*DIFFERENCES BETWEEN MITOCHONDRIAL AND CYTOPLASMIC TRANSFER RNA AND AMINOACYL TRANSFER RNA SYNTHETASES FROM RAT LIVER**

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The mitochondria of yeast,[1] *Neurospora*,[2] and *Tetrahymena*[3] have been shown to contain transfer RNA (tRNA). Barnett *et al.*[4] have found, in addition, that *Neurospora* mitochondria contain 15 aminoacyl-tRNA synthetases, at least three of which acylate only mitochondrial tRNA.

The purpose of this communication is twofold. First we will present evidence showing that, as with eukaryotic microbial systems, the mitochondria of mammalian cells contain species of tRNA which are distinct from those of the cytoplasm. Second, we will show that cytoplasmic leucyl- and tyrosyl-tRNA synthetases cannot acylate tRNA's which are exclusively mitochondrial. These observations add support to the suggestion that the protein-synthesizing machinery of the mitochondrion may be of different genetic origin than that of the cytoplasm.

Materials and Methods.—Isolation of mitochondria: Rat liver mitochondria were prepared as described previously,[5] with the following modifications to assure maximal sedimentation of nuclear and microsomal fragments. Homogenization was performed in a filter-sterilized solution of 0.3 *M* sucrose, 0.002 *M* ethylenediaminetetraacetate (EDTA), 0.025 *M* tris(hydroxymethyl)aminomethane (tris)-HCl buffer pH 7.3 (0.3 *M* SET), which contained 0.006 *M* mercaptoethanol when mitochondria were prepared as a source of synthetases (0.3 *M* SET-SH). Two successive low-speed centrifugations at 700 × *g* were performed. The twice-washed mitochondrial pellets (8,000 × *g*) were sedimented successively on two linear gradients of filter-sterilized sucrose solutions (1.03 *M*–1.91 *M* SET or SET-SH) at 22,000 rpm (Spinco rotor SW 25.1) for 90 min each. Mitochondria were collected strictly from the center of the main band; this sacrificed a less dense fraction of mitochondria that possibly contained microsomal fragments. The final mitochondrial pellets were suspended and resedimented either in 0.3 *M* sucrose, 0.001 *M* $MgCl_2$, 0.025 *M* tris-HCl pH 7.4 (for extraction of RNA) or in 0.01 *M* KCl, 0.01 *M* $MgCl_2$, 0.1 *M* tris-HCl 7.8, 0.006 *M* mercaptoethanol (for extraction of synthetases). The yield of mitochondrial protein from the livers of ten rats was 200–300 mg.

Bacterial counts: Aliquots of the final mitochondrial preparations were plated on Difco brain-heart infusion agar (for counts of viable bacteria) and added in suspension to Difco antibiotic medium to grow bacteria for subsequent isolation. Colony counts after 24–48 hr revealed 3–50 viable bacteria/mg mitochondrial protein.

Preparation of tRNA: Cytoplasmic tRNA was prepared by phenol extraction of rat liver homogenates from which the mitochondria had been removed by centrifugation. RNA was precipitated by the addition of 2 vol of 95% ethanol. The precipitates were dissolved in 0.02 *M* tris-HCl buffer pH 7.5 containing 0.15 *M* NaCl (TBS). The solution was made 1 *M* with respect to NaCl and ribosomal RNA precipitated overnight at 4°C. The tRNA was precipitated from the supernate with ethanol and redissolved in TBS. Following 5 cycles of precipitation and re-solution, the tRNA was stored at a concentration of 1 mg/ml at −80°C. Mitochondria were suspended in TBS containing 1% sodium dodecyl sulfate, and the mitochondrial nucleic acids were extracted with phenol until no protein was found at the phenol-TBS interphase. RNA and DNA were precipitated with ethanol, as described above, and redissolved in TBS. $MgCl_2$ was added to the final RNA

solution to 0.005 M, and electrophoretically purified DNase (Worthington Biochemical Corp., Freehold, N. J.) was added to a concentration of 10 μg/ml. The solution was incubated at 37°C for 15 min and extracted with phenol. RNA's other than tRNA were precipitated with 1 M NaCl as described above, and tRNA, isolated after repeated precipitation by ethanol, was stored as described for cytoplasmic tRNA.

Preparation of synthetases: Cytoplasmic synthetases were prepared from supernates of homogenized rat livers after centrifugation at 110,000 $\times$ g for 3 hr, according to the method of Taylor *et al.*[6]

For preparing mitochondrial synthetases, the mitochondria were suspended in a solution containing 0.1 M tris pH 7.8, 0.01 M KCl, 0.01 M $MgCl_2$, and 0.006 M mercaptoethanol. The suspension was sonicated in eight 15-sec bursts at 4°C on a Branson sonifier with a microtip adjusted to maximum output. The debris was removed by centrifugation at 100,000 $\times$ g for 1 hr. The supernate was passed through a Sephadex G-100 column equilibrated with the buffer used for suspending the mitochondria. The protein eluting in the void volume was collected and stored at −80°C.

Acylation of tRNA: Reaction mixtures used for all experiments contained the following in μmoles/ml: tris-HCl pH 7.8, 100; $MgCl_2$, 10; KCl, 10; adenosine 5′-triphosphate, 1.0; cytidine 5′-triphosphate, 0.1; and mercaptoethanol, 6.0. Each 0.35-ml reaction mixture contained 1.0 mg of synthetase protein, 100 μg tRNA, the appropriate H^3 or C^{14} amino acid, and 50 mμmoles of the remaining 19 nonradioactive amino acids. Under these conditions, the amount of amino acid incorporated into tRNA increased linearly with the addition of increasing amounts of tRNA up to at least 100 μg of tRNA per reaction mixture. Reaction mixtures were incubated for 10 min at 37°, at which time acylation was found to be complete. The incubations were terminated by the addition of 3 ml of cold 0.2 M sodium acetate buffer, pH 5.2, containing 0.2 M NaCl and 0.05 M $MgCl_2$. For comparative methylated albumin Kieselguhr (MAK) chromatography, two reaction mixtures were pooled after the addition of the cold buffer. Three drops of 10% glycogen solution and 300 μg of carrier yeast tRNA (General Biochemical Corp.) were added and the mixture was extracted with phenol. The aminoacyl-tRNA's were precipitated with ethanol and redissolved in the buffer three times. After the final precipitation, the tRNA's were dissolved in 0.02 M tris-HCl buffer pH 7.0 containing 0.2 M NaCl and applied to the MAK columns.

MAK chromatography: MAK was prepared according to the method of Mandell and Hershey.[7] Columns 0.8 $\times$ 20 cm were washed with 0.02 M tris-HCl buffer pH 7.0 containing 0.2 M NaCl until all excess protein was eluted. The tRNA prepared as described above was applied and washed into the column with 16 ml of 0.02 M tris-HCl pH 7.0 buffer containing 0.2 M NaCl. The tRNA was eluted with a linear gradient of 0.3 M to 0.7 M NaCl in 0.02 M tris-HCl buffer pH 7.0. A total of 200 ml of elution fluid was used, and 1.1-ml samples were collected at a rate of 1.5 ml per min. Samples were precipitated and prepared for scintillation counting as described by Taylor *et al.*[6]

Results.—Cochromatography on MAK columns of cytoplasmic and mitochondrial tRNA's acylated by their homologous synthetases: Figure 1 shows the results of cochromatographing mitochondrial H^3-leucyl-tRNA and cytoplasmic C^{14}-leucyl-tRNA. Only that portion of the gradient in which leucyl-tRNA was eluted is shown here. The two profiles are clearly different. The major species of cytoplasmic leucyl-tRNA elutes in region I. A minor species is found in region II. The mitochondrial leucyl-tRNA elutes as only a minor species in region I, whereas major species appear in regions II and III. Cytoplasmic leucyl-tRNA contains no species eluting in region III of the profile.

In Figure 2 are seen the elution profiles of cytoplasmic C^{14}-tyrosyl-tRNA and mitochondrial H^3-tyrosyl-tRNA. Again the profiles are quite distinct. Cytoplasmic tRNA elutes as a single peak in region I of the tyrosine profile, while

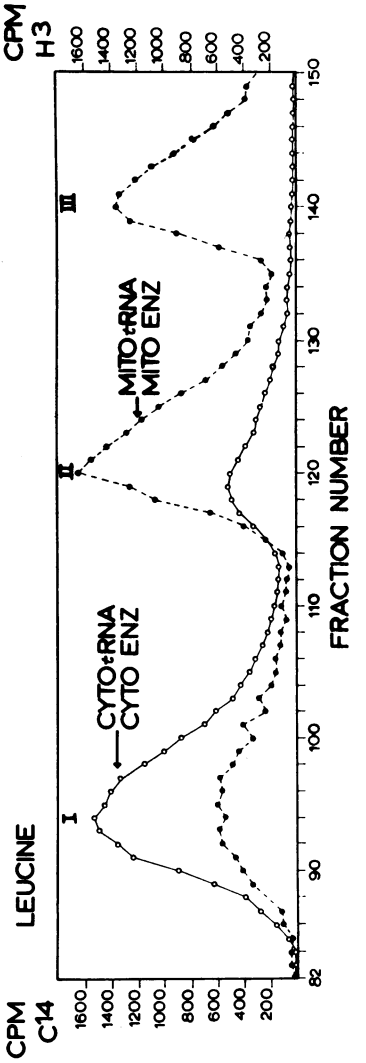

FIG. 1.—MAK chromatography of mitochondrial and cytoplasmic leucyl-tRNA acylated by their homologous synthetases. Mitochondrial tRNA (*broken line, closed circles*) was acylated with 40 μc of H^3-leucine (44 c/mmole). Cytoplasmic tRNA (*solid line, open circles*) was acylated with 4 μc C^{14}-leucine (25 mc/mmole). Procedures for MAK chromatography are described in *Materials and Methods*.

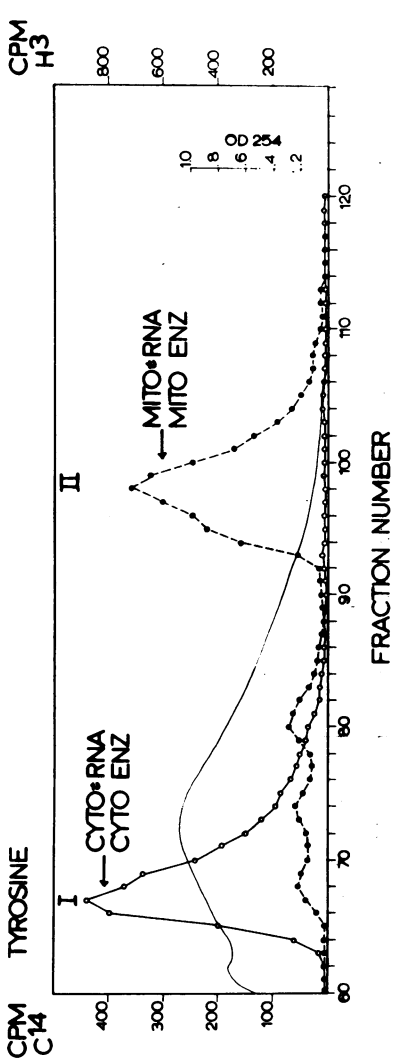

FIG. 2.—MAK chromatography of mitochondrial and cytoplasmic tyrosyl-tRNA acylated by their homologous synthetases. Mitochondrial tRNA (*broken line, closed circles*) was acylated with 25 μc H^3-tyrosine (47 c/mmole), and cytoplasmic tRNA (*solid line, open circles*) was acylated with 5 μc of C^{14}-tyrosine (368 mc/λ-mmole). A smooth line representing typical optical density tracing is shown here. Note the positions of the major mitochondrial and cytoplasmic peaks with respect to the OD tracing.

mitochondrial tyrosyl-tRNA elutes as a series of minor peaks in region I and a major peak in region II.

In contrast to the profiles of leucyl and tyrosyl-tRNA, Figure 3 shows that phenylalanyl-tRNA's from mitochondria and cytoplasm produce similar elution profiles. In this instance there appear to be only slight differences between cytoplasmic and mitochondrial phenylalanyl-tRNA.

MAK chromatography of cytoplasmic and mitochondrial tRNA's acylated by heterologous synthetases: Barnett *et al.*[4] have shown that mitochondrial aspartyl-, phenylalanyl-, and leucyl-tRNA synthetases from *Neurospora* acylate only their respective mitochondrial tRNA's. It was therefore of interest to examine the MAK profiles of mitochondrial and cytoplasmic tRNA's acylated by heterologous synthetases.

Figure 4 shows the results of cochromatography of mitochondrial leucyl-tRNA acylated with cytoplasmic synthetases and cytoplasmic leucyl-tRNA acylated with its homologous synthetase. The two profiles are nearly identical. This indicates that cytoplasmic leucyl-tRNA synthetase cannot acylate mitochondrial $tRNA^{Leu}$ eluting in region III of the profile (Fig. 1). Mitochondrial $tRNA^{Leu}$ II is acylated only as a minor species by cytoplasmic synthetase, even though it is a major species when acylated by its homologous synthetase.

When cytoplasmic $tRNA^{Leu}$ was acylated by mitochondrial synthetase and cochromatographed with mitochondrial tRNA acylated by its homologous synthetase, the profiles seen in Figure 5 were obtained. The mitochondrial synthetase preparation was able to acylate cytoplasmic $tRNA^{Leu}$ I and II. The elution profiles reflect those obtained upon acylation with the cytoplasmic synthetase (Fig. 1).

When compared with that of Figure 2, the mitochondrial tyrosyl-tRNA profile in Figure 6 shows that cytoplasmic synthetase acylated mitochondrial $tRNA^{Tyr}$ eluting in region I, but not that eluting in region II. It may be that the cytoplasmic synthetase could acylate only one of what appears to be a complex group of $tRNA^{Tyr}$ which elutes in that region. The resulting profile does not display the heterogeneity observed following acylation by mitochondrial synthetase (Fig. 2).

When the cytoplasmic tyrosyl-tRNA was acylated by mitochondrial synthe-

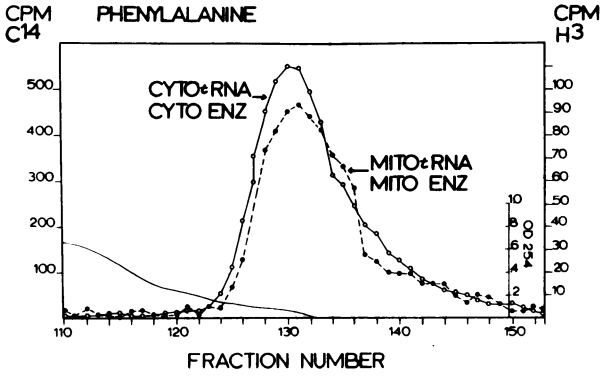

FIG. 3.—MAK chromatography of mitochondrial and cytoplasmic phenylalanyl-tRNA acylated by their homologous synthetases. Mitochondrial tRNA (*broken line, closed circles*) was acylated with 40 μc of H^3-phenylalanine (4.25 c/mmole), and cytoplasmic tRNA (*solid line, open circles*) was acylated with 4 μc of C^{14}-phenylalanine (366 mc/mmole).

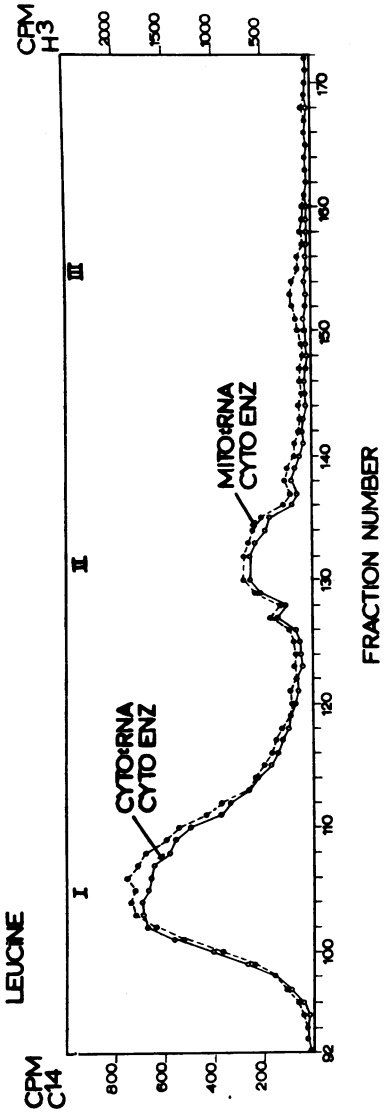

FIG. 4.—MAK chromatography of mitochondrial leucyl-tRNA acylated by cytoplasmic synthetase and cytoplasmic leucyl-tRNA acylated by its homologous synthetase. Mitochondrial tRNA (*broken line, closed circles*) was acylated with 40 μc of H^3-leucine (44 c/mmole), and cytoplasmic tRNA (*solid line, open circles*) was acylated with 4 μc C^{14}-leucine (251 mc/mmole).

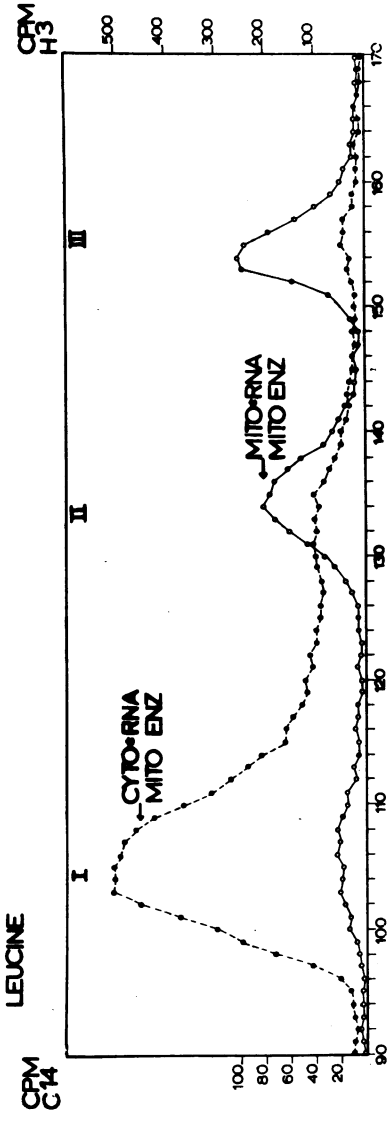

FIG. 5.—MAK chromatography of cytoplasmic leucyl-tRNA acylated by mitochondrial synthetase, and mitochondrial leucyl tRNA acylated by its homologous synthetase. Cytoplasmic tRNA (*broken line, closed circles*) was acylated with 40 μc of H^3-leucine (44 c/mmole), and mitochondrial tRNA (*solid line, open circles*) was acylated with 10 μc of C^{14}-leucine (251 mc/mmole).

tase and cochromatographed with cytoplasmic tRNA acylated by its homologous synthetase, the profiles seen in Figure 7 were obtained. The only region of the profile showing increased activity over background is that of region I. This indicates that our mitochondrial synthetase preparation was able to acylate cytoplasmic tRNA eluting in this region. The activity in region II was found to be due to a small amount of contaminating mitochondrial tRNA present in the mitochondrial synthetase used in this experiment.

Discussion.—MAK chromatography shows that rat liver mitochondria contain major species of $tRNA^{Leu}$ and $tRNA^{Tyr}$ not found in the cytoplasm. Simpson[8] has also found that rat liver mitochondria contain species of $tRNA^{Leu}$ which are distinct from those of the cytoplasm. The degree to which minor mitochondrial tRNA species may represent cytoplasmic contamination has not been determined. However, treatment of the purified mitochondria with snake venom phosphodiesterase prior to RNA extraction does not eliminate any of the minor peaks.

Barnett *et al.*[4] have shown that certain mitochondrial synthetases of *Neurospora* are unable to acylate cytoplasmic tRNA's. Our results demonstrate that cytoplasmic synthetases are unable to acylate species of tRNA which are exclusively mitochondrial, thus showing some degree of specificity. Rat liver mitochondrial synthetases, however, appear to be able to acylate cytoplasmic tRNA's. The interchangeability of certain mitochondrial and cytoplasmic tRNA's and synthetases shown here may reflect cross-contamination of the synthetases, or it may be a true reflection of a limited degree of specificity found within the liver cell. Purification of the synthetases will be necessary before a choice can be made between these two alternatives. It is obvious, however, that some degree of specificity does exist.

The presence of bacteria in mitochondrial preparations can affect the results of experiments using these preparations.[9-12] Although all solutions used to prepare the mitochondria were filter-sterilized, our mitochondrial preparations contained a total of 10^3 to 10^4 bacteria per 300 mg of mitochondrial protein. There is little likelihood that enough material could be extracted from so few bacteria to affect the results reported here.

In addition, the MAK profiles of leucyl- and tyrosyl-tRNA from the contaminating bacteria were found to be quite different from those of the mitochondria.

We were unable to alter the profiles of mitochondrial and cytoplasmic tRNA's by heating them to 75°C in EDTA or 65°C in the presence of Mg^{++} prior to acylation. Therefore, the additional peaks of mitochondrial tRNA appear not to be due to physical alterations such as those described for bacterial $tRNA^{Try}$ by Gartland and Sueoka[13] and for yeast $tRNA^{Leu}$ by Lindahl *et al.*[14] This also eliminates the possibility that the new mitochondrial tRNA's might be the products of partial ribonuclease digestion.[15] Such molecules would be irreversibly denatured at 75°C in EDTA.

Whether the tRNA's of the mitochondria respond to different codons than does cytoplasmic tRNA or whether their different structures are necessary adaptations in order that they may interact with mitochondrial ribosomes or transfer factors is not known. Also, the origin of the unique mitochondrial tRNA's and

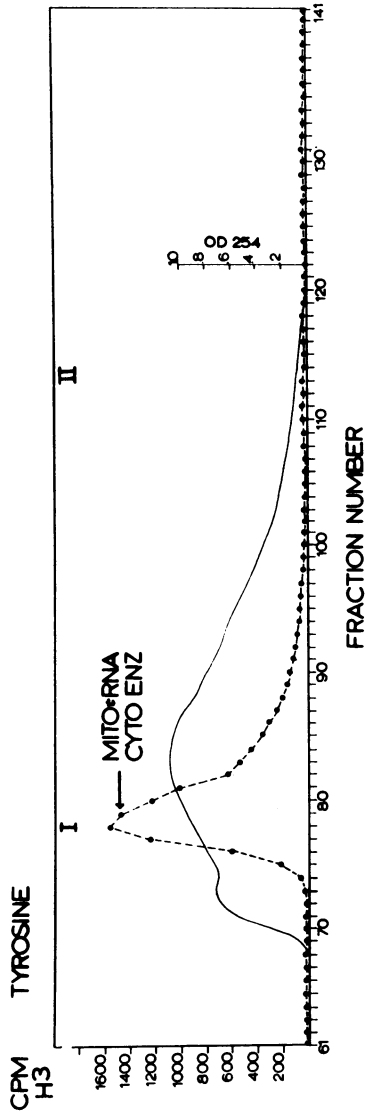

FIG. 6.—MAK chromatography of mitochondrial tyrosyl-tRNA acylated by cytoplasmic synthetase. Acylation was performed with 25 μc H^3-tyrosine (47 c/mmole). The solid line represents a typical optical density tracing. Note the position of the peak with respect to the OD tracing.

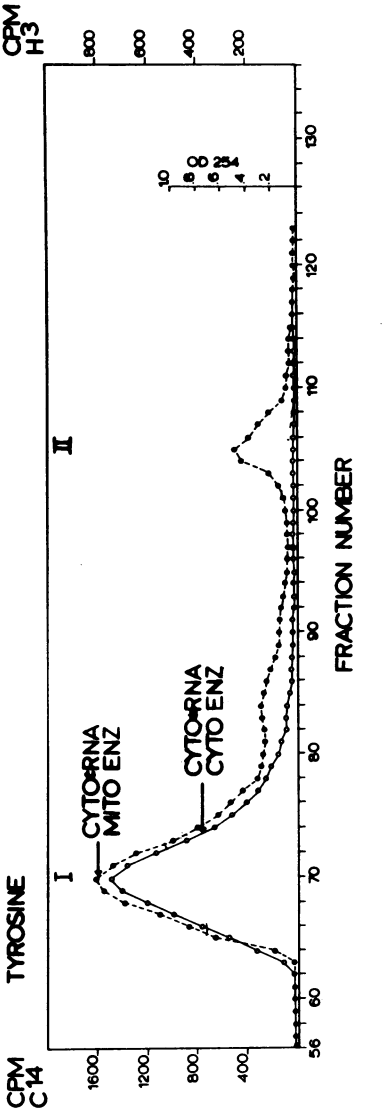

FIG. 7.—MAK chromatography of cytoplasmic tyrosyl-tRNA acylated by mitochondrial synthetase and cytoplasmic tRNA acylated by its homologous synthetase. Cytoplasmic tRNA (*broken line, closed circles*) was acylated with mitochondrial enzyme using 25 μc H^3-tyrosine (47 c/mmole). Cytoplasmic tRNA (*solid line, open circles*) was acylated by its homologous enzyme with 5 μc C^{14}-tyrosine (251 mc/mmole).

synthetases is not known. The observations reported here, however, would suggest that the protein-synthesizing apparatus of mitochondria may be of different genetic origin from that of the cytoplasm.

Summary.—Co-chromatography of rat liver mitochondrial and cytoplasmic aminoacyl-tRNA's on MAK columns has revealed differences between tRNA's from the two sources. Mitochondria contain species of $tRNA^{Leu}$ and $tRNA^{Tyr}$ not found in the cytoplasm. Cytoplasmic aminoacyl-tRNA synthetases cannot acylate species of $tRNA^{Leu}$ or $tRNA^{Tyr}$ that are exclusively mitochondrial.

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[1] Wintersberger, E., *Biochem. Z.*, **341**, 409 (1965).
[2] Barnett, W. E., and D. H. Brown, these PROCEEDINGS, **57**, 452 (1967).
[3] Suyama, Y., and J. Eyer, *Biochem. Biophys. Res. Commun.*, **28**, 746 (1967).
[4] Barnett, W. E., D. H. Brown, and J. L. Epler, these PROCEEDINGS, **57**, 1775 (1967).
[5] Nass, S., M. M. K. Nass, and U. Hennix, *Biochim. Biophys. Acta*, **95**, 426 (1965).
[6] Taylor, M. W., C. A. Buck, G. A. Granger, and J. J. Holland, *J. Mol. Biol.*, **33**, 809 (1968).
[7] Mandell, J. D., and A. D. Hershey, *Anal. Biochem.*, **1**, 66 (1960).
[8] Simpson, M. V., personal communication.
[9] Lado, P., and M. Schwendimann, *Italian J. Biochem.*, **15**, 279 (1966).
[10] Wheeldon, L., *Biochem. Biophys. Res. Commun.*, **24**, 407 (1966).
[11] Beattie, D. S., R. E. Basford, and S. B. Koritz, *J. Biol. Chem.*, **243**, 3366 (1967).
[12] Sandell, S., H. Low, and A. von der Decken, *Biochem. J.*, **104**, 575 (1967).
[13] Gartland, W., and N. Sueoka, these PROCEEDINGS, **55**, 948 (1966).
[14] Lindahl, T., A. Adams, and J. R. Fresco, these PROCEEDINGS, **55**, 914 (1966).
[15] Nishimura, S., and G. D. Novelli, *Biochim. Biophys. Acta*, **80**, 574 (1964).