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The Relationship of Protein and Lipid Synthesis During the Biogenesis of Mitochondrial Membranes

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Summary. Rat liver mitochondria were fractionated into inner and outer membrane components at various times after the intravenous injection of ^{14}C-leucine or ^{14}C-glycerol. The time curves of protein and lecithin labeling were similar in the intact mitochondria, the outer membrane fraction, and the inner membrane fraction. In rat liver slices also, the kinetics of ^{3}H-phenylalanine incorporation into mitochondrial KCl-insoluble proteins was identical to that of ^{14}C-glycerol incorporation into mitochondrial lecithin. These results suggest a simultaneous assembly of protein and lecithin during membrane biogenesis.

The proteins and lecithin of the outer membrane were maximally labeled *in vivo* within 5 min after injection of the radioactive precursors, whereas the insoluble proteins and lecithin of the inner membrane reached a maximum specific activity 10 min after injection.

Phospholipid incorporation into mitochondria of rat liver slices was not affected when protein synthesis was blocked by cycloheximide, puromycin, or actinomycin D. The injection of cycloheximide 3 to 30 min prior to ^{14}C-choline did not affect the *in vivo* incorporation of lecithin into the mitochondrial inner or outer membranes; however, treatment with the drug for 60 min prior to ^{14}C-choline resulted in a decrease in lecithin labeling. These results suggest that phospholipid incorporation into membranes may be regulated by the amount of newly synthesized protein available.

When mitochondria and microsomes containing labeled phospholipids were incubated with the opposite unlabeled fraction *in vitro*, a rapid exchange of phospholipid between the microsomes and the outer membrane occurred. A slight exchange with the inner membrane was observed.

A fundamental problem in understanding mitochondrial biogenesis is the mechanism by which the component proteins and lipids are assembled to form membranes. It is well known that mitochondria contain two distinct membranes (inner and outer) which differ both chemically and enzymatically (Parsons, Williams, Thompson, Wilson, & Chance, 1967). The biosynthesis of the protein components of these membranes appears to involve two independent sites of synthesis; one is mitochondrial involved in the synthesis of some insoluble proteins, perhaps "structural" proteins of the inner

membrane (Beattie, Basford, & Koritz, 1967; Neupert, Brdiczka, & Bücher, 1967), and one is extramitochondrial involved in the synthesis of the proteins of the outer membrane, the soluble proteins, and the remaining proteins of the inner membrane (Beattie, Basford, & Koritz, 1966; Freeman, Haldar, & Work, 1966; Gonzalez-Cadavid & Campbell, 1967), which are transferred into the mitochondrial structure in a subsequent step (Beattie, 1968*a*; Kadenbach, 1967). Membranes also consist of phospholipids which appear to be mainly synthesized in the microsomes, although two recent reports (Stoffel & Schiefer, 1968; Kaiser & Bygrave, 1968) have suggested that isolated mitochondria have the ability to synthesize phospholipids. In contrast, McMurray and Dawson (1969) have concluded that mitochondria have only a very limited ability, if any, to synthesize phospholipids.

Although the pathways and sites of protein and phospholipid biosynthesis have been characterized in some detail, little is known about membrane biogenesis from these component molecules, regardless of their intracellular site of synthesis. The problem of the biogenesis of mitochondrial membranes has been approached in this study by comparing the rates of protein and phospholipid incorporation into rat liver mitochondria in vivo and in slices. The time curves of protein and lecithin labeling were identical in intact mitochondria and in the purified inner and outer membrane fractions, suggesting a simultaneous assembly of protein and lecithin (a major mitochondrial phospholipid) during membrane formation. The proteins and phospholipids of the outer membrane fraction were labeled more rapidly than those of the inner membrane fraction. This result provides further evidence that the outer membrane may be the first component synthesized during mitochondrial biogenesis (Beattie, 1969). Phospholipid incorporation also appeared to be regulated by the amount of newly synthesized protein available. When protein synthesis was blocked by cycloheximide in vivo or in slices, phospholipid incorporation into the membranes continued for a short time until previously synthesized protein was no longer available for phospholipid binding.

In addition, the in vitro exchange of phospholipids between microsomes and mitochondria reported by Wirtz and Zilversmit (1968) was confirmed. The greatest exchange occurred in the outer membrane fraction; however, there was significant exchange with the inner membrane fraction.

Materials and Methods

Adult male rats weighing approximately 175 g received 20 μc of uniformly labeled ^{14}C-L-leucine or 40 μc of ^{14}C-1,3-glycerol by intravenous injection and were killed at 5, 10, 15, 20, 30, and 45 min after injection. Liver mitochondria were prepared in 0.25 M

sucrose containing 0.01 M Tris, pH 7.4, washed four times and separated into three fractions (inner membrane-matrix, outer membrane, and a soluble fraction) by the digitonin method of Schnaitman and Greenawalt (1968). Microsomes were prepared by centrifuging the first mitochondrial supernatant at $12{,}000 \times g$ for 10 min. The pellet was discarded and the supernatant was centrifuged at $144{,}000 \times g$ for 1 hr to sediment microsomes. The inner membrane-matrix fraction was fractionated by sonication to yield an insoluble membranous fraction and a matrix fraction as previously described (Beattie, 1969). The completeness of the separation was monitored by the use of specific enzyme markers; succinic dehydrogenase for the inner membrane, monoamine oxidase and rotenene-insensititive nicotinamide adenine nucleotide dehydrogenase (NADH)-cytochrome *c* reductase for the outer membrane, isocitric dehydrogenase for the matrix, and glucose-6-phosphatase and NADPH-cytochrome *c* reductase for the microsomes (Beattie, 1968*b*). A major problem in interpretation of radioactive labeling experiments was the rather high contamination (8 %) of the outer membrane fraction with microsomal membranes as indicated by the marker enzymes. The other submitochondrial fractions had lower amounts of contamination. In all experiments reported in this study, the specific activities of the various submitochondrial fractions were corrected for contamination due to microsomes using the two marker enzymes to determine the percent microsomal contamination of each fraction (Beattie, 1969).

Rat liver slices were prepared and incubated in Krebs-Ringer bicarbonate solution and the radioactive precursor as previously described (Beattie, 1968*a*). Mitochondria were prepared from the slices in 0.25 M sucrose and fractionated into water-soluble, 0.6 N KCl-soluble and KCl-insoluble protein fractions (Beattie et al., 1966).

In other experiments, 5 mg of cycloheximide (in 0.9 % aqueous saline) per 100 g of body weight was injected intravenously 3, 15, 30, and 60 min prior to the injection of 25 μc of ^{14}C-choline. The animals were killed after 10 min. Liver mitochondria were prepared and fractionated into inner and outer membrane fractions as described above.

Phospholipid Exchange In Vitro

The in vitro exchange of phospholipids between microsomes and mitochondria was tested using the conditions of Wirtz and Zilversmit (1968). Labeled mitochondria and microsomes were prepared from animals which had received 40 μc of ^{14}C-glycerol by intraperitoneal injection 16 hr prior to sacrifice or 25 μc of ^{14}C-choline by intravenous injection 1 hr prior to sacrifice. The isolated mitochondria and microsomes were resuspended in unlabeled $100{,}000 \times g$ supernatant to a protein concentration of 30 mg/ml. Equal portions of the resuspended mitochondria and microsomes were incubated with the same amount of supernatant fraction for 30 min at 30 °C and in an icebath. One set of flasks contained labeled mitochondria and unlabeled microsomes; the second set contained unlabeled mitochondria and labeled microsomes. At the end of the incubation, the suspensions were centrifuged at $15{,}000 \times g$ for 10 min. The pellet containing mitochondria was resuspended two times in sucrose and centrifuged at $6{,}500 \times g$ for 10 min. The supernatant from the $15{,}000 \times g$ centrifugation was centrifuged at $100{,}000 \times g$ for 60 min to prepare microsomes.

Glucose-6-phosphatase was used as a measure of microsomal contamination in freshly isolated mitochondria and in the mitochondrial pellet reisolated after an incubation at 0 and 30 °C under these conditions (Table 1). The extent of microsomal contamination decreased from 3.8 % in the freshly isolated mitochondria to values of 2.6 and 0.6 % in mitochondria isolated after incubation at 0 and 30 °C, respectively. The activity of this enzyme in the microsomal fraction did not decrease after incubation for 30 min at 30 °C. Similar results were obtained when NADPH-cytochrome *c* reductase was used as a microsomal marker.

Table 1. *Glucose-6-phosphatase activity in mitochondria and microsomes before and after incubation*

Location of activity	Conditions	Specific activity[a] (mμmoles/min/mg)	Contamination (%)
Microsomes	Before incubation[b]	151	—
	After incubation at 30 °C	148	—
Mitochondria	Before incubation	5.7	3.8
	After incubation at 0 °C	3.9	2.6
	After incubation at 30 °C	0.95	0.6

[a] Glucose-6-phosphatase activity in the various fractions was determined at 37 °C by the method of Hübscher and West (1965).

[b] Incubation of mitochondria and microsomes and their subsequent reisolation was as described in the text.

Lipid Analyses

Lipids from the microsomes, the intact mitochondria, and the submitochondrial fractions were extracted with 20 volumes of chloroform-methanol (2:1 v/v) overnight at 4 °C. The lipid extracts were washed twice with 0.2 volumes of 0.9 % NaCl according to Folch, Lees, and Sloane Stanley (1957). The chloroform layer was taken to near dryness under a stream of nitrogen at 40 °C and redissolved in a small volume of 2:1 chloroform-methanol.

Individual phospholipids were separated by thin layer chromatography on silica gel. Plates were activated at least 1 hr at 110 °C before chromatography. The developing solvent was chloroform-methanol-water (65:25:4). For better separation of cardiolipin from the solvent front, the plates were developed in chloroform-ethyl acetate-glacial acetic acid (80:20:5) prior to development in chloroform-methanol-water. Standard solutions of lecithin, phosphatidyl ethanolamine, phosphatidyl inositol, and cardiolipin were used in each run. The bands were visualized by exposure to iodine vapor and scraped off the plate with a spatula for counting and phosphorus determination after overnight elution of the lipids from the gel with chloroform-methanol. In some experiments, the gel was scraped directly into a counting vial, eluted, and then counted. The radioactivity in the various phospholipids was determined by liquid scintillation counting in 10 ml of toluene containing 0.4 % PPO and 0.01 % POPOP. The scintillation counter used had an efficiency for ^{14}C of greater than 90 %. Lipid phosphorus was determined by the method of Ames and Dubin (1960). The distribution of the various phospholipids in the inner and outer membrane fractions prepared by the digitonin method was qualitatively similar to that reported by Parsons et al. (1967) and by Stoffel and Schiefer (1968). The mitochondrial cardiolipin was located primarily in the inner membrane, whereas phosphatidyl inositol was largely in the outer membrane.

Protein Analyses

Proteins were prepared for counting by previously described methods (Beattie et al., 1967). Protein concentrations were determined by the Biuret method of Gornall, Bardawill, and David (1949) or by the method of Lowry, Rosebrough, Farr, and Randall (1951).

Materials

Uniformly labeled ^{14}C-L-leucine (250 mc/mmole), ^{14}C-1,3-glycerol (6 mc/mmole), choline ^{14}C-methyl (10 mc/mmole), and generally labeled ^{3}H-phenylalanine (5 c/mmole) were obtained from New England Nuclear (Boston, Mass.); cycloheximide and puromycin from Sigma; actinomycin D from Merck, Sharpe and Dohme; chromatographically pure standards of lecithin, phosphatidyl ethanolamine, phosphatidyl inositol and cardiolipin from Applied Science Laboratories (State College, Pa.). Digitonin obtained from Sigma Chem. Co. (St. Louis, Mo.) was recrystalllized from hot ethanol prior to use. All solvents were of spectrophotometric grade or were redistilled prior to use.

Results

When either radioactive leucine or glycerol was injected intravenously into rats, the specific radioactivity of the various protein and lipid components of intact mitochondria reached a maximum within 20 to 30 min (Fig. 1). The proteins and the lecithin were labeled within 20 min after injection, whereas phosphatidyl ethanolamine was labeled more slowly than these components during the first 10 min after injection and continued to gain radioactivity at a significant rate for 30 min.

A similar pattern of labeling was observed in the purified inner and outer membrane fractions. The time curves of incorporation of lecithin and protein into the insoluble inner membrane fraction (Fig. 2) were identical

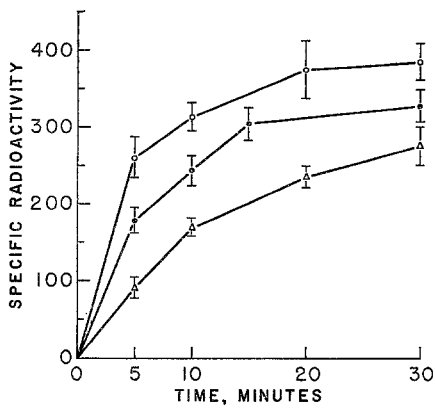

Fig. 1

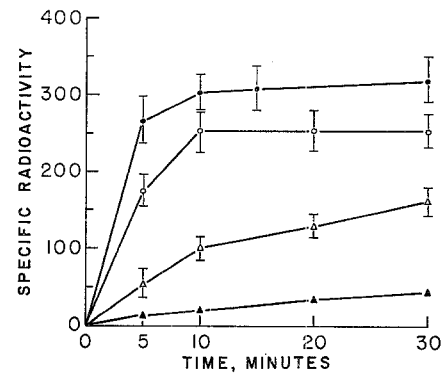

Fig. 2

Fig. 1. Time curve of leucine incorporation into protein (•——•) and glycerol incorporation into lecithin (○——○) or phosphatidyl ethanolamine (△——△) in intact mitochondria at various times after intravenous injection of the radioactive precursors

Fig. 2. Time curve of leucine incorporation (•——•) into the insoluble membranous proteins obtained from a sonicated inner membrane preparation, and glycerol incorporation into lecithin (○——○), phosphatidyl ethanolamine (△——△), or cardiolipin (▲——▲) of intact inner membrane-matrix preparations at various times after intravenous injection of the radioactive precursors

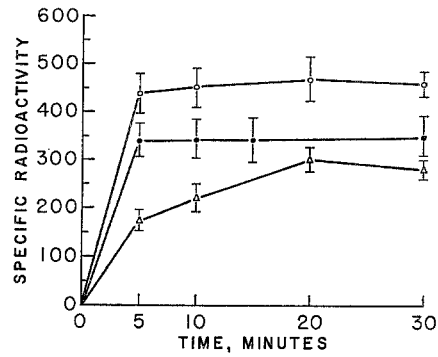

Fig. 3. Time curve of leucine incorporation into protein (•——•) and glycerol incorporation into lecithin (○——○) or phosphatidyl ethanolamine (△——△) of outer membranes at various times after intravenous injection of the radiactive precursors

and reached a maximum specific activity 10 min after injection of the radioactive precursors. After this time, no further incorporation into these components was observed. In addition, no decrease in specific radioactivity occurred for 30 min. Phosphatidyl ethanolamine was labeled more slowly in this fraction and was still being labeled at a significant rate 30 min after injection. Cardiolipin, located primarily in the inner membrane (Parsons et al., 1967) was labeled at a slow linear rate throughout the times studied. A slow in vivo synthesis of cardiolipin was also observed by Taylor, Bailey, and Bartley (1967) and by Gross, Getz, and Rabinowitz (1969), who reported that mitochondrial cardiolipin was still being labeled 3 days after injection of ^{32}P at which time phosphatidyl ethanolamine and lecithin had undergone considerable decay.

The lecithin and protein of the outer membrane fraction were rapidly labeled in vivo, reaching their maximum specific activity within 5 min after injection (Fig. 3). In this fraction, phosphatidyl ethanolamine was again labeled more slowly and reached a maximum specific activity 20 min after injection. Phosphatidyl inositol, present in the outer membrane fraction, was labeled very poorly and hence was not plotted.

The kinetics of labeling of the intact mitochondria and the purified inner and outer membrane fractions did not resemble that of the microsomes (Fig. 4). Protein and lecithin in the microsomes reached a maximum specific activity 15 min after administration of the radioactive precursors at which time their specific activity decreased. Phosphatidyl ethanolamine in the microsomes was maximally labeled 5 min after injection and remained constant throughout the times studied.

The inability to distinguish differences in the kinetics of labeling of protein and lecithin in vivo may have resulted from the rapidity with which

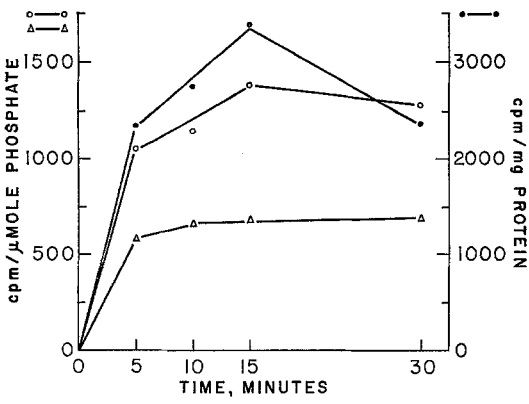

Fig. 4

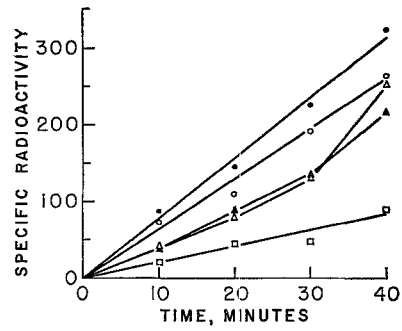

Fig. 5

Fig. 4. Time curve of leucine incorporation into protein (•——•) and glycerol incorporation into lecithin (○——○) or phosphatidyl ethanolamine (△——△) of the microsomes

Fig. 5. Time curve of labeling of mitochondrial protein and phospholipids in rat liver slices incubated at 22 °C in 2.0 ml Krebs-Ringer-bicarbonate containing 4 μc of ^{14}C-glycerol and 5 μc of ^{3}H-phenylalanine. Mitochondria were prepared and fractionated into various protein fractions and extracted with chloroform-methanol for lipid analysis. KCl-insoluble proteins (•——•), water-soluble proteins (△——△), lecithin (○——○), phosphatidyl ethanolamine (▲——▲), and cardiolipin (□——□). Protein radioactivity is expressed as cpm/mg protein and lipid radioactivity as cpm/μmole phosphate

these processes occur in the intact animal. Hence, incorporation was studied in rat liver slices in which the biosynthetic processes can be slowed by performing incubations at 22 °C. The rate of ^{3}H-phenylalanine incorporation into the KCl-insoluble proteins of the mitochondria was linear with time (Fig. 5), as was that of ^{14}C-glycerol into lecithin. A slight lag occurred in the labeling of the water-soluble and KCl-soluble proteins at short times of incubation. After 30 min, the rate of labeling of these two soluble protein fractions was more rapid than that of the insoluble proteins such that their specific activity approached that of the KCl-insoluble proteins after a 40-min incubation. Previous observations had suggested an initial synthesis of the insoluble proteins as a prerequisite for the subsequent integration of the soluble proteins into the mitochondrial structure (Beattie, 1968*a*). The time curve of phosphatidyl ethanolamine labeling in the slices was more complex, indicating that this phospholipid was incorporated at a continually increasing rate. These kinetics were observed in several different experiments. Cardiolipin was also labeled at a slow linear rate in the slices.

The possible relationship between protein and phospholipid incorporation into mitochondrial membranes was tested experimentally by studying

Table 2. *Effect of inhibitors of protein synthesis on phospholipid labeling in rat liver slices*[a]

Exp.	Additions to complete system	Final incubation time (min)	Mitochondria cpm/μmole P_i			Microsomes cpm/μmole P_i	
			Lecithin	Phosphatidyl ethanolamine	Cardiolipin	Lecithin	Phosphatidyl ethanolamine
1	None	20	315	444	—	669	1,150
	Puromycin	20	308	455	—	798	1,585
2	None	20	550	610	—	1,305	1,800
	Cycloheximide	20	557	615	—	1,075	1,315
	None	40	1,100	992	—	2,385	2,195
	Cycloheximide	40	1,380	1,560	—	2,415	2,460
3	None	30	500	445	89	—	—
	Cycloheximide	30	566	438	67	—	—
	None	60	573	426	87	—	—
	Cycloheximide	60	592	416	70	—	—

[a] Liver slices (400 mg) were preincubated 30 min in 2.0 ml of Krebs-Ringer-bicarbonate containing 600 μg/ml of puromycin and 100 μg/ml of cycloheximide where indicated. The slices were then removed from the beakers, rinsed with saline and placed in fresh Krebs-Ringer-bicarbonate containing 1.0 μc of ^{14}C-glycerol and the same amount of drug where indicated. The final incubation time is indicated for each experiment.

phospholipid incorporation when protein synthesis had been blocked by specific inhibitors. Concentrations of cycloheximide which completely inhibit amino acid incorporation into mitochondrial protein in rat liver slices (Beattie, 1968*a*) had no effect on the incorporation of glycerol into lecithin and phosphatidyl ethanolamine of mitochondria and of microsomes (Table 2) even after a 30-min preincubation of the slices with the drug prior to addition of the glycerol. In some experiments, a significant stimulation of lecithin and phosphatidyl ethanolamine incorporation into the mitochondria and microsomes was observed under these conditions. In contrast, an approximately 25% inhibition of cardiolipin synthesis was observed in the presence of cycloheximide. Puromycin also had no effect on glycerol incorporation into mitochondrial phospholipids and a slight stimulation of incorporation into microsomal phospholipids in rat liver slices (Table 2).

The effects of actinomycin D on amino acid incorporation into the various mitochondrial protein fractions and on glycerol incorporation into the major mitochondrial phospholipids are compared in Table 3. Neither a 20-min nor a 60-min preincubation with actinomycin had any inhibitory

Table 3. *Effects of actinomycin on protein and phospholipid synthesis in mitochondria of rat liver slices*[a]

Component	Specific activity[b]					
	20-min preincubation			60-min preincubation		
	Control	+Actinomycin	% Control	Control	+Actinomycin	% Control
Lecithin	895	1,075	120	556	684	123
Phosphatidyl ethanolamine	607	710	117	411	497	121
Cardiolipin	133	174	131	114	145	127
Water-soluble proteins	480	137	28.5	595	86	14.4
KCl-soluble proteins	366	110	30	480	29	6.0
KCl-insoluble proteins	461	183	40	430	118	27.4

[a] Liver slices were preincubated for either 20 or 60 min with actinomycin (10 μg/ml) prior to the final incubation of 30 min with actinomycin (10 μg/ml) and either 1.0 μc of glycerol or 0.5 μc of u.l. (^{14}C) leucine. Mitochondria were reisolated and extracted with chloroform-methanol for lipid analysis or fractionated by the procedure of Beattie et al., (1966) to yield the various submitochondrial protein fractions.

[b] Specific activity for phospholipids expressed as cpm/μmole P_i and for proteins as cpm/mg protein.

effect on the labeling of the phospholipids. In fact, a slight stimulation was observed. Amino acid incorporation, however, was severely inhibited by preincubation with actinomycin. The inhibition by actinomycin was greater in the soluble protein fractions than in the KCl-insoluble protein fraction. It is of some interest that this latter fraction contains those proteins which are synthesized within the mitochondria (Beattie, 1968*a*). This result suggests that actinomycin D has a more rapid inhibitory effect on those proteins synthesized extramitochondrially, i.e., those synthesized under control of mRNA made in the nucleus rather than on those synthesized within the mitochondria, which are under control of mitochondrial mRNA. This result was anticipated because Shanmugram and Bhargave (1968) had reported that ^{3}H-actinomycin D was taken up first into the nuclei and then into the mitochondria of rat liver slices.

The lack of inhibition of phospholipid synthesis observed in the slices may have resulted because sufficient protein was still available for phospholipid binding, or alternately because newly synthesized protein need not be

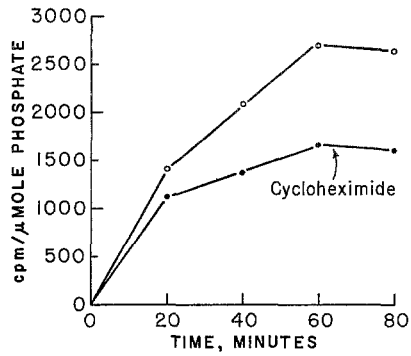

Fig. 6

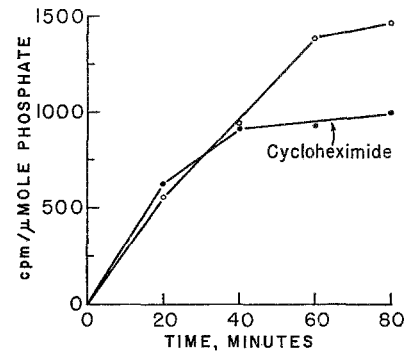

Fig. 7

Fig. 6. Time curve of ^{14}C-glycerol incorporation into lecithin of microsomes in rat liver slices from control rats (o——o) and from rats which had received cycloheximide (5 mg/100 g body weight) 1 hr prior to sacrifice (•——•). Incubations were performed as described in Table 2

Fig. 7. Time curve of ^{14}C-glycerol incorporation into lecithin of mitochondria in rat liver slices from control rats (o——o) and from rats which had received cycloheximide 3 (•——•). Incubations were performed as described in Table 2

present for lipid synthesis to occur. This problem was approached by using slices obtained from rats which had received cycloheximide by intravenous injection 1 hr prior to sacrifice. Cycloheximide has been reported to inhibit protein synthesis within 10 sec after injection (Ray, Lieberman, & Lansing, 1968). Glycerol incorporation into lecithin of the microsomal fraction was inhibited at all times of incubation in the slices obtained from the cycloheximide-treated rats as compared to slices from normal rats (Fig. 6). The extent of inhibition was low after a 20-min incubation but much greater at all subsequent times. It should be noted that there was a linear rate of lecithin synthesis in the control slices for 60 min, whereas the rate of lecithin synthesis in the slices from the cycloheximide-treated rats increased very little after the first 20 min. Identical results were obtained with phosphatidyl ethanolamine labeling in the slices from rats receiving the drug.

Somewhat different results were obtained in the labeling of mitochondrial phospholipids under these conditions. During the first 40 min of incubation, no differences in the extent of lecithin labeling were observed between the slices from the cycloheximide-treated rats and the control rats (Fig. 7). After 40 min, however, no further incorporation into lecithin occurred in the slices from the cycloheximide-treated rats, although the control slices continued to incorporate glycerol into lecithin for 60 min at which time the rate tapered off.

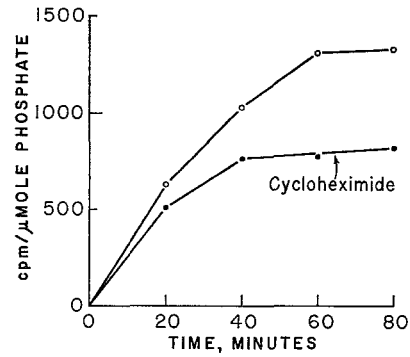

Fig. 8. Time curve of ^{14}C-glycerol incorporation into phosphatidyl ethanolamine in rat liver slices from control rats (o——o) and from rats which had received cycloheximide (•——•). Incubations were performed as described in Table 2

The amount of glycerol incorporated into phosphatidyl ethanolamine in the slices from the cycloheximide-treated rats was almost the same as that in the control slices after a 20-min incubation (Fig. 8). At longer incubation times, however, a significant inhibition of glycerol incorporation into phosphatidyl ethanolamine was observed. Again, the labeling of phosphatidyl ethanolamine ceased after 40 min of incubation in the slices from the cycloheximide-treated rats, whereas in the control slices a linear rate of phosphatidyl ethanolamine synthesis was observed for 60 min or longer.

Another approach to this problem was an in vivo one in which the extent of phospholipid synthesis was studied in rats which had received cycloheximide at various time intervals prior to ^{14}C-choline. On each day that an experiment was performed, two rats were injected with cycloheximide for a certain time before the intravenous injection of ^{14}C-choline. The animals were sacrificed 10 min later. Two control rats were also injected with radioactive choline and sacrificed after 10 min. In this way, the incorporation of ^{14}C-choline into the cycloheximide-treated rats could be compared to that of control rats kept under identical conditions. As shown in Table 4, the injection of cycloheximide 3 and 15 min before choline had no effect on the extent of lecithin labeling in the intact mitochondria, the inner or outer membrane fractions, or the microsomes. Treatment with the drug for 30 min prior to the phospholipid precursor caused a 27% inhibition of ^{14}C-choline incorporation into lecithin of the microsomes, but was still without effect on lecithin labeling in the mitochondrial membranes. In contrast, when the animals received cycloheximide 60 min before choline, the labeling of all fractions was decreased. Under these conditions, the

Table 4. *Effect of cycloheximide on ^{14}C-choline incorporation into lecithin of mitochondrial fractions and microsomes in vivo*[a]

Fraction	Lecithin (cpm/μmole P_i)			
	Time of cycloheximide treatment (min)			
	3	15	30	60
Mitochondria	2,710 ± 490 (6)	2,500 (2)	3,830 (2)	3,300 ± 362 (3)
+ Cycloheximide	2,790 ± 330 (4)	2,730 (2)	3,310 (2)	2,310 ± 217 (3)
Inner membrane	1,270 ± 344 (6)	1,020 (2)	845 (2)	955 ± 30 (3)
+ Cycloheximide	1,560 ± 235 (4)	930 (2)	970 (2)	810 ± 95 (4)
Outer membrane	2,690 ± 273 (6)	4,700 (2)	4,660 (2)	4,570 ± 534 (3)
+ Cycloheximide	3,360 ± 452 (4)	4,760 (2)	4,870 (2)	3,144 ± 257 (3)
Microsomes	11,400 (2)	10,300 (2)	12,700 (2)	12,900 ± 1,300 (3)
+ Cycloheximide	14,300 (2)	10,200 (2)	9,470 (2)	8,310 ± 1,370 (3)

[a] Rats weighing 175 g were injected with cycloheximide (5 mg/100 g body weight) at times ranging from 3 to 60 min prior to ^{14}C-choline injection. Mitochondria were isolated and fractionated into inner and outer membrane fractions as described in the text. The numbers in parentheses indicate the number of rats involved in each group. The data are expressed as the mean ± the standard error of the mean.

inner membrane fraction was inhibited 15%, the outer membrane fraction 30%, and the microsomes 36%.

The in vitro exchange of phospholipids between mitochondria and microsomes was tested using the conditions of Wirtz and Zilversmit (1968). Mitochondria which had been reisolated after an incubation with ^{14}C-glycerol-labeled microsomes were fractionated into inner and outer membrane fractions by the digitonin procedure of Schnaitman and Greenawalt (1968). The results (Table 5) indicate an exchange of lecithin and phosphatidyl ethanolanine between the microsomes and the mitochondria representing approximately 20% of the unincubated microsomes. The small exchange observed during a 30-min incubation at 0 °C was subtracted from that obtained during the incubation at 30 °C so that the above values represent net exchange. The inner membrane fraction prepared from these mitochondria contained a similar amount of labeled lecithin and phosphatidyl ethanolamine representing 20% of the unincubated microsomes. The outer membrane fraction contained almost twice as much labeled lecithin and phosphatidyl ethanolamine, an amount equivalent to an exchange of greater than 30%.

Similar results were obtained when the microsomes and mitochondria were labeled with ^{14}C-choline (Table 6). The inner membrane fraction contained an amount of labeled lecithin representing an exchange of 24%

Table 5. *Exchange of phospholipids between microsomes labeled with ^{14}C-glycerol and unlabeled mitochondria*[a]

Fraction	Radioactivity			
	Lecithin		Phosphatidyl ethanolamine	
	cpm/μg P_i	% nonincubated microsomes	cpm/μg P_i	% nonincubated microsomes
Mitochondria	82	21.0	72	20.6
Inner membrane	78	20.0	69	19.7
Outer membrane	124	31.8	125	35.7
Microsomes, before incubation	390	–	350	–

[a] Unlabeled mitochondria resuspended in 100,000 × *g* supernatant were incubated for 30 min with labeled microsomes, resuspended in 100,000 × *g* supernatant, obtained from animals which had received 40 μc of ^{14}C-glycerol by intraperitoneal injection 16 h prior to sacrifice. The specific activities presented above are those obtained in a 30-min incubation at 30 °C minus those obtained in a 30-min incubation at 0 °C.

Table 6. *Exchange of lecithin between microsomes and mitochondria in vitro*[a]

Experiment and temperature	Inner membrane		Outer membrane	
	cpm/μmole P_i	% of non-incubated	cpm/μmole P_i	% of non-incubated
Exp. 1 (0 °C)	75.6	3.3	357.1	15.5
(30 °C)	555.4	24.0	833.5	36.1
Exp. 2 (0 °C)	1,168	100	1,504	100
(30 °C)	919	82.0	1,153	74

[a] In experiment 1, labeled microsomes obtained from animals which had received 25 μc of ^{14}C-choline by intravenous injection 30 min prior to sacrifice were incubated with unlabeled mitochondria for 30 min at 0 and 30 °C. In experiment 2, labeled mitochondria were incubated with unlabeled microsomes for 30 min at 0 and 30 °C. The mitochondria were reisolated after the incubation as described in the text and fractionated into inner and outer membranes by the digitonin method of Schnaitman and Greenawalt (1968).

and the outer membrane fraction an exchange of 36% of the unincubated microsomes. In this experiment, the amount of exchange into the inner membrane fraction during incubation at 0 °C was minimal; however, during 0 °C incubation, the outer membrane fraction contained considerable labeled lecithin, an amount equivalent to 15% of the unincubated microsomes. During the incubation of ^{14}C-choline-labeled mitochondria with unlabeled microsomes, a decrease in radioactivity of about 20 and 25% was observed in the inner and outer membrane fractions, respectively.

Discussion

The time course of protein and phospholipid incorporation into the two membranes of the mitochondria has been studied in an attempt to clarify the mechanism of membrane biogenesis. Protein and lecithin were labeled in vivo with identical kinetics in the intact mitochondria and in the purified inner and outer membrane fractions. Each of these membranes had a unique labeling pattern, but in each case the time course of lecithin labeling paralleled that of the protein. A similar situation occurred in rat liver slices. Lecithin was labeled at a linear rate as were the KCl-insoluble proteins, which represent the most tightly membrane-bound proteins of the mitochondria. These results suggest that protein is incorporated into the mitochondrial membranes simultaneously with lecithin.

Similar conclusions were drawn by Dallner, Siekivitz, and Palade (1966*a*) in a study of the biogenesis of endoplasmic reticulum membranes. Their results suggested that new membrane is formed from lipid and protein in the rough endoplasmic reticulum and subsequently transferred to the smooth-surfaced part of the system. These workers also proposed that the "basic" membrane thus formed might then serve as a framework on which constitutive enzymes might be added in a series of successive steps (Dallner, Siekivitz, & Palade, 1966*b*).

The biogenesis of mitochondrial membranes may proceed by an analogous mechanism. Work from several laboratories has indicated that the great bulk of mitochondrial proteins is synthesized outside the mitochondria (Roodyn & Wilkie, 1968; Beattie et al., 1967; Henson, Weber, & Mahler, 1968) and transferred into the mitochondrial structure in a subsequent step (Beattie, 1968*a*; Gonzalez-Cadavid & Campbell, 1967; Kadenbach, 1967). Phospholipid biosynthesis also appears to occur mainly in the endoplasmic reticulum, although several recent reports (Bygrave & Kaiser, 1968; Stoffel & Schiefer, 1968) indicate that the outer mitochondrial membrane fraction may contain the enzymes of phospholipid biosynthesis. Hence, phospholipids must also be transferred from their sites of synthesis to the mitochondrial membranes. Kadenbach (1968) has direct evidence that the transfer of ^{32}P-phospholipids from labeled microsomes to unlabeled mitochondria proceeds with kinetics identical to that of the transfer of ^{14}C-proteins under his experimental conditions. He concluded from these results that proteins are transferred into the mitochondria as a phospholipid-protein complex. Several advantages to such a mechanism are immediately apparent. A masking of the activity of these enzymes outside the mitochondrial structure might be accomplished by formation of such a complex. In

addition, a phospholipid-protein complex might pass more easily through the mitochondrial membrane than a pure protein molecule.

The possible relationship between protein and lipid incorporation into mitochondrial membranes was tested directly by studying phospholipid incorporation when protein synthesis was blocked by specific inhibitors. During short-term experiments, lipid incorporation into the various membranes does not appear to require the simultaneous synthesis of new protein. The injection of cycloheximide for periods of time from 3 to 30 min prior to the administration of ^{14}C-choline did not inhibit the in vivo incorporation of lecithin into the mitochondrial inner or outer membrane fractions; however, a slight inhibition of lecithin incorporation into the microsomal fraction was observed in animals which had received cycloheximide 30 min prior to the phospholipid precursor. In rat liver slices, also, no inhibition of glycerol incorporation into lecithin or phosphatidyl ethanolamine of the mitochondria or the microsomes was observed when protein synthesis was blocked by puromycin, cycloheximide, or actinomycin D. Cycloheximide did cause a slight inhibition of cardiolipin labeling. When protein synthesis had been blocked for a sufficiently long time, however, a decrease in lipid incorporation was observed. The injection of cycloheximide 60 min prior to ^{14}C-choline resulted in a 15% decrease in lecithin incorporation into the inner membrane fraction, a 30% decrease in the outer membrane fraction, and a 36% decrease in the microsomes. It appears that lecithin incorporation into the outer membrane is more sensitive to cycloheximide than is lecithin incorporation into the inner membrane fraction. This may reflect the chemical and enzymatic similarity of the outer membrane and the endoplasmic reticulum (Parsons et al., 1967) and suggests that their biogenesis may proceed by a similar mechanism. Also, the inner membrane contains those proteins synthesized by the cycloheximide-insensitive system of the mitochondria (Beattie et al., 1967).

These results suggest that the continued incorporation of lipids in the presence of inhibitors of protein synthesis during short-term experiments may have resulted because sufficient protein was still available to form a lipoprotein complex with newly synthesized phospholipid molecules even when the synthesis of new protein has been blocked. This view is supported by the studies of glycerol incorporation in rat liver slices obtained from animals which had received cycloheximide 1 hr prior to sacrifice. The kinetics of lecithin and phosphatidyl ethanolamine incorporation into mitochondria was similar in the slices from the cycloheximide-treated rats and the control rats during incubations of 20 to 40 min. At longer incubation times, no further incorporation occurred in the slices of the treated rats, although the

control slices continued to incorporate at a linear rate for 60 to 80 min. Thus, initially sufficient protein might be available for phospholipid incorporation into the membranes, whereas during longer times the amount of available protein becomes limiting. Similar results were obtained by Schiefer (1969) in vivo. Cycloheximide caused a slight inhibition of lecithin incorporation into intact mitochondria and purified cytochrome oxidase when sufficient time had elapsed so that previously synthesized protein was no longer available for the binding of the newly synthesized phospholipid. Another possible explanation for the inhibition of lipid synthesis only after relatively long-term treatment with cycloheximide is that this inhibition is an indirect one on lipid synthesis resulting from a decrease in energy or other precursors following inhibition of protein synthesis.

The results of the present investigation indicate that phosphatidyl ethanolamine incorporation into the mitochondrial membranes proceeds more slowly than lecithin incorporation. In an in vivo study of phospholipid synthesis, Stein and Stein (1969) observed that labeling of mitochondrial lecithin after ^{14}C-choline administration was more rapid than labeling of mitochondrial phosphatidyl ethanolamine after ^{14}C-ethanolamine administration. They concluded that both phospholipids were synthesized in the microsomes at a similar rate but that phosphatidyl ethanolamine was transported into the mitochondria at a slower rate than was lecithin.

The time sequence with which the various phospholipid classes are labeled in vivo appears to depend on the radioactive precursor used. Various studies with ^{32}P had indicated that mitochondrial phosphatidyl ethanolamine was labeled more rapidly than mitochondrial lecithin (McMurray & Dawson, 1969; Taylor et al., 1967). These observations are difficult to reconcile with the results of the present study in which ^{14}C-glycerol was used as a precursor and with the aforementioned study of Stein and Stein (1969) in which ^{14}C-choline and ^{14}C-ethanolamine were used as phospholipid precursors. In addition, McMurray and Dawson (1969) reported a constant rate of labeling of mitochondrial lipids for 60 min after the intravenous injection of ^{32}P, in contrast to the results presented here in which lecithin and phosphatidyl ethanolamine reached a maximum specific activity within 30 min after intravenous injection of ^{14}C-glycerol. Recently, Schiefer (1969) has also reported that mitochondrial lecithin was maximally labeled 30 min after intravenous injection of ^{14}C-choline, after which time the specific activity slightly decreased. Perhaps these differences arise from changes in the phosphate pool rather than in phospholipid biosynthesis per se.

The exchange of phospholipids between mitochondria and microsomes in vitro reported by Wirtz and Zilversmit (1968) has been confirmed in this

study in which intracellular fractions labeled with either ^{14}C-glycerol or ^{14}C-choline were used. This exchange was not due to microsomal contamination in the mitochondrial fraction, since no gain in glucose-6-phosphatase activity in the mitochondrial fraction and no loss from the microsomal fraction were observed after a 30-min incubation at 30 °C (Table 1). Significantly more radioactive phospholipids were transferred in or out of the outer membrane fraction than the inner membrane fraction. This result was not unexpected in light of the greater accessibility of the outer membrane to the microsomes as well as the close similarity of the outer mitochondrial membrane and the endoplasmic reticulum (Parsons et al., 1967). However, a significant exchange of phospholipids between the inner membrane and the microsomes did occur. A role for this exchange in the cell may be suggested by the experimental evidence of this paper. If the vast majority of mitochondrial proteins and lipids are synthesized in the endoplasmic reticulum, as most evidence indicates, a mechanism must exist for their transport into the mitochondrial structure. It has been demonstrated that the in vitro exchange requires a heat-labile supernatant factor presumably a protein (Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969). This supernatant factor may thus fulfill the role of the lipoprotein carrier proposed by Dawson (1966) to carry phospholipids through the cytoplasm during intracellular transfers of phospholipids between membranes and may also function in transfer of lipoprotein complexes during membrane biosynthesis.

These experiments also indicate a time sequence for formation of the two mitochondrial membranes. The various protein and phospholipid components of the outer membrane fraction were labeled in vivo at a much more rapid rate than those of the inner membrane fraction. It should be noted that the injection of a radioactive precursor represents essentially a pulse label. Bergeron and Droz (1969) have shown that the radioactivity in the trichloroacetic acid-soluble fraction of the plasma after intravenous injection of ^{3}H-leucine decreased rapidly to reach 2 to 6% of the injected dose within 2 min. In addition, Work (1968) has calculated that proteins are synthesized in the ribosomes within 30 sec after in vivo introduction of the label. Hence, the time in which a given membrane fraction reaches its maximum labeling would be largely a function of the transport of the newly synthesized membrane components from their sites of assembly in the endoplasmic reticulum. Experiments in vivo (Beattie et al., 1966) and in vitro (Kadenbach, 1967; Beattie, 1968*a*) have suggested that the transport process is relatively slow compared to the synthetic process. The time sequences reported here may suggest that the outer mitochondrial membrane is formed prior to the inner membrane fraction. Yu, Lukins, and Linnane (1968), in

studies of mitochondrial biogenesis in yeast, have also proposed that the formation of the outer membrane structure, which is not synthesized by the mitochondria, may precede the formation of the inner membrane or cristae. This follows logically from the close proximity of the outer membrane and the endoplasmic reticulum in the cell and their biochemical similarities (Parsons et al., 1967).

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