MODIFIED NUCLEOTIDES OF MITOCHONDRIAL TRANSFER RIBONUCLEIC ACIDS

BY

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Abstract

^{32}P-labeled low molecular weight RNA was extracted from mitochondria of KB cells. Thin layer chromatography was used to separate and quantitate the various nucleotides. The amount of label incorporated into modified nucleotides of mitochondrial LMW RNA was 5.6% of the total amount incorporated into all nucleotides of this RNA. The value obtained in the same way for cytoplasmic LMW RNA was 10.9%. This is the only data available on modified nucleotide content of mitochondrial LMW RNA.

Abbreviations

tRNA -- transfer ribonucleic acid

rRNA -- ribosomal ribonucleic acid

mRNA -- messenger ribonucleic acid

Ap -- adenylic acid

Up -- uridylic acid

Cp -- cytidylic acid

Gp -- guanidylic acid

ψp -- pseudouridylic acid

Py -- pyrimidine

Pu -- purine

N -- nucleoside (or nucleotide)

hU -- dihydrouridine

LMW -- low molecular weight

DEAE -- diethylaminoethyl

TLC -- thin layer chromatography

SDS -- sodium dodecyl sulfate

RSB -- reticulocyte standard buffer

EDTA -- ethylenediaminetetraacetic acid

MAK -- methylated albumin kieselguhr

cpm -- counts per minute

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Introduction and Statement of the Problem

The semi-autonomy of mitochondria was first noticed when it was observed that these organelles undergo binary fission at times which are not coincident with the mitotic fission of the cell. It was also found that certain genes affecting these organelles (such as the petite mutants of yeasts) were inherited in a non-Mendelian way. The observation of cytoplasmic inheritance suggested that the cell contained some amount of genetic material which was located elsewhere than in the chromosomes of the nucleus. This suggestion was dramatically verified by Margit Nass when she isolated DNA from the mitochondria.

In the years following this discovery it has been found that mitochondria contain not only their own self-replicating DNA, but also all other materials that are necessary for a complete protein synthesizing system. These materials include ribosomes which differ in molecular weight from those of the cytoplasm, and transfer RNAs which differ from those of the cytoplasm in the pattern of their elution from methylated albumin kieselguhr columns. The mitochondria also contain their own aminoacyl-tRNA synthetases, enzymes which form bonds between tRNA molecules and amino acids.

Hybridization studies have shown that ribosomal RNAs and at least some of the tRNAs of the mitochondria are transcribed from mitochondrial DNA.

Methylated albumin kieselguhr chromatography elution profiles of mitochondrial tRNA exhibit certain peaks which are not found in the elution profiles of cytoplasmic tRNA. These peaks represent species of mitochondrial tRNA which can be acylated only by mitochondrial aminoacyl-tRNA synthetases. These observations have led to the generalization that mitochondria possess a protein synthesizing system which is distinct from that of the cytoplasm.

It is important to note that the mitochondrial genome probably does not contain sufficient DNA to code for all of the elements of this distinct mitochondrial protein synthesis system. Some of the necessary components may be nuclear coded and then transported into the mitochondria.

For the total cell to function efficiently, the activities of the mitochondrion must be coordinated with the activities of the nucleus and the cell as a whole. Many experiments have shown that the mitochondrial protein synthesizing system, and the mitochondrial tRNA profile specifically, is profoundly affected by the conditions of the cell. It also seems logical that some feedback mechanism must operate whereby the metabolic activities of the mitochondria produce an effect on the activities of the remainder of the cell. It has been suggested that a mutual rhythm exists between the cellular mitotic cycle and the division cycle of the mitochondria.

It is doubtful that a satisfactory model for the coordination of the biosynthetic and mitotic activities of the cell as a whole can be formulated without reference to the role of the mitochondria. A clear understanding of the function of the mitochondrial protein synthesis and its relationship to nuclear controlled protein synthesis will be crucial to the understanding of the role of the mitochondria in control of the cell cycle and biosynthetic activities. Central to this problem of mitochondrial protein synthesis is the question of the mitochondrial tRNA, its origin, biosynthesis, structure and function.

It is known that the mitochondrial tRNA differs from the cytoplasmic tRNA in the pattern of its elution from methylated albumin kieselguhr columns, but no data is available on specific structural differences between mitochondrial and cytoplasmic tRNA. It is the purpose of this research to obtain comparative data on the nucleotide composition of mitochondrial and cytoplasmic tRNA as a first step in determining the structural differences between these two populations of nucleic acid molecules.

Literature Review

Transfer ribonucleic acid acts to transfer an amino acid onto a growing polypeptide chain in the process of ribosomal protein synthesis. The amino acid is bound through an ester linkage to the free 2' or 3' hydroxyl group of the 3' terminal AMP residue of the tRNA. The structure of this bond is illustrated in Figure 1. The formation of the aminoacyl bond between the tRNA molecule and the amino acid is catalyzed by a class of enzymes referred to as aminoacyl-tRNA synthetases (E.C. 6.1.1). Structurally different tRNA molecules that accept the same amino acid are referred to as isoaccepting tRNAs.

All ribonucleic acids are polymers of ribonucleotides composed of a phosphoric acid group, a ribose moiety and a purine or pyrimidine base. Transfer ribonucleic acid differs from other major classes of ribonucleic acids in two salient respects: low molecular weight, and a higher proportion of the so-called modified nucleosides.

Modified nucleosides are characterized by chemical alteration of the four major bases (adenine, guanine, cytosine and uracil). The enzymes that catalyze these alterations are referred to as modifying enzymes. 10 to 20% of the nucleotides that make up tRNA molecules contain modified bases. Ribosomal RNA contains fewer modified bases and messenger RNA is apparently without modified bases.[1]

Available evidence suggests that the biosynthetic precursor of transfer RNA is transcribed from DNA with a

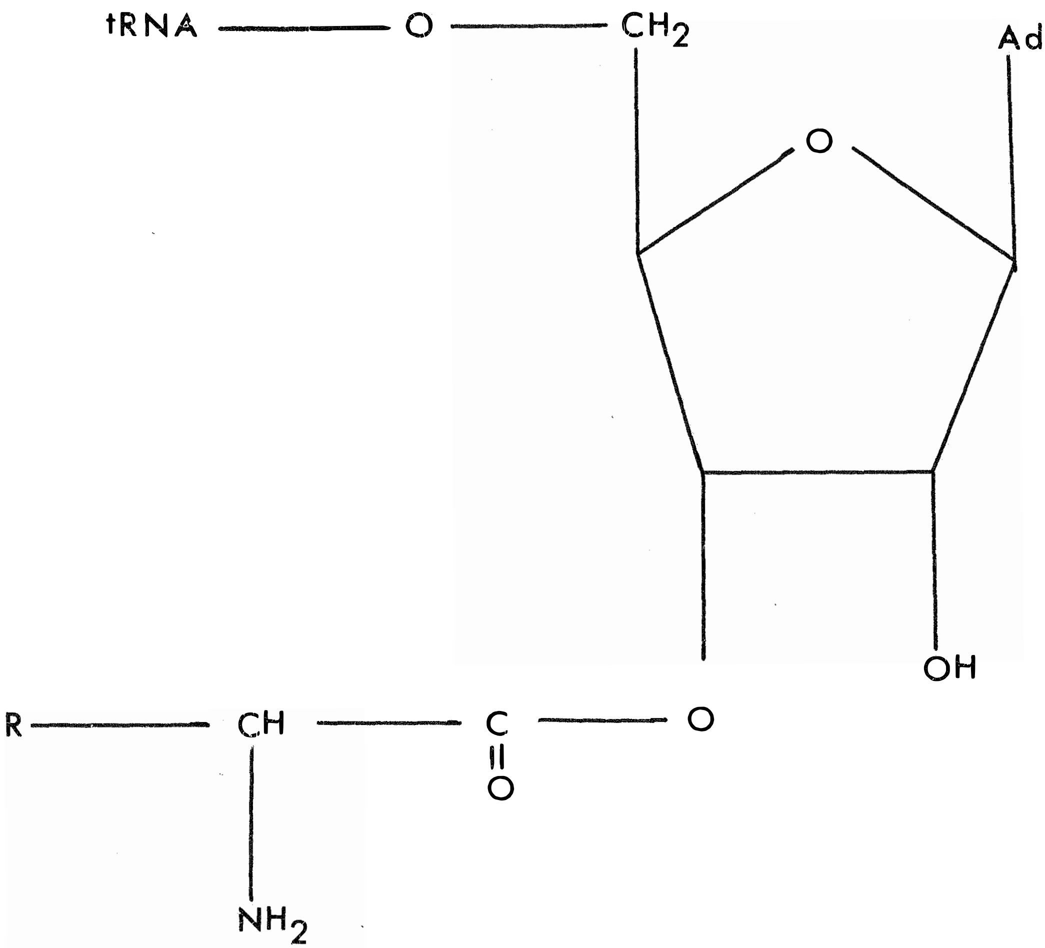


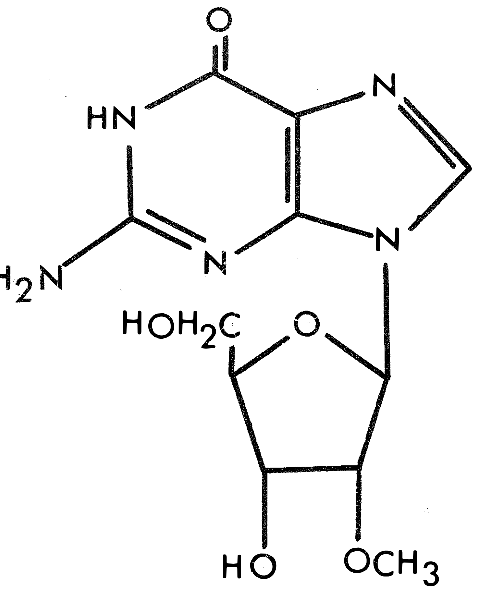
Figure 1

The ester bond between the 2' or 3' hydroxyl group of the tRNA molecule and its specific amino acid.

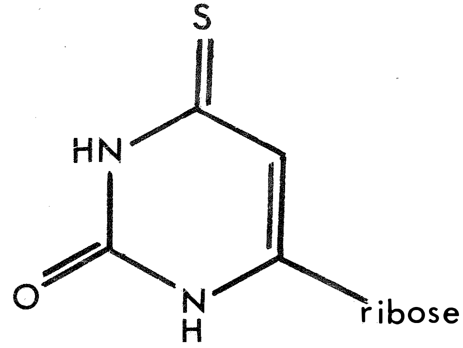
slighly higher molecular weight than the final tRNA product.[2] After transcription, part of the chain is cleaved, and some of the nucleic acid bases are enzymatically modified at the polynucleotide level. Figure 2 illustrates some of these modified nucleosides. Before the mature tRNA molecules become active in accepting and transferring amino acids, they assume a complex secondary and tertiary structure. A general pattern for the secondary structure originally proposed by Holley is shown in Figure 3. The tertiary structure is a matter which is currently under investigation in a number of laboratories.[3]

Because they are physically and chemically different from their parent nucleosides, the modified nucleosides may play a major role in determining the secondary and tertiary structure of the tRNA molecule and thereby in determining its biological activities.[4] Since the modified nucleosides may help to determine the activity of tRNA it is suspected that enzymatic modification of nucleosides in tRNA may be one aspect of control and regulation of protein synthesis.[5] For this reason a great deal of study has been devoted to the modified nucleosides in tRNA. This research has centered on a number of basic questions: How are the modified nucleosides formed? Where are they located on the tRNA molecule? What function do they have?

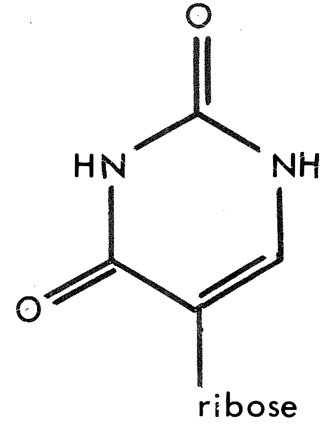
For some of the modified nucleosides the enzymatic reaction that results in their formation is known. In all cases studied it appears that the modification occurs at the



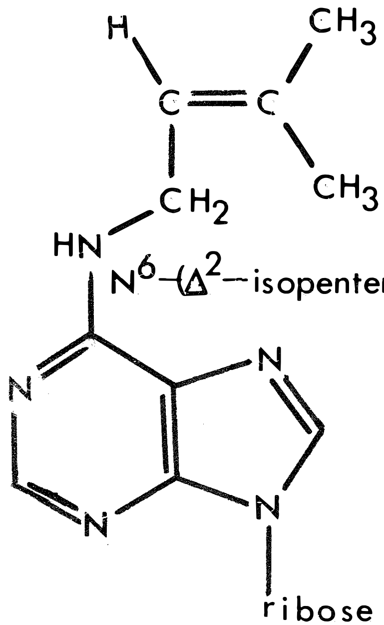
2′—O — methylguanosine



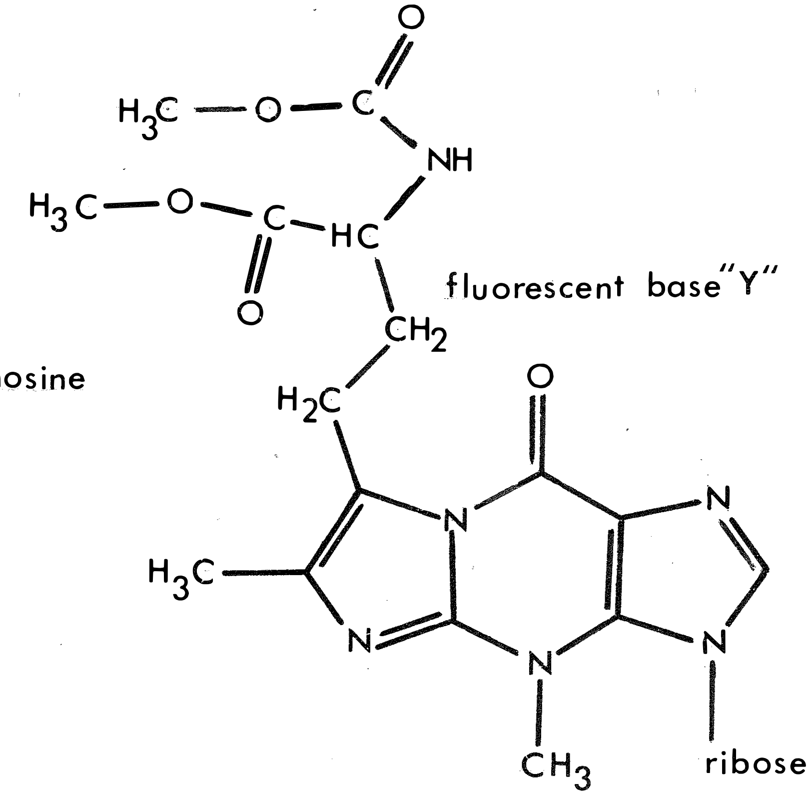
4 — thiouracil



pseudouracil



N^{6}–(Δ^{2}–isopentenyl) adenosine

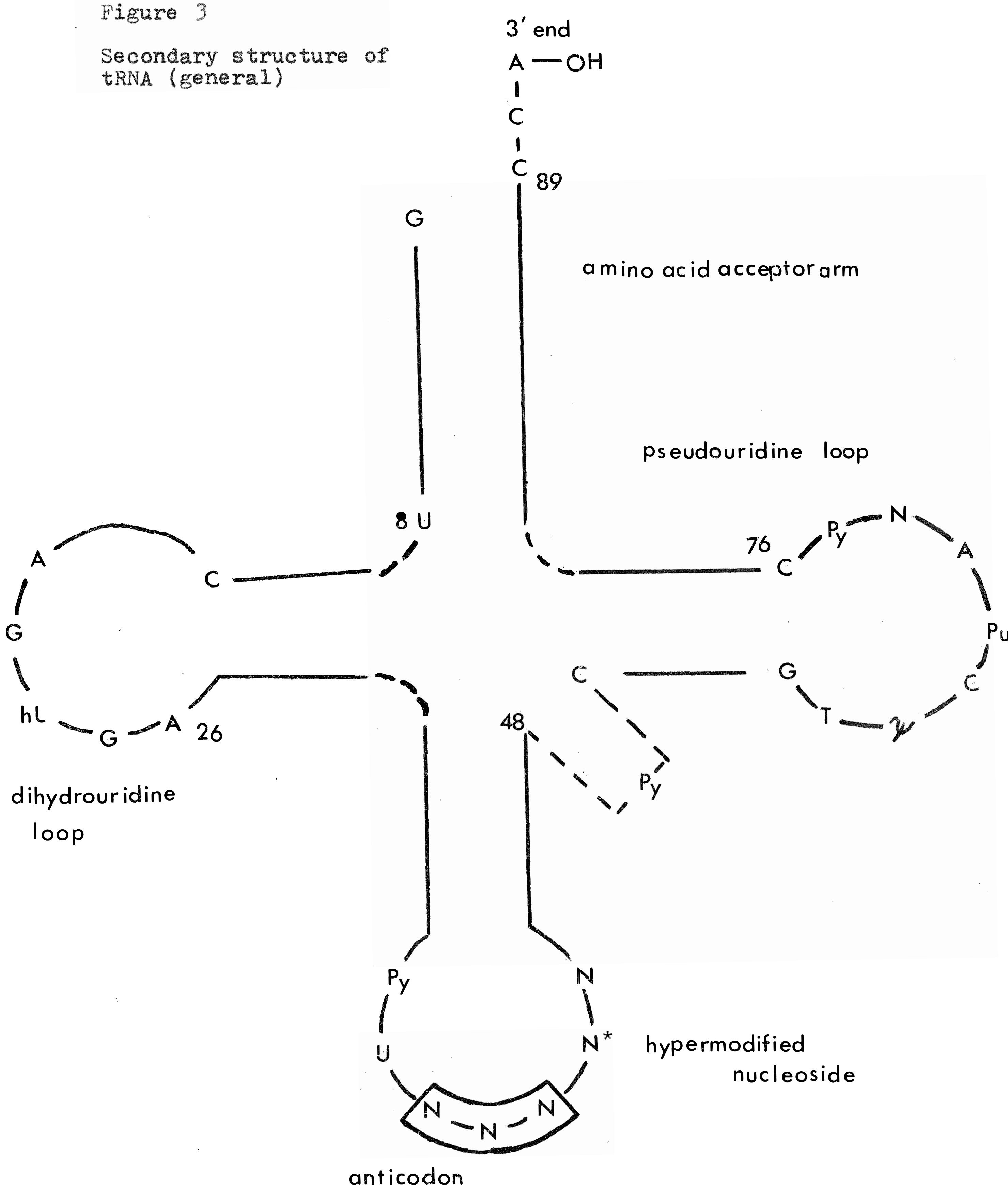


fluorescent base "Y"

Figure 2
Some modified nucleosides

Figure 3

Secondary structure of tRNA (general)



polynucleotide level. The best known examples of modifying enzymes are the methylases involved in the biosynthesis of the methylated components of tRNA.[6] Kline et al reported the isolation from yeast and rat liver of an enzyme which transfers isopentenyl pyrophosphate groups to adenosine residues in tRNA to produce N^6-(Δ^2-isopentenyl)adenosine residues within the tRNA molecule. This enzyme acts at the macromolecular level since it uses tRNA as a substrate.[7] An enzyme has been found which transfers sulfur from cysteine to tRNA to produce 4-thiouridine residues in tRNA. This enzyme also acts on the polynucleotide level.[8]

The polynucleotide sequence of a number of tRNAs has been determined.[9-11] These sequences show that the modified nucleosides make up 10 to 20% of the total tRNA structure.[1]

The modified nucleosides are distributed throughout the tRNA chain with no obvious pattern, however some interesting generalizations can be made about their locations. For some species of tRNA certain modified bases are always found on the 3' side of the anticodon group.[12] The yeast alanine tRNA originally sequenced by Holley has 1-methyl inosinic acid next to the anticodon.[9] A table of nucleosides adjacent to the first letter of the anticodon of twelve species of tRNA from yeast, rat liver and <u>E. coli</u> has been published.[13] These adjacent nucleotides include unmodified adenosine and five or six species of modified nucleosides including N^6-(Δ^2-isopentenyl)adenosine which is adjacent to the anticodon in five of the twelve tRNA species listed.

Pseudouridine is found in the so-called pseudouridine loop of almost all tRNAs whose sequence is known.[9-11] In some cases lack of modification of the nucleotide adjacent to the anticodon results in inactivity of the molecule in binding to ribosomes.[14]

In general the function of the modified nucleosides within the tRNA molecule is not known. They may be involved in the regulation of protein synthesis through their effect on the conformation and activity of tRNA.[4-5]

The isoaccepting species of tRNA present in a cell may vary depending in various ways on the state of the cell. In some cases it has been shown that infecting viruses code for their own species of tRNA. Phage T4 codes for a species of leucyl-tRNA which appears in chromatographic profiles of E. coli tRNA after infection of the bacterium with this virus.[15] The number of isoaccepting tRNAs present in a cell also depends on cellular growth conditions. Doi et al chromatographed tRNA from Bacilus subtilis using methylated albumin kieselguhr columns. They found that the iso-accepting tRNA peaks observed for seryl-tRNA from bacteria grown in rich broth differed from those of bacteria grown on so-called sporulation medium.[16]

Isoleucyl-tRNA was isolated from aerobically and an-aerobically grown E. coli cells by Kwan et al. These investigators compared the pattern of elution of these tRNAs from DEAE-Sephadex columns and found that the isoleucyl-tRNA of anaerobically grown cells contained different species relative to the isoleucyl-tRNA of aerobically grown cells. They also found that the isoleucyl-tRNA of anaerobically grown cells had a lower requirement for Mg^{++} for binding to ribosomes than isoleucyl-tRNA of aerobically grown cells.[17]

The tRNA profiles may vary depending on the state of differentiation of the cell. Holland et al found chromatographic differences between tRNA from fibroblasts as compared to the tRNA from most differentiated cells.[18] Yang and Comb found differences in the MAK column chromatographic patterns of lysyl-tRNA from fertilized and unfertilized sea urchin eggs and from various stages in the early development of sea urchin embryos.[19]

Some of the peaks they observed in the MAK column chromatographs of the soluble fractions (cytoplasm) from an early stage in embryogenesis, later appeared in particulate (ribosomal and mitochondrial) fractions as the embryos developed. They refer to this apparent migration as "changes in the distribution of the multiple forms of lysyl-tRNA among different cellular components."[19]

Malignant cells have a different proportion of tRNA species than their non-malignant counterparts. The specific activity of methylases which were isolated from a variety of tumors by Tsutsui et al was found to be much greater than the specific activity of methylases from normal cells.[20] The elution patterns from MAK columns of phenylalanyl-, seryl-, and tyrosyl-tRNA derived from Ehrlich ascites tumor cells showed major differences when compared with normal mouse tissue.[21]

The tRNA content varies between cells involved in different specific antibody responses. Two different mouse plasma cell tumors were compared by Yang and Novelli. One was a producer of immunoglobulin-A and the other a producer of immunoglobulin-G. Marked differences were found in their seryl-tRNAs.[22] This may be related to the fact that serine is one of the few amino acids which differs in amount in the active fragment of different antibody immunoglobulins.[23]

All of these differences between tRNAs derived from different sources suggest, but do not prove, that tRNA is involved in the regulation of cellular activities and possibly differentiation. It is clear that tRNA patterns are very sensitive to any major functional and/or metabolic change in a cell. Varying degrees of modification of tRNA may play a part in changing these isoaccepting tRNA chromatographic patterns. However, there are other possible ways to account for these changes. They may represent changes in the rate of transcription of DNA which codes for tRNA precursors or changes in processing of different isoacceptor precursors, or changes in the rate of degradation of different isoacceptors.

The mitochondria contain a population of tRNA molecules which is different from the cytoplasmic population. MAK column chromatography reveals the presence of tRNA species in the mitochondria which are chromatographically similar to some tRNA species in the cytoplasm and other tRNA species which apparently have no cytoplasmic counterparts. These "exclusively mitochondrial" tRNA species apparently cannot be aminoacylated by cytoplasmic synthetases. They can be aminoacylated only by synthetases found in the mitochondria.[24]

Whether the mitochondrial genes code for their own synthetases is still an open question. It has been demonstrated that a nuclear gene is involved in the synthesis of the mitochondrial leucyl-tRNA synthetase in Neurospora. In a strain of Neurospora with a nuclear gene mutation affecting the cytoplasmic leucyl-tRNA synthetase, the mitochondria possess very little leucyl-tRNA synthetase activity.[25]

The mitochondria appear to have an independent protein synthesizing system which is to some extent genetically autonomous from the protein synthesizing system in the cytoplasm. A discussion of the informational content of mitochondrial DNA has been given by Nass.[26] Since the mitochondrial genome is probably too small to code for all the aminoacyl-tRNA synthetases, if indeed it codes for any, the autonomy of the mitochondrial protein synthesizing system may be incomplete.

Materials and Methods

Cells and Medium:

KB cells, a human epithelial transformed line carried continuously in the laboratory, were routinely grown in monolayer cultures in 75 cm^2 plastic Falcon flasks in Eagle's minimal essential medium with 5% horse serum and 100 units of penicillin and 100 mcg streptomycin per ml. The medium was changed daily and the cells grew with a generation time of approximately 24 hours. Cells were grown in suspension in Eagle's minimal essential medium with 5% horse serum and maintained at about 2-5 X 10^5 cells per ml. Medium and special phosphate-free medium as well as serum were purchased from Grand Island Biological Company.

Radioactive Materials:

Carrier-free ^{32}P-labeled phosphoric acid was purchased from New England Nuclear Corporation, as were ^{14}C- and ^{3}H-labeled amino acids. Toluene-based counting solution was prepared by dissolving four grams of Omnifluor (New England Nuclear) in one liter of toluene. 10 ml of this solution was poured into each of the counting vials. Bray's counting solution was made as described.[27] Radioactive samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer model 3320.

Chromatographic Materials:

Diethylaminoethyl cellulose (Whatman DE23), purchased from Reeve-Angel, was washed according to manufacturer's instructions.

Thin layer plates were prepared from Sigmacell Type 19, microcrystalline cellulose (Sigma Chemical Company). The cellulose was washed three or four times in distilled, deionized water until the supernatant was clear and free of yellow color. Thin layer plates (250 micrometers thick) were prepared using a Brinkmann thin layer spreader.

The 3' monophosphoric acids of each of the four major bases were purchased from Sigma Chemical Company as was pseudouridine 3' monophosphate. All other chemicals were reagent grade.

Labeling of Cells:

Cells were radioactively labeled with phosphorus-32 by suspending them in phosphate-free medium (2-5 X 10^5 cells per ml) for approximately one hour and then adding 10 microcuries per ml of ^{32}P-labeled carrier-free phosphoric acid. After incubation for approximately 24 hours the cells were recovered by centrifugation at 3,000 x g for ten minutes.

Isolation of Mitochondria:

Mitochondria were prepared according to the procedure of Vesco and Penman.[28] Cells (approximately 2.5 X 10^8) were washed with 10 ml of phosphate buffered saline and suspended in 10 ml "RSB" buffer (0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris pH 7.5, 0.01 M beta-mercaptoethanol). The cell suspension was homogenized with approximately twenty strokes of a

Dounce homogenizer.

The homogenized cell suspension was centrifuged at 1000 x g for 10 minutes to remove the nuclear pellet. The supernatant was centrifuged again at the same speed. The supernatant from this second centrifugation was centrifuged at 10,000 x g to obtain a mitochondrial pellet. The supernatant from this centrifugation, called "cytoplasmic supernatant," was saved for the isolation of cytoplasmic RNA. The mitochondrial pellet was washed three times by resuspension in about 2 ml sucrose buffer (0.25 M sucrose, 0.001 M EDTA, 0.01 M Tris pH 7.5, 0.01 M beta-mercaptoethanol) and centrifugation at 10,000 x g. The final thrice-washed mitochondrial pellet was then usually suspended in 1 ml RSB buffer and frozen. This pellet was used as the source of mitochondrial RNA.

Isolation of Mitochondrial RNA

The mitochondrial pellet in RSB buffer obtained above was diluted to approximately 4 ml by addition of 2 ml 0.1 M Tris pH 7.5. Approximately one O.D. unit of rat liver RNA was added as a carrier and 300 microliters of 10% sodium dodecyl sulfate (SDS) was added to a final concentration of 1% SDS. Mitochondria were mixed thoroughly with the SDS and then extracted with an equal volume of water-saturated phenol, centrifuged, and the aqueous layer withdrawn. The phenol layer was then re-extracted with one ml of 0.1 M Tris pH 7.5.

The combined aqueous layers containing the mitochondrial RNA were loaded on a DEAE-cellulose column (0.5 X 1.5 cm). The column was washed and the RNA isolated as for the cytoplasmic sample described below. Typically about 5 X 10^5 counts per minute of ^{32}P-labeled low molecular weight RNA was obtained from the mitochondria of a 500 ml cell suspension culture (approximately 2.5 X 10^8 cells).

Isolation of Cytoplasmic RNA:

The "cytoplasmic supernatant" resulting from the centrifugation in which the initial mitochondrial pellet was obtained was extracted with an equal volume of water-saturated phenol. The aqueous layer was loaded on a DEAE-cellulose column (0.5 X 1.5 cm) previously equilibrated with low salt buffer (0.1 N NaCl in 0.1 M Tris pH 7.5). After the sample was loaded on the column, the column was washed to remove non-RNA ^{32}P with five to ten column volumes of low salt buffer until the radioactive phosphorus-32 level was less than 100 counts per minute in a 50 microliter sample in Bray's counting solution.

The low molecular weight RNA was then eluted with high salt buffer (1.0 N NaCl in 0.1 M Tris pH 7.5) and 2 ml fractions were collected. Ten microliters of each fraction was spotted on Whatman 3MM filter paper disks and the disks were washed three times in 5% trichloroacetic acid (15 minutes per wash) at ice bath temperature to precipitate the RNA. The papers were then washed successively 15 minutes in ethyl alcohol-ether (1:1) and finally in ether. The filter papers were dried and placed in counting vials with 10 ml of toluene-based counting solution. The fractions containing the radioactive label were combined and precipitated with 2½ volumes of 95% ethyl alcohol. Preparation of rat liver RNA from a liver homogenized in Tris pH 7.5 buffer and extracted with phenol was similar to the preparation of cytoplasmic RNA described above.

Hydrolysis of RNA and Thin Layer Chromatography of Nucleotides:

Samples of both mitochondrial and cytoplasmic low molecular weight RNA were hydrolyzed in a total volume of 50 microliters containing 0.2 mM EDTA, 10 mM NaAc pH 4.5, and 0.05 units of T2 ribonuclease (Sigma). The samples were incubated at 37 degrees for approximately 18 hours. After hydrolysis, the samples were evaporated under a stream of air to a small volume (approximately 5 to 10 microliters) and spotted onto thin layer cellulose plates.

Thin layer plates were chromatographed in a two dimensional solvent system according to Nishimura.[12] The first solvent system was isobutyric acid: concentrated NH_3: H_2O, 50:1:29 (v/v/v). The second was isopropanol: concentrated HCl: H_2O, 70:15:15 (v/v/v).

After chromatography, the thin layer plates were dried and placed in casettes and exposed for three or four days with Kodak X-Omat x-ray film. The outlines of areas darkened on the film were traced onto the corresponding areas of the TLC plates. The radioactive spots were scraped off and placed into separate counting vials containing the toluene-based counting fluid.

The counts obtained were assigned to the nucleotides identified with the spots from which they originated.

Identification of Nucleotides:

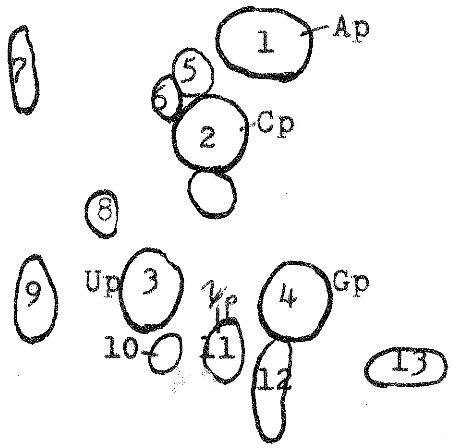
Initial identification of nucleotides was based on Nishimura. These identifications were confirmed for the four major bases and for pseudouridine by chromatography against authentic samples.

Results

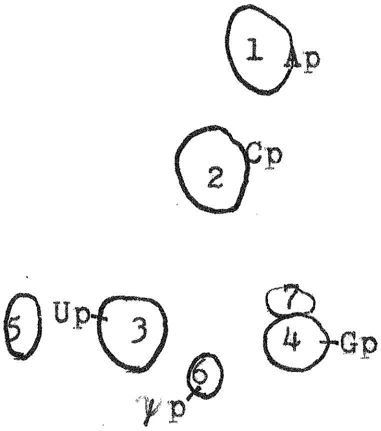
Visual inspection of the autoradiographic patterns produced by the thin layer chromatography of cytoplasmic and mitochondrial low molecular weight RNA reveals that in addition to the four major bases, both samples contain a number of modified nucleotides (see Fig. 4). The number of nucleotides visualized in this way varied from one experiment to another. The variation was slight in the case of the cytoplasmic samples and considerable in the case of the mitochondrial samples. As many as ten or as few as six different nucleotides could be visualized in the mitochondrial samples. The cytoplasmic samples usually exhibited 13 or 14 different nucleotides.

The cytoplasmic samples showed a greater variety of nucleotides than the mitochondrial samples even in samples where the total ^{32}P radioactivity used in the thin layer chromatography was less for the cytoplasmic than for the mitochondrial low molecular weight RNA. This observation suggests that there may be a greater variety of modified nucleotides in cytoplasmic tRNA than is present in mitochondrial tRNA.

As noted in the materials and methods section, the only modified nucleotide positively identified is pseudouridylic acid. This nucleotide was found in all samples of both mitochondrial and cytoplasmic LMW RNAs. Nishimura has published a thin layer chromatographic pattern obtained from hydrolyzed E. coli tRNA with identification of fifteen mod-



Cytoplasmic RNA



Mitochondrial RNA

Figure 4
Typical autoradiographic pattern obtained from thin layer chromatographic plates of hydrolyzed cytoplasmic and mitochondrial low molecular weight RNA.

ified nucleotides. By comparing this E. coli chromatographic profile with those obtained for cytoplasmic and mitochondrial low molecular weight RNA it is possible to tentatively identify some of the other modified nucleotides. However, Nishimura's patterns for E. coli RNA too different from those obtained for KB cell RNA to permit any strong conclusions from such a comparison.

The data from all of the experiments conducted are shown in Tables 1 and 2. These data were obtained by counting the ^{32}P radioactivity contained in the spots scraped from the TLC plates as described under Materials and Methods. Some experiments were eliminated because of poor resolution of the thin layer chromatograph. Samples were counted for two, five or ten minutes and the total counts obtained were divided by the counting time to obtain counts per minute. The counts from all nucleotides other than the four major nucleotides and pseudouridylic acid were summed and included in the sixth column of Figures 1 and 2. These Figures show the results for 13 experiments for the cytoplasmic samples and 8 experiments for the mitochondrial samples. The total shown in the last column includes the counts from all nucleotides, both modified and unmodified.

Because the total ^{32}P radioactivity counted varied from one experiment to the next it is not possible to compare these results directly. For this reason the results tabulated in Tables 1 and 2 are expressed in Tables 3 and 4 as decimal fractions normalized to Up. This permits a direct

Table 1

Nucleotide Content of Cytoplasmic RNA -- counts per minute in a given nucleotide*

experiment number	Ap	Cp	Up	Gp	Ψp	others	total
1.	2,180	4,140	2,480	3,820	274	878	13,800
2.	1,730	3,280	2,100	4,040	226	854	12,200
3.	2,200	4,230	4,490	3,890	382	473	15,700
4.	3,960	7,300	3,430	8,050	804	2,060	25,600
5.	14,800	27,500	17,400	22,500	2,750	12,300	97,300
6.	3,250	5,340	2,810	6,000	486	1,550	19,400
7.	3,700	6,550	3,390	7,500	643	2,060	23,800
8.	1,940	3,670	1,900	4,150	330	714	12,700
9.	845	1,650	745	1,540	162	1,190	6,100
10.	2,960	5,160	3,030	5,900	570	1,560	19,200
11.	1,370	1,940	998	1,800	89	794	6,990
12.	211	410	184	388	34	119	1,350
13.	1,270	2,230	1,190	2,200	219	149	7,260

* The figures given are the number of counts per minute obtained when the spots corresponding to particular nucleotides are counted in the scintillation counter. Data are rounded off to three significant figures.

Table 2

Nucleotide Content of Mitochondrial RNA -- counts per minute in a given nucleotide

experiment number	Ap	Cp	Up	Gp	ψp	others	total
1.	994	1,080	982	602	52	137	3,850
2.	988	880	1,000	790	108	276	4,040
3.	670	645	565	540	47	76	2,540
4.	2,500	2,120	2,190	1,780	174	225	8,990
5.	2,080	1,690	1,430	1,320	139	227	6,890
6.	1,025	1,440	1,090	920	67	86	4,120
7.	2,500	2,530	2,490	2,370	212	289	10,400
8.	1,690	1,640	1,500	1,770	166	357	7,100

comparison of the results.

The first three columns in both Tables 3 and 4 express the three major nucleotides Ap, Cp and Gp as ratios to Up. The fourth column expresses the content of pseudouridylic acid as a ratio to Up. The fifth column gives the content of all modified nucleotides including pseudouridylic acid as a ratio to the total counts from all nucleotides both modified and unmodified. The last column is the uridylic acid content. The figures for modified nucleotide content were obtained by dividing the cpm for all modified nucleotides by the total cpm for all nucleotides in each experiment.

The averages of the results from all experiments are shown in the bottom line of Tables 3 and 4. The average modified nucleotide content of the cytoplasmic LMW RNA is 0.109 or approximately 11%. The corresponding figure for the mitochondrial LMW RNA is 0.056 or approximately 6%. The meaning of these figures will be considered in the following section.

Table 3

Nucleotide Content of Cytoplasmic RNA expressed as ratios.

	Ap/Up	Cp/Up	Gp/Up	ψp/Up	modified* / total	Up / total
1.	0.879	1.67	1.54	0.110	0.0833	0.180
2.	0.824	1.56	1.92	0.108	0.0885	0.172
3.	0.490	0.942	0.866	0.0851	0.0545	0.286
4.	1.15	2.13	2.35	0.234	0.112	0.134
5.	0.851	1.58	1.29	0.158	0.155	0.179
6.	1.16	1.90	2.14	0.173	0.105	0.145
7.	1.09	1.93	2.21	0.190	0.113	0.142
8.	1.02	1.93	2.18	0.174	0.0819	0.149
9.	1.15	2.21	2.07	0.217	0.221	0.122
10.	0.977	1.70	1.95	0.188	0.111	0.158
11.	1.37	1.94	1.80	0.0892	0.126	0.143
12.	1.15	2.23	2.11	0.184	0.113	0.136
13.	1.07	1.87	1.85	0.184	0.0507	0.184
av.	1.01	1.81	1.87	0.161	0.109	0.162

* all modified nucleotides including ψp.

Table 4

<u>Nucleotide Content of Mitochondrial RNA expressed as ratios.</u>

	Ap/Up	Cp/Up	Gp/Up	Ψp/Up	$\frac{\text{modified}}{\text{total}}$	$\frac{\text{Up}}{\text{total}}$
1.	1.01	1.10	0.613	0.0530	0.0490	0.255
2.	0.988	0.880	0.790	0.108	0.0950	0.248
3.	1.19	1.14	0.956	0.0832	0.0484	0.222
4.	1.14	0.968	0.813	0.0795	0.0444	0.243
5.	1.45	1.18	0.923	0.0972	0.0531	0.207
6.	0.940	1.32	0.844	0.0615	0.0371	0.265
7.	1.00	1.02	0.952	0.0851	0.0482	0.239
8.	1.13	1.09	1.18	0.111	0.0737	0.211
av.	1.11	1.09	0.884	0.0848	0.056	0.236

Discussion

It has been demonstrated that the modified nucleotide content of a low molecular weight RNA sample from mitochondria of KB cells differs only slightly from the modified nucleotide content of a low molecular wight RNA sample from the cytoplasm of the same cells. These LMW RNA samples may be contaminated with some RNA other than transfer RNA, such as 5S RNA and degraded messenger and ribosomal RNA. Such contamination would reduce the modified nucleotide content of the samples. More than half of the cytoplasmic sample must be tRNA because the 11% figure for the modified nucleotide content is within the 10 to 20% range known to be characteristic of tRNA based on primary sequences. The mitochondrial sample may be contaminated to a greater or lesser extent than the cytoplasmic sample. This possibility must be considered in comparing the modified nucleotide content of the two samples. The data may suggest that the modified nucleotide content of the mitochondrial tRNA is slightly lower than that of the cytoplasmic tRNA but this remains unproved because of the possibility that the apparent differences are due to differences in the level of contamination of the two samples.

The data for mitochondrial and cytoplasmic LMW RNA is summarized in Table 5. This table also gives data from Attardi[29] for comparison. Attardi's data for HeLa cell RNA is the only data in the literature for nucleotide content of mitochondrial LMW RNA. Attardi's data for Gp+Cp content

Table 5

Summary of Data

	Gp+Cp content	ψp content	modified content
Mitochondrial	47%	2.3%	6%
Cytoplasmic	60%	2.6%	11%

Data from Attardi[30]

	Gp+Cp content	methylated nucleotide content
Mitochondrial	43.6%	4.6%
Cytoplasmic	60.8%	10.1%

compare closely with the data obtained in this research. Attardi's "estimated number of methyl groups per 100 nucleotides" is comparable to the percent modified nucleotide content found in these experiments.

Low molecular weight RNA samples obtained from mitochondria of mammalian cells have been shown to contain a variety of modified nucleotides. The amount of these modified nucleotides present in the mitochondria is not greatly different from the amount found in cytoplasmic LMW RNA. The amount of pseudouridylic acid found in mitochondrial LMW RNA is almost identical to the amount found in cytoplasmic LMW RNA.

Inasmuch as the mitochondrial tRNA is sequestered inside an organelle, one might have expected that there would be little opportunity for enzymatic modification, and that the mitochondrial tRNA would therefore be greatly undermodified. Our data indicate that such is not the case. The mitochondrial tRNA may be modified to a slightly lesser extent than the cytoplasmic both in terms of the variety of molecular modification and in the total percentage of nucleosides modified, but it is not likely that the percentage of nucleosides modified could be many times less than in the cytoplasm, since the Gp Cp content of the mitochondrial LMW RNA suggests that contamination with cytoplasmic tRNA is minimal.

It must be remembered that the mitochondrial samples contain all isoaccepting species of tRNA found in the mitochondria, at least some of which are transcribed from mitochondrial genes. It is obvious that no conclusions can be drawn from this data with respect to the percentage of nucleosides modified in any single isoaccepting species of mitochondrial tRNA.

The highly modified RNA molecules present in the mitochondria enter the mitochondria from the cytoplasm, or become enzymatically modified within the mitochondria. Fully modified cytoplasmic tRNA species may enter the mitochondria and become part of the mitochondrial tRNA population. The tRNA isoaccepting species transcribed from mitochondrial genes may be (1) modified within the mitochondrion or (2) sent out into the cytoplasm for modification and then returned or (3) sent out into the cytoplasm and not returned, or (4) remain permanently unmodified. The true situation may be some combination of these possibilities.

If the tRNA molecules are modified within the mitochondrion, then the modifying enzymes must be present inside the organelle. The question of the presence of modifying enzymes within the mitochondrion has yet to be answered. If modifying enzymes are found within the mitochondrion, the question will present itself as to whether these enzymes are coded by nuclear or mitochondrial genes. Nass has shown that the mitochondrial DNA has about 15,000 base pairs, which is sufficient to code for approximately 30 polypeptide chains of molecular weight 20,000.[26] No mitochondrial gene products other than tRNA and rRNA have been positively identified, however Nass suggests that the mitochondrial DNA may code for some components of the inner mitochondrial membrane, some cytochromes, and some RNAs of mitochondrial ribosomes.[26] In view of the limited coding capacity of mitochondrial DNA, it is probable that not all the enzymes which modify nucleosides within mitochondrial tRNA precursors are mitochondrial coded.

The degree of modification of mitochondrial tRNAs will have implication for their secondary and tertiary structure as well as for the question of how they bind to mitochondrial ribosomes. The secondary structure illustrated in Figure 3 is based on primary sequences of specific tRNA species. As no mitochondrial tRNA has yet been sequenced it is not known whether mitochondrial tRNA species will conform to a similar pattern.

The mitochondria possess their own protein synthesizing system, part of which is coded by mitochondrial genes, and part presumably enters the mitochondria from the cytoplasm. If modification of tRNA is necessary for binding to ribosomes, as the work of Gefter[14] suggests, then the modifying enzymes may also be considered part of a complete protein synthesizing system. To understand the dependence of the mitochondrial protein synthesizing system on the nuclear genome it will be necessary to know how mitochondrial tRNAs come to be modified.

As a first step toward understanding of the role of modification of tRNA in mitochondrial protein synthesis, this research has shown that mitochondrial tRNA is indeed modified. Further experiments must be done to determine whether modifying enzymes are present within the mitochondrion and whether these enzymes act to modify mitochondrial coded RNA molecules. The broader question of the role of mitochondrial isoaccepting tRNA species may be explored if a way can be found to selectively inactivate certain mitochondrial isoacceptors. Future experiments such as these should help to establish the function of the modified nucleotides which this research has demonstrated to exist within mitochondrial tRNA.

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