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Atypical Pattern of Utilization of Amino Acids for Mitochondrial Protein Synthesis in HeLa Cells*

(chloramphenicol/endoplasmic reticulum/amino-acid pools/mitochondrial tRNA)

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ABSTRACT **The capacity of HeLa cell mitochondria, either isolated or in intact cells, to incorporate different labeled amino acids into proteins was investigated. Eight amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, and lysine), which include most of the charged polar ones, showed a very low amount, if any at all, of chloramphenicol-sensitive incorporation, relative to that expected for an "average" HeLa-cell protein. By contrast, the most hydrophobic amino acids (leucine, isoleucine, valine, phenylalanine, and methionine) were the most actively incorporated by HeLa mitochondria. The available evidence suggests that pool effects cannot account for this general pattern of utilization of amino acids; furthermore, this pattern is in good agreement with the known hydrophobic properties of proteins synthesized in mitochondria.**

There is abundant evidence that mitochondria from different organisms possess a specific protein-synthesizing system utilizing distinctive ribosomes and tRNA species (for reviews, see refs. 2 and 3). At least some of these tRNA species are coded for by mitochondrial DNA (mit-DNA) (for a review, see ref. 4). In rat-liver mit-DNA, the presence of genes for four tRNA species ($tRNA^{Leu}$, $tRNA^{Phe}$, $tRNA^{Tyr}$, and $tRNA^{Ser}$) has been shown by RNA–DNA hybridization with amino acid–tRNA complexes labeled in the amino-acid moiety (5). RNA–DNA hybridization experiments have indicated the presence of 12 sites for 4S RNA in HeLa mit-DNA (6, 7) and 15 sites in *Xenopus laevis* oocyte mit-DNA (8). These observations have raised the question of whether animal-cell mitochondria utilize for protein synthesis an incomplete set of endogenous tRNA species, or whether the missing tRNA species are imported from the cytoplasm. In the present work, the capacity of HeLa mitochondria to incorporate different amino acids into proteins is investigated.

We found that the various labeled amino acids are utilized by HeLa mitochondria in proportion strikingly different from that observed for protein synthesis by endoplasmic reticulum (ER) polysomes or expected for incorporation into an "average" HeLa-cell protein, with very low , if any at all, incorporation of the charged polar amino acids and a predominant utilization of the most hydrophobic ones.

MATERIALS AND METHODS

HeLa Cells Were Grown in suspension culture (9).

Preparation of Subcellular Fractions. For analysis of mitochondrial protein synthesis *in vitro* or *in vivo*, unless otherwise specified, a 5000 × *g* crude mitochondrial fraction, enriched in mitochondria relative to other membrane components (10), was used. For analysis of protein synthesis on rough ER polysomes, a 8100 × *g* fraction, containing the bulk of mitochondria and rough ER elements (11), was used.

In Vitro Incubation and Analysis of In Vitro Products. Conditions of incubation of the various subcellular fractions for protein synthesis, and methods of measurement of radioactivity and protein have been described (10). The radioactive amino acid tested was added at 5 μCi/ml. Each incubation mixture for mitochondrial protein synthesis contained 0.1 mM of each amino acid except the radioactive one.

Three incubation mixtures were set up for each amino acid: for the zero time point, for 60-min incubation, and for 60-min incubation in the presence of 100 μg/ml of chloramphenicol (CAM) [to inhibit mitochondrial protein synthesis (10, 12, 13)] or 300 μg/ml of cycloheximide (CH) [to inhibit cytoplasmic protein synthesis (10, 13, 14)], depending on whether mitochondrial or cytoplasmic protein synthesis was analyzed. As a standard, the CAM-sensitive or CH-sensitive incorporation of [^{3}H]leucine was routinely tested in each experiment with a given subcellular fraction. Incorporation of [^{3}H]cysteine was tested by use of [^{3}H]cystine in the presence of a reducing agent (2 mM mercaptoethanol or 1 mM dithiothreitol), and compared to that of [^{3}H]leucine under the same conditions.

In Vivo Labeling and Analysis of In Vivo Products. HeLa cells were labeled for 1 hr with the tritiated amino acid at 0.5 μCi/ml in modified Eagle's medium (15) lacking the amino acid being tested, with 5% dialyzed calf serum, in the presence of 200 μg/ml of emetine [another inhibitor of cytoplasmic protein synthesis (16–18)], or emetine and 200 μg/ml of CAM, or CAM alone (added 5 min before the label). A [^{3}H]leucine incorporation control was done routinely.

The 5000 × *g* or 8100 × *g* membrane fraction was prepared as described above, dissolved in 1 N NaOH, heated for 1 hr at 37°, neutralized with 1 N HCl, brought to 0.5% sodium dodecyl sulfate, and analyzed for protein content and acid-precipitable radioactivity.

RESULTS

In vitro experiments

The 8100 × *g* and the 5000 × *g* crude mitochondrial fractions from HeLa cells support, under appropriate conditions, protein synthesis characterized as mitochondrial on the basis of its sensitivity to CAM and resistance to CH and RNase (10). The proteins thus synthesized exhibit the same profile on

Abbreviations: mit-DNA, mitochondrial DNA; ER, endoplasmic reticulum; CAM, chloramphenicol; CH, cycloheximide.

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polyacrylamide gel electrophoresis as the products of mitochondrial protein synthesis *in vivo* (19).

Fig. 1*a* shows an experiment testing the effects of increasing concentrations of CH on mitochondrial protein synthesis supported by the 5000 × *g* fraction. Up to 300 μg/ml of CH, there is a constant low sensitivity of amino-acid incorporation to the drug (about 12%), presumably due to protein synthesis on contaminating rough ER (11). At higher concentrations of CH, there is a progressive depression of amino-acid incorporation, which suggests inhibition of incorporation by mitochondria; the mechanism of this effect, however, was not further analyzed.

Fig. 1*b* shows the sensitivity to increasing concentrations of CAM of mitochondrial protein synthesis supported by the 8100 × *g* fraction, which was used here to better detect any effect of the drug on contaminating rough ER. Inhibition of protein synthesis by the drug increases rapidly up to about 100 μg/ml of CAM, and continues to increase more slowly at higher drug concentrations, even beyond the 86% level expected to represent mitochondrial protein synthesis, on the basis of the effects of 150 μg/ml of CH. These results suggest a certain sensitivity to CAM of protein synthesis on the rough ER contaminant; this sensitivity may be due to the particular incubation conditions used for mitochondrial protein synthesis, since CAM has very little, if any, effect on cytoplasmic protein synthesis supported either by the same membrane fraction or by free polysomes (Fig. 1*c*).

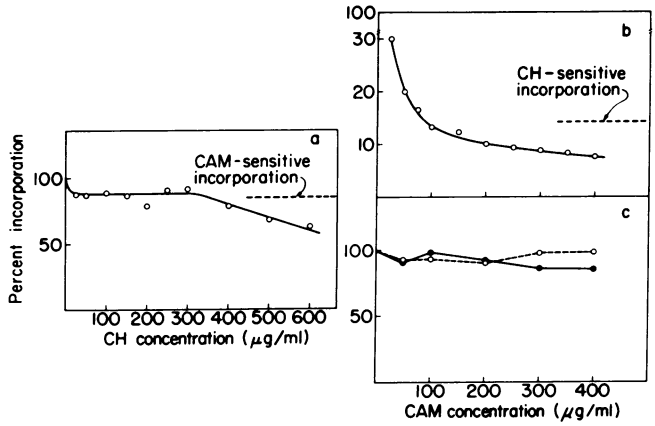

FIG. 1. Sensitivity to different concentrations of CAM or CH of protein synthesis supported by various subcellular fractions. (*a*) 350 μg of protein of a 5000 × *g* crude mitochondrial fraction were incubated with increasing concentrations of CH or 100 μg/ml of CAM, under conditions for mitochondrial protein synthesis (10). 100% corresponds to 21,030 cpm/mg of protein. (*b*) 430 μg of protein of a 8100 × *g* crude mitochondrial fraction were incubated with increasing concentrations of CAM or 150 μg/ml of CH, under conditions for mitochondrial protein synthesis (10). 100% corresponds to 25,540 cpm/mg of protein. (*c*) 380 μg of a 8100 × *g* crude mitochondrial fraction and 0.1 ml (200 μg of protein) of the 229,000 × *g* supernatant (for rough ER protein synthesis, ●——●), or 0.2 ml (200 μg of protein) of the 13,300 × *g* supernatant (for free polysome protein synthesis, ○- - -○), were incubated with increasing concentrations of CAM, under conditions for cytoplasmic protein synthesis (10). 100% corresponds to 17,200 and 15,970 cpm/mg of protein for rough ER and free polysome protein synthesis, respectively.

TABLE 1. *Utilization of different labeled amino acids for protein synthesis in HeLa cell mitochondria and rough endoplasmic reticulum*

Amino acid*	Relative incorporation: Mitochondria, In vitro	Mitochondria, In vivo	Rough ER	Expected for an "average" HeLa cell protein†	Intracellular concentration of free amino acids in HeLa cells† (mM)
[^{3}H]Leu (50.5)	100	100	100	100	0.73
[^{3}H]Ala (51)	8.1 (1.1-11.9)		110	95.5	1.43
[^{3}H]Arg (11)	4.8 (0-14.0)	1.9	240	58.4	0.03
[^{3}H]Asp (26)	2.5 (0.8-4.1)		125	108 {96.4§	1.27
[^{3}H]AspNH_2 (18)	12.7 (11.3-15.0)		420	108 {11.4§	0.15
[^{3}H]CySH (3.9)	0¶	0¶	33.6	13.4	<0.05
[^{3}H]Gly (11.1)	2.6 (2.4-2.7)		1.7	89.9	0.79‖
[^{3}H]Glu (15)	3.6 (3.4-3.9)		4.9	129 {73.8§	10.8
[^{14}C]GluNH_2 (0.218)	3.2 (2.6-3.8)		52.2	129 {55.4§	8.1
[^{3}H]His (35)	9.7 (8.1-11.2)	8.5 (6.2-9.5)	73.2	23.6	0.26
[^{3}H]Ile (39)	177 (169-185)	290	84.2	59.6	1.00
[^{3}H]Lys (41.6)	4.5 (0.2-6.9)	1.3	143	89.9	0.29
[^{3}H]Met (0.67)	22.6 (12.0-35.8)		29.9	22.5	0.19
[^{3}H]Phe (50.5)	19.4 (18.8-20.0)		54.8	41.6	0.52
[^{3}H]Pro (45.7)	14.3 (3.2-30.9)		260	69.7	0.80
[^{3}H]Ser (2.23)	12.6 (10.3-14.8)		120	56.2	0.03
[^{3}H]Thr (1.82)	19.7 (12.8-26.6)		43.1	55.1	0.96
[^{3}H]Try (4.05)	12.4 (4.6-20.2)	90.7 (73.3-108)	68.8		<0.1
[^{3}H]Tyr (50.4)	7.0 (2.4-9.2)	8.9 (5.6-12.2)	70.6	30.3	0.81
[^{3}H]Val (17.2)	53.0 (39.9-77.5)		72.4	80.9	0.79

Mitochondria. In vitro: The specific activity (cpm/mg of protein) corresponding to the CAM-sensitive incorporation for each amino acid has been normalized with respect to that obtained for the CAM-sensitive [^{3}H]leucine incorporation in the same experiment. Averages and ranges of 2–4 experiments are given. *In vivo:* The emetine-resistant and CAM-sensitive incorporation values pertaining to 1–3 experiments for each amino acid are presented as described above.

Rough ER. The CH-sensitive incorporation values for different amino acids are presented as described above.

* In parentheses, specific activity in Ci/mmol.

† It is assumed that the relative rates of incorporation for the various amino acids are proportional to their mole percent in an "average" HeLa-cell protein [from published data (15)]. No data for tryptophan content of HeLa-cell proteins are available.

‡ See ref. 20.

§ Tentatively estimated from the relative sizes of the corresponding free amino-acid pools (20).

¶ Incorporation of [^{3}H]CySH in the control was lower than that obtained in the presence of CAM.

‖ The pool of glutathione (20), which turns over fairly rapidly (21), probably enlarges the effective pool of glycine.

In Table 1, CAM-sensitive incorporation for each tritiated amino acid is expressed as percent of the CAM-sensitive incorporation of the [^{3}H]leucine in the same preparation. To better evaluate the significance of the relative values thus obtained for the various amino acids against the pattern of amino-acid utilization for overall HeLa-cell protein synthesis, we compared the above values to those expected for an "average" HeLa-cell protein on the basis of its amino-acid composition (assuming a proportionality between amino-acid content and relative rate of incorporation, independent of any

In the following experiments, the 5000 × *g* fraction was used to investigate the incorporation *in vitro* into protein of the 20 most common amino acids. As a criterion for mitochondrial protein synthesis, sensitivity to CAM at 100 μg/ml was used (Fig. 1*b*).

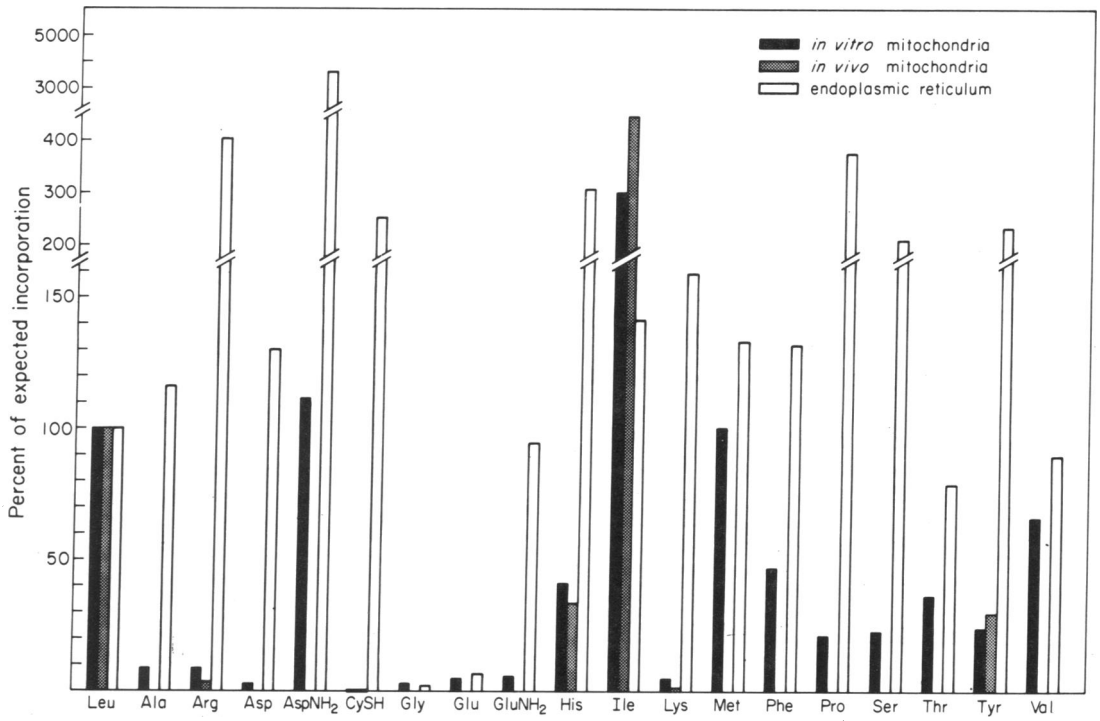

FIG. 2. Percent incorporation of various amino acids by mitochondria or ER relative to that expected for the synthesis of an "average" HeLa-cell protein. The data are derived from Table 1.

pool effects). Some labeled amino acids (alanine, arginine, aspartic acid, glutamic acid, glutamine, cysteine, glycine, and lysine) are incorporated at less than 10% of the amount expected for an "average" HeLa-cell protein (Fig. 2). Incorporation of other amino acids (asparagine, histidine, proline, serine, and tyrosine), though fairly low compared to that of leucine (7–14%) (Table 1), appears to be substantial relative to that expected for an "average" HeLa-cell protein (from 20 to more than 100%). Finally, other amino acids are incorporated to a considerable absolute extent, although less than leucine (valine, phenylalanine, methionine, and threonine), or even more than leucine (isoleucine).

CAM sensitivity ranged between 57 and 87% for the various amino acids actively incorporated by the mitochondria (71–87% for leucine), between 26 and 65% for the amino acids with moderate incorporation, and between 0 and 44% for the amino acids with very low incorporation [the higher values observed for glycine (59%) and glutamic acid (56%) are probably related to the extremely low apparent incorporation by the ER observed for these amino acids (see below)].

Due to differences in specific activity, each amino acid had been tested at the same level of radioactivity, but not at the same molar concentration. The effects of amino-acid concentration in the incubation mixture on the measured incorporation were investigated for two amino acids with high specific activity, [^{3}H]leucine and [^{3}H]phenylalanine. For both amino acids, the amount of label incorporated, for a constant amount of radioactivity added, remained unchanged over a wide range of specific activities, while it decreased progressively beyond a molar concentration of 1 μM (Fig. 3). Since addition of a mixture of unlabeled amino acids causes only a moderate stimulation, if any at all, of mitochondrial protein synthesis *in vitro* (22–24), a likely explanation of the above results is that, with an increasing concentration of the amino acid, there is a proportional increase in its uptake. This increase would substantially compensate for the decrease in its specific activity, until the amount of amino acid taken in becomes no longer negligible relative to the intramitochondrial pool.

Assuming that one can extrapolate to all amino acids the behavior shown by [^{3}H]leucine and [^{3}H]phenylalanine, since the specific activity of most of the amino acids used in this work fell in the plateau region of Fig. 2, no correction was made for the data of Table 1. However, the incorporation reported for [^{3}H]methionine, [^{3}H]threonine, [^{3}H]tryptophan, [^{3}H]serine, and [^{3}H]cysteine may have been in this way underestimated. [^{14}C]Glutamine incorporation was compared to that of [^{14}C]leucine at the same specific activity.

The time course of *in vitro* incorporation by HeLa mitochondria was analyzed for [^{3}H]leucine, [^{3}H]proline, and [^{3}H]glycine, three amino acids differing widely in the extent of their utilization. An almost linear net incorporation for 60 min followed by a plateau was observed for [^{3}H]leucine and [^{3}H]proline, whereas [^{3}H]glycine incorporation apparently ceased after 30 min. This result suggests that the relative incorporation at 60 min of these amino acids, and presumably of all (Table 1), was not influenced substantially by the kinetics of the process.

In order to compare the relative incorporation into protein of the various amino acids by HeLa-cell mitochondria with their utilization by the rough ER polysomes, the 8100 $\times$ *g* crude mitochondrial fraction was incubated with each labeled amino acid in the presence of the soluble fraction, under conditions allowing cytoplasmic protein synthesis (10). The sensitivity to CH of amino-acid incorporation, under these conditions, was 83–86% for [^{3}H]leucine and ranged between 70 and 93% for the other amino acids. Amino-acid incorporation by rough ER polysomes follows a completely

different pattern from that observed in the proteins synthesized by mitochondria (Table 1 and Fig. 2). Excluding [^{3}H]glutamic acid and [^{3}H]glycine, whose incorporation is presumably affected by the large soluble pools (Table 1), the amino acids that are utilized by mitochondria to a very low extent are incorporated by rough ER polysomes in substantial amounts. Incorporation of the different amino acids by the ER polysomes is more similar to that expected for an "average" HeLa-cell protein than that found for mitochondria.

In vivo experiments

For a few amino acids that are not synthesized by HeLa cells it was possible to perform labeling experiments *in vivo*. Mostly, amino acids that showed very low incorporation *in vitro* were tested, using emetine to block cytoplasmic protein synthesis (16–18). Under these conditions, incorporation of [^{3}H]leucine into mitochondrial protein was 90–95% sensitive to chloramphenicol.

In general, the pattern *in vivo* of utilization of the amino acids tested reproduced fairly closely the pattern *in vitro* (Table 1 and Fig. 2). Thus, [^{3}H]arginine, [^{3}H]lysine, and [^{3}H]cysteine showed a relative incorporation even lower than that observed *in vitro*. Confirmed also was the low incorporation of [^{3}H]histidine and [^{3}H]tyrosine, and the high incorporation of [^{3}H]isoleucine. [^{3}H]Tryptophan showed a relative incorporation *in vivo* much higher than *in vitro*, presumably due to the small size of the cytoplasmic tryptophan pool (20).

The extremely low incorporation observed *in vivo* with [^{3}H]arginine, [^{3}H]lysine, and [^{3}H]cysteine raised the possibility that these are not used at all for mitochondrial protein synthesis. The experiments reported in Table 2 suggest that this may be the case, at least for arginine and lysine. In fact, 1-hr or 2-hr incubation of the cells in medium lacking these two amino acids resulted in more than 30% and more than 60% decrease, respectively, in [^{3}H]leucine incorporation into proteins synthesized by cytoplasmic polysomes, with substantially no change or only a moderate decrease (about 27% after 2-hr starvation) in its incorporation by mitochondria. Since complete inhibition of cytoplasmic protein synthesis by a 2-hr pretreatment of the cells with emetine caused about 64% depression of mitochondrial protein synthesis (Table 2),

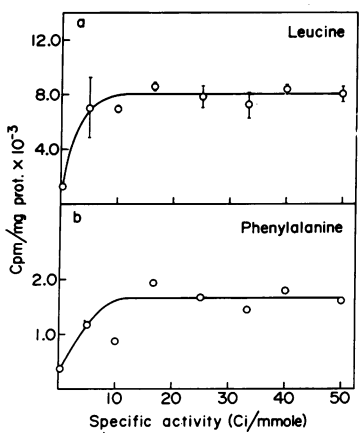

FIG. 3. Effect of amino-acid concentration on measured CAM-sensitive incorporation of [^{3}H]leucine or [^{3}H]phenylalanine. The 5000 × *g* mitochondrial fraction was incubated, under conditions for mitochondrial protein synthesis (10), in the presence of 5 μCi/ml of [^{3}H]leucine (*a*) or [^{3}H]phenylalanine (*b*) and decreasing amounts of the corresponding unlabeled amino acid.

TABLE 2. *Effect of arginine and lysine starvation on mitochondrial and cytoplasmic protein synthesis*

Experiment	Preincubation		Relative incorporation %		
	Medium	Length (hr)	Mitochondria	ER polysomes	Free polysomes
1	Control	1	100.0	100.0	100.0
	Arg, Lys-deficient	1	95.9	68.0	68.8
2	Control	2	100.0	100.0	100.0
	Arg, Lys-deficient	2	72.7	38.1	37.3
	Control + emetine	2	35.9	-	-

In Exp. 1, HeLa cells were preincubated for 1 hr in medium lacking leucine (control) or leucine, arginine, and lysine, then tested for mitochondrial protein synthesis in the presence of 200 μg/ml of emetine, or for cytoplasmic protein synthesis in the presence of 200 μg/ml of CAM. In Exp. 2, both control medium and arginine, lysine deficient medium contained 10 μM leucine, and a 2-hr preincubation was done. In addition, mitochondrial protein synthesis was also tested in a culture preincubated for 2 hr in control medium containing 200 μg/ml of emetine. The data reported pertain to the CAM-sensitive and CH-sensitive incorporation for mitochondria and cytoplasmic polysomes, respectively.

it seems reasonable to interpret the small effect on mitochondrial protein synthesis of 2-hr arginine and lysine starvation as due to the same phenomenon.

DISCUSSION

The work reported here indicates that, in HeLa cells, amino acids are utilized for mitochondrial protein synthesis in proportion strikingly different from that observed for protein synthesis by ER-bound polysomes or expected for incorporation into an "average" HeLa-cell protein.

Eight labeled amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, and lysine) were incorporated by mitochondria at less than 10% of the amount expected for an "average" HeLa-cell protein (15) or a "structural-protein" fraction from a crude mitochondrial preparation of these cells (Vaughan and Attardi, in preparation), assuming a proportionality between incorporation and amino-acid content, independent of any pool effects. For three of these amino acids (arginine, lysine, and cysteine) even lower incorporation was found by labeling experiments *in vivo*.

The possibility of pool effects in these incorporation experiments needs to be seriously considered. Of the eight amino acids mentioned above, five are either not synthesized by HeLa cells [arginine and lysine (25)] or are synthesized extramitochondrially [cysteine, glycine, and glutamine (26)]. A large intramitochondrial pool of any of them, if it reflects a high rate of utilization, could presumably be maintained only through a high rate of uptake; this should allow adequate labeling of the pools. This argument does not hold for the three of the eight amino acids that are synthesized intramitochondrially [glutamic acid, aspartic acid, and alanine (27)]. Furthermore, proteolytic enzymes (28) could conceivably produce relatively large pools of amino acids by degradation of cytoplasmically synthesized mitochondrial proteins. Unfortunately, there are no data on the free amino-acid pools of HeLa-cell mitochondria. However, the published values for the intramitochondrial free amino-acid pools in rat liver (22) indicate that, at least for four amino acids (argi-

nine, cysteine, lysine, and aspartic acid), the low incorporation observed cannot be accounted for by a large intramitochondrial pool. It should be noticed that the general pattern of utilization of amino acids by HeLa mitochondria agrees well with the known hydrophobic character of the proteins synthesized in these organelles (see, for a review, ref. 31), arguing against drastic pool effects.

On the other hand, the low relative CAM-sensitive incorporation observed *in vitro* with the amino acids mentioned above, with the exception of [^{3}H]cysteine, may not represent mitochondrial protein synthesis at all, but may be due to a partial inhibition by CAM of protein synthesis on contaminating ER-bound polysomes. Such a phenomenon may be related to the inhibitory effects that CAM, at relatively high concentrations (>50 μg/ml), has on respiration and ATP formation (29) and to the demonstrated capacity of ATP produced by mitochondria to support protein synthesis on contaminating ER elements (30). In agreement with this interpretation, a considerably lower CAM-sensitive incorporation was observed in the *in vivo* experiments. If the above interpretation were correct, the present data could mean that some at least of the amino acids exhibiting low incorporation are not utilized at all for protein synthesis by HeLa mitochondria. The possible lack of effect on mitochondrial protein synthesis of the starvation of HeLa cells for arginine and lysine could be explained, though not uniquely, by this interpretation. Conclusive evidence on the question of the the real utilization of these "minor" amino acids could only come from an amino-acid analysis of purified proteins synthesized in mitochondria.

The amino acids that are utilized to a very low extent, if at all, for mitochondrial protein synthesis in HeLa cells include most of the charged polar amino acids (arginine, aspartic acid, lysine, and, possibly, glutamic acid) and some of the neutral polar amino acids (cysteine and glutamine). Furthermore, histidine and some other neutral polar amino acids (tyrosine, serine, and threonine) appear to be utilized to a low to moderate extent, whereas the strongly hydrophobic amino acids, like leucine, isoleucine, valine, phenylalanine, and methionine, are actively incorporated.

The available evidence indicating the presence in rat-liver mitochondria of organellespecific $tRNA^{Leu}$, $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Val}$, and $tRNA^{Phe}$ (32), and in HeLa mitochondria of $tRNA^{Met}_f$ (33), is in agreement with the amino-acid incorporation pattern observed here, and strongly suggests that these tRNA species are involved in transfer of the corresponding amino acids. As concerns the origin of the tRNA species utilized for HeLa mitochondrial protein synthesis, the present work has not provided a conclusive answer to the more general question raised by the RNA–DNA hybridization data (6–8), namely whether animal-cell mitochondria use for protein synthesis only the amino acids corresponding to the incomplete set of endogenous tRNA species.

Neurospora mitochondria apparently contain a complete or almost complete set of tRNA species (34), and in yeast mtDNA the presence of at least 20 4S RNA genes has been reported (35, 36). It will be interesting to see whether in these organisms the pattern of amino-acid utilization for mitochondrial protein synthesis is different from that described here for HeLa cells, and, if so, whether this difference can be correlated with an evolutionary change in the spectrum of the proteins synthesized in mitochondria.

Apart from the question of the number of amino acids utilized by HeLa-cell mitochondria and from the possible evolutionary implications of the present findings, this work has for the first time attempted a characterization in terms of amino-acid composition of the proteins synthesized in mitochondria and has provided a chemical basis for the known hydrophobic properties of these proteins (31). Furthermore, the striking difference observed in the pattern of amino-acid utilization by the mitochondrial and the cytoplasmic protein-synthesizing systems in HeLa cells will represent a valuable tool for identification of proteins synthesized in animal mitochondria in the absence of inhibitors.

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