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Study of Protein Synthesis in Rat Liver Mitochondria: Use of Cycloheximide

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1. Effects of short-term and long-term administration of cycloheximide on rat liver mitochondrial protein synthesis have been examined and were found to be different.

2. Long-term administration of cycloheximide resulted in inhibition of total cellular protein synthesis including that of mitochondria while, at short-term intervals, 8–10% of mitochondrial protein synthesis was cycloheximide-resistant.

3. The inhibitory effect was also reflected in terms of protein synthesizing ability of mitochondria *in vitro*, the inhibition becoming apparent at 40 min and showing progressive increase with time.

4. The observed inhibition of mitochondrial protein synthesis by cycloheximide was not due to either inhibition of energy metabolism or alteration of amino-acid pool.

5. Cycloheximide did not enter mitochondria or sonic preparation under conditions *in vitro*. On the other hand, after administration of [^{3}H]cycloheximide, significant quantities of the label were found to be associated with mitochondria and mitoribosomes.

6. These results indicated that cycloheximide reached the site of action in mitochondria under conditions *in vivo* but was unable to do so *in vitro*.

7. The results are discussed to elucidate the possible mechanisms involved in the inhibition of truly mitochondrial protein synthesis by cycloheximide.

In recent years it has become apparent that mitochondria have their own genetic apparatus and an intrinsic protein-synthesizing machinery including DNA, RNAs, ribosomes and enzymes involved in transcription and translation processes [1–5]. However, the amount of genetic information contained in mitochondrial DNA is limited and most of the mitochondrial proteins are coded by nuclear genes, synthesized on cytoplasmic ribosomes and subsequently transferred to these organelles [1–5]. During the past few years, there have been major advances in our knowledge of nucleo-cytoplasmic interactions during mitochondrial biogenesis [5,6]. Excellent work has been recently carried out on these lines by Mason and Schatz [7] and Tzagoloff *et al.* [8] in yeast. These studies emphasize the complex interrelationship between mitoribosomal and cytoribosomal protein-synthesizing systems during the final assembly of a mitochondrion.

Site-specific inhibitors such as cycloheximide and chloramphenicol serve as useful tools to study the coordination between these two protein-synthesizing systems. While chloramphenicol inhibits bacterial and mitochondrial protein synthesis [2], cycloheximide is known to be a potent inhibitor for cytoplasmic protein synthesis without having any effect on protein synthetic ability of mitochondria *in vitro* [2]. However, *in vivo* the effect of cycloheximide on mitochondrial protein synthesis often gives variable results [9–14]. In addition, most of the studies involving use of cycloheximide have been carried out with lower eukaryotes such as yeast [6,8,13–15], *Neurospora* [12,16,17], locust flight muscle [11,18], *Tetrahymena* [19] *etc.* and Krebs ascites tumor cells [10] and there are only a few reports pertaining to the use of this antibiotic in the study of mitochondriogenesis in mammalian system [20–23].

Present report deals with the effect of cycloheximide on mitochondrial protein synthesis in rat liver, under conditions *in vivo* and *in vitro*. These studies indicate that the effects of short-term and long-term cycloheximide administration on mitochondrial protein synthesis are different. At short intervals, cycloheximide does not inhibit truly mitochondrial protein synthesis. On the other hand, long-term treatment with cycloheximide results in the inhibition of truly mitochondrial protein synthesis, as reflected in terms of their protein-synthesizing ability *in vitro*. The results are discussed in relation to the effect of cycloheximide on mitochondrial protein synthesis *per se*

and also in relation to the interdependence of mitochondrial and cytoplasmic protein synthesis during mitochondrial biogenesis.

MATERIALS AND METHODS

Chemicals

ATP, ADP, sodium dodecylsulfate, sodium succinate, acrylamide, *N,N'*-methylenebisacrylamide, L-amino acids, cycloheximide, deoxycholate, Triton X-100, 2,5-diphenyloxazole, (PPO), 1,4-bis(-4-methyl-5-phenyloxazol-2-yl)-benzene (dimethyl-POPOP) and bovine serum albumin were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). [^{3}H]Cycloheximide and DL-[1-^{14}C]leucine (specific radioactivity 47.9 mCi/mmol) were obtained from Isotope Division of this Research Centre. [^{3}H]Cycloheximide was further purified by thin-layer chromatography using butan-1-ol/water (95/5, v/v) as a solvent system and identified by co-chromatography of an authentic sample. Under these conditions, cycloheximide has a R_F value of 0.45. The final product had a specific radioactivity of 93.8 mCi/mmol.

All other chemicals used were Analar grade from BDH Chemicals Ltd (Dorset, Poole, UK).

Animals

Female albino rats of Wistar strain weighing between 150–180 g were used throughout the experiments. Cycloheximide was dissolved in 0.9% NaCl and administered intraperitoneally as indicated. Control animals received appropriate amounts of saline.

Preparation of Subcellular Fractions

Animals were killed by decapitation and livers were quickly removed and chilled in 0.25 M sucrose containing 10 mM Tris/HCl, pH 7.4 and 1 mM EDTA (isolation medium). Mitochondria were isolated essentially according to the procedure described previously [24]. A 10% homogenate made in isolation medium was centrifuged at $650 \times g$ for 10 min to sediment nuclei. Nuclei-free supernatant was centrifuged at $6500 \times g$ for 10 min and resulting mitochondrial pellet was subsequently washed three times by suspending in isolation medium and centrifuging at $6500 \times g$ for 10 min. Mitochondria-free supernatant was centrifuged at $18000 \times g$ for 10 min to remove residual mitochondrials and the supernatant obtained was spun at $105000 \times g$ for 1 h to obtain microsomes. Microsomes were washed once by suspending in isolation medium and centrifuging at $105000 \times g$ for 1 h.

For studies on subcellular distribution of [^{3}H]-cycloheximide, the homogenate was filtered through four layers of cheese cloth to remove unbroken cells and cell debris. Rest of the fractionation procedure was as described above, except that total mitochondria were obtained by centrifuging at $10000 \times g$ for 10 min. Nuclear pellet was washed once.

Cytoribosomes were prepared according to the method of Wettstein *et al.* [25] and mitoribosomes were prepared according to the method of O'Brien [26].

All operations were carried out at 0–4 °C.

Incorporation Studies

DL-[1-^{14}C]Leucine. For studies *in vivo*, 20 μCi of DL-[1-^{14}C]leucine (dissolved in 0.9% NaCl)/100 g body weight was administered intraperitoneally and animals were sacrificed at the time indicated. Incorporation of DL-[1-^{14}C]leucine by mitochondria *in vitro* was carried out as described previously [27]. For measurement of radioactivity samples were spotted on Whatman 3MM filter paper strips (7 cm × 1.8 cm), dried in a current of air and processed for counting [27]. Radioactivity was counted using a scintillator containing 0.3% PPO and 0.01% dimethyl POPOP in toluene, in a Beckman LS-100 liquid scintillation spectrometer at 95% efficiency.

Uptake of [^{3}H]Cycloheximide

Experiments in vitro. For studies on uptake *in vitro* of [^{3}H]cycloheximide, three-times-washed mitochondria were incubated in a medium used for DL-[1-^{14}C]-leucine incorporation [27], except that in place of DL-[1-^{14}C]leucine, 1 μCi of [^{3}H]cycloheximide with or without unlabelled cycloheximide (500 μg/ml) was added. Uptake of [^{3}H]cycloheximide was measured up to 30 min. The reaction was stopped by chilling the mixture at the end of incubation periods. Mitochondria were recovered by centrifugation and were washed 3 times using isolation medium.

Experiments in vivo. For subcellular distribution studies rats received intraperitoneally 30 μCi of [^{3}H]cycloheximide along with 2 mg unlabelled cycloheximide/100 g body weight and were sacrificed after 1 h. Subcellular fractions were isolated as described above. For ^{3}H counting, samples were directly added to vials containing 10 ml of scintillator consisting of 0.3% PPO and 0.01% dimethyl POPOP in toluene/Triton X-100 (2/1, v/v) [28] and counted in a Beckman LS-100 liquid scintillation spectrometer at 37% efficiency.

Protein determination was done by Lowry's method [29].

Determination of Mitochondrial Protein Content

Mitochondria were washed three times and, therefore, could not be recovered quantitatively because of operational losses. Hence the method of Gross [30]

was used to determine the mitochondrial total protein content in a given weight of tissue. This method entails the estimation of specific activity of the enzyme succinate dehydrogenase in mitochondria, as well as the total enzyme activity in the whole homogenate [31]. The total mitochondrial protein content is derived by rule of three computation. Succinate dehydrogenase activity was determined by the method of Caplan and Greenwalt [32].

Oxidative Phosphorylation

ADP/O ratio and respiratory control index were determined in a Gilson Oxygraph (model KM, Gilson Medical Electronics, Middleton, Wis., U.S.A.) using succinate as a substrate as described previously [24]. ADP/O ratios and respiratory control index were calculated as described by Chance and Williams [33].

Determination of Amino-Acid Pool

Proteins were precipitated with 10% cold trichloroacetic acid (0–4 °C), centrifuged and washed once with 10% cold trichloroacetic acid (0–4 °C). The supernatant and washings were pooled and were extracted three times with ether to remove trichloroacetic acid. The trichloroacetic acid soluble material was estimated by ninhydrin assay according to Alberti and Bartley [34].

For measurement of radioactivity, the ether-extracted samples were spotted on Whatman 3MM filter paper strips and directly counted using a toluene-based scintiallator as described above for proteins.

Dodecylsulfate-Polyacrylamide Gel Electrophoresis

Dodecylsulfate-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [35] with some modifications. Details are as described elsewhere [36].

RESULTS

Effects of Cycloheximide on DL-[1-^{14}C]*Leucine Incorporation* in vivo

In preliminary studies, effects of cycloheximide on microsomal and mitochondrial protein synthesis *in vivo* were examined. In these experiments, the animals received 0.2 mg cycloheximide/100 g body weight [37,38]. DL-[1-^{14}C]Leucine was injected at different time intervals as indicated in Table 1. It was observed that cycloheximide inhibited microsomal as well as mitochondrial protein synthesis to the extent of 85% and 90% respectively, by the end of 1 h. The inhibition increased at 2 h, being about 95% in both the systems and declined thereafter. Interestingly, at 8 h period mitochondrial protein synthesis was still inhibited to the extent of 75%, whereas the inhibition of microsomal protein synthesis was only 41%. No inhibition was evident at the end of 48 h which may probably be due to detoxification of the drug.

Table 2 shows the effect of cycloheximide concentration on mitochondrial and microsomal protein synthesis *in vivo*. DL-[1-^{14}C]Leucine was injected intraperitoneally 2 h after cycloheximide administration and the animals were killed 1 h later. It can be seen that cycloheximide concentration as low as 50 µg/100 g body weight brought about 50% inhibition in both microsomes as well as mitochondria; higher concentrations resulted in increased inhibition. Even under these conditions, almost equal inhibition of mitochondrial and microsomal protein synthesis was evident. In fact, the inhibition of mitochondrial protein synthesis was always slightly higher as compared to microsomes. Administration of cycloheximide (1–5 mg/100 g body weight) for 2 h practically resulted in stoppage of total cellular protein synthesis including that of mitochondria.

Table 3 gives the effect of shorter time administration of cycloheximide (2 mg/100 g body weight) on

Table 1. *Effect of cycloheximide on* DL-[1-^{14}C]*leucine incorporation* in vivo
Rats received 20 µCi of DL-[1-^{14}C]leucine/100 g body weight intraperitoneally at different time intervals after cycloheximide (0.2 mg/100 g body weight) administration intraperitoneally and were killed 1 h later. Isolation of subcellular fractions and method for counting of radioactivity are as detailed in the text. The control animals did not receive cycloheximide. Results are given as typical of four independent experiments

Time	Incorporation of DL-[1-^{14}C]leucine			Inhibition		
	homogenate	mitochondria	microsomes	homogenate	mitochondria	microsomes
h	counts × min^{-1} × mg protein^{-1}			%		
Control	1692	814	3069			
1	152	122	319	91.0	85.0	89.6
2	96	31	185	94.3	96.1	94.0
8	738	203	1808	56.4	75.1	41.1
48	1845	786	2740	–	–	–

Table 2. *Effect of cycloheximide concentration on* DL-*[1-^{14}C]leucine incorporation* in vivo
DL-[1-^{14}C]Leucine (20 μCi/100 g body weight) was injected 2 h after cycloheximide administration and animals were killed 1 h later. Other details are as in Table 1. The results are given as mean of 4 independent experiments ± S.E.M.

Cyclohex-imide	Incorporation of DL-[1-^{14}C]leucine			Inhibition		
	homogenate	mitochondria	microsomes	homogenate	mitochondria	microsomes
mg/100 g body weight	counts × min^{-1} × mg protein^{-1}			%		
0.0	1692 ± 121	814 ± 81	3289 ± 255			
0.025	1506 ± 184	687 ± 32	3108 ± 111	11.0	15.6	5.5
0.050	809 ± 143	397 ± 68	1659 ± 105	52.2	51.2	49.6
0.100	523 ± 15	210 ± 23	1174 ± 17	69.1	74.2	64.3
0.150	114 ± 2	49 ± 2	227 ± 12	93.3	94.0	93.1
1.00	–	24 ± 2	150 ± 16	–	97.1	95.4
5.00	–	20 ± 1	68 ± 3	–	97.5	97.9

Table 3. *Effect of short-term administration of cycloheximide on* DL-*[1-^{14}C]leucine incorporation* in vivo
Rats received 20 μCi of DL-[1-^{14}C]leucine/100 g body weight after 15 min of cycloheximide (2 mg/100 g body weight) administration intraperitoneally and were sacrificed at different time intervals as indicated. Other details are as in Table 1. Results are given as typical of 4 independent experiments

Time after label administration	Inhibition		
	homogenate	mitochondria	microsomes
min	%		
2	88.0	83.5	89.5
5	90.0	87.5	92.0
8	95.0	89.0	96.0
15	96.0	90.0	96.0

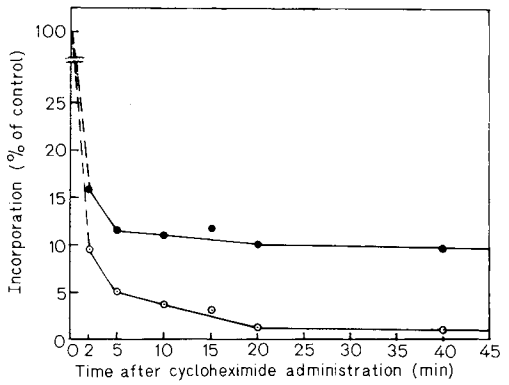

Fig. 1. *Effect of simultaneous injection of cycloheximide and* DL-*[1-^{14}C]leucine.* Animals were simultaneously administered with 2 mg cycloheximide and 20 μCi of DL-[1-^{14}C]leucine/100 g body weight and sacrificed at the time indicated. Control animals received only DL-[1-^{14}C]leucine. Results are expressed as percentage incorporation of control; (O——O) microsomes; (●——●) mitochondria

mitochondrial and microsomal protein synthesis. DL-[1-^{14}C]Leucine incorporation was studied for 2, 5, 8 and 15 min after 15 min of cycloheximide administration. It can be seen that under these conditions about 8–10% of mitochondrial protein synthesis was cycloheximide resistant (Table 3). Similar results were noted when cycloheximide and DL-[1-^{14}C]leucine were injected simultaneously (Fig. 1). On the other hand the microsomal protein synthesis was almost completely inhibited. However, as studied earlier (Table 2), when DL-[1-^{14}C]leucine incorporation was examined after 2 h of cycloheximide treatment, practically complete (98%) inhibition of total cellular protein synthesis, including mitochondrial, was noted. It thus became apparent that the short-term (Table 3 and Fig. 1) and long-term (Table 2) effects of cycloheximide on mitochondrial protein synthesis *in vivo* were dissociable. While the short-term effect shows cycloheximide-resistant mitochondrial protein synthesis which amounts to 8 to 10%, the long-term effects bring about inhibition of total cellular protein synthesis including that of mitochondria.

Effect of Cycloheximide Administration on Mitochondrial Protein Synthesis in vitro

To examine whether the observed inhibition of mitochondrial protein synthesis *in vivo* is also reflected under conditions *in vitro*, protein synthetic ability of mitochondria isolated from cycloheximide administered animals was studied. Results given in Table 4 indicate that mitochondria isolated from cycloheximide-treated animals show practically no decrease in their protein synthetic ability *in vitro* up to about 20 min, by the end of 40 min about 60% inhibition is observed which increases to about 75% by 2 h. Since the protein synthesizing ability *in vitro* is a measure of the truly mitochondrial protein synthesis [2,27], these results, taken together with those outlined in Tables 1 and 2, clearly substantiate

Table 4. *Effect of cycloheximide administration on mitochondrial protein synthesis* in vitro
Rats received 2 mg cycloheximide/100 g body weight intraperitoneally and were killed at different time intervals as indicated in the table. Incorporation of DL-[1-^{14}C]leucine *in vitro* was carried out for 1 h using 3-times-washed isolated mitochondria as described in the text. Incorporation by isolated mitochondria from control animals (zero time) was 271 ± 22 counts × min^{-1} × mg protein^{-1}. Results are averages of 6 independent experiments. + sign indicates stimulation in the incorporation activity *in vitro*

Time after cycloheximide administration	Inhibition
min	%
0	
5	+ 9.1
10	+ 6.8
15	+ 1.6
20	10.0
40	61.4
60	71.3
120	74.7

Table 5. *Effect of cycloheximide* in vivo *on mitochondrial oxidative metabolism*
Animals were sacrificed 2 h after cycloheximide administration (2 mg/100 g body weight). Rates of respiration in presence and absence of ADP were determined using succinate as substrate [24]. Calculation of ADP/O ratio and respiratory control index was according to Chance and Williams [33]. Results are given as mean of 5 independent experiments ± S.E.M.

Cyclo-hexi-mide	ADP/O ratio	Rate of oxidation		Respiratory control index
		+ ADP	− ADP	
mg/100 g body weight		nmol O_2 × min^{-1} × mg protein^{-1}		
0	1.58 ± 0.18	56.0 ± 3.40	14.6 ± 0.34	3.84
2	1.50 ± 0.03	64.8 ± 3.15	15.3 ± 0.10	4.23
5	1.53 ± 0.12	55.5 ± 3.21	14.8 ± 0.29	3.75

the inference that at long-term intervals, cycloheximide indeed inhibits the protein-synthesizing ability of mitochondria.

The observed inhibition of mitochondrial protein synthesis *in vivo* as well as *in vitro* after cycloheximide treatment may be due to several reasons. These include: interference with energy metabolism of mitochondria; alteration of amino-acid pool size; inhibition of mitochondrial protein synthesis *per se*; and interdependence of mitochondrial and microsomal protein synthesis. The first three possibilities were tested in the subsequent studies. The alternative possibility of interdependence is considered under 'Discussion'.

Mitochondrial Oxidative Metabolism

Table 5 gives the ADP/O ratio and respiratory control index for mitochondria isolated from normal and cycloheximide-treated animals. Cycloheximide concentrations (2 and 5 mg/100 g body weight) which almost completely inhibit total cellular protein synthesis (Table 2), did not show any effect on ADP/O ratios, rate of oxygen consumption in presence and absence of ADP and respiratory control index. Thus, it appears that even at these high concentrations cycloheximide does not interfere with energy metabolism of mitochondria.

Amino-Acid Pool Size

Preliminary experiments to correlate the decreased incorporation with amino acid pool of mitochondria indicated that the specific activity of the mitochondrial pool was always higher in the cycloheximide-treated rats (Table 6). The pool-size, however, could not be assessed correctly because of losses during washings (Table 6). As an alternative and nearest approach, we tried to correlate the observed changes with the homogenate pool.

Table 6. *Effect of cycloheximide administration on amino acid pool of mitochondria*
Animals were administered 2 mg cycloheximide/100 g body weight intraperitoneally 2 h prior to injection of 20 μCi of DL-[1-^{14}C]leucine/100 g body weight and were sacrificed 1 h later. Control animals received only DL-[1-^{14}C]leucine. Amino acids were estimated by 'ninhydrin' method [34] as described in the text. Results are given as mean ± S.E.M. of 4 independent experiments performed in duplicate

Mitochondria	Amino acid content		Incorporation of DL-[1-^{14}C]leucine	
	normal	cycloheximide-treated	normal	cycloheximide-treated
	μg × mg mitochondrial protein^{-1}		counts × min^{-1} × μg amino acid^{-1}	
Once-washed	10.92 ± 0.28	9.11 ± 0.60	5.51 ± 0.44	8.13 ± 0.89
Thrice-washed	8.82 ± 0.53	6.36 ± 0.61	4.21 ± 0.21	6.80 ± 0.71

Table 7. *Effect of cycloheximide administration on amino-acid pool size of liver homogenate*
The experimental details are as described in Table 6. Mitochondrial protein content was determined by the method of Gross [30] as detailed in the text and values are given as typical of 5 independent experiments. Other results are given as mean ± S.E.M. of 4 independent experiments performed in duplicate

Group	Amino-acid content	Mitochondrial proteins	Incorporation of DL-[1-^{14}C]leucine		Relative specific activity (*a*/*b*)
			a	*b*	
	$\mu g \times g$ liver^{-1}	$mg \times g$ liver^{-1}	counts $\times$ min^{-1} $\times$ mg mitochondrial protein^{-1}	counts $\times$ min^{-1} $\times \mu g$ amino acid^{-1}	
Control	6366 ± 291	41.8	1048.7 ± 21.5	8.18 ± 0.29	128.20
Cycloheximide-treated	10789 ± 718	41.2	39.9 ± 1.9	17.19 ± 0.98	2.32

It was observed that administration of cycloheximide resulted in about 70% increase in the pool size of the homogenate and the specific activity of the pool became approximately two-fold (Table 7). However, in spite of the increase in the pool size and in its specific activity the incorporation into mitochondria decreased to the extent of 96%. This is also reflected in terms of a tremendous decrease in the relative specific activity from 128 to 2.3 (Table 7). Mitochondrial protein content of the liver, however, remained unaffected under these conditions as is to be expected. Thus notwithstanding the limitations that the homogenate pool can not strictly be related to the mitochondrial pool, the results obtained in the present experiment with mitochondria and homogenate indeed indicate that the observed decrease in mitochondrial protein synthesis may not be due to the alterations of the pool size. The possibility of cycloheximide inhibiting mitochondrial protein synthesis *per se* was next examined.

The foregoing studies (Tables 1, 2 and 4) indicated that cycloheximide may indeed be affecting truly mitochondrial protein synthesis. On the other hand, it is known that cycloheximide when added *in vitro*, does not inhibit the protein-synthesizing activity of isolated mitochondria [27] (Table 8). These observations raised a possibility that such a differential effect of cycloheximide under conditions *in vivo* and *in vitro* may be due to permeability barrier across the mitochondrial membranes. This was tested by examining effect of cycloheximide addition to sonicated mitochondria on their protein-synthesizing activity.

Results in Table 8 show that sonication by itself resulted in about 55% decrease in the incorporation by mitochondria *in vitro*. This may be because of loss of structural integrity. However, addition of cycloheximide to sonicated mitochondria and/or sonication of mitochondria in presence of cycloheximide did not significantly affect their incorporation ability *in vitro*. It may be that even under these conditions,

Table 8. *Effect of cycloheximide on DL-[1-^{14}C]leucine incorporation in vitro by sonicated mitochondria*
Mitochondria were sonicated at 0–4 °C in a MSE ultrasonic disintegrator at 1.8 kHz for 2 min (30 s, 4 times) in the absence or presence of cycloheximide. Final concentration of cycloheximide in incubation medium was 500 µg/ml. Incorporation *in vitro* was carried out as described in the text at 30 °C for 1 h. Results are given as typical of 5 independent experiments

Mitochondria	Incorporation of DL-[1-^{14}C]leucine	Inhibition
	counts $\times$ min^{-1} $\times$ mg protein^{-1}	%
Control	261	
Control + cycloheximide	243	5.7
Sonicated	117	55
Sonicated + cycloheximide	115	56
Sonicated in presence of cycloheximide	147	44

cycloheximide fails to reach the site of action and perhaps the transfer is mediated by a carrier. In order to verify this possibility, experiments were carried out using 105000 $\times$ *g* supernatant in the incubation system. However, addition of cycloheximide along with cell-sap or addition of cell-sap from cycloheximide-treated animals, also did not affect the incorporation ability of the mitochondria *in vitro* (Table 9). These results thus indicated that cycloheximide fails to reach the site of action under conditions *in vitro* probably because the carrier may function only *in vivo*; alternatively, cycloheximide may be entering mitochondria *in vivo* independently of a hypothetical carrier. These possibilities were further explored by following the uptake of [^{3}H]cycloheximide *in vitro* and *in vivo*.

Studies on uptake of [^{3}H]cycloheximide by mitochondria *in vitro*, in the absence or presence of unlabelled cycloheximide (500 µg/ml) are summarised in Tables 10 and 11, respectively. It was observed that negligible amount of radioactivity is associated with mitochondria, the activity being practically the

Table 9. *Effect of cell sap on* DL-[1-^{14}C]*leucine incorporation* in vitro *by mitochondria*
Animals were injected with 2 mg cycloheximide/100 g body weight intraperitoneally 1 h prior to sacrifice. Incorporation of DL-[1-^{14}C]-leucine *in vitro* into mitochondrial proteins was carried out for 1 h at 30 °C. Results are typical of 5 independent experiments

Addition	Incorporation of DL-[1-^{14}C]leucine	Inhibition
	counts × min^{-1} × mg protein^{-1}	%
None	253.0	
+ 0.2 ml cell sap (from control animal)	248.0	2.0
+ 0.2 ml cell sap (from cycloheximide-treated animal)	254.0	0.0
+ 0.5 ml cell sap (from cycloheximide-treated animal)	250.0	1.3

Table 10. *Uptake of* [^{3}H]*cycloheximide* in vitro *by mitochondria*
1 μCi of [^{3}H]cycloheximide was added to the mitochondrial protein-synthesizing system *in vitro* as indicated in the text except that DL-[1-^{14}C]leucine was omitted and cold leucine was included in the amino acid mixture. Uptake was studied at 30 °C for up to 30 min. At the end of incubation, mitochondria were washed three times with 0.25 M sucrose containing 10 mM Tris/HCl, pH 7.4 and 1 mM EDTA. Radioactivity was counted in a toluene/Triton scintillator system as indicated in the text. Results are given as mean ± S.E.M. of 4 independent experiments

Incubation time	Uptake of [^{3}H]cycloheximide	Uptake
min	counts × min^{-1} × mg protein^{-1}	%
0	143.0 ± 7.20	0.197
5	225.0 ± 17.20	0.219
15	253.0 ± 5.37	0.255
30	309.0 ± 6.90	0.351

Table 11. *Uptake of* [^{3}H]*cycloheximide* in vitro *by mitochondria in presence of unlabelled cycloheximide*
Uptake of [^{3}H]cycloheximide *in vitro* was carried out as indicated in Table 10 except that 500 μg of unlabelled cycloheximide/ml of incubation medium was added along with 1 μCi of [^{3}H]cycloheximide. Other details are as in Table 10. Results are given as mean ± S.E.M. of 4 independent experiments

Incubation time	Uptake of [^{3}H]cycloheximide	Uptake
min	counts × min^{-1} × mg protein^{-1}	%
0	198.0 ± 2.8	0.226
5	316.0 ± 8.6	0.296
15	356.0 ± 16.0	0.311
30	386.0 ± 13.1	0.333

Table 12. *Association of* [^{3}H]*cycloheximide* in vivo *with subcellular fractions of rat liver*
Animals received intraperitoneally 30 μCi of [^{3}H]cycloheximide (+ 2 mg unlabelled cycloheximide)/100 g body weight 1 h prior to sacrifice. Mitoribosomes and cytoribosomes were isolated as described in the text. Radioactivity was counted in a toluene/Triton scintillator system as described in the text. Recovery of [^{3}H]cycloheximide in the subcellular fractions was calculated as a percentage by comparing the total number of counts in the fractions with those found in total liver homogenate. Results are given as mean ± S.E.M. of 3 independent experiments

Fraction	Uptake of [^{3}H]cycloheximide	Recovery
	counts × min^{-1} × mg protein^{-1}	%
Homogenate	2310.3 ± 405.0	100.0
Nuclei	192.7 ± 27.1	2
Mitochondria	306.0 ± 30.4	4
Microsomes	825.6 ± 90.1	8
Cell sap	5869.6 ± 245.0	75
Mitoribosomes	97 ± –	–
Cytoribosomes	412 ± –	–

same in the absence (Table 10) and in the presence (Table 11) of the carrier cycloheximide. This probably indicates a simple tritium exchange. This possibility is further supported by the fact that the exchange level at 0 h and 30 min shows only a marginal difference (Tables 10 and 11). These results therefore substantiate the view that cycloheximide does not enter mitochondria under conditions *in vitro*.

The possibility of cycloheximide entering into the mitochondria under conditions *in vivo* was then investigated by studying subcellular distribution of injected [^{3}H]cycloheximide. The results in Table 12 show that although most of the label (75%) is present in cell sap, about 4% and 8% of it is associated with mitochondria and microsomes, respectively (Table 12). It is therefore apparent that cycloheximide may be entering into mitochondria under conditions *in vivo*.

Preliminary studies revealed that significant radioactivity was associated with both, cytoribosomes and mitoribosomes (Table 12). The specific activities, however, were less than those of microsomes and mitochondria. This is understandable in view of the fact that fractionation of ribosomes involved treatment with detergents and loss of radioactivity may have occurred during this treatment. However, the fact remains that significant activity is still associated with both cytoribosomes and mitoribosomes (Table 12).

DISCUSSION

The inhibitory effect of cycloheximide on ribosomal protein synthesis is well recognised [2,4]. Recently, however, it is becoming increasingly evident that besides its effect on ribosomal protein synthesis, cycloheximide may exert multiple effects. Thus, it has been reported that cycloheximide inhibits synthesis of 16 S ribosomal RNA in L cells [39] and in

rat liver [40] and of nuclear RNA [41] and chromatin [42] in HeLa cells. In *Chlorella*, cycloheximide is known to inhibit selectively nuclear DNA synthesis [43]. Inhibition of both mitochondrial as well as cytoplasmic ribosomal RNA species in *Tetrahymena* by cycloheximide has been reported [19]. Changes in the ultrastructure of rat liver cells within 6 h after administration of cycloheximide have also been noted [44]. Results presented in this paper bring out yet another aspect of cycloheximide action.

It is clear from the present studies that the short-term and long-term effects of cycloheximide on mitochondrial protein synthesis are dissociable (Tables 2 and 3, Fig. 1). After short-term treatment with cycloheximide, up to 8–10% of mitochondrial protein synthesis is discernible (Table 3, Fig. 1), an observation consistent with that of several other workers [10–12, 20–22]. This has been shown to be identical to the protein-synthesizing ability *in vitro* of isolated mitochondria and, therefore, taken to represent the truly mitochondrial protein synthesis [2]. On the other hand, long-term treatment with cycloheximide leads to practically complete stoppage of truly mitochondrial protein synthesis (Table 2), which is also reflected in their protein synthesizing activity *in vitro* (Table 4). The results, therefore, besides supporting the observations that mitochondria can synthesize only 8–10% of their proteins [2–6], also point to the complexities in the use of cycloheximide. In this context, it is of interest to note that Kroon and Arendzen have observed that 'in case of side effects of antibiotics influencing at one or another level with mitochondrial biogenesis one should seriously consider whether the arrest of mitochondrial development is the cause underlying the toxic signs' [45].

Inhibition of mitochondrial protein synthesis after cycloheximide treatment has also been reported recently in lower eukaryotes. Ibrahim *et al.* [46] observed a 50% decrease in the protein synthesis by yeast mitochondria *in vitro* [46]. In their experiments, partially derepressed cells were grown in cycloheximide for 3 h followed by transfer to fresh medium for 1–2 h prior to the isolation of mitochondria. In *Tetrahymena*, even a 5-min incubation with cycloheximide could lead to decreased incorporation activity of the isolated mitochondria. After the cells had been treated for 30 min, more than 50% of the activity was lost [19].

The possibility of a cycloheximide effect on truly mitochondrial protein synthesis being due to interference with energy metabolism or arising as a result of changes in the amino acid pool seems unlikely in view of the data presented (Tables 5 and 6 and 7 respectively). Under conditions *in vitro*, cycloheximide did not enter the site of action, even in the presence of added cell sap (Table 9). However, when injected *in vivo*, sizable amounts of cycloheximide were found to be associated with mitochondria and microsomes (4% and 8%, respectively). Interestingly, significant activity is found to be associated with mitoribosomes and cytoribosomes (Table 12). Contrary to the observation reported here, Ashwell and Work [10] found that an assayable amount of cycloheximide was associated with mitochondria after incubation with the antibiotic *in vitro*. We are unable to explain the reason for such a discrepancy at the present time. However, from our present results (Tables 10–12) it would seem that cycloheximide is able to reach the site of action (presumably mitoribosomes) only under *in vivo* conditions. These studies, however, do not indicate whether the process of cycloheximide association with mitochondria *in vivo* involves a carrier or is independent of it. It is also likely that [^{3}H]cycloheximide may be entering into mitochondria non-specifically and without having any inhibitory action *per se* [47] and that the observed inhibition of mitochondrial protein synthesis *in vivo* might be due to interdependence of mitochondrial and microsomal protein-synthesizing systems. Such a line of thinking is further borne out by observations reported in Table 4. One interesting feature of these findings is that cycloheximide did not inhibit mitochondrial protein synthesis up to about 40 min, after its administration. Probably these results illustrate the dependence of mitochondrial protein synthesis on cytoplasmically synthesized partner proteins. It may be that there is enough of partner proteins to last for up to 40 min after cycloheximide administration, when the effect of cycloheximide becomes apparent. In other words, some cytoplasmically synthesized product(s) required for continuation of mitochondrial protein synthesis [15] might be short-lived and getting exhausted within 40 min or so. Interdependence of the mitochondrial and cytoplasmic protein-synthesizing systems during biogenesis of mitochondrial enzymes such as cytochrome oxidase [48–50] and ATPase [8] has been amply emphasized by Schatz and Mason in their recent review [6].

With a view to finding out the possibility whether the synthesis of partner proteins is inhibited after cycloheximide treatment, dodecylsulfate-polyacrylamide gel electrophoresis was carried out using various subcellular components such as whole mitochondria, mitochondrial membranes, microsomes and cell sap isolated from normal as well as cycloheximide-injected animals (2 mg/100 g body weight for 1 h). Preliminary studies, however, indicated that the possible short-lived protein product(s) could not be detected by this method. No differences were observed in the number of bands as well as peak height in the protein profiles (microdensitometric scans) obtained for normal and cycloheximide-treated animals in mitochondria, mitochondrial membrane proteins as well as microsomal and cell sap proteins (Fig. 2). Probably, such product(s) may exist in trace quantitites

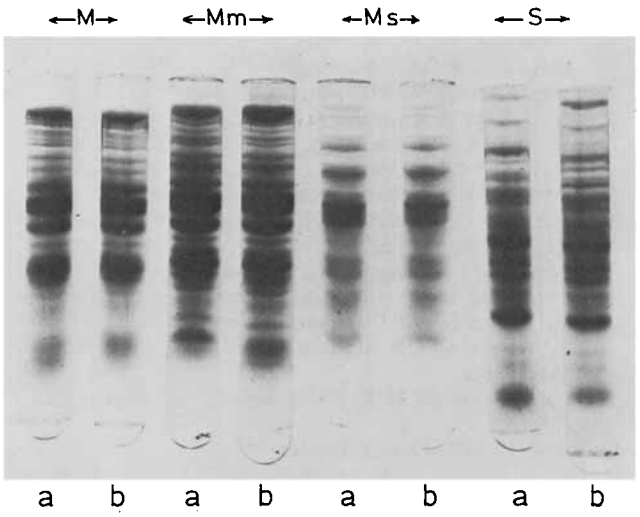

Fig. 2. *Typical dodecylsulfate-polyacrylamide gel photograph showing polypeptide patterns obtained for mitochondria (M), mitochondrial membranes (Mm), microsomes (Ms) and supernatant (S) from (a) normal and (b) cycloheximide-treated rats.* Other details are as described in the text

and/or escape detection because of the fact that they may have the same molecular weight as that of other proteins which makes it difficult to detect them or separate them from other subunit proteins.

The possibility that under the influence of cycloheximide, the hydrophobic proteins (products of mitochondrial protein synthesis) could not leave the mitoribosomes unless they are pulled off by corresponding cytoplasmically synthesized subunit proteins and remain attached to mitoribosomes thereby making them unavailable for further protein synthesis has also to be be considered in this context [6].

Alternately, the short-lived product might be a messenger-RNA, a translation product of mitochondrial DNA [51], whose half-life has been shown to be about 15 min in yeast [52]. It could also be a ribosomal RNA, since, cycloheximide is known to inhibit the synthesis of both mitochondrial as well as cytoplasmic ribosomal RNA species in *Tetrahymena* [19]. These possibilities, however, have not been verified experimentally and need further exploration.

The possibility that metabolites of cycloheximide may be responsible for the inhibition of mitochondrial protein synthesis seems to be unlikely in view of the experiments outlined in Table 9 where mitochondria were incubated with cell sap from cycloheximide-treated animals and had no affect on their protein-synthesizing ability *in vitro*.

Although in the present investigations the reason for inhibition of mitochondrial protein synthesis by cycloheximide could not be pin-pointed, the results have ruled out interference with energy metabolism and amino-acid pool as possible causes. Besides, these studies have clearly dissociated short-term and long-term effects of cycloheximide, thus emphasizing the complexity of inhibitor action as pointed out by Kroon and Arendzen [45]. Needless to say, further exploration of cycloheximide effects on mitochondrial RNA metabolism would help to shed more light on the mechanism of inhibition of mitochondrial protein synthesis by cycloheximide.

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