*EVIDENCE FOR THE INVOLVEMENT OF A NUCLEAR GENE IN THE PRODUCTION OF THE MITOCHONDRIAL LEUCYL-tRNA SYNTHETASE OF NEUROSPORA**

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The mitochondria of *Neurospora* have been shown to contain many elements of the machinery of protein synthesis that are distinctly different from those of the rest of the cell. The differences observed in the structural and functional specificity of mitochondrial and "cytoplasmic" ribosomes,[1] transfer RNA's,[2] and several aminoacyl-tRNA synthetases[3] suggest that protein synthesis in mitochondria and the cytoplasm may be regulated by distinctly different control mechanisms. In view of this, it has become important to determine whether the structure of the various elements of mitochondrial protein synthesis is specified by the mitochondrial genome.

The availability of a strain of *Neurospora* 45208t, bearing a nuclear gene mutation that results in the production of the major "cytoplasmic" leucyl-tRNA synthetase with a two- to threefold higher K_s for leucine than normal[4] offered a unique opportunity to determine whether the structure of the corresponding mitochondrial leucyl-tRNA synthetase is determined by the same or an independent genetic element. If, for example, the mitochondrial leucyl-tRNA synthetase were indeed specified by the mitochondrial genome, strain 45208t should produce, during growth, a normal mitochondrial leucyl-tRNA synthetase. It was surprising to find, as we show below, that instead of a normal mitochondrial leucyl-tRNA synthetase or an enzyme with an altered affinity for leucine the mitochondria of 45208t contain, at best, very little leucyl-tRNA synthetase activity while possessing a near normal amount of phenylalanyl-tRNA synthetase activity.

Materials and Methods.—Biological: STD8A of *Neurospora crassa* was the wild-type strain used and 45208t-2-15A the mutant. Both strains were grown in 6-liter batches of synthetic medium with 1.0% sucrose as the carbon source and supplemented with 75 mg leucine per liter. Routinely flasks were inoculated with about 10^5 conidia and incubated with aeration for 40 hr at 34°. Although the growth of 45208t is restricted at 34° in the absence of leucine, growth is near normal in liquid medium supplemented with leucine. Cultures of the mutant were checked for genetic homogeneity before use by testing them for growth on leucine-containing medium for 48 hr at 39°. At this temperature, 45208t hardly grows even when provided with leucine. The mutant is fairly unstable upon prolonged vegetative subculture and frequent purification by conidial reisolation is required.

Preparation of enzyme extracts: Mycelia were collected by filtration and washed with water. All subsequent operations were carried out at 0–4°. To each gm wet weight of mycelia 10 ml of 0.05 *M* tris(hydroxymethyl)aminomethane HCl, pH 7.2 (Tris buffer) containing 5×10^{-4} *M* dithiothreitol (DTT) were added and the suspension was homogenized at 0–4° with a PT20 Polytron for 2 min, then sonicated intermittently for about 5 min per 100 ml of extract with a Branson 20-kc sonifier. Cell debris and subcellular particles were removed first by centrifugation at 8,000 × *g* for 15 min, then by centrifugation for 1 hr at 80,000 × *g*. Ten mg protamine sulfate per 100 mg protein was added to the clear supernatant and the resulting precipitate removed by centrifugation at 8,000 × *g*. Protein was precipitated from the supernatant by the slow addition of solid ammonium sul-

fate until 75% saturation was obtained. After standing overnight at 0° the precipitate was collected by centrifugation and suspended in 55% ammonium sulfate saturated Tris buffer with 5×10^{-4} *M* DTT. Extraction of protein soluble in 55% ammonium sulfate was allowed to proceed for 1 hr after which the insoluble protein was again collected by centrifugation and stored at −20°.

Isolation of mitochondria: The relatively crude preparations of mitochondria used in these studies were prepared by a modified version of the method of Küntzel and Noll[1] using 0.01 *M* Tris HCl, pH 7.2, 0.01 *M* $MgCl_2$, 0.1 *M* NH_4Cl, and 0.44 *M* sucrose. This method was chosen because others that prescribed the use of versene to stabilize isolated mitochondria seemed to lead to excessive loss of mitochondrial leucyl-tRNA synthetase. Care was taken to keep bacterial contamination minimal by using sterilized sucrose buffer and freshly "scrubbed" equipment. Fewer than 20 bacteria per mg protein were found upon plating mitochondrial preparations on nutrient agar. Mycelia grown and collected as described above were suspended in 5 ml of sucrose buffer per gm wet weight and an equivalent weight of acid-washed Superbrite glass beads 080-5005 (Minnesota Mining and Manufacturing Co.) were added. The mixture was then homogenized in a Lourdes blender for 1 to 2 min and ground in 75-ml batches for 2 min at 10,000 rpm in a Gifford-Wood Mini-Mill with a 0.003-in. gap setting. Debris was removed by centrifugation for 10 min at 1,500 *g* and the crude mitochondria sedimented by centrifugation at 27,000 *g* for 30 min. The pellet obtained was resuspended in about one half the original volume of sucrose buffer, centrifuged at 13,000 *g* for 1 hr. The resulting pellet was found to contain a significant amount of membranous material as determined by electron microscopy. Further purification by differential centrifugation resulted in excessive losses of the mitochondrial leucine tRNA synthetase. The mitochondria obtained were frozen, then thawed by the addition of Tris buffer, pH 7.2, with 5×10^{-4} *M* DTT and sonicated for 5 min as above. Debris was removed by centrifugation at 105,000 *g* for 1 hr. Solid ammonium sulfate was added to the supernatant until 70% saturation was obtained and the precipitate collected by centrifugation and stored at −20°.

Preparation of Neurospora tRNA: Transfer RNA was prepared from mycelia grown, harvested, and washed as described above. The mycelia were then suspended in Tris-HCl buffer containing 0.01 *M* magnesium acetate and 1.5 *M* NaCl (1 gm wet weight mycelia per 10 ml buffer) and homogenized in a Polytron for 1 min at 0°. The homogenate was shaken vigorously for 1 hr at room temperature with an equal volume of phenol saturated with buffer, then filtered through Miracloth and centrifuged. The tRNA was precipitated from the aqueous layer after the addition of 0.1 volume of 20% potassium acetate and 2.5 volumes of ethanol. After standing for 16 hr at −20° the precipitate was collected by centrifugation and was then discharged of esterified amino acids by incubation in 0.1 *M* glycine-NaOH buffer at pH 10 for 1 hr at 37°. Insoluble material was removed by centrifugation, and the tRNA was precipitated by the addition of an equal volume of 20% potassium acetate and 3 volumes of ethanol. After collection by centrifugation the tRNA was dissolved in 0.2 *M* NaCl and applied to a diethylaminoethyl (DEAE) cellulose column that had been pre-equilibrated with 0.2 *M* NaCl. After the column had been exhaustively washed with 0.2 *M* NaCl, the tRNA was eluted with 1.0 *M* NaCl and precipitated by the addition of 3 volumes of ethanol. The precipitate was collected by centrifugation and stored at −20°. *E. coli* B tRNA was purchased from General Biochemicals. *Neurospora* mitochondrial tRNA and mitochondria-free "cytoplasmic" tRNA were gifts of Dr. W. E. Barnett. Oak Ridge National Laboratory, to whom we are deeply indebted.

Aminoacyl-tRNA synthetase assays: The tRNA synthetases were assayed by a modified version of the method of Manns and Novelli[5] using a reaction mixture containing 12.5 μmoles Tris HCl pH 7.2, 4.5 μmoles magnesium acetate, 0.025 μmole DTT, 0.625 μmole ATP, 1.0 mμmole H^3 phenylalanine or 4,5 H^3-leucine (5 c/mmole), 200 gm tRNA, and 0.1 ml of enzyme preparation or column fraction in a total volume of 0.2 ml. The reaction mixture was incubated at 37° for 15 min, then 0.1 ml was removed and applied onto a filter paper disk. The disk was immediately dried in a stream of hot air, plunged into

cold 10% trichloroacetic acid, washed as described by Kelmers *et al.*,[6] then dried and counted by liquid scintillation at about 20% efficiency.

Results.—Two distinct leucyl-tRNA synthetases are consistently found after chromatography on hydroxylapatite of protein obtained from whole cell extracts of the wild-type strain of *Neurospora*. As illustrated in Figure 1, the major and minor leucyl-tRNA synthetase activities can be distinguished by their relative efficiency in the leucine charging of the tRNA's of *E. coli* and *Neurospora*. The first and minor species of leucyl-tRNA synthetase to elute from the column appears to charge *E. coli* tRNA more effectively than *Neurospora* tRNA. As indicated below, this enzyme is the mitochondrial leucyl-tRNA synthetase and, as first demonstrated by Barnett *et al.*,[3] it charges with leucine tRNA isolated from mitochondria some 10–20 times more effectively than cytoplasmic tRNA. The enzyme's apparent preference for *E. coli* tRNA in the experiments reported here is a consequence of the fact that mitochondrial tRNA constitutes only a small

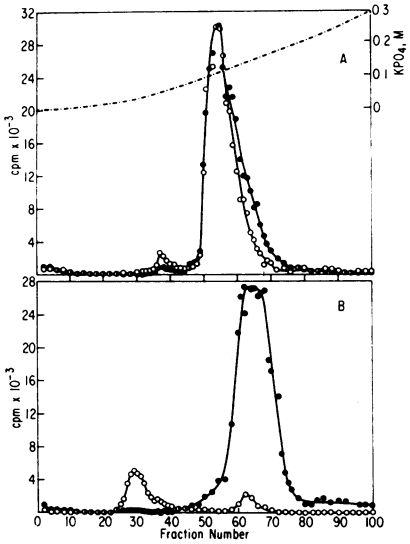

FIG. 1.—The hydroxylapatite chromatographic distribution of the tRNA synthetases of wild-type mycelia of *Neurospora*.

(*A*) Leucyl-tRNA synthetase activity assayed with *Neurospora* tRNA (●); assayed with *E. coli* tRNA (○).

(*B*) Phenylalanyl-tRNA synthetase activity assayed in the same chromatogram with *Neurospora* tRNA (●); assayed with *E. coli* tRNA (○).

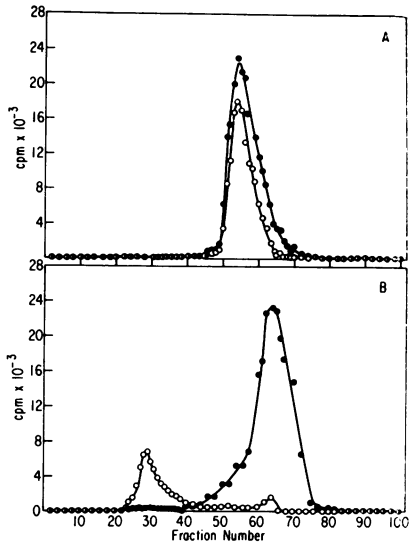

FIG. 2.—Hydroxylapatite chromatographic distribution of the tRNA synthetases of 45208t mycelia.

(*A*) Leucyl-tRNA synthetase activity assayed with *Neurospora* tRNA (●); assayed with *E. coli* tRNA (○).

(*B*) Phenylalanyl-tRNA synthetase activity assayed with *Neurospora* tRNA (●); assayed with *E. coli* tRNA (○).

Chromatographic Procedure (Figs. 1–4).—Chromatography was performed on a 1.5 × 15.0-cm column of hydroxylapatite with a 0.5-cm layer of coarse kieselguhr at the top and bottom. The column was pre-equilibrated with 0.005 *M* KPO_4 buffer, pH 7.7 containing 5×10^{-4} *M* DTT and 30 mg of protein, desalted by passage through a column of Sephadex G50, was chromatographed using a 0.005–0.3 *M* KPO_4 pH 7.7 gradient. The gradient illustrated was applied to the column with an ISCO Dialagrad pump. The flow rate was 0.5 ml per minute and 5.0-ml fractions were collected.

fraction of the tRNA isolated from *Neurospora* mycelia that was used in the assays for enzymatic activity. The resemblance of *E. coli* and mitochondrial tRNA as substrates for the mitochondrial tRNA synthetase proved fortuitous for mitochondrial tRNA is less readily obtainable than *E. coli* tRNA.

The second, somewhat heterogeneous peak of leucyl-tRNA synthetase activity to elute from the column contains the bulk of the "cytoplasmic" enzyme. Characteristically, under conditions of assay employed here, the enzyme eluting in the early part of the second peak is about equally effective in charging *E. coli* and *Neurospora* tRNA, while the activity that elutes in the trailing part of the distribution charges *Neurospora* tRNA more effectively than *E. coli* tRNA. It is not yet clear whether the heterogeneity of the pattern reflects the involvement of two distinctly different leucyl-tRNA synthetases or whether some structural transition occurs during extraction and chromatography that alters substrate specificity and chromatographic mobility. The same heterogeneity has been observed on DEAE-cellulose chromatography. Rechromatography on either hydroxylapatite or DEAE-cellulose of that part of the peak that charges *E. coli* tRNA effectively has resulted in the preferential loss of a considerable fraction of the *E. coli* tRNA charging activity. Because of excessive losses of enzyme activity during rechromatography, however, it has not been possible to distinguish between the possibility of the involvement of an interconversion of two forms of leucyl-tRNA synthetase or the loss of a labile enzyme with a high specificity for *E. coli* tRNA.

Figure 1*B* is the distribution of the phenylalanyl-tRNA synthetases in the same chromatogram. Again there are two chromatographically and functionally distinct synthetases. The first to elute is the mitochondrial synthetase which, like the corresponding leucine-specific enzyme, charges effectively *E. coli* tRNA. This enzyme has been found to charge mitochondrial tRNA with phenylalanine and to be almost completely ineffective in charging cytoplasmic tRNA. Again, the apparent poor activity displayed by the enzyme against *Neurospora* tRNA is due to the relatively low concentration of mitochondrial tRNA in the mycelial tRNA preparation used in the analysis of these chromatograms. The bulk of the cytoplasmic phenylalanyl-tRNA synthetase activity is eluted in a single peak but unlike the leucine-specific cytoplasmic enzyme it is highly specific for *Neurospora* tRNA. Barnett and Epler[7] have shown that the *Neurospora* phenylalanyl-tRNA synthetase (probably the mitochondrial enzyme), in addition to charging *E. coli* phenylalanine-specific tRNA, charges the valine and alanine tRNA's anomalously with phenylalanine. It seems likely then, that the general functional similarity displayed by *E. coli* and mitochondrial tRNA is a result of some structural similarity of the two tRNA's. It is probable therefore, that a considerable fraction of the enzyme activity observed may involve anomalous charging of the tRNA's of *E. coli* other than those specific for leucine and phenylalanine.

The chromatographic pattern of the leucyl-tRNA synthetase activity of 45208t is illustrated in Figure 2*A*. The most striking feature of the chromatogram is the virtual absence of the minor mitochondrial enzyme. At most, only a trace of mitochondrial synthetase has been found in extracts of 45208t grown under a wide variety of incubation temperatures and lecuine supplementation.

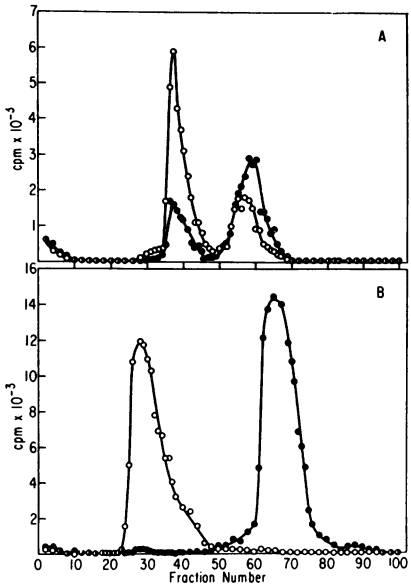

FIG. 3.—Hydroxylapatite chromatographic distribution of the tRNA synthetases in a crude wild-type mitochondrial preparation.

(*A*) Leucyl-tRNA synthetase assayed with *Neurospora* tRNA (●); assayed with *E. coli* tRNA (○).

(*B*) Phenylalanyl-tRNA synthetase activity assayed with *Neurospora* tRNA (●); assayed with *E. coli* tRNA (○).

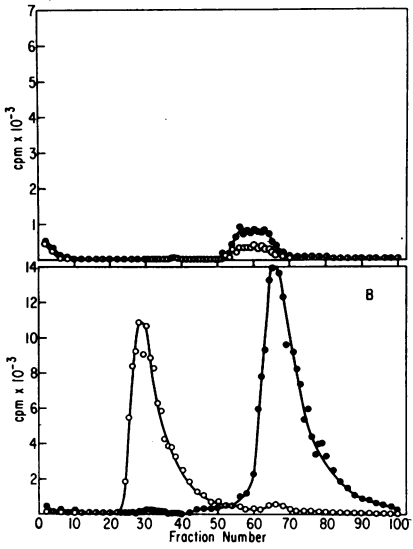

FIG. 4.—Hydroxylapatite chromatographic distribution of the tRNA synthetases in crude 45208t mitochondrial preparations.

(*A*) Leucyl-tRNA synthetase assayed with *Neurospora* tRNA (●); assayed wtih *E. coli* tRNA (○).

(*B*) Phenylalanyl-tRNA synthetase assayed with *Neurospora* tRNA (●); assayed with *E. coli* tRNA (○).

Forty mg of protein was applied to the column instead of the usual 30; otherwise the chromatographic procedure was identical to that described above.

Virtually all of the leucyl-tRNA synthetase activity of 45208t elutes from the column at the position characteristic of the cytoplasmic enzyme. However, the activity profile of the synthetase against *E. coli* and *Neurospora* tRNA is indicative of a characteristic difference between the 45208t and the wild-type cytoplasmic leucyl-tRNA synthetase. It has been consistently observed that, after chromatography, the 45208t enzyme charges *E. coli* tRNA less effectively than does the wild-type enzyme. No such difference in charging efficiency is observed in extracts of the two strains before chromatography. Thus it appears that the *E. coli* tRNA charging function of the 45208t synthetase is more labile to chromatography than is the corresponding wild-type enzyme.

Figure 2*B* is the elution pattern obtained for the 45208t phenylalanyl-tRNA synthetase. The chromatographic behavior of the mitochondrial and cytoplasmic phenylalanyl-tRNA synthetases of the mutant is virtually identical to that of the synthetases of the wild-type strain.

In order to improve detection of the missing minor leucyl-tRNA synthetase of 45208t, and to establish the fact that the minor peak is indeed the mitochondrial enzyme, mitochondria were obtained by differential centrifugation and enzyme

extracts of the purified mitochondria were subjected to chromatographic analysis. It soon became obvious, however, that although it was possible to obtain purified wild-type mitochondrial preparations containing only the leucyl and phenylalanyl-tRNA synthetases that corresponded to the minor enzyme species of mycelial extracts, a large fraction of the mitochondrial leucine tRNA synthetase was lost during repeated differential centrifugation. Such losses of mitochondrial specific enzymes during fractionation of *Neurospora* mitochondria is fairly commonplace[8] and could only obscure any attempt to determine the extent of the deficiency of the mitochondrial leucyl-tRNA synthetase in 45208t. Hence, chromatographic analysis was performed on the synthetases prepared from crude mitochondrial preparations obtained by only two rapid cycles of differential centrifguation.

The chromatographic distribution of the leucyl-tRNA synthetases in extracts of such crude mitochondrial preparations obtained from the wild-type strain is presented in Figure 3*A*. It is clear that the first, or minor, leucyl-tRNA synthetase of mycelial extracts has been enriched two- to threefold as compared to extracts of whole cells and is the major leucyl-tRNA synthetase present in such mitochondrial preparations. Characteristically, the cytoplasmic leucyl-tRNA synthetase activity of such preparations appears disproportionately reduced about tenfold, with a somewhat greater loss of the *E. coli* tRNA charging ability.

The distribution of the phenylalanyl-tRNA synthetases in the same chromatogram is illustrated in Figure 3*B*. Again the minor phenylalanyl-tRNA synthetase of mycelial extracts is enriched about twofold in the crude mitochondrial preparation and, in contrast to the corresponding leucyl-tRNA synthetase, the activity of the cytoplasmic phenylalanyl-tRNA synthetase is reduced by an equivalent amount. It is not clear whether the differential loss of the cytoplasmic leucyl and phenylalanyl-tRNA synthetase activity reflects the relative stability of the two enzymes or their relative ability to bind to subcellular particles contaminating mitochondrial preparations.

The identification of the minor leucyl and phenylalanyl-tRNA synthetases of mycelial extracts and the corresponding synthetases that are enriched in mitochondrial preparations as mitochondrial enzymes is based primarily on the fact that they show a high degree of substrate specificity by charging mitochondrial tRNA with phenylalanine and leucine 10–20 times more effectively than cytoplasmic tRNA. Furthermore, these enzymes, as shown by Barnett *et al.*,[3] are the only leucyl and phenylalanyl-tRNA synthetases that can be found in highly purified preparations of mitochondria. Failure to obtain greater enrichment of these mitochondrial synthetases in the crude preparations used here probably resulted from the fact that the mitochondrial preparations were heavily contaminated with cell membranes and other cytoplasmic particles as determined by electron micrography.

The chromatographic behavior of the leucyl-tRNA synthetases obtained from a similarly prepared crude mitochondrial preparation of 45208t is illustrated in Figure 4*A*. Despite the fact that 40 mg, rather than the usual 30 mg, of protein was chromatographed, only a trace of activity was found in the region of the chromatogram characteristic of the mitochondrial leucyl-tRNA synthetase.

In addition to the deficiency of mitochondrial leucyl-tRNA synthetase, the recovery of cytoplasmic synthetase activity was only one quarter of that expected on the basis of enzyme recovery from wild-type mitochondria. (It should be pointed out that the small amount of activity that is eluted near the start of the chromatogram is independent of extract source and is due, apparently, to the presence of some small amount of enzyme associated with nucleic acid that does not adsorb to hydroxylapatite.)

The distribution of the phenylalanyl-tRNA synthetases in the crude mitochondrial fraction of 45208t is illustrated in Figure 4*B*. Although the recovery of synthetase activity was about 30 per cent less than expected on the basis of the recovery of the enzymes from similar preparations of wild-type mitochondria, both cytoplasmic and mitochondrial phenylalanyl tRNA synthetase are present in the expected proportions. The somewhat reduced amount of phenylalanyl-tRNA synthetase activity recovered is consistent with previously published data on the general production of defective protein by 45208t.

Discussion.—The virtual absence of detectable mitochondrial leucyl-tRNA synthetase activity from mycelial and mitochondrial extracts of strain 45208t as well as the relative instability and altered K_s for leucine displayed by the cytoplasmic leucyl-tRNA synthetase[4] suggest strongly that the cytoplasmic and mitochondrial leucyl-tRNA synthetases are altered functionally as a consequence of the same mutation. Since the 45208t phenotype segregates formally as a mutant gene at the *leu-5* locus on linkage group V, it seems quite likely that the structure of the mitochondrial leucyl-tRNA synthetase as well as the cytoplasmic synthetase are to a large measure specified by the same nuclear gene. However, it is clear from the difference in substrate specificity and chromatographic mobility that the two enzymes differ in some rather crucial respects. As a consequence either the structure of the cytoplasmic enzyme is modified in some way upon incorporation into the mitochondrion or the nuclear gene message is altered during or after translation by the mitochondrial protein synthetic apparatus. Whatever the mechanism, it is interesting to speculate that the imposition of mitochondrial tRNA specificity on aminoacyl-tRNA synthetases produced as a consequence of nuclear gene activity may be one way in which independent regulation of mitochondrial and cytoplasmic protein synthesis is maintained by an organelle possessing a relatively small genome. In a sense, the suggestion that the mitochondrial leucyl-tRNA synthetase is a modified version of the cytoplasmic enzyme is analogous to the observed alteration of the host valyl-tRNA synthetase upon infection of *E. coli* with T4.[9]

The apparent deficiency of mitochondrial leucyl-tRNA synthetase in 45208t raises some doubt as to the validity of the previously offered explanations of the temperature-sensitive conditional lethal phenotype of the mutant. Since the mutant, when grown at high temperature, was found to produce a variety of structurally altered enzymes and since the temperature effect was markedly reversed by added leucine, it seemed reasonable to relate amino acid misrecognition at elevated temperatures to the alteration of the leucine-binding property of the mutant leucyl-tRNA synthetase. This explanation seemed adequate to account for both the temperature sensitivity of the mutant and the requirement of exog-

enously supplied leucine for rapid growth at intermediate temperatures. However, in view of the observations reported here it might be possible to account for the conditional lethal phenotype of the mutant by the paucity of mitochondrial leucyl-tRNA synthetase activity. It is important to point out, however, that the data obtained do not permit the distinction between low levels of enzyme production, low activity, or the production of an enzyme exquisitely unstable to the extraction and chromatographic procedures employed. However, extracts of 45208t grown at 24° contain no more mitochondrial leucyl-tRNA synthetase than extracts obtained from the mutant grown at 34°. It seems clear then that the deficiency in mitochondrial leucyl-tRNA synthetase activity displayed by 45208t is not primarily due to a uniquely high-temperature sensitivity of the enzyme's activity or its synthesis.

Summary.—The mitochondria of mutant strain 45208t of *Neurospora* have been found to be almost devoid of mitochondrial leucyl-tRNA synthetase activity. Since the mutant also produces a somewhat unstable cytoplasmic leucyl-tRNA synthetase with an altered K_s for leucine and the mutation in 45208t is at the *leu*-5 locus on linkage group V, it is quite likely that at least part of the structure of the mitochondrial and cytoplasmic enzymes is specified by the same nuclear gene.

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