SECTION III

STUDY OF PROTEIN SYNTHESIS IN RAT LIVER MITOCHONDRIA:
USE OF CYCLOHEXIMIDE
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 - 4). 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 - 4). During the past few years, there have been major advances in our knowledge of nucleo-cytoplasmic interactions during mitochondrial biogenesis (5). Excellent work has been recently carried out on these lines by Schatz and Mason (5) and Tzagoloff et al (6) in yeast. These studies emphasize the complex interrelationship between mito-ribosomal and cyto-ribosomal protein synthesizing systems during final assembly of a mitochondrion.

Site specific inhibitors such as cycloheximide (CH) and chloramphenicol (CAP) serve as useful tools to study the coordination between these two protein synthesizing systems. CH is a potent inhibitor for cytoplasmic protein synthesis without having any effect on in vitro protein synthetic ability of mitochondria (2). However, in vivo, effect of CH on mitochondrial protein synthesis often gives variable results (7 - 12). In addition, most of these studies have been carried out either with yeast (5, 6, 11, 12), Neurospora (10, 13, 14), locust flight muscle (9, 15), Tetrahymena (16), Krebs ascites tumor cells (8), etc. and there are few reports pertaining to the use of this antibiotic in the study of mitochondriogenesis in mammalian system (17 - 20).
Present report deals with the effect of CH on mitochondrial protein synthesis in rat liver, under in vivo and in vitro conditions. These studies indicate that the effects of short and long-term CH administration on mitochondrial protein synthesis are different. At short intervals, CH does not inhibit truly mitochondrial protein synthesis. On the other hand, long-term treatment with CH results in the inhibition of truly mitochondrial protein synthesis, as reflected in terms of their in vitro protein synthesizing ability. The results are discussed in relation to effect of CH on mitochondrial protein synthesis per se and also the interdependence of mitochondrial and cytoplasmic protein synthesis during mitochondrial biogenesis.

MATERIALS AND METHODS

Chemicals:

ATP, ADP, SDS, sodium succinate, acrylamide, N,N'-methylene bisacrylamide (Bis), L-amino acids, cycloheximide, glucose-6-phosphate, 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 (47.9 mCi/m mole) were obtained from Isotope Division of this Research Centre. Tritiated cycloheximide was further purified by thin layer chromatography using n-butanol : water (95 : 5 v/v) as a solvent.
system and identified by co-chromatography of authentic sample. Under these conditions, CH has a Rf value of 0.45.

All other chemicals used were Analar grade from BDH Chemicals Ltd., Dorset, Poole, U.K.

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. 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 according to procedure of Katyare et al. (21). A 10% homogenate made in isolation medium was centrifuged at 650 x g for 10 min to sediment nuclei. Nuclei-free supernatant was centrifuged at 6,500 x g for 10 min and resulting mitochondrial pellet was washed three times by suspending in isolation medium and centrifuging at 6,500 x g for 10 min. Mitochondria-free supernatant was centrifuged at 18,000 x g for 10 min to remove residual mitochondria and the supernatant obtained was spun at 105,000 x g for 1 hr to obtain microsomes. Microsomes were washed once by suspending in isolation medium and centrifuging at 105,000 x g for 1 hr.
For studies on subcellular distribution of $^3$H-CH, 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 10,000 x g for 10 min. Nuclear pellet was washed once.

Preparation of cyto-ribosomes:

Cyto-ribosomes were prepared according to the method of Wettstein et al. (22). A 10% homogenate was made in 0.25 M sucrose containing 50 mM Tris-HCl pH 7.6, 25 mM KCl and 5 mM MgCl$_2$ (TMK-buffer). Nuclei and mitochondria were removed by centrifugation and mitochondria-free supernatant was treated with deoxycholate at a final concentration of 0.1%, layered over discontinuous gradient (3 ml of 1 M sucrose in TMK-buffer + 4 ml of 0.5 M sucrose in TMK-buffer + 3 ml of sample) and centrifuged at 105,000 x g for 2½ hr. Pellet obtained was suspended in TMK-buffer and used as 'total' cyto-ribosomes.

Preparation of mito-ribosomes:

Mito-ribosomes were prepared according to the method of O'Brien (23). Four times washed mitochondria were resuspended at a concentration of 1 to 5 mg protein per ml in the standard buffer (50 mM KCl, 50 mM NH$_4$Cl, 5 mM Tris-HCl, pH 7.6 and 20 mM MgCl$_2$) and lysed by addition of 0.1 volume of 10 or 20% Triton X-100.
This suspension was centrifuged for 10 min at 60,000 x g to sediment a 'membrane residue' fraction. The resulting supernatant containing released ribosomes, was layered over 2 ml of standard buffer containing 24% sucrose and centrifuged for 3 hr at 230,000 x g for 3 hr to sediment the crude mitochondrial ribosomal fraction.

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

Mitochondrial preparations were tested for microsomal contamination from time to time using glucose-6-phosphatase as a marker enzyme (Section II.1).

Incorporation studies:

DL(1-¹⁴C) leucine:

For in vivo studies, 20 µCi of DL(1-¹⁴C) leucine (dissolved in 0.9% NaCl)/100 g body weight was administered intraperitoneally and animals were sacrificed at the time indicated.

In vitro incorporation of DL(1-¹⁴C) leucine and measurement of radioactivity were carried out as described in Section II.1.

³H-cycloheximide:

For subcellular distribution studies rats received (IP) 30 µCi of ³H-CH along with 2 mg cold CH/100 g body weight and were sacrificed after one hr. Subcellular fractions were isolated and radioactivity was counted (Details described below).
For studies on \textit{in vitro} uptake of $^3$H-CH, three times washed mitochondria were incubated in a medium used for DL(1-$^{14}$C) leucine incorporation (Section II.1), except that in place of DL(1-$^{14}$C) leucine, $1\mu$Ci of $^3$H-CH with or without cold CH (500\,\mu\text{g/ml}) was added. Uptake of $^3$H-CH was measured up to 30 min. The reaction was stopped by chilling the incubated mixture at the end of incubation periods. Mitochondria were recovered by centrifugation and were washed 3 times using 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA.

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 Triton X-100 : toluene (1:2 v/v) (24) and counted in a Beckman LS-100 liquid scintillation spectrometer at 37\% efficiency.

Protein determination was done by Lowry's method (25).

\textbf{Determination of mitochondrial protein content:}

Mitochondria were washed three times and, therefore, could not be recovered quantitatively because of operational losses. Hence, method of Gross (26) was used to determine the mitochondrial total protein content in a given weight of tissue. This method entails the estimation of succinate dehydrogenase activity per mg protein in mitochondria, as well as of their total enzyme activity in whole homogenate. The total mitochondrial protein content is derived by rule of three computation. Succinate dehydrogenase activity was determined by the method of Caplan and Greenwalt (27).
Oxidative phosphorylation:

ADP/O ratio and respiratory control index (RCI) were determined in a Gilson Oxygraph model KM (Gilson Medical Electronics, Middleton, Wis., U.S.A.) using succinate as a substrate. The reaction mixture consisted of 16 mM potassium phosphate buffer, pH 7.4, 38 mM NaCl, 40 mM KCl, 12 mM KF, 6 mM MgCl₂, 10 mM succinate and 4 - 5 mg of mitochondrial proteins in a total volume of 2.0 ml (28). The measurements of respiration rates were carried out at 25°C.

A small aliquot of ADP (20 μl, 0.2 - 0.4 μmoles) was added from time to time and rates of respiration in presence of added ADP and after its depletion were determined. ADP/O ratios and RCI were calculated as described by Chance and Williams (29).

Determination of amino acids pool:

Proteins were precipitated with 10% TCA, centrifuged and washed once with 10% TCA. The supernatant and washings were pooled and were extracted three times with ether to remove TCA. The TCA soluble material was estimated by ninhydrin assay according to Alberti and Bartley (30).

SDS-polyacrylamide gel electrophoresis:

SDS-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (31) with some modifications. Details are as described in Section II.1.
RESULTS

In vivo effects of cycloheximide on DL(1-14C) leucine incorporation:

In preliminary studies, effects of CH on microsomal and mitochondrial protein synthesis in vivo were examined. In these experiments, the animals received 0.2 mg CH/100 g body weight (17 - 19). 20 μCi of DL(1-14C) leucine/100 g body weight was injected at different time intervals as indicated in the Table 1. The animals were killed one hr later and radioactivity in the protein was determined. The results in Table 1 show that CH inhibited microsomal as well as mitochondrial protein synthesis to the extent of 85% and 90%, respectively by the end of one hr. The inhibition increased at two hr, being about 95% in both the systems and declined thereafter. The inhibition decreased by 8 hr and no inhibition was evident at the end of 48 hr which may probably be due to detoxification of the drug. At all intervals studied, inhibition of mitochondrial and microsomal protein synthesis was almost equal.

Table 2 shows the effect of CH concentration on in vivo mitochondrial and microsomal protein synthesis. DL(1-14C) leucine was injected (IP) 2 hr after CH administration and animals were killed one hr later. CH 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
Table 1

**EFFECT OF CYCLOHEXIMIDE IN VIVO ON DL(1-\(^{14}\)C) LEUCINE INCORPORATION**

<table>
<thead>
<tr>
<th>Time hr</th>
<th>Specific radioactivity (cpm/mg of protein)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenate</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>0</td>
<td>1692</td>
<td>814</td>
</tr>
<tr>
<td>1</td>
<td>152</td>
<td>122</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>738</td>
<td>203</td>
</tr>
<tr>
<td>48</td>
<td>1845</td>
<td>786</td>
</tr>
</tbody>
</table>

Rats received 20 \(\mu\)Ci of DL(1-\(^{14}\)C) leucine/100 g body weight intraperitoneally at different time intervals after CH (0.2 mg/100 g body weight) administration (IP) and were killed one hour later. Isolation of subcellular fractions and method for counting of radioactivity are as detailed in the text.
Table 2

EFFECT OF CYCLOHEXIMIDE CONCENTRATION ON DL(1⁻¹⁴C) LEUCINE INCORPORATION IN VIVO

<table>
<thead>
<tr>
<th>Cycloheximide (mg/100 g.b.wt.)</th>
<th>Specific radioactivity (cpm/mg of protein)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenate</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>0</td>
<td>1692</td>
<td>814</td>
</tr>
<tr>
<td>0.025</td>
<td>1502</td>
<td>719</td>
</tr>
<tr>
<td>0.050</td>
<td>884</td>
<td>404</td>
</tr>
<tr>
<td>0.100</td>
<td>-</td>
<td>207</td>
</tr>
<tr>
<td>0.150</td>
<td>112</td>
<td>47</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
<td>20</td>
</tr>
</tbody>
</table>

Leucine-1⁻¹⁴C (20 μCi/100 g.b.wt.) was injected 2 hour after CH administration and animals were killed one hour later. Other details are as in Table 1.
fact, the inhibition of mitochondrial protein synthesis was always slightly higher as compared to microsomes. Administration of CH (1 - 5 mg/100 g body weight) for 2 hr practically resulted in stoppage of total cellular protein synthesis including that of mitochondria.

In the next set of experiments, the effect of CH was examined at shorter time intervals. CH (2 mg/100 g body weight) was administered simultaneously with label (20 µCi DL(1-14C) leucine/100 g body weight) and the results are shown in Figure 1. It was noted that under these conditions about 10% mitochondrial protein synthesis is CH-resistant, whereas complete inhibition of microsomal protein synthesis was observed within 15 - 20 minutes.

Table 3 gives the effects of shorter time administration of CH (2 mg/100 g body weight) on mitochondrial and microsomal protein synthesis. In these experiments, after 15 min of CH (2 mg/100 g body weight) administration, DL(1-14C) leucine incorporation was studied for 2, 5 and 15 minutes. It can be seen that when incorporation was examined for shorter intervals (2 - 15 min), 8 - 10% CH resistant mitochondrial protein synthesis was observed (Table 3). However, as studied earlier (Table 2), when the animals were first administered with CH and DL(1-14C) leucine incorporation was examined after 2 hr of CH treatment, inhibition of total cellular protein synthesis, including mitochondrial, was noted. It thus became apparent that the short term (Fig. 1 and Table 3) and long-term (Table 2) effects of CH on mitochondrial protein synthesis
Fig. 1. Effect of CH administration on incorporation in vivo of D L(1-14C) leucine by mitochondria and microsomes. Animals were injected (i.p.) with 7 mg of CH and 20 μC of D L(1-14C) leucine/100 g., weight simultaneously and sacrificed after different time intervals. Other details are as described in the text.
### Table 3

**EFFECT OF SHORT-TERM ADMINISTRATION OF CYCLOHEXIMIDE IN VIVO ON DL(1-\(^{14}\)C) LEUCINE INCORPORATION**

<table>
<thead>
<tr>
<th>Time after label administration (min)</th>
<th>Inhibition (%)</th>
<th>Homogenate</th>
<th>Mitochondria</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>88</td>
<td>83.5</td>
<td>89.5</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>90</td>
<td>87.5</td>
<td>92</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>95</td>
<td>89</td>
<td>96</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>96</td>
<td>90</td>
<td>96</td>
</tr>
</tbody>
</table>

Rats received 20 \(\mu\)Ci of DL(1-\(^{14}\)C) leucine/100 g body weight after 15 minutes of CH (2 mg/100 g body weight) administration and were sacrificed at different time intervals as indicated. Other details are as in Table 1.
in vivo were dissociable. While the short term effect shows CH-resistant mitochondrial protein synthesis which amounts to 8 to 10%, the long term effects brings about inhibition of total cellular protein synthesis including that of mitochondria.

Effect of cycloheximide administration on in vitro mitochondrial protein synthesis:

To examine whether the observed in vivo inhibition of mitochondrial protein synthesis is also reflected under in vitro conditions, protein synthetic ability of mitochondria isolated from CH administered (2 mg/100 g body weight) animals was studied and results are given in Table 4. Mitochondria isolated from CH-treated animals show practically no decrease in their in vitro protein synthetic ability up to about 20 min; by the end of 40 min, about 60% inhibition is observed which increases to about 75% by 2 hr. Since the in vitro protein synthesizing ability is a measure of the truly mitochondrial protein synthesis, these results, taken together with those outlined in Tables 1 and 2, clearly substantiate the inference that at long-term intervals, CH indeed inhibits the protein synthesizing ability of mitochondria.

The observed inhibition of mitochondrial protein synthesis in vivo as well as in vitro after CH treatment may be due to several reasons. These include: i) interference with energy metabolism of mitochondria; ii) alteration of amino acid pool size; iii) inhibition of mitochondrial protein synthesis per se by CH; and
Table 4

EFFECT OF CYCLOHEXIMIDE ADMINISTRATION ON IN VITRO MITOCHONDRIAL PROTEIN SYNTHESIS

<table>
<thead>
<tr>
<th>Time after CH administration (min)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+ 9.1</td>
</tr>
<tr>
<td>10</td>
<td>+ 6.8</td>
</tr>
<tr>
<td>15</td>
<td>+ 1.6</td>
</tr>
<tr>
<td>20</td>
<td>10.0</td>
</tr>
<tr>
<td>40</td>
<td>61.4</td>
</tr>
<tr>
<td>60</td>
<td>71.3</td>
</tr>
<tr>
<td>120</td>
<td>74.7</td>
</tr>
</tbody>
</table>

Rats received 2 mg CH/100 g body weight intraperitoneally and were killed at different time intervals as indicated in the table. In vitro incorporation was carried out using three times washed isolated mitochondria as described in the text. Incorporation by mitochondria isolated from control animals (0 time) was 271 ± 22.1 cpm/mg of protein/hr. + sign indicates stimulation in the in vitro incorporation activity.
iv) interdependence of mitochondrial and microsomal protein synthesis. The first three possibilities were tested in the subsequent studies. The alternate possibility of interdependence is considered under 'Discussion'.

Mitochondrial oxidative metabolism:

Table 5 gives the ADP/O ratio and RCI for mitochondria isolated from normal and CH treated animals. CH concentrations (2 and 5 mg/100 g body weight) which almost completely inhibit total cellular protein synthesis (Table 2), do not show any effect on ADP/O ratios, rate of oxygen consumption in presence and absence of ADP and the RCI. Thus, it appears that even at these high concentrations, CH does not interfere with energy metabolism although its effect on mitochondrial protein synthesis is apparent at much lower concentrations (Tables 1 and 2).

Amino acids pool size:

Results of amino acids pool size and mitochondrial protein content in control and CH-treated animals (2 mg CH/100 g body weight for 1 hr) are given in Table 6. It is difficult to estimate the amino acids pool size in mitochondria as it may be expected that amino acids pool will decrease after washings. Hence, amino acids in homogenate were estimated and this has been taken to represent changes in the amino acids pool of mitochondria. Administration of CH (2 mg/100 g body weight) resulted in about
Table 5

EFFECT OF CYCLOHEXIMIDE IN VIVO ON MITOCHONDRIAL OXIDATIVE METABOLISM

<table>
<thead>
<tr>
<th>Cycloheximide (mg/100 g b.wt.)</th>
<th>ADP/O ratio</th>
<th>Rate of oxidation m/u moles of O$_2$/min per mg of protein</th>
<th>RCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ ADP</td>
<td>- ADP</td>
</tr>
<tr>
<td>0</td>
<td>1.58 ± 0.182</td>
<td>56.0 ± 3.4</td>
<td>14.6 ± 0.34</td>
</tr>
<tr>
<td>2</td>
<td>1.50 ± 0.03</td>
<td>64.8 ± 3.15</td>
<td>15.3 ± 0.10</td>
</tr>
<tr>
<td>5</td>
<td>1.53 ± 0.12</td>
<td>55.5 ± 3.21</td>
<td>14.8 ± 0.29</td>
</tr>
</tbody>
</table>

Animals were sacrificed 2 hrs after CH administration (2 mg/100 g body weight). Rates of respiration in presence and absence of ADP were determined using succinate as substrate. Calculation of ADP/O ratio and RCI was according to Chance and Williams (29). Results are given as mean of five independent experiments ± SEM.
Table 6

EFFECT OF CYCLOHEXIMIDE ADMINISTRATION ON AMINO ACIDS POOL SIZE

<table>
<thead>
<tr>
<th>Group</th>
<th>Amino acids (μg/g liver)</th>
<th>Mitochondrial proteins (mg/g liver)</th>
<th>Specific radioactivity of DL(1-14C) leucine cpm/mg of Mitochondrial proteins (a)</th>
<th>Specific radioactivity of Amino acid pool (b)</th>
<th>Relative Sp. activity (a/b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5930</td>
<td>41.8</td>
<td>814.0</td>
<td>4.4</td>
<td>185.0</td>
</tr>
<tr>
<td>+ CH</td>
<td>7100</td>
<td>41.2</td>
<td>31.0</td>
<td>9.1</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Animals were intraperitoneally administered 2 mg CH/100 g body weight one hour prior to the injection of 20μCi DL(1-14C) leucine/100 g body weight and they were sacrificed one hour later. Control animals received only DL(1-14C) leucine. Amino acids were estimated by 'ninhydrin' method (30). Mitochondrial protein contents were determined by the method of Gross (26) as detailed in the text.
20% increase in the pool size and the specific radioactivity of the pool became about 2 fold compared to the control. On the other hand, the incorporation of DL(1-\(^{14}\)C) leucine into mitochondrial proteins *in vivo* was inhibited to the extent of 96% (Table 6), in spite of the increase in its specific radioactivity. This is reflected in a tremendous decrease in the relative specific activity from 185 to 3.4. Mitochondrial protein content of liver, however, was unaffected under these conditions, as is to be expected. These results therefore rule out the possibility that the observed effect may be due to alteration in the pool size. The possibility of CH inhibiting mitochondrial protein synthesis *per se* was then examined next.

**Inhibition of mitochondrial protein synthesis *per se***:

The foregoing studies (Tables 1, 2 and 4) indicate that CH may indeed be affecting truly mitochondrial protein synthesis. On the other hand, addition of CH *in vitro* does not inhibit the protein synthesizing activity of isolated mitochondria (Section II.1 and Table 7 of this section). To examine the possibility whether such a differential effect of CH under *in vivo* and *in vitro* conditions is due to permeability barrier across the mitochondrial membranes, effect of CH addition to sonicated mitochondria on their protein synthesizing activity was tested.

Results in Table 7 show that sonication by itself resulted in about 55% decrease in the *in vitro* incorporation by
Table 7

EFFECT OF CYCLOHEXIMIDE ON DL(1-14C) LEUCINE INCORPORATION IN VITRO BY SONICATED MITOCHONDRIA

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Specific radioactivity (cpm/mg of protein)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>261</td>
<td>-</td>
</tr>
<tr>
<td>Control + CH</td>
<td>243</td>
<td>-</td>
</tr>
<tr>
<td>Sonicated</td>
<td>117</td>
<td>55</td>
</tr>
<tr>
<td>Sonicated + CH</td>
<td>115</td>
<td>56</td>
</tr>
<tr>
<td>Sonicated in presence of CH</td>
<td>147</td>
<td>44</td>
</tr>
</tbody>
</table>

Mitochondria were sonicated in absence or presence of CH to give concentration of 500 μg/ml in the final reaction mixture. Sonication was carried out at 0 - 4°C in a MSE ultrasonic disintegrator at 1.8 KC for 2 min (30 sec, 4 times). Wherever indicated, CH was added at a final concentration of 500 μg/ml. In vitro incorporation was carried out as described in the text at 30°C for 1 hr.
mitochondria. This may be because of loss of structural integrity. However, addition of CH to sonicated mitochondria and/or sonication of mitochondria in presence of CH did not significantly affect their in vitro incorporation ability. Probably, under these conditions, CH fails to reach the site of action and a carrier may be necessary for its transfer. In order to verify this possibility, experiments were carried out using 105,000 x g supernatant to see if this contained a carrier which transports CH across mitochondrial membranes.

Addition of CH along with cell sap or addition of cell sap from CH treated animals, also did not affect the in vitro incorporation (Table 8). This may mean that CH fails to reach the site of action under in vitro conditions probably because the carrier may function only in vivo; alternately, CH may be entering mitochondria in vivo independent of a hypothetical carrier. These possibilities were further explored by following the in vitro and in vivo uptake of \(^3\text{H}\)-CH.

Studies on in vitro uptake of \(^3\text{H}\)-CH by mitochondria, in the presence or absence of cold CH (500 \(\mu\)g/ml) are summarised in Tables 9 and 10, respectively. Under these conditions, negligible amount of radioactivity is associated with mitochondria, the activity being practically the same in the presence (Table 10) and in the absence (Table 9) of the carrier CH. This probably indicates a simple tritium exchange. This possibility is further supported by the fact that the exchange level at 0 hr and 30 min shows only a marginal difference (Tables 9 and 10). These results therefore substantiate the view that CH does not enter mitochondria under in vitro conditions.
Table 8

EFFECT OF CELL SAP ON DL(1-14C) LEUCINE INCORPORATION IN VITRO BY MITOCHONDRIA

<table>
<thead>
<tr>
<th>Additions</th>
<th>Specific radioactivity (cpm/mg of protein)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>253</td>
<td>-</td>
</tr>
<tr>
<td>+ 0.2 ml cell sap (control rat)</td>
<td>248</td>
<td>2.00</td>
</tr>
<tr>
<td>+ 0.2 ml cell sap (CH-rat)</td>
<td>254</td>
<td>0.00</td>
</tr>
<tr>
<td>+ 0.5 ml cell sap (CH-rat)</td>
<td>250</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Animals were injected with 2 mg CH/100 g body weight one hour prior to sacrifice. In vitro incorporation of DL(1-14C) leucine into mitochondrial proteins was carried out for one hour at 30°C.
Table 9

IN VITRO UPTAKE OF $^3$H-CYCLOHEXIMIDE BY ISOLATED MITOCHONDRIA

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Specific radioactivity (cpm/mg of protein)</th>
<th>Uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>143.0 ± 7.20</td>
<td>0.197</td>
</tr>
<tr>
<td>5</td>
<td>225.0 ± 17.20</td>
<td>0.219</td>
</tr>
<tr>
<td>15</td>
<td>253.0 ± 5.37</td>
<td>0.255</td>
</tr>
<tr>
<td>30</td>
<td>309.0 ± 6.9</td>
<td>0.351</td>
</tr>
</tbody>
</table>

1 $\mu$Ci of $^{3}$H$^{14}$-leucine was added to the mitochondrial protein synthesizing system in vitro as indicated in the text except 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 text.
<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Specific radioactivity (cpm/mg of protein)</th>
<th>Uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>198.0 ± 2.8</td>
<td>0.226</td>
</tr>
<tr>
<td>5</td>
<td>316.0 ± 8.6</td>
<td>0.296</td>
</tr>
<tr>
<td>15</td>
<td>356.0 ± 16.0</td>
<td>0.311</td>
</tr>
<tr>
<td>30</td>
<td>386.0 ± 13.1</td>
<td>0.333</td>
</tr>
</tbody>
</table>

*In vitro* uptake of $^3$H-Cycloheximide was carried out as indicated in Table 9 except that 500 μg of cold Cycloheximide was added along with 1 μCi of $^3$H-Cycloheximide. Other details are as in Table 9.
The possibility of CH entering into the mitochondria under *in vivo* conditions was then investigated by studying subcellular distribution of injected $^3$H-CH. In these studies, animals were sacrificed one hr after administration of 30 μCi of $^3$H-CH along with 2 mg cold CH/100 g body weight. The results are shown in Table 11. Though most of the label (75%) is found to be present in cell sap, about 4% and 8% of it is associated with mitochondria and microsomes, respectively. These results thus indicate that CH may be entering into mitochondria under *in vivo* conditions.

In preliminary studies, association of $^3$H-CH with cyto- and mito-ribosomes was examined. These studies revealed that significant radioactivity was associated with both, cyto-ribosomes and mito-ribosomes (Table 11). The specific activities, however, were less than those of microsomes and mitochondria. This is understandable in view of the fact that fractionation of ribosomes involves 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 cyto-ribosomes and mito-ribosomes (Table 11).

**DISCUSSION**

The inhibitory effect of CH on ribosomal protein synthesis is well recognized (2, 4). Recently, however, it is becoming increasingly evident that besides its effect on ribosomal protein
### Table 11

ASSOCIATION OF $^{3}$H-CYCLOHEXIMIDE WITH SUBCELLULAR FRACTIONS OF RAT LIVER

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Specific radioactivity (cpm/mg of protein)</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>118.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>150.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Microsomes</td>
<td>703.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Cell sap</td>
<td>9058.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Mito-ribosomes</td>
<td>97.0</td>
<td>–</td>
</tr>
<tr>
<td>Cyto-ribosomes</td>
<td>412.0</td>
<td>–</td>
</tr>
</tbody>
</table>

Animals received (I.P) 30 µCI of $^{3}$H-CH (+ 2 mg cold CH)/100 g body weight one hour prior to sacrifice. Mito-ribosomes and cyto-ribosomes were isolated as described in the text. Radioactivity was counted in a toluene-triton scintillator system.
synthesis, CH may exert multiple effects. Thus, it has been reported that CH inhibits synthesis of 16S ribosomal RNA in L cells (32), and of nuclear RNA in He La cells (33) and in rat liver (34). In Chlorella, CH is known to inhibit selectively nuclear DNA synthesis (35). Inhibition of both mitochondrial as well as cytoplasmic ribosomal RNA species in Tetrahymena by CH has been reported (16). Results presented in this section bring out yet another aspect of CH action.

It is clear that the short-term and long-term effects of CH on mitochondrial protein synthesis are dissociable (Tables 2 and 3; Fig.1). After short-term treatment with CH, up to 8 - 10% of mitochondrial protein synthesis is discernible (Fig.1, Table 3), an observation consistent with that of several other workers (2, 17 - 19). 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 CH leads to practically complete stoppage of truly mitochondrial protein synthesis, as is evident from their in vitro protein synthesizing activity (Table 4). The results, therefore, besides supporting the observations that mitochondria can synthesize only 8 - 10% of their proteins (2 - 5), also point to the complexities in the use of CH. 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' (36).

Inhibition of mitochondrial protein synthesis after CH treatment has also been reported recently in lower eukaryotes. Ibrahim et al. observed a 50% decrease in the in vitro protein synthesis by yeast mitochondria (37). In their experiments, partially derepressed cells were grown in CH for 3 hr followed by transfer to fresh medium for 1 - 2 hr prior to the isolation of mitochondria. In Tetrahymena, even a 5 min incubation with CH 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 (16).

The possibility of CH effect on truly mitochondrial protein synthesis being due to interference with energy metabolism or arising as a result of changes in the amino acids pool seems unlikely in view of the data presented (Tables 5 and 6, respectively). Under in vitro conditions, CH did not enter the site of action, even in the presence of added cell sap (Table 7). However, when injected in vivo, sizable amounts of CH were found to be associated with mitochondria and microsomes (4 and 8%, respectively). Interestingly, significant activity is found to be associated with mitochondrial and cyto-ribosomes (Table 11). Contrary to the observations reported here, Ashwell and Work (8) found that a sizable amount of CH was associated with mitochondria after in vitro incubation with the antibiotic. However, it should be pointed out that no
experimental details were provided and it is likely that CH was
losely associated with mitochondria. It would therefore seem
that, under in vivo conditions, CH is able to reach site of action -
presumably mito-ribosomes. These studies, however, do not indicate
whether the process of CH association with mitochondria in vivo
involves a carrier or is independent of it. It is also likely
that $^{3}H$-CH may be entering into mitochondria nonspecifically and
without having any inhibitory action per se (38) 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 CH did not inhibit mitochondrial
protein synthesis up to about 40 min after its administration.
This suggests dependence of mitochondrial protein synthesis on
cytoplasmically synthesized partner proteins. Such an inter­
dependence of the two protein synthesizing systems during biogenesis
of mitochondrial enzymes has been emphasized by Schatz and Mason
in a recent review (5). It may be that there is enough of partner
proteins to last for up to 40 min after CH administration, when
the effect of CH becomes apparent. In other words, some cyto­
plasmically synthesized product(s) required for continuation of
mitochondrial protein synthesis might be short-lived and getting
exhausted within 40 min or so. The possible short-lived products
could be protein in nature, involved in mitochondrial protein
synthesizing machinery.
With a view to find out this possibility whether the synthesis of partner proteins is inhibited after CH treatment, SDS-gel electrophoresis was carried out using various subcellular components such as whole mitochondria, mitochondrial membranes, microsomes and cell sap. In preliminary studies, SDS-polyacrylamide gel electrophoresis failed to detect the possible short-lived protein product(s). No differences were observed in the number of peaks as well as peak height in the protein profiles obtained from normal and CH-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 quantities and escape detection or 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 CH, the hydrophobic proteins - products of mitochondrial protein synthesis - could not leave the mito-ribosomes unless they are pulled off by corresponding cytoplasmically synthesized subunit proteins and remain attached to mito-ribosomes thereby making them unavailable for further protein synthesis has also to be considered (5).

Alternately, the short-lived product might be a messenger-RNA, a translation product of mitochondrial DNA (39), whose half-life has been shown to be about 15 min in yeast (40). It could also be a ribosomal RNA, since, CH is known to inhibit the synthesis of both mitochondrial as well as cytoplasmic ribosomal
Fig. 2. Typical SDS-polyacrylamide gel photographs showing polypeptide patterns obtained from mitochondria (M), mitochondrial membranes (Mm), supernatant (S) and (Ms) microsomes from normal (N) and cycloheximide (CH) treated rats. Other details are as described in the text.
RNA (r-RNA) species in Tetrahymena (16). These possibilities, however, have not been verified experimentally and need further exploration.

It is also likely that under the in vivo conditions a metabolite of CH may be formed which could be responsible for the observed inhibition of the mitochondrial protein synthesis. To elucidate this possibility, experiments were carried out in which CH was added to mitochondria-free supernatant and effect of this system (mitochondria-free supernatant plus CH) was studied on in vitro protein synthesizing ability of mitochondria. Results of these studies revealed that there was no inhibition, indicating that possibly a metabolite of CH may not be responsible for the inhibition of mitochondrial protein synthesis.

Although in the present investigations the reason for inhibition of mitochondrial protein synthesis by CH could not be pin-pointed, the results have ruled out interference with energy metabolism and amino acids pool as possible causes. Besides, these studies have clearly dissociated short and long-term effects of CH, thus emphasizing the complexity of inhibitor action as pointed out by Kroon and Arendzen (36). Needless to say, further exploration of CH effects on mitochondrial RNA synthesis would help to shed more light on mechanism of inhibition of mitochondrial protein synthesis by CH.
SUMMARY

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

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

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

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

5. CH did not enter mitochondria or sonic preparation under \textit{in vitro} conditions. On the other hand, after administration of $^3$H-CH significant quantities of $^3$H-CH were found to be associated with mitochondria and mito-ribosomes.

6. These results indicated that CH reached the site of action in mitochondria under \textit{in vivo} conditions but was unable to do so \textit{in vitro}. 
7. The results have been discussed to elucidate the possible mechanisms involved in the inhibition of truly mitochondrial protein synthesis by CH.
REFERENCES