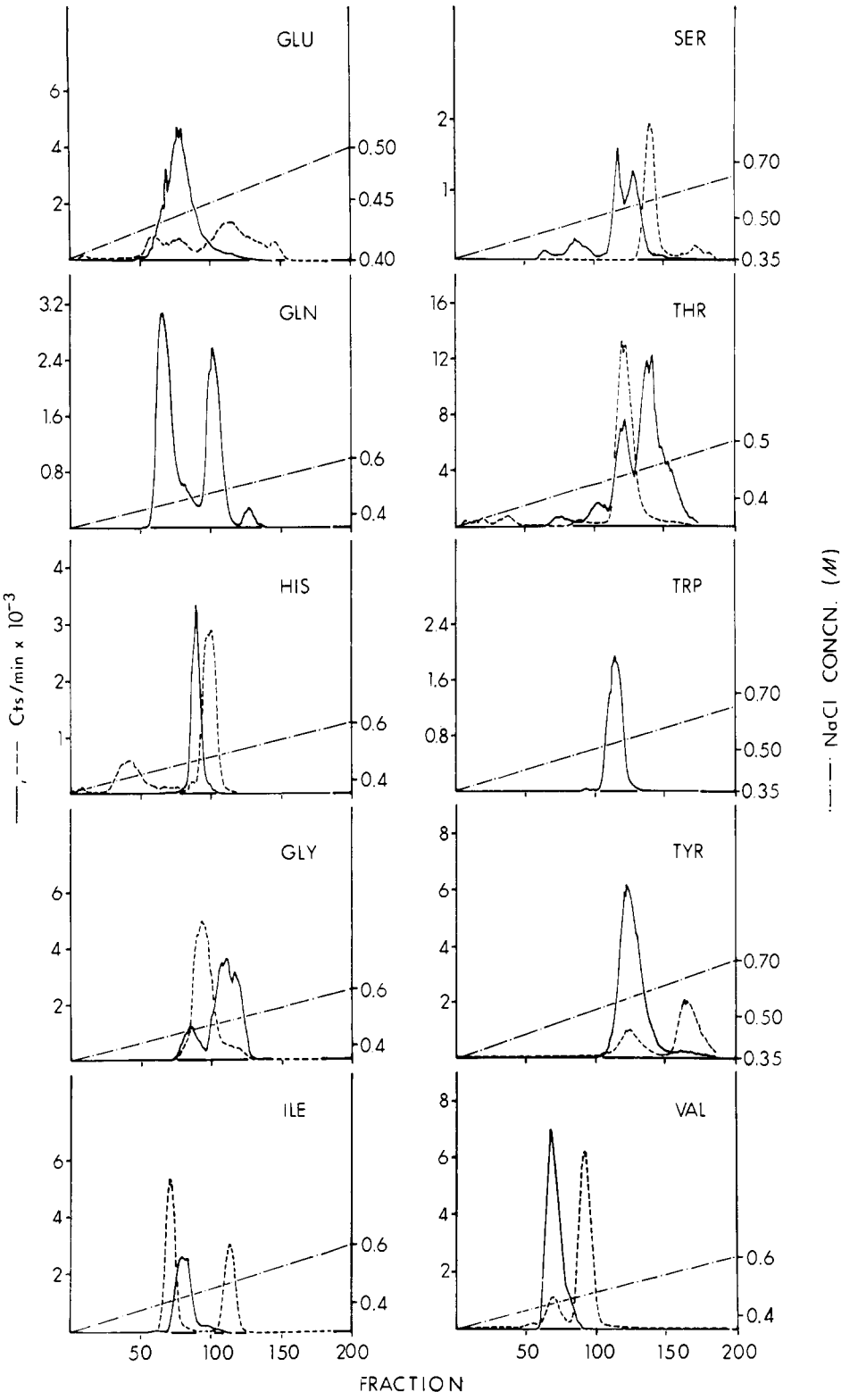


FIGURE 3: Cochromatography (reversed phase) of cytoplasmic and mitochondrial tRNAs from *Neurospora*. All preparations utilized tRNAs extracted from the isolated mitochondrial and cytoplasmic fractions. The preparations of isoleucyl-tRNA^{Ile}, leucyl-tRNA^{Leu}, phenylalanyl-tRNA^{Phe}, and seryl-tRNA^{Ser} were acylated with the cytoplasmic and mitochondrial enzymes. In all other preparations, a crude whole-cell



synthetase preparation containing both the cytoplasmic and mitochondrial activities was used. (——) Cytoplasmic [^{3}H]aminoacyl-tRNA except with [^{14}C]glutaminyl-tRNA^{Gln}. (– – – – –) Mitochondrial [^{14}C]aminoacyl-tRNA. Test gradients of 0.35–0.65 or 0.70 M NaCl were run initially. The chromatography was then repeated or run with a modified gradient to achieve a satisfactory separation.

graphs are shown for cytoplasmic–mitochondrial comparisons of 15 different amino acids. Cytoplasmic species only are shown for asparagine, cysteine, glutamine, proline, and tryptophan. In these cases, low activity of enzyme preparations prevented comparison with the mitochondrial species. In all cases, there appear to be one or more mitochondrial species that are chromatographically distinct from the cytoplasmic species. In some cases, there is a mitochondrial peak coincident with a cytoplasmic activity. It is conceivable that this is due to contamination of the mitochondrial tRNA by cytoplasmic tRNA (or the reverse). Yet, when a specific mitochondrial enzyme is used (*e.g.*, with phenylalanine), the coincident peak is present.

A comparison of the data from countercurrent distribution (Figure 1) and from reversed-phase chromatography (Figure 3) points to the superior resolution and facility of the reversed-phase system. However, a precaution should be used in the interpretation of "postcharged" fractionations where multiple peaks do occur; for it is known that molecules lacking a terminal base may exhibit properties different from those of the intact molecule, although they may be reparable upon treatment with crude enzyme preparations (RajBhandary *et al.*, 1966; Makman and Cantoni, 1966). With respect to a "precharged" RNA preparation, artifacts due to "nicked" molecules or partially degraded samples could show different properties and cannot be dismissed. Certainly, the purity of amino acid used must be considered and the proper precautions and/of controls carried out.

In summary, the survey described here characterizes the tRNAs of *Neurospora crassa* by countercurrent distribution and reversed-phase chromatography. *Neurospora* contains many isoaccepting species. A portion of this multiplicity can be ascribed to the occurrence of distinct species of tRNAs in the cytoplasm and the mitochondria. The differences in these translational macromolecules supply additional evidence for a unique protein-synthesizing system in *Neurospora* mitochondria.

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