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Synthesis of Rat Liver Mitochondrial Proteins after the Administration of a Nonlethal Dose of Cycloheximide (40334)

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Since the earliest report that mitochondria incorporate labeled amino acids into polypeptides *in vitro* (1), major efforts have been directed toward the isolation and characterization of mitochondrially synthesized polypeptides (2–4). From carefully designed *in vitro* systems, a few of the products of mitochondrial protein synthesis have been identified. Most of these proteins were low molecular weight, hydrophobic, chloroform–methanol-extractable inner-membrane proteins (5–12). Understanding of the mitochondrial protein synthetic system has also been aided by the use of cycloheximide and chloramphenicol in *in vitro* and *in vivo* systems (13–16). Most often studies with cycloheximide *in vivo* were carried out with lethal doses (10–100 mg/kg) which cause irreversible metabolic and cellular changes (17–20). Thus, it is difficult to distinguish normal physiological events from toxic effects of the antibiotic. With a nonlethal dose of cycloheximide (2 mg/kg) we have demonstrated that the incorporation of radioactive label into low molecular weight mitochondrial trichloroacetic acid-insoluble material is stimulated in the absence of cytoplasmic protein synthesis whereas the synthesis and/or incorporation of large cytoplasmically synthesized proteins into mitochondria requires the presence of cytoplasmic protein synthesis. The differential labeling patterns of these mitochondrial proteins presented in this report extend the cooperative nature of cytoribosomal and mitoribosomal proteosynthetic systems observed with mammalian cells in culture to the intact rat.

Materials and Methods. The experiments were performed on male Wistar rats (210 ± 10 g). Maintenance and treatment of the animals were carried out as previously described (21). Mitochondria (3× washed) of the control and cycloheximide-treated rat liver were isolated in separate tubes under identical conditions according to the procedure described (15).

Extractions of mitochondrial proteins were performed with 0.05 *M* Na_2HPO_4 buffer at various pH values containing 0.05 *M* β-mercaptoethanol. The specific pH values were produced by the dropwise addition of either sodium hydroxide (5 *N*) or concentrated phosphoric acid to 0.05 *M* β-mercaptoethanol in 0.05 *M* Na_2PO_4. Proteins were extracted sequentially with buffers of decreasing pH values (7.5, 6.5, 5.5, 4.5) and with buffers of increasing pH values (8.5, 9.5, 10.5, 11.5). For each extraction, the pellet was stirred at 4° for 10 min in the appropriate buffer and then centrifuged for 30 min at 27,000 *g*. The final pellet from each series (4.5P and 11.5P) was solubilized in 1% SDS/0.1 *M* β-mercaptoethanol/10 m*M* Tris–HCl buffer, pH 7.5, at 95 to 100° and dialyzed against 0.01 *M* sodium phosphate buffer (pH 7.2) containing 0.01 *M* β-mercaptoethanol and 0.1% SDS. After dialysis, separation of proteins was carried out immediately on 10% polyacrylamide gels containing 0.1% SDS according to the procedure of Dehlinger and Schimke (22).

For amino acid incorporation, groups of four animals were injected ip with [^{3}H]leucine (40–60 Ci/mmole) or ^{3}H-labeled protein hydrolysate (mixture 3130-08, Schwarz/Mann) 1 hr before sacrifice. Samples containing radioactivity were determined as described by Ch'ih *et al.* (23). Protein was determined by the method of Lowry *et al.* (24). To eliminate interfering substances such as β-mercaptoethanol, all samples were treated with 10% trichloroacetic acid and the precipitates were redissolved in 0.1 *N* NaOH before protein determination.

Results and discussion. The incorporation of [^{3}H]leucine or ^{3}H-labeled protein hydrolysate into liver mitochondria and submitochondrial fractions during cycloheximide treatment (2 mg/kg body wt) were similar to rat kidney (25), with an inhibition at 2 hr and stimulation at 24 hr (26). Prior to the determination of ^{3}H-labeled protein hydrolysate radioactivity in the gel slices of the various

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submitochondrial protein fractions separated by the SDS–PAGE system, absorbance profiles were obtained and showed no differences between control and treated animals. A typical gel scan of the insoluble fractions is shown in Fig. 1.

As shown in Fig. 2, the major peaks of the radioactivity profiles from the insoluble protein fractions of the control corresponded well with their respective absorbance patterns (Fig. 1). In the insoluble mitochondrial protein fractions (pH 11.5 and 4.5) from animals treated for 2 hr there was no inhibition of label incorporation into the low molecular weight region. In contrast, during cycloheximide-stimulated synthesis (24 hr), there were peaks of equal or, in most instances, greater incorporation than in the corresponding control fractions. As to the high molecular weight region (Fig. 2), incorporation of label into these polypeptides was significantly inhibited in the absence of cytoplasmic protein synthesis. These results obtained from *in vivo* experiments demonstrate (i) that sublethal levels of cycloheximide will transiently suppress synthesis and/or incorporation of large cytoplasmically synthesized proteins into mitochondria and (ii) synthesis and/or incorporation of this material seems to be stimulated during the recovery phase.

As to the incorporation of radioactive label into the soluble fractions (Figs. 3 and 4), labeling of high molecular weight polypeptides was inhibited at 2 hr after cycloheximide treatment and stimulated at 24 hr. Radioactivity exhibited by the materials migrated to

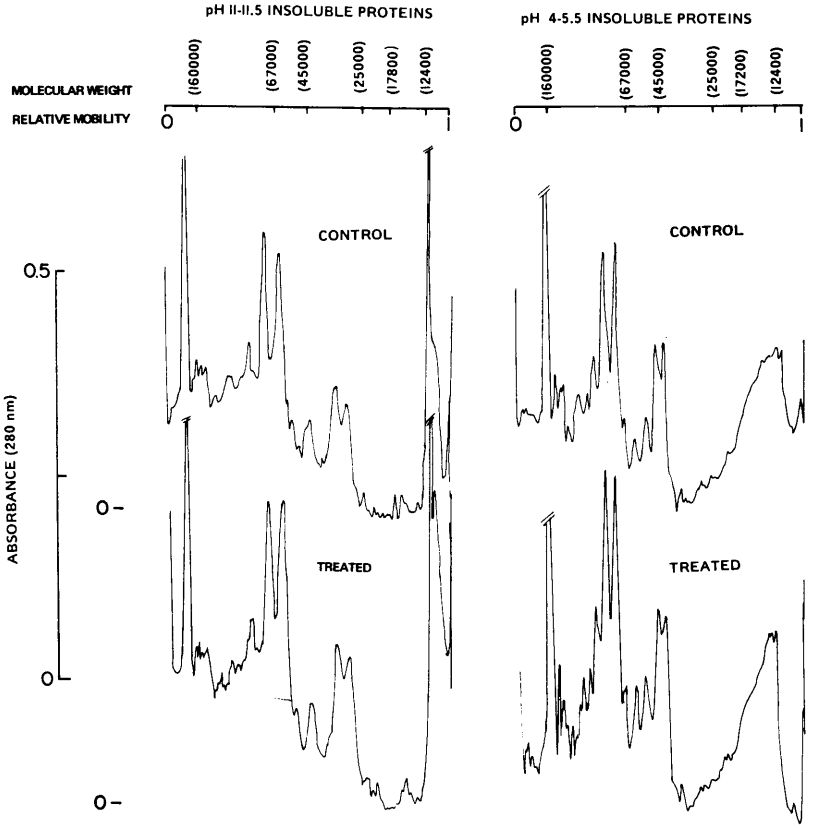

FIG. 1. Electrophoretic distribution of insoluble polypeptides isolated from normal and cycloheximide-treated mitochondria. Isolation of mitochondria and submitochondrial protein fractions and method for SDS–polyacrylamide gel electrophoresis are as detailed in the text. Proteins (75 μg) were separated at 3 mA/gel for 90 min in the anodal direction at room temperature. Molecular weight markers were: γ-globulin (160,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), myoglobin (17,000), and cytochrome *c* (12,400). Relative mobilities of the standard proteins when plotted against log(molecular weight) gave a linear relationship. The correlation coefficient was 0.995, which was highly significant. Standard proteins were run as markers with each set of gels and a variation of molecular weight of 2000 was observed among the various runs.

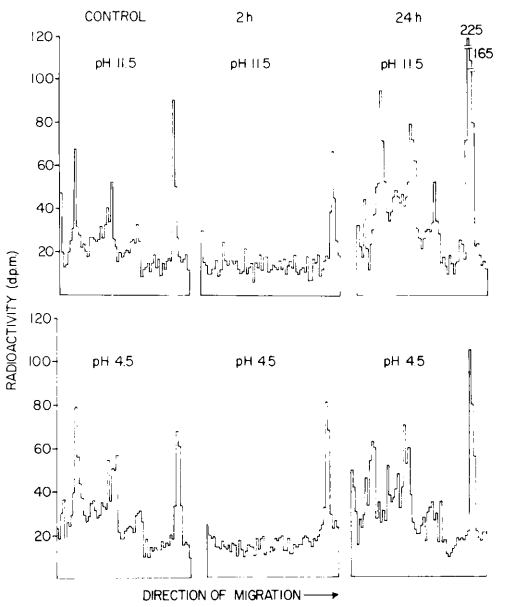

FIG. 2. Radioactivity profiles of mitochondrial insoluble proteins separated by SDS–polyacrylamide gel electrophoresis. ^{3}H-labeled protein hydrolysate (4 mCi/kg body wt) was given 60 min prior to sacrifice. Gels were sliced into 1.25 ± 0.25 mm slices by using the DE 113 horizontal gel slicer (Hoefer Scientific Instruments); each slice was solubilized in 0.5 ml of NCS solubilizer at 50° for 16 hr, and 10 ml of scintillation cocktail (Yorktown Research) was added before radioactivity counting. Protein (75 μg) was applied to the gel in each case; for other details see the legend to Fig. 1.

the front of the gel (low molecular weight region), however, showed two- to fivefold stimulation in pH 5.5, 6.5, 7.5, and 8.5 fractions during both the inhibitory and recovery phase. The labeled material in the low molecular weight range (less than 12,000) present in the aqueous extracts may represent materials other than polypeptides (i.e., aminoacyl-tRNA, or phospholipids) and there it is not possible to assess the actual contribution of mitochondrial protein synthesis to this region of the radioactivity profile.

Results presented in this paper extended the findings with *in vitro* and cultured cell systems into intact animals and suggest that high molecular weight polypeptides are either synthesized by the cytoribosomal system or the formation of functional membrane proteins requires the cooperation of both protein synthetic systems (2–12). Since in our extraction procedure lipid solvents such as chloroform and methanol were avoided, the high molecular weight products observed in the 24-hr treated animals may represent the crosslinked proteolipids of the mitochondrial membrane as discussed by O'Brien (4). Furthermore, the SDS–PAGE separation of the various polypeptides present in submitochondrial fractions was carried out immediately with freshly extracted samples, without storage, avoiding both aggregation and degradation (4); thus, the low molecular weight materials were presumably not the result of proteolysis. The cycloheximide-resistant radioactivity appeared in the low molecular weight region of the SDS gel may suggest that it is the product of mitochondrial protein synthesis because gels were routinely stained for protein and scanned at 550, 280, and 260 nm, and consistent patterns were obtained in all cases. However, the correlation between this material and mitochondrially synthesized polypeptides requires further experimentation.

Employing lethal doses of cycloheximide *in vivo* (5, 7, 11–16), the reversal of inhibition of cytoplasmic protein synthesis and the presence of labeled high molecular weight polypeptides can never be seen because the cytoplasmic protein synthetic system is irreversibly inhibited and the animals die within a few hours (17–20); thus, the use of high cycloheximide doses as well as the use of mitochondria in *in vitro* studies eliminates the coupling between the cytoplasmic and mitochondrial systems, thereby disallowing any effects this relationship may exert on mitochondrial translation products.

In conclusion, the interdependency of mitochondrial and cytoplasmic protein synthetic systems has been demonstrated in lower eucaryotes and cultured mammalian cells. Employing cycloheximide at a nonlethal dose provides a direction for an evaluation of a similar response in the living animal. There is no doubt that coordination between mitochondrion and cell sap involves important regulatory mechanism which may not be easily resolved by the *in vivo* approach, but carefully designed experimentation with whole animals may offer some insights for future investigation in the area of mitochondrial biogenesis.

Summary. Following *in vivo* treatment of

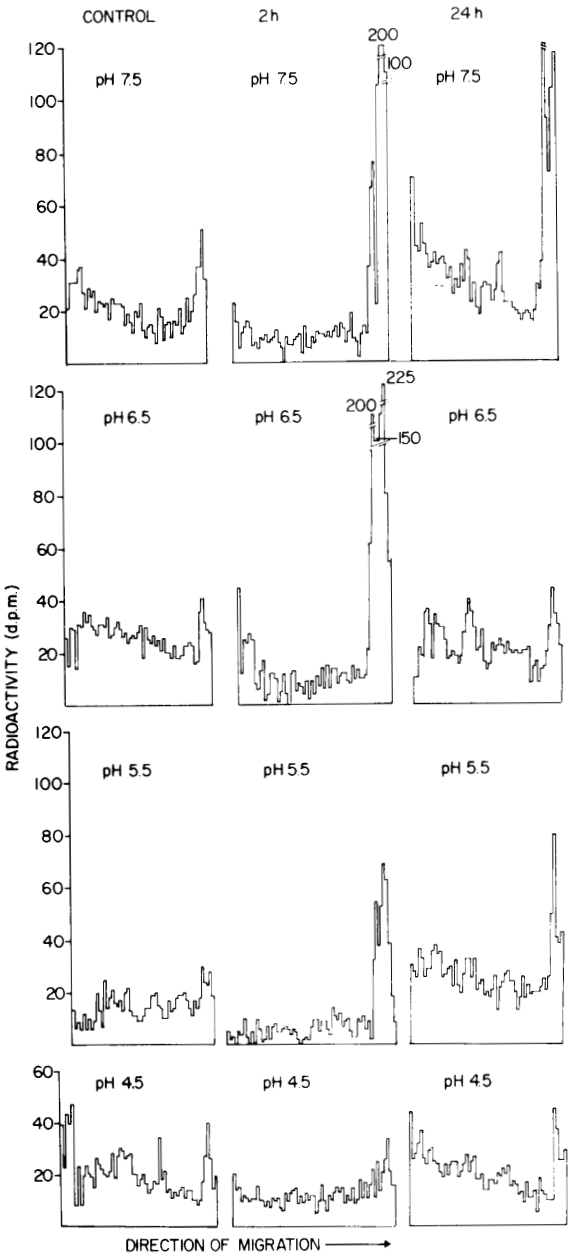

FIG. 3. Radioactivity profiles of soluble proteins extracted with acidic buffers from control and cycloheximide-treated (2 and 24 hr) mitochondria. For details see the legends to Figs. 1 and 2.

rats with a nonlethal dose of cycloheximide (2 mg/kg body wt), analysis of the newly synthesized liver mitochondrial polypeptides by SDS–PAGE system showed: (i) sublethal levels of cycloheximide did transiently suppress synthesis and/or incorporation of large cytoplasmically synthesized proteins into mitochondria; (ii) synthesis and/or incorporation of this material was stimulated during the recovery phase. The differential labeling patterns of these mitochondrial proteins observed *in vivo* during cycloheximide treatment substantiate the cooperative nature of the cytoribosomal protein synthetic system to the

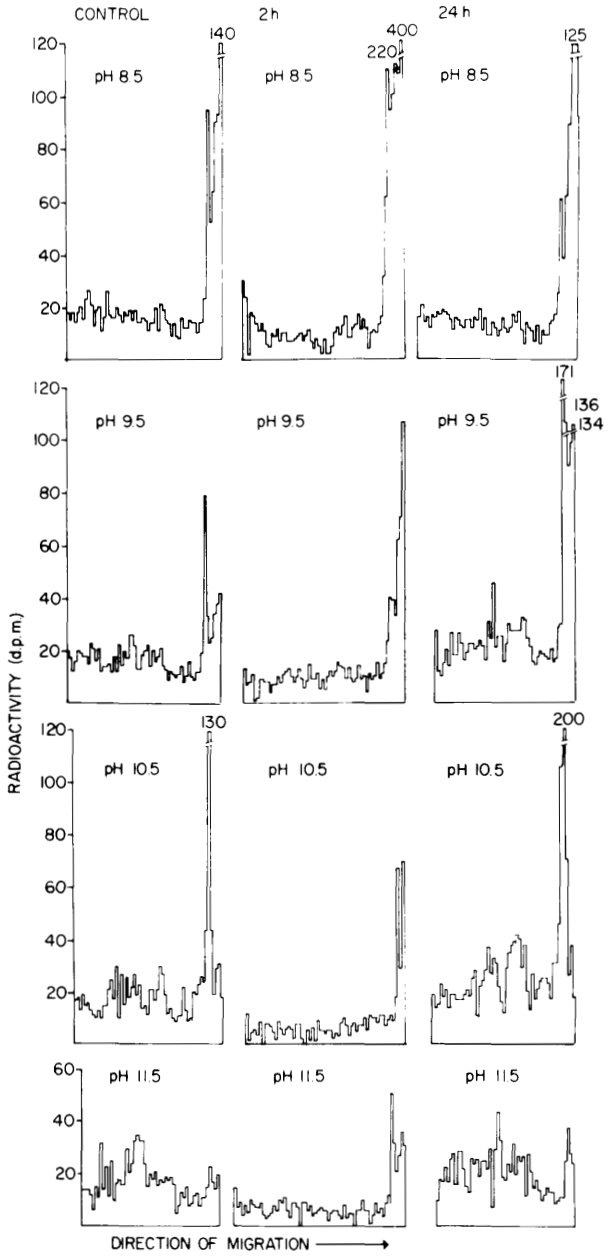

FIG. 4. Radioactivity profiles of soluble proteins extracted with alkaline buffers from control and cycloheximide-treated (2 and 24 hr) mitochondria. For details see the legends to Figs. 1 and 2.

formation of functional mitochondrion observed with mammalian cells in culture.

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