*MITOCHONDRIAL TRANSFER RIBONUCLEIC ACIDS**

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In the course of studying the transfer ribonucleic acids (tRNA) and aminoacyl-RNA synthetases of *Neurospora crassa*, it was observed[1-4] that two synthetases are present for both aspartic acid and phenylalanine. In addition, the two synthetases for each amino acid were found to differ functionally as evidenced by their different specificities in acylation. In each case the "minor" synthetase acylates a single subspecies of the tRNA's for one of the amino acids.

This report presents evidence that the species of aspartic acid tRNA and phenylalanine tRNA acylated by the "minor" synthetases are of mitochondrial origin. It is also shown that mitochondria contain amino acid-acceptor tRNA's for 17 other amino acids. A preliminary report of these studies has been presented.[5]

Experimental Procedure.—Strains: *Neurospora crassa* wild-type strain OR23-1a was used.

Preparation of mitochondria: Hyphae in the exponential phase of growth were collected from Vogel's[6] synthetic medium supplemented with: Difco yeast extract, 2.5 gm per liter; Difco casamino acids, 1.5 gm per liter; glucose, 15 gm per liter; Dow P-200 polyglycol, 0.02 ml per liter. The mitochondria were isolated by differential centrifugation as described by Hall and Greenawalt.[7] Following partial disruption of hyphae, cellular debris and nuclei were removed by centrifugation ($1500 \times g$), and tRNA from this low-speed supernatant (S_1) is considered as whole or unfractionated tRNA. A cytoplasmic or soluble fraction and a mitochondrial fraction were subsequently obtained by centrifugation of the S_1 at $8000 \times g$. tRNA was isolated directly from the cytoplasmic fraction. To further reduce contamination with other subcellular matter, the mitochondria were subjected to sucrose gradient zonal centrifugation using a B-IV zonal rotor.[8] The gradient (1000 ml) used was 10–30 per cent sucrose with a 55 per cent sucrose cushion (500 ml). Preparations were centrifuged at 40,000 rpm for one hour and 40-ml aliquots collected.

In the preparation of mitochondria, sterile solutions were used throughout in order to eliminate bacterial contamination.

Preparation of transfer RNA: tRNA was prepared from the various fractions by a modification of the method of Holley *et al.*[9] Material was suspended in 0.01 *M* Tris-HCl buffer (pH 7.5) containing 1 per cent sodium dodecyl sulfate and 0.005 *M* magnesium acetate. Following the addition of an equal volume of water-saturated phenol, the preparation was shaken for one hour at 4°C, centrifuged, and the nucleic acids were precipitated by the addition of 0.1 vol of 2 *M* potassium acetate and 3 vol of ethanol. The tRNA was then isolated by elution from DEAE-cellulose.

Preparation of enzymes: The two aspartyl-RNA synthetases were prepared by DEAE-cellulose column chromatography, and the two phenylalanyl-RNA synthetases by hydroxyl apatite chromatography as described[3] previously. Following chromatography, fractions containing the separated synthetases were made to 20 per cent glycerol and stored at −20°C.

Radioactive materials: Uniformly labeled C^{14}-L-amino acids were obtained from the New England Nuclear Corporation.

Measurement of aminoacyl-RNA formation: The assay reaction mixture contained (in addition to enzyme and tRNA) per ml: 50.0 μmoles Tris-HCl buffer (pH 7.5); 0.5 μmole ATP; 10.0 μmoles magnesium acetate; 5.0 μmoles β-mercaptoethanol; and 1.0 μc C^{14}-amino acid. Reactions were performed at 30°C for 30 minutes in a final volume of either 0.25 ml or 0.5 ml. C^{14}-aminoacyl-RNA was assayed by a modification[10] of the filter paper disk method of Bollum.[11]

Results and Discussion.—Multiple aminoacyl-RNA synthetase systems have been described[3, 4] for aspartic acid and phenylalanine in *Neurospora.* These systems are characterized by the presence of two synthetases for a single amino acid, each of which acylates a unique tRNA or set of tRNA's. Thus, questions are raised concerning the nature of their role within the cell, such as whether both sets of synthetases and their tRNA's for a given amino acid participate freely in the synthesis of *all* cellular proteins, or whether one set of components is restricted in use to the synthesis of a particular class of proteins. In the present investigation, we have examined the tRNA's for both aspartic acid and phenylalanine for the possibility that certain species of tRNA for either amino acid may be associated with mitochondria rather than simply being soluble components of the cytoplasm. The procedures described by Hall and Greenawalt[7] have been used for the preparation of mitochondria (followed by zonal centrifugation, see Fig. 1) and cytoplasmic fractions.

The results of analyzing tRNA's from these preparations for aspartic acid-acceptor activity with the two aspartyl-RNA synthetases are shown in Table 1. It is apparent from these data that the species of tRNA which is acylated by aspartyl-RNA synthetase II (asp syn II) is associated with the mitochondrial fraction. Evidence for the association of one of the phenylalanine tRNA's with the mitochondria is shown in Table 2. In this case, with phenylalanyl-RNA synthetase I (phe syn I), a tenfold increase in the phenylalanine-acceptor activity of mitochondrial tRNA over the activity of whole tRNA was observed.

While it seems clear that the tRNA's acylated by asp syn II and phe syn I are associated with the mitochondrial fraction, the mitochondria also contain tRNA's capable of accepting aspartic acid from *both* aspartic acid enzymes, and phenylalanine from *both* phenylalanine enzymes. One would not expect cytoplasmic

TABLE 1

ASPARTIC ACID-ACCEPTOR ACTIVITIES OF FRACTIONATED *Neurospora* tRNA PREPARATIONS

tRNA	Specific Activity ($\mu\mu$moles aspartyl-RNA formed per A_{260} unit)	
	Aspartyl-RNA synthetase I	Aspartyl-RNA synthetase II
Whole tRNA	2.2	0.1
Cytoplasmic tRNA	1.1	0.0
Mitochondrial tRNA	1.1	5.5

TABLE 2

PHENYLALANINE-ACCEPTOR ACTIVITIES OF FRACTIONATED *Neurospora* tRNA PREPARATIONS

tRNA	Specific Activity ($\mu\mu$moles phenylalanyl-RNA formed per A_{260} unit)	
	Phenylalanyl-RNA synthetase I	Phenylalanyl-RNA synthetase II
Whole tRNA	0.09	3.3
Cytoplasmic tRNA	0.06	2.1
Mitochondrial tRNA	1.03	1.9

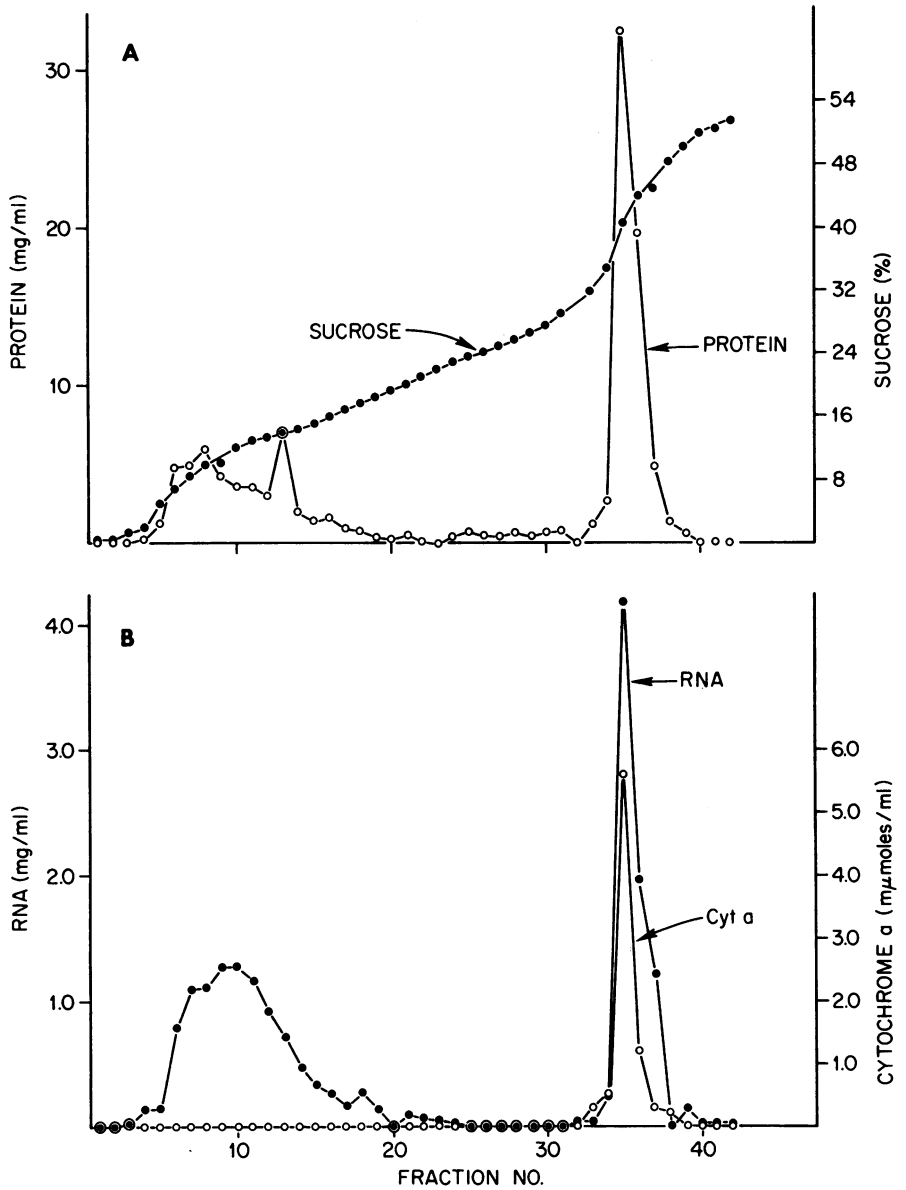

FIG. 1.—Sedimentation profile of *Neurospora* mitochondria. A mitochondrial suspension (155 ml) was subjected to sucrose gradient zonal centrifugation as described in *Experimental Procedure*. Fractions (40-ml) were collected and assayed for protein by using a modification of the method of Lowry *et al.*[16] RNA was determined by the method of Ogur and Rosen,[17] and cytochrome *a* was determined by the difference in absorbance at 6080 and 6300 Å.[18] (*A*) Protein concentration (○); per cent sucrose (●). (*B*) Cytochrome *a* (○); RNA (●).

tRNA's in mitochondria if indeed they contain their own unique set of tRNA's. The most probable explanation for this observation is that asp syn I and phe syn II are capable of acylating mitochondrial tRNA's as well as those from the cytoplasm.

To examine the nature of the association between these tRNA's and mitochondria,

we performed two types of experiments. First, mitochondria were prepared by sucrose gradient zonal centrifugation and treated with venom phosphodiesterase under conditions which destroy 95–100 per cent of the acceptor activity of added tRNA. The results of this treatment on the acceptor activity of tRNA subsequently isolated from the mitochondria may be seen in Table 3. It is apparent

TABLE 3

ACCEPTOR ACTIVITY OF tRNA ISOLATED FROM MITOCHONDRIA TREATED WITH SNAKE VENOM PHOSPHODIESTERASE

tRNA	Specific Activity ($\mu\mu$moles aspartyl-RNA formed per A_{260} unit)	
	Aspartyl-RNA synthetase I	Aspartyl-RNA synthetase II
Control mitochondrial tRNA	1.0	2.1
tRNA from phosphodiesterase-treated mitochondria	1.4	4.3

Mitochondria were prepared by zonal centrifugation as described in *Experimental Procedure* and immediately treated with 500 μg snake venom phosphodiesterase (Worthington Biochemical Corp.) per ml at pH 7.5 and 30°C for 10 min. Following this treatment, the mitochondria were heated to 90°C for 3 min to inactive the nuclease, and the tRNA was isolated as described in *Experimental Procedure* for assay.

that the relationship between the tRNA and mitochondria is such that the molecules are not susceptible to enzymatic attack. In a second approach, C^{14}-aspartyl-RNA was mixed with freshly prepared mitochondria and subjected to sucrose gradient centrifugation. Figure 2 shows that there is no tendency for the tRNA to bind to, or to become associated with, mitochondria. Both of these observations support the concept that the tRNA is an integral part of the mitochondria.

Mitochondrial tRNA (prepared from venom phosphodiesterase-treated mito

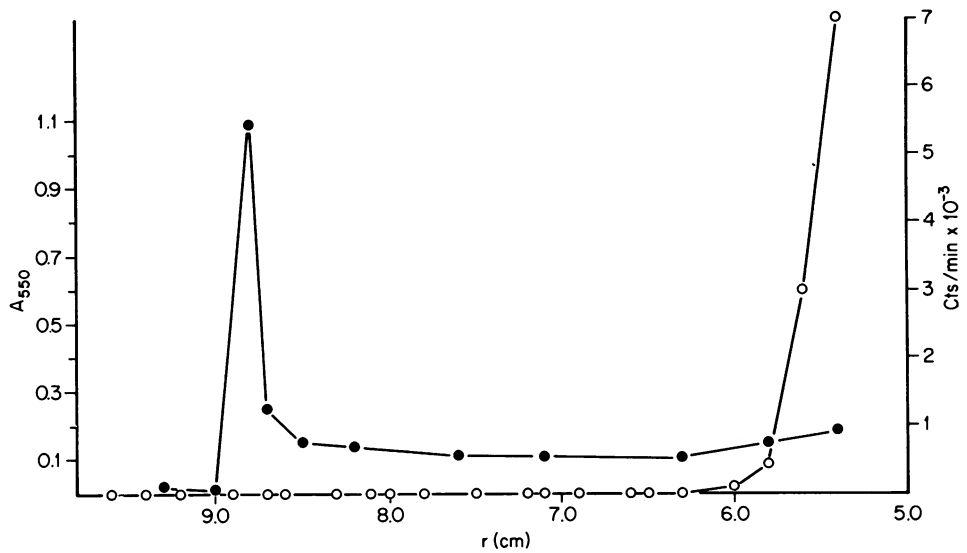

FIG. 2.—Sedimentation profiles of a mixture of mitochondria and C^{14}-aspartyl RNA. Mitochondria were prepared as in *Experimental Procedure*. Mitochondrial tRNA was acylated with C^{14}-aspartic acid by aspartyl-RNA synthetase II, and the C^{14}-aspartyl RNA isolated as previously described.[3] Of the mixture 0.5 ml was layered on 3.5 ml of 30% sucrose with a 55% cushion (1.0 ml) and centrifuged for 15 min at 15,000 rpm in a SW39 Spinco rotor. Three-drop samples were collected and analyzed for cytochrome *c* (absorbance at 5500 Å following reduction with sodium hyposulfite, ●). C^{14}-aspartyl-RNA (○) was determined by the standard filter paper disk technique.[10, 11]

TABLE 4

AMINO ACID-ACCEPTOR ACTIVITIES OF MITOCHONDRIAL AND UNFRACTIONATED *Neurospora* tRNA'S

Amino acid	Specific Activity ($\mu\mu$moles aminoacyl-RNA formed per A_{260} unit) Whole tRNA	Mitochondrial tRNA
Alanine	9.0	4.8
Arginine	9.7	6.7
Asparagine	4.7	1.6
Glutamic acid	6.6	1.2
Glutamine	15.3	3.4
Glycine	13.8	5.1
Histidine	6.1	6.0
Isoleucine	10.5	4.6
Leucine	7.2	3.6
Lysine	10.7	1.7
Methionine	5.5	2.0
Proline	5.3	0.8
Serine	7.8	3.1
Tryptophan	2.9	9.3
Tyrosine	4.6	1.6
Valine	13.1	4.7

tRNA was prepared from mitochondria treated with snake venom phosphodiesterase as described in Table 3 for assay. Unfractionated tRNA represents tRNA isolated from whole hyphal preparations.

chondria) has also been assayed for acceptor activity for other amino acids. It may be seen in Table 4 that acceptor RNA is present for all amino acids tested although usually with somewhat lower specific activities than those for whole cell tRNA. Thus, it appears that mitochondria contain a full complement of amino acid-acceptor tRNA's.

The experiments reported here demonstrate that certain *Neurospora* tRNA's are associated with the mitochondrial fraction. The possibility exists that these tRNA's are localized in some organelle or membrane structure which the present methods do not distinguish from mitochondria. We feel this is unlikely since the mitochondria have been examined by electron microscopy and there is little or no contamination with other material. Figure 3 shows a typical electron photomicrograph of a cross section of the mitochondria.

Pertinent to this report is the observation that cytoplasmic and mitochondrial protein syntheses have been shown[12–14] to be basically different in yeast. Cytoplasmic protein synthesis in this organism is unaffected by a number of antibiotics, including chloramphenicol.[15] Examination of yeast mitochondria, however, revealed that the formation of several mitochondrial enzymes was inhibited by these compounds, thus leading the investigators to conclude that two distinct systems operate in yeast.

The observations reported here raise the possibility that mitochondria contain a complete unique set of tRNA's, distinct from those which participate in cytoplasmic protein synthesis. We are currently pursuing this issue as well as the corollary question of whether mitochondria contain unique aminoacyl-RNA synthetases.

Summary.—Mitochondrial preparations from *Neurospora crassa* have been examined for the presence of transfer RNA's and the following observations made: (1) Mitochondria contain tRNA for all 18 amino acids tested, (2) one species of aspartic acid tRNA appears to be uniquely associated with mitochondria rather than the cytoplasm, and (3) the mitochondrial tRNA fraction is greatly enriched for one of the species of phenylalanine tRNA. These tRNA's appear to be an integral part of the mitochondrion since they are not susceptible to venom phos-

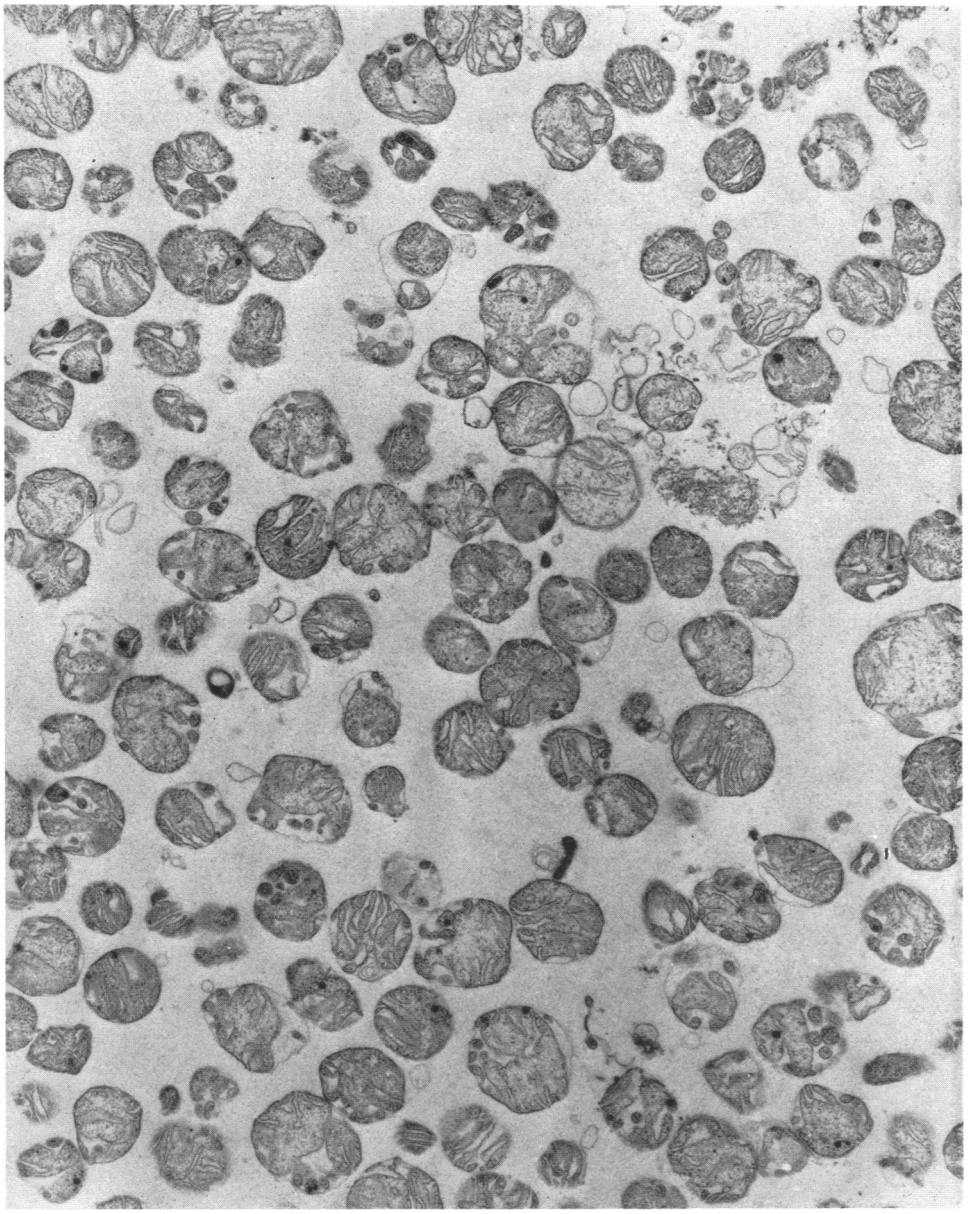

FIG. 3.—Electron photomicrograph of *Neurospora* mitochondria. Mitochondria from sucrose gradient zonal centrifugation were suspended in fixative containing 2% OsO_4, 0.005 *M* EDTA, 0.15% bovine serum albumin, 41% sucrose, and 0.04 *M* potassium phosphate buffer (pH 7.6), dehydrated through a graded series of alcohols and embedded in Epon 812. Sections were stained with 3% uranyl acetate for 17 hr followed by lead citrate for 5 min. ×1425.

phodiesterase digestion prior to mitochondrial disruption, and isolated tRNA does not exhibit an affinity to bind to or associate with the mitochondria under the conditions of their isolation.

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