BRAIN AND LIVER MITOCHONDRIAL PROTEIN SYNTHESIS: POTASSIUM DEPENDENT CHLORAMPHENICOL INHIBITION

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SUMMARY

Protein synthesis in vitro in mitochondrial preparations from mouse brain and mouse liver is sensitive to inhibition by chloramphenicol if potassium is the predominant cation in the incubations, but not when the media are enriched with respect to sodium ion and are potassium ion poor.

All preparations of brain mitochondria examined to date also exhibit sensitivity to inhibition by cycloheximide, whereas this is not found with liver mitochondria.

Mitochondria from every source that has been examined possess the capacity for the in vitro incorporation of amino acids into structural (inner membrane) protein utilizing a system which is strikingly similar to bacterial protein synthesis. For example, the inhibition of this process by chloramphenicol and the resistance to inhibition by cycloheximide has served to distinguish mitochondrial protein synthesis from eucaryotic cytoplasmic ribosomal systems (1-6). Mitochondria isolated from brain have provided an exception to these observations, as resistance to inhibition by chloramphenicol and sensitivity to acetoxycycloheximide have been reported (7).

As noted in a recent review (8) the failure of chloramphenicol to inhibit protein synthesis in mitochondria derived from brain may require a complex genetic explanation. If brain mitochondria differ in this fundamental way from all other mitochondria, and if mitochondria

are indeed self replicating and inherited, an assumption would be required that the fertilized ovum contains both "types" of mitochondria and that there exists some mechanism to insure that the daughter cell receives each type, just as each cell receives one of each type of chromosome in ordinary mitotic karyokinesis. An alternative explanation might be that in some manner the brain mitochondrion has differentiated, replacing its chloramphenicol-sensitive ribosomes with ribosomes sensitive to cycloheximide. Whatever the explanation, this interesting difference deserves more study.

We report here that the conditions ordinarily employed (3) for in vitro mitochondrial protein synthesis (i.e., a medium rich in potassium ion and relatively sodium ion poor) permitted a synthesis of protein by both liver and brain mitochondria which was partially sensitive to chloramphenicol. On the other hand, the incubation conditions employed in the previously mentioned report (7) (a medium rich in sodium ion and relatively potassium ion poor), while supporting a synthesis of protein, did not permit chloramphenicol inhibition with either brain or liver mitochondria. These observations suggest that the reported insensitivity of brain mitochondria to chloramphenicol is attributable to the incubation conditions, and that a potassium rich environment is required for inhibition by this antibiotic.

MATERIALS AND METHODS

Reagents of the highest available purity were obtained from commercial sources. All equipment and glassware were autoclaved prior to use. All reagents were passed through a 0.45 μ millipore filter. Swiss mice, Webster strain, were sacrificed by decapitation and the brains and/or livers were removed as rapidly and as aseptically as possible. The tissues were homogenized at 0° by hand in an all glass homogenizer in 10 volumes of 0.33 M sucrose. The homogenates were centrifuged at 0° at 700 x g for 15 minutes. Mitochondria were

sedimented from the supernatant fluid at 8000 x g for 10 minutes and washed with 0.33 M sucrose five times. The final washed mitochondrial pellets were suspended by gentle hand homogenization in 0.33 M sucrose and incubated under the conditions described below.

Incubations were at 30° in a water bath with mechanical shaking for 45 minutes in the media described in the accompanying tables. Reactions were terminated by the addition of an equal volume of ice cold 10 per cent trichoroacetic acid which was 10 mM with respect to nonradioactive leucine. After standing on ice for 15 minutes, the residues were collected by centrifugation at 9000 x g for 15 minutes. The precipitates were resuspended by vigorous mechanical agitation in 2 ml of 5 per cent trichloroacetic acid which was 10 mM with respect to leucine. After heating at 90° for 15 minutes the precipitates were collected by centrifugation and washed generally four times or until the radioactivity in a nonincubated control was near background levels. The precipitates were transferred quantatively with water to glass scintillation counting vials. After drying in a stream of air, the residues were dissolved in hyamine, and radioactivity was assayed in a toluene phosphor in a liquid scintillation counter. Quench corrections were by external standardization or by channels' ratio.

In experiments designed to measure the uptake of chloramphenicol by isolated mitochondria, ^{14}C-chloramphenicol was incubated for 0,5,10,20, and 30 minutes at 30° in the two media (omitting ^{14}C-leucine) described in Table I. The incubations were terminated by immediate immersion in ice, and the mitochondrial pellets were collected by centrifugation at 9000 x g. The pellets were washed twice with 0.33 M sucrose, transferred to counting vials, dried, dissolved, and counted, employing the methods described above. Following similar incubations with ^{14}C-chloramphenicol a search was made for the possible formation of acetylated derivatives of the antibiotic following the methods of Shaw (9). Protein estimations

were by the method of Lowry, et al (10).

RESULTS

Isolated liver and brain mitochondria carried out an incorporation of ^{14}C-L-leucine into material insoluble in trichloroacetic acid in media enriched with either potassium or sodium as the predominant cation. (Table I). The synthesis in liver mitochondria was not inhibited by cycloheximide at a level which is strongly inhibitory to higher organism ribosomal systems (5,6) and to brain mitochondrial preparations.

TABLE I

THE EFFECT OF Na^+ AND K^+ ON CHLORAMPHENICOL INHIBITION OF MITOCHONDRIAL PROTEIN SYNTHESIS

Incubation Conditions	Brain Mitochondria		Liver Mitochondria	
	CPM/mg	% Inhibition	CPM/mg	% Inhibition
K^+	1214	-	497	--
K^+ + Chloramphenicol	934	23	296	40
K^+ + Cycloheximide	621	49	456	8
Na^+	1386	-	578	--
Na^+ + Chloramphenicol	1430	0	552	4
Na^+ + Cycloheximide	786	43	544	6

Liver mitochondria (2.24 mg protein) or adult brain mitochondria (0.8 mg protein) were incubated for 45 minutes at 30° in a total volume of 1 ml. containing either 0.13 M KCL or 0.13 M NaCl, 0.01M $MgCl_2$, 0.005 M K-succinate, 0.005 M Na phosphate, pH 7.6, 0.002 M ATP, 0.035 M Tris-HCl pH 7.6, 1 µc of ^{14}C-L-leucine (specific activity 278 µc/µm) and 100 µg of D-threo-chloramphenicol or cycloheximide, when present. Preparation of mitochondria and assay of radioactivity in trichloroacetic acid-insoluble material was as described in Materials and Methods.

Inhibition of the synthetic process by D-threo-chloramphenicol was observed only in media enriched in potassium ion, whereas no inhibition was observed in the sodium enriched medium. The per cent inhibition by chloramphenicol of liver mitochondrial protein synthesis varied in

individual preparations to as high as 90 per cent, generally averaging about 50 per cent. Inhibition of brain mitochondria by chloramphenicol generally was from 25 to 40 per cent.

Repeated attempts to lower the sensitivity of brain mitochondria to cycloheximide by exhaustive washings were not successful. If the cycloheximide-inhibited activity represents cytoplasmic ribosomal contamination, the binding of these ribosomes by brain mitochondria must be considerably greater than for liver mitochondria. Furthermore, incubations of the brain mitochondrial preparations with ribonuclease A, 10 µg/ml., did not affect the results.

Experiments with young (14 day old) mouse brains also revealed the dependency on the potassium enviroment for chloramphenicol inhibitability. The lowering of the potassium to sodium ratio resulted in a graded amount of inhibition (Table II).

The uptake of ^{14}C-chloramphenicol by mitochondria was found to be somewhat greater in the sodium ion enriched medium. For example, incubation

TABLE II

THE INFLUENCE OF Na/K RATIO ON CHLORAMPHENICOL INHIBITION OF YOUNG MOUSE BRAIN MITOCHONDRIA

INCUBATION CONDITIONS	CPM/mg	% INHIBITION
0.111 M K^+	830	--
0.111 M K^+ + CAM	597	28
0.111 M K^+ + 0.05 M Na^+	1029	--
0.111 M K^+ + 0.05 Na^+ + CAM	898	13
0.120 M Na^+ + 0.055 M K^+	854	--
0.120 M Na^+ + 0.055 M K^+ + CAM	836	2

Brain mitochondria (3.0 mg protein) were incubated for 45 min. at 30° in 1.0 ml containing 0.01 M $MgCl_2$, 0.006 M phosphate, 0.035 M Tris-HCl pH 7.6, 0.005 M succinate, 0.002 M ATP, 1 µc of ^{14}C-L-leucine, 100 µg chloramphenicol (CAM) (when present) and total K^+ or Na^+ as shown.

of mitochondria with 0.5 μc (0.16 μ moles) of ^{14}C-chloramphenicol gave a time and temperature dependent uptake of 9541 CPM/mg. in the sodium-enriched medium and of 7462 CPM/mg. in the potassium enriched medium. These findings cannot be considered as evidence that the antibiotic has equal access to its site of action, since this site is unknown. They only suggest that the difference between sensitivity to the antibiotic in the two media is not attributable in any gross way to a dependency upon potassium ion for uptake of chloramphenicol or to an inhibition of the process by sodium ion.

The possibility was considered that the resistance to chloramphenicol in the sodium medium might be explained by the sodium ion activation of an acetylating enzyme which might render the antibiotic inactive, as was shown to occur in chloramphenicol resistant bacteria (9). A search for acetylated derivatives of chloramphenicol, by thin layer chromatography as described by Shaw (9), after incubation of mitochondria with ^{14}C-chloramphenicol in both media, failed to support this possibility.

DISCUSSION

We have shown that the inhibition of *in vitro* mitochondrial protein synthesis by chloramphenicol took place in a potassium ion enriched medium, and could not be demonstrated in a potassium-poor medium. Vasquez (11) has shown that bacterial protein synthetic systems are sensitive to chloramphenicol *in vitro* only under conditions that mimic *in vivo* conditions, specifically a high potassium ion concentration. His further demonstration that the binding of chloramphenicol to the 50 S subunit of the ribosome required potassium ion and did not take place in a sodium ion enriched environment suggests that a similar explanation for our findings is reasonable (12). The site of synthesis of protein within the mitochondria is not known with certainty, however recent findings in yeast mitochondria suggest that the mitochondrial ribosomes are internal to the inner membrane (13). If this is the case for brain and liver mitochondria, as seems

likely, our findings may be attributable to some indirect effect, because under our incubation conditions the mitochondrial inner membrane is reported to be impermeable to potassium and sodium (14).

Our attempts to find a difference between chloramphenicol uptake, or to find a possible chloramphenicol inactivating system in the sodium ion environment were unsuccessful. Future experiments, perhaps with isolated inner membrane preparations, may eventually lead to an explanation for the differences which we have reported here.

Inhibition of protein synthesis by cycloheximide has been reported to represent a distinguishing feature of brain mitochondria (7), and the results of our studies would support this observation. Protein synthetic activity sensitive to this antibiotic may be attributable to ribosomes of the type normally found in the cytoplasm of eucaryotic organisms. Additional studies are required before it will become clear whether the brain mitochondrion does indeed differ in this fundamental way from other mitochondria, and in fact does possess two types of ribosomes, or whether the cycloheximide inhibited activity is only a measure of an unusual degree of cytoplasmic ribosomal contamination.

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