Biogenesis of Mitochondrial Membranes: Biochemical and Morphological Evidence of Two Protein-Synthesizing Systems[1] (34787)

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In vitro studies of isolated mitochondria indicate that the incorporation of amino acids occurs only in certain insoluble proteins of the inner membrane (1), and that this is inhibited by chloramphenicol and not by cycloheximide (2). Whereas, *in vivo* experiments have demonstrated that amino acids are incorporated into the inner and outer membranes, and matrical proteins (3). In view of the striking chemical and functional similarities between the outer mitochondrial membrane and the endoplasmic reticulum, the effects of chloramphenicol and cycloheximide, and the failure of isolated mitochondria to incorporate amino acids into their outer membrane, it has been suggested that in the intact cell the proteins of the outer membrane are synthesized by the endoplasmic reticulum (4). At present direct *in vivo* experimental evidence of the existence of two protein-synthesizing systems, one intramitochondrial, the other extramitochondrial, is lacking.

The present *in vivo* experiments were undertaken in an attempt to establish whether the protein-synthesizing systems responsible for the formation of inner and outer membranes are localized at different sites by their differential sensitivity to chloramphenicol and cycloheximide. The results strongly suggest that inner and outer mitochondrial membrane incorporate labeled leucine under the control of two protein-synthesizing systems; evidence is given that cycloheximide, a well-known inhibitor of endoplasmic protein synthesis, produces an exclusive inhibition of outer membrane synthesis; chloramphenicol on the other hand, causes inhibition of both inner and outer membrane biosynthesis.

Materials and Methods. Male rats weighing 220–250 g were starved for 7 days and subsequently refed for 3 days, maneuvers which have been shown previously to cause quantitative depletion of hepatic mitochondria followed by their restitution (5). The active mitochondriogenesis which takes place during refeeding provides the most suitable metabolic conditions for the study of mitochondrial membrane biosynthesis. Animals were refed Purina Chow pellets supplemented with 2.5% chloramphenicol. Cycloheximide was administered to appropriate animals by daily intraperitoneal injection at a dose of 1.5 mg/kg body weight. Three experimental groups were employed in these studies: animals receiving chloramphenicol alone, cycloheximide alone, and those refed stock diet. On the day of sacrifice, each animal received 100 μCi of ^{3}H-leucine (sp act 2 Ci/mmole) injected intraperitoneally, followed by injection of 1 mg/100 g body weight of unlabeled leucine in saline 25 min later. After 25 min had elapsed the animals were killed, and mitochondria were isolated from livers, special care being taken to reduce microsomal contamination (6). This resulted in a relatively low yield of mitochondria, but the microsomal contamination was reduced to an insignificant level, as proven both by electron microscopy and assay of glucose-6-phosphatase activity. Fractionation of mitochondria into inner and outer membrane was accomplished by a modification of the method of swelling–shrinking followed by sonication and discontinuous density-gradient cen-

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trifugation (7). Purity of the outer and inner membrane subfractions was ascertained by spectrophotometric assays of monoamine-oxidase, for outer membrane, and beta-hydroxybutyric and succinic dehydrogenases for inner membrane. Cross contamination of outer and inner membranes, as calculated by the specific activities of the above-mentioned enzymes in each submitochondrial fraction was always less than 4%. Matrical proteins were isolated from the inner membrane fraction by sonication for 110 sec with a Branson sonifier set at 100 W followed by centrifugation at 120,000*g* for 45 min. Radioactivity of the various fractions was measured by a modification of Beattie's method (8) in a Packard Tri-Carb scintillation counter. Proteins were determined by the Lowry method. The data obtained were analyzed statistically by the one-tailed *t* test. For morphological studies 0.5–1 mm^2 fragments of livers or pellets of mitochondria and submitochondrial fractions were either fixed in 5% buffered glutaraldehyde followed by secondary fixation in 2% osmium tetroxide or fixed in 2% buffered osmium tetroxide. These were dehydrated, embedded in epon, and prepared for electron microscopy in the routine fashion.

Results and Discussion. Table I shows leucine incorporation into the various submitochondrial fractions as well as into whole sonicated mitochondria. It appears that the inner membrane is highly sensitive to chloramphenicol, whereas it is not affected by cycloheximide. The latter, on the contrary, selectively inhibits leucine incorporation into outer membrane by 34% ($p<.005$), as compared to only 0.4% inhibition of the inner membrane incorporation.

In view of the *in vitro* ineffectiveness of cycloheximide on amino acid incorporation by isolated mitochondria, the selective inhibition observed in the present *in vivo* experiment points out that outer membrane biosynthesis is related and dependent on the protein-synthesizing system localized in the endoplasmic reticulum. Moreover, the insensitivity of the inner membrane to cycloheximide shows that the biosynthetic system of

TABLE I. Incorporation of ^{3}H-Leucine into Various Mitochondrial Fractions. Incorporation Expressed as Percentage of Change from Normal Is Shown in Parentheses.

Experiment	Radioactivity of mitochondrial fractions (cpm/mg protein ± SE)				Ratios of incorporation	
	Sonicated mitochondria	Outer membrane	Inner membrane	Matrical proteins	Outer membrane / Inner membrane	Matrical proteins / Inner membrane
Control	1536 ± 302	1983 ± 40	1970 ±182	1452 ± 153	1.022	0.737
Cycloheximide	1606 ± 162 (+4)	1323 ± 111 (—34)	1964 ± 52 (—0.4)	1075 ± 166 (—26)	0.671	0.545
Chloramphenicol	882 ± 25 (—43)	1286 ± 207 (—36)	989 ± 50 (—50)	922 ± 46 (—37)	1.314	0.936

the inner membrane is different from that of outer membrane, and localized within the mitochondrion itself.

The action of chloramphenicol seems to be more complex and less selective than that of cycloheximide. As shown in Table I, chloramphenicol inhibits leucine incorporation into both membranes, although incorporation into inner membrane was inhibited 50% ($p<.01$), whereas, that of outer membrane was inhibited by 34% ($p<.01$). The apparent discrepancy between the earlier reports of *in vitro* experiments (2, 9) and the present *in vivo* results suggests that in the intact cell chloramphenicol may affect both mitochondrial and extramitochondrial protein-synthesizing systems. In this regard it is noteworthy that this antibiotic also inhibited leucine incorporation into matrical proteins.

The existence of a close relationship between the mitochondrial and extramitochondrial protein-synthesizing systems is suggested by the studies of Roodyn *et al.* (10) who showed that stimulation of hepatic mitochondrial biosynthesis by thyroid hormone in thyroidectomized rats is accompanied by strikingly parallel temporal and quantitative stimulation of protein synthesis in mitochondria and microsomes. This apparent dependence may be better understood by the work of Freeman and Haldar (11, 12) who have shown that chloramphenicol inhibits NADH oxidation in mitochondria, an event which leads to decreased ATP production, inhibition of RNA synthesis, and impaired cytoplasmic protein synthesis. Similar conclusions were drawn from studies on regenerating liver by Firkin and Linnane (13) and by Kroon and De Vries (14). Unpublished experiments in this laboratory have shown that the specific activity of succinic dehydrogenase in mitochondria isolated from chloramphenicol-treated refed rats similar to those reported in this communication is reduced by 40% as compared to controls ($p < .01$).

Thus, the inhibition of outer membrane synthesis by chloramphenicol in our experiments may be the end result of impaired energy generation by the inner membrane which in turn leads to decreased extramitochondrial protein synthesis.

However, it should be pointed out that the question whether inhibition of extramitochondrial protein synthesis by chloramphenicol actually follows or is concomitant to inhibition of inner membrane function remains to be established.

The apparent differential inhibition of leucine incorporation into the inner and outer mitochondrial membranes by chloramphenicol may serve to explain large blebs of the outer membrane reported in an earlier study by one of us (15) on hepatic mitochondria of animals fed a diet containing 1.5% chloramphenicol as shown in Fig. 1. Although this finding was encountered in a limited number of mitochondria in any one hepatocyte, it was present in all hepatocytes of chloramphenicol-treated rats regardless of the type of chemical fixative employed. Since extensive study of the livers of nontreated animals did not reveal any comparable abnormality of outer membrane morphology, it was concluded that the alteration was due to an effect of chloramphenicol. The blebs may be redundant membrane resulting from a greater rate of growth of the outer membrane as compared to that of the inner membrane.

Preliminary studies on the effect of the simultaneous administration of both antibiotics show that leucine incorporation into both membranes is inhibited by 80%, and blebs of the outer membrane were not present. Our

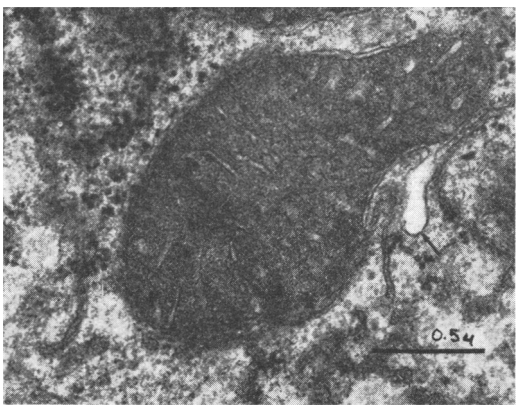

FIG. 1. Electron micrograph of a hepatic mitochondrion from a rat refed a diet containing 1.5% chloramphenicol. The arrow points to a bleb on the outer membrane which is interpreted as redundant membrane (×53,000).

observations on the biosynthesis of matrical proteins suggest that these may also be synthesized in the endoplasmic reticulum, since the rate of incorporation into them is significantly affected by cycloheximide.

Summary. The *in vivo* incorporation of ^{3}H-leucine into inner and outer mitochondrial membrane has been investigated in rats during enhanced liver mitochondriogenesis.

Administration of cycloheximide for 3 days (1.5 mg/kg body weight, intraperitoneally) caused a 34% inhibitin of incorporation into outer membrane, as compared to controls, but was without effect on the incorporation into inner membrane. Chloramphenicol administered orally for 3 days (2.5%) inhibited incorporation into inner membrane and to a lesser degree into outer membrane.

The results reported support the conclusion that biosynthesis of outer mitochondrial membrane is dependent on protein synthesis localized in the endoplasmic reticulum, whereas the system associated with synthesis of the inner membrane appears to be localized within the mitochondrion.

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Received Dec. 3, 1969. P.S.E.B.M., 1970, Vol. 134.