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Inter-Membrane Transfer of Phosphatides between Microsomes and Mitochondria of 10-Day Old Rat Brain

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INTER-MEMBRANE TRANSFER OF PHOSPHATIDES BETWEEN MICROSOMES AND MITOCHONDRIA OF 10-DAY OLD RAT BRAIN

by

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ABSTRACT OF DISSERTATION

Entitled Inter-membrane Transfer of Phosphatides between Microsomes and Mitochondria of 10-day old Rat Brain submitted by Viesturs Argots Cernaks Liepkalns in partial fulfillment of the requirement for the degree of Doctor of Philosophy, February, 1973.

A soluble, heat-labile and non-dialyzable factor in developing rat brain was discovered which transferred phospholipids from microsomes to mitochondria. Double label experiments indicated that the whole phosphoglyceride moiety was being transferred. Donor microsomes were labeled in vivo with 1-C^{14}18:2ω6 or 1-C^{14}18:3ω3 or Pi^{32} by intracerebral injection. Specific activity of mitochondrial phosphatides was dependent on dialyzed supernatant protein concentration, and time of incubation at 37° with labeled microsomes. Specificity of transfer was suggested in that the class, type and degree of unsaturation of labeled phosphoglycerides transferred was dependent on the compound used to label the donor microsomes. Thus, the ratio of specific activity of mitochondrial phosphatidyl choline to phosphatidyl ethanolamine was higher when 1-C^{14}18:2ω6 was used to label the donor microsomes than when 1-C^{14}18:3ω3 was used. Response of mitochondrial phosphatide specific activity to soluble transfer factor concentration up to 2 mg/ml was exponential. The molecular weight of this phosphoglyceride transfer factor from developing rat brain is greater than 10,000 m.w. A mechanism involving "collision time" between the subcellular particles was proposed.

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INTRODUCTION

The mitochondrion is an organelle whose main function in most mammalian cells is to provide the cellular machinery with its quota of energy in the form of ATP. This ATP upon splitting of its phosphate bond releases 7.5 Kcal/mole of energy barriers (Lehninger, 1965). Understanding the role of mitochondria in the central nervous system (C.N.S.) has been the goal of a community of neurochemists for some time. The nervous tissue uses more O_2 per unit dry weight than any other mammalian tissue (Kety, 1957), although cerebral mitochondria prefer not to oxidize fatty acids under normal conditions (Cahill, 1970).

Mitochondria, generally, are composed of an outer membrane, an inner membrane, an intra-mitochondrial matrix and an intra-mitochondrial space. With the advent of more sophisticated differential and density centrifugation techniques, it has been possible to assign operational as well as morphological definitions to a sub-mitochondrial fraction. Enzymatic activities have been constantly observed by workers to isolate coincidently with one sub-mitochondrial fraction or another. As the volume of data increased, the commensurate segregation of these enzymatic activities has become in itself the criterion for the presence and purity of a submitochondrial

fraction. Mitochondria, in general, host a number of enzyme systems. Among these are the dehydrogenases, the electron transport chain, ATP coupling enzymes, the fatty acid oxidases protein synthesases, transaminases, glycolytic enzymes and monoamine oxidase (Abood, 1970). Some of the enzyme systems have been localized (Schnaitman and Greenawalt, 1968). Monoamine oxidase (MAO) and kynurenine hydroxylase in the outer membrane; succinate-cytochrome reductase, cytochrome oxidase, NADP isocitrate dehydrogenase, β-hydroxy butyrate dehydrogenase all in the inner membrane; adenylate kinase in the intra-mitochondrial space and malate dehydrogenase in the inner mitochondrial matrix. Fatty acid elongation and desaturation activities have been reported in liver, brain and heart mitochondria in both inner and outer membrane fractions (Colli et al., 1969), (Aeberhard and Menkes, 1968), (Whereat et al., 1969).

Discussions relating the molecular structure of the mitochondrion to its function are intimately related to problems facing membrane biochemists in general. As a semi-autonomous, membrane enclosed organelle the mitochondrion has been studied from the point of view of, for example, intra-membrane transport (Carafoli et al., 1971), structural proteins (Schnaitman, 1969) and lipid-protein interaction

(Tzagaloff et al., 1967).

Any theory of membrane function and structure must account for the role of phosphatides therein. Phospholipids make up as much as 30% of the dry weight of brain mitochondria (Lapetina et al., 1968). Evidence is accumulating that the localization and juxtaposition of individual phosphatides is related to the compartmentalization of enzymatic activities. Individual phosphatides are themselves made up of a spectrum of species by virtue of their fatty-acyl moieties. Recently the field of lipid-dependent enzymes has expanded to the extent that the "polar head" group and the fatty-acyl composition of lipid co-factors have been shown to influence the rates of these enzymes (Porcellati and Arienti, 1970). Thus, at least one role of phosphatides in the membrane has come to light: that of providing a membrane-bound ("particulate") enzyme with the proper milieu, or perhaps to maintain the enzyme in the proper conformation(s).

It has been shown that the molecular structure of fatty acids affects the degree of their interaction in monolayers (Ladbrooke and Chapman, 1969). It is not at all surprising then that the transport of metabolites (including ions) across membranes might be influenced by the fatty-acyl composition of the membrane's phosphatide species as well as the charge of polar groups (Tinoco and McIntosh, 1970). In the case of the mitochondrion, transported

metabolites would include those required for its continuing function as a member ("the battery") of the cellular community as well as metabolites ("building blocks") for its own differentiation and maintenance. The recent exciting observations regarding the presence in the mitochondrion of protein synthesizing machinery (Luck and Reich, 1964), (DNA, tRNA's, ribosomes) has led to speculation on how much of itself the mitochondrion is capable of regenerating and on the biogenesis of the mitochondria during development of the cell (Ashwell and Work, 1970).

A number of laboratories have suggested that the mitochondria may be distant cousins of entrapped bacteria which adapted to the intracellular environment of primeval cells to the mutual benefit (Ashwell and Work, 1970). Although the length (and coding capability) of intramitochondrial DNA precludes its coding for all the proteins required for the differentiation of mitochondria (Freeman et al., 1967) some endogenous proteins are certainly coded for and synthesized intra-mitochondrially (Work et al., 1968).

Meanwhile, and in parallel, lipid biochemists have turned to the question of the origin of lipid components of mitochondrial membranes (Dawson, 1966). Models of biological membranes entail lipid-protein complexes (Wallach, 1968) and some workers in bacterial systems have reported the formation of lipid-protein

complexes as prior steps to membrane function (Weiser and Rothfield, 1968).

Lipid Composition of Mammalian Brain Mitochondria (Lapetina et al., 1968)

Approximately 60% of the dry weight of mitochondria isolated from the brain (cerebral cortex) is protein and 37% is lipid. Two per cent is proteolipid. Of the lipid material, 60-75% is phospholipid. The following tables give the percentages of each phospholipid class expressed as per cent of total lipid phosphate.

TABLE 1 (Lapetina et al., 1968)

PHOSPHOLIPIDS OF GUINEA PIG FOREBRAIN MITOCHONDRIA

PHOSPHATIDYL CHOLINE (PC)	36.1
PHOSPHATIDYL ETHANOLAMINE (PE)	35.8
PHOSPHATIDYL SERINE (PS)	Trace
SPHINGOMYELIN (Sph)	Not Detectable (ND)
PHOSPHATIDYL INOSITOL (PI)	3.6
PHOSPHATIDIC ACID (PA)	4.1
CARDIOLIPIN (CL)	18.1

TABLE 2 (Skrbic and Cumings, 1970)

PHOSPHOLIPIDS OF HUMAN BRAIN MITOCHONDRIA

PC	40
PE	23
PS	5.0
Sph	3.7
PI	5.5
PA	.6
CL	11.1
PE (plasmalogen)	9.7
Alkyl ethers	2.2

Forty per cent of rat brain mitochondrial fatty acids are saturated and 60% are unsaturated. Table 3 is a profile of the fatty acid composition of rat brain mitochondria expressed as per cent of total fatty acids.

TABLE 3 (Abood, 1970)

14:0	.7
15:0	0
16:0	12.1
16:1	1.0
16:2	0
18:0	24.5
18:1	21.9
18:2	3.1
18:3	0
20:4	11.4
22:6	14.4
24:0	2.5

Table 4 reports the fatty acid composition of mitochondrial PC from the 1 week and the adult rat brain expressed as % of total.

TABLE 4 (Skrbic and Cumings, 1970)

	1-Week Old	Adult
14:0	2.5	.1
16:0	55.2	47.1
16:1	5.4	1.0
18:0	4.2	12.2
18:1	21.8	30.5
20:1	0	.5
20:4	4.5	5.6
22:6	2.6	1.6

It must be kept in mind that the composition of membranous elements of tissue is affected (although C.N.S. tissue less than other tissue) to some degree by the diet and age of the animal. But even so I would call your attention to the predominance of the phosphatides PC and PE and the fatty acids 16:0, 18:0, 18:1ω9, 20:4ω6, 22:6ω3.

Phospholipid Synthetic Pathways and their Localization

In view of reports of high levels of these lipid species in mitochondria, from the point of view of mitochondrial (and membrane) biogenesis we must ask whether the mitochondrion is capable of de novo synthesis of these compounds; whether the mitochondria contain enzyme systems for de novo synthesis of phosphatides and elongation, desaturation and acylation of fatty acids.

Phosphatidyl choline and PE are synthesized similarly; via CDP intermediates:

(ETHANOLAMINE) CHOLINE + ATP ⟶ (P-ETHANOLAMINE) P-CHOLINE + AMP + Pi

(P-ETHANOLAMINE) P-CHOLINE + CTP ⟶ (CDP-ETHANOLAMINE) CDP-CHOLINE (Sung and Johnstone, 1967) + (PPi)

(CDP-ETHANOLAMINE) CDP-CHOLINE + DIGLYCERIDE ⟶ PC + CMP (Kennedy) and Weiss, 1956)

PA obtains from acylation of glycerol phosphate:

$$\text{GLYCEROL-3-P} + 2\ \text{ACYL CoA} \longrightarrow \text{PHOSPHATIDIC ACID}$$

(Possmayer et al., 1969)

$$\text{PHOSPHATIDIC ACID (PA)} \xrightarrow{\text{Phosphatidate Hydrolase}} \text{DIGLYCERIDE (DG)} + \text{Pi}$$

(Agranoff, 1962)

Phosphatidyl Inositol (PI) is synthesized through the CDP-DIGLYCERIDE intermediate:

$$\text{CTP} + \text{PA} \longrightarrow \text{CDP-DIGLYCERIDE} + \text{PPi}$$

$$\text{I} + \text{CDP-DIGLYCERIDE} \longrightarrow \text{PI} + \text{CMP}$$

(Paulus and Kennedy, 1960)

A Ca^{+2} stimulated base exchange enzyme has been reported which catalyzes the exchange of serine, ethanolamine and perhaps choline moieties among the phosphatides.

$$P_2E + P_1S \overset{Ca^{+2}}{\rightleftharpoons} P_1E + P_2S$$

(Pils and Hubscher, 1961)

Workers for the most part have been unable to observe sufficient levels of lipid synthesizing enzymes in pure mitochondrial preparations to account for the levels of the major phosphatides with the notable exception of phosphatidic acid and cardiolipin (diphosphatidyl glycerol) synthesizing enzymes.

$$\text{CDP-DG} + \text{GLYCEROL-3-P} \longrightarrow \text{PHOSPHATIDYL-GLYCEROL-P} + \text{CMP}$$

$$\text{PHOSPHATIDYL GLYCEROL-P} \longrightarrow \text{PG} + \text{Pi}$$

$$\text{PG} + \text{CDP-DG} \longrightarrow \text{CARDIOLIPIN} + \text{CMP}$$

(Sarzala et al., 1970)

Differential and density centrifugation techniques coupled with assays have determined the intracellular localization of the above enzymatic activities. Synthesis of CDP-choline and CDP-ethanolamine occurs in vitro in soluble fractions of brain and liver (Fiscus and Schneider, 1966). However, the final steps in PC, PI and PE synthesis probably occur in the endoplasmic reticulum (Hill and Lands, 1970). The phospholipid base-exchange enzymes of rat brain are located in a post-mitochondrial particulate fraction (Kanfer, 1972).

Subcellular Localization of Fatty-Acid Metabolism

Purified mature rat brain mitochondria differ from microsomes and supernatant fractions in precursor requirements for fatty acid synthesis. Whereas microsomes were not able to incorporate C^{14} from C^{14} acetyl CoA into fatty acids, mitochondria apparently displayed a fatty acid synthetic capacity which incorporated radioactivity from both C^{14} acetyl CoA and C^{14} malonyl CoA^{16} (Aeberhard and Menkes, 1968). When mitochondrial or microsomal fractions were incubated without ATP in the presence of the proper C^{14}-2-carbon unit precursor, the products of synthesis were mainly 14:0, 16:0 and 18: 0 fatty acids with carboxyl to alkyl C^{14} ratios of 1/6 indicating de novo synthesis. When these particulate fractions were incubated in the presence of ATP, long chain and unsaturated fatty acids were synthesized including 18:0, 18:1, 20:4, 24:0 and 22:6 with carboxyl carbon C^{14}

to alkyl C^{14} ratios of 1/2. It seems likely that ATP was required to activate endogenous precursors (primers) for the elongation system of both particulate fractions. Incorporation of only 29% of the fatty acids into phospholipid synthesized from acetyl CoA and ATP by brain mitochondria was explained by the anaerobic conditions of incubation. Phospholipid synthesis from free fatty acids requires aerobic conditions. Interestingly, these authors were unable to detect any fatty acid synthesis in the myelin fraction.

Rat liver mitochondria can elongate fatty acids in vitro (Colli et al., 1969). This mitochondrial elongation system required ATP or an acyl-CoA primer, reduced pyridine nucleotide and acetyl-CoA. ATP could satisfy the requirement for fatty acyl CoA primer and deacylation of acetyl CoA synthetase activity as measured by CoASH disappearance could be overcome by addition of acyl-CoA primer. Triton-extracted submitochondrial particles which are deficient in bound pyridine nucleotide could entertain elongation via reducing equivalents from either NADH or NADPH. Furthermore, loss of reduced NADH or NAD oxidoreductase activity did not impair reduced nucleotide pyridine nucleotide stimulated incorporation of C^{14} from C^{14} acetyl-CoA into long chain fatty acids. Solubilization of malonyl CoA decarboxylase activity nullified the ability of C^{14}-malonyl CoA to serve as a condensing unit for fatty elongation by submitochondrial particles. The elongation from medium chain precursors (decanoyl-

CoA) and long-chain precursors (palmitoyl-CoA) was somewhat localized in inner and outer membrane fraction respectively.

A de novo fatty acid synthesizing activity was located in the inner membrane of rabbit heart mitochondria by Whereat et al. (1969). These authors also confirm the location of a fatty acid elongation system in the outer membrane. Requirements for fatty acid synthesis in both inner and outer membrane fractions included ATP, CoA, acetate and reduced pyridine nucleotide. Differences in requirements for inner and outer membrane fatty acid synthesis were also observed. Inner membrane de novo synthesis required citrate and NADH, with limited further stimulation by added NADPH. Reducing equivalents for outer membrane elongation could be obtained from NADH and NADPH. However an amount of NADH stimulated elongation to a greater extent than an equivalent sum of NADH plus NADPH. The authors contend that the outer mitochondrial membrane acts as a shuttle for reducing equivalents from the cytosol in the form of elongated fatty acids which in turn are oxidized in the matrix. The intramitochondrial respiratory chain then reoxidizes the intramitochondrial NADH.

Rat liver and brain mitochondria were found to elongate 22:0 to 24:0 and 22:1ω9 to 24:1ω9 in vitro by Boone and Wakil (1970). A

sonicated suspension of brain mitochondria yielded a 100,000xg particulate fraction which had greater elongation specific activity as measured by C^{14} incorporation from C^{14}-acetyl CoA, NADH, NADPH, ATP and acyl CoA. When acyl CoA was omitted, C^{14} from C^{14}-acetyl CoA was incorporated into stearic and other longer chain, and polyunsaturated fatty acids.

That acetyl CoA and not malonyl CoA was the 2-carbon unit donator in the mitochondrial elongation system was demonstrated in a number of ways. Malonyl CoA decarboxylase activity failed to inhibit elongation. In a series of incubations with increasing amounts of acetyl CoA and a constant amount of C^{14} malonyl CoA, dilution of C^{14} in the fatty acid products occurred to an extent expected if acetyl CoA were the immediate precursor. Malonyl CoA did not dilute incorporation of C^{14} acetyl CoA. Avidin did not inhibit C^{14} incorporation into long chain fatty acids from C^{14} acetyl CoA.

Specificity for the primer fatty acids was expressed in two ways. Short chain acyl CoAs served as better substrates for elongation than longer chain acyl CoA's and monounsaturated acyl CoA's served as better substrates than their saturated homologs. The author concludes either that the elongation system is specific for short-chain and monounsaturated acyl CoA's or that under the

conditions of their experiments the elongation system has greater access to the more soluble acyl CoA's.

A reduced pyridine nucleotide-supported desaturating activity in hen liver microsomes has been described by Jones et al. (1969). Lipid dependence of the desaturase activity was suspected when acetone-extracted microsomes lost their capability to desaturate stearyl CoA to oleyl CoA. Sixty per cent restoration of activity was accomplished by the addition of a mixture of phospholipid, triglyceride and free fatty acids obtained from a lipid extract of the hen liver microsomal fraction. The phospholipid requirement could be satisfied by lecithin. Complete restoration of desaturase activity by the addition of lipid to the acetone extracted microsomes was not achieved since the lipid dependent acyl-CoA hydrolase activity was completely restored and stearyl CoA was being hydrolyzed at a rate comparable with its desaturation. NADH cytochrome C reductase lipid dependency was also tested and was similar to that of the desaturase save for the lack of a triglyceride requirement. However, NADPH-cytochrome C reductase activity was not dependent on lipid. Since NADPH can also satisfy the desaturase activity requirement for reduced pyridine nucleotide, the authors claim the participation of a NADPH-cytochrome reductase activity is coupled to the desaturase activity via the cytochrome b_5 reductase of the hen liver microsome electron transport system.

Microsomes from rat liver are able to elongate and desaturate fatty acid precursors in the presence of TPNH, CoA, ATP, Mg^{+2} and malonyl CoA (Mohrauer et al., 1967). Linoleic acid was converted to 20:2ω6 under anaerobic conditions. Under aerobic conditions 18:3ω6, 20:3ω6 and 20:4ω6 were formed. Hence microsomal elongation activity can be "uncoupled" from desaturase activity. This property of in vitro systems has been used to study the regulation of fatty acid synthesis. An interrelationship exists between the various fatty acids and the presence of one polyunsaturated fatty acid (PFA) influences the elongation and desaturase mechanisms on another PFA. Thus, evidence accumulated to date indicates that the extent of elongation and desaturation of 18:2ω6 or 18:3ω3 is controlled by fatty acids already present in the medium (Brenner and Peluffo, 1966), (Nervi et al., 1968). Alpha linolenic acid inhibits desaturation of 18:2ω6 to 18:3ω6 (Brenner and Peluffo, 1969). Oleic acid is also inhibitory but not to the same degree.

Introduction of Structural Specificity into Phosphatides

Elongated fatty acids of the ω3 and ω6 families such as 22:6ω3 and 20:4ω6 are introduced into phosphatides exclusively via the deacylation-acyltation cycle (Kanoh, 1969), Waite et al., 1969). Shorter-chain fatty acids, primarily 16:0 (palmitic acids) are

introduced via de novo synthesis. Specificity is expressed toward the acyl-CoA's at the level of de novo synthesis (Possmayer et al., 1969), that is, the acylation of glycerol phosphate. Acyl-CoA, acyl transferase and the lipase A_1 and A_2 combine to "retailor" phosphatide acyl composition after de novo synthesis (Lennarz, 1970), (Hill and Lands, 1970). The presence of short chain or long-chain acyl transferases in the outer mitochondrial membrane of rat liver has been seriously questioned (Eibl et al., 1969). Lysophosphatidyl choline: acyl-CoA acyl transferase and lysophosphatidyl ethanolamine: acyl CoA acyl transferase have been reported in microsomal fractions of liver and brain (Hill and Lands, 1970). Thus, the redress of fatty-acyl compositions subsequent to de novo synthesis would seem to occur mainly in the endoplasmic reticulum although mitochondria have the capability of primary introduction of shorter chain fatty-acyl moieties through the acyl CoA: Glycerol-3 phosphate acyl transferases.

The net result of fatty acyl redistributions is that unsaturated fatty acyl groups tended to be retained in the β position and saturated fatty acyl moieties tended to be retained in the α position (Hill and Lands, 1970) of the glycerol backbone. Enrichment of 18:0 and 20:4 in the PC fraction of cerebral lipids and 22:6 in the PE fraction can also be explained

by the specificity of deacylation: acylation cycles, formation of the CDP-diglycerides and the selective utilization of diglycerides for the synthesis of PC and PE species (McMurray and Magee, 1972).

The question of intermembrane exchange between "microsomes" (the endoplasmic reticulum) and mitochondria is relevant from the point of view of the phospholipid classes since it is likely that nitrogen-containing bases of phospholipid classes are incorporated into mitochondria only as moieties of the whole phosphatide molecule (see below). The same relevancy may hold for the phosphatide species with certain fatty acyl moieties which have been sculptured by the enzyme cycles of the endoplasmic reticulum. For the endoplasmic reticulum seems from the literature to be the main theater of lipid synthesis and modification.

INTER-MEMBRANE TRANSFER OF PHOSPHOLIPIDS

Since Dawson accurately predicted inter-membrane transport of phospholipids in 1966, a number of laboratories have contributed to the volume of evidence which indicates that, in vitro at least, transfer and exchange of labeled lipid molecular species can occur. These studies were inspired by the finding that the endoplasmic reticulum (E.R.) of liver cells houses enzymes (e.g. CDP-choline:1, 2-diglyceride choline phospho-transferase (Wilgram and Kennedy, 1963)

required for de novo synthesis of phospholipids, enzymes which have not yet been observed in mitochondrial preparations (Sarzala et al., 1970). Thus, the working hypothesis on the part of most workers at this point is that, in vivo, the E.R. is the site of de novo synthesis of phospholipid molecules. These compounds then get distributed to other intracellular membranous sites which contain phosphoglycerides, although themselves incapable of phosphoglyceride synthesis.

In in vitro experiments, lipid fractions of microsomes were usually labeled in vivo and then incubated in vitro with unlabeled mitochondria. Some of the compounds used to label moieties of the phosphoglyceride in vivo have been ^{32}P (Akiyama and Sakagami, 1968), ^{14}C-glycerol (Wirtz and Zilversmit, 1968), 1-^{14}C choline (McMurray and Dawson, 1969) and 1-^{14}C palmitate (Sauner and Levy, 1971) and ^{14}C-acetate (Abdelkader and Mazliak, 1970). Wirtz and Zilversmit (1968) first observed that the presence of a soluble heat-labile and non-dialyzable factor was required to stimulate transfer of lipid-soluble ^{32}P radioactivity from liver microsomes to mitochondria in vitro. Jungawala and Dawson (1970) have investigated the incorporation of ^{14}C choline into isolated rat liver mitochondria. They concluded that under the proper incubating conditions mitochondrial incorporation of ^{14}C choline into lipid-extractable radioactivity is

dependent upon the presence of supernatant (100,000 xg) and microsomal fraction. Experiments by Dawson's group also indicated that "^{14}C incorporation" by the mitochondrial fraction alone observed by other workers (Kaiser, 1968) may have been due to contaminating microsomes. Sauner and Levy (1971) observed the incorporation of lipid-soluble ^{32}P and ^{14}C glycerol into liver mitochondria only in the presence of microsomes. However, incorporation of 1-^{14}C palmitate occurred in the absence of microsomes. McMurray and co-workers (1969) also were able to accomplish incorporation of ^{32}P *in vitro* into major phosphoglycerides of rat liver mitochondria by the addition of microsomes and a heat-labile supernatant factor. Extrapolation to zero microsomal contamination based on enzyme marking revealed that only phosphatidic acid may have incorporated ^{32}P directly. The incorporation by mitochondria of lipid-soluble ^{14}C from CDP-choline (1-^{14}C) required a heat-labile supernatant factor and was not energy dependent.

Jungawala and Dawson (1970) have studied phospholipid exchange within isolated liver cells. In cells which were pulse-labeled with $P^{32}i$, Me-^{14}C choline, ^{14}C-ethanolamine or U-^{14}C inositol, the labels were chased from microsomes to mitochondria. The authors concluded that the site of *de novo* synthesis of phospholipids in the liver cell is the endoplasmic reticulum and that the

mitochondria obtain newly synthesized phosphatides from the endoplasmic reticulum.

Stein and Stein (1969) used radioautography to localize the fate of the newly synthesized phosphatides, PC and PE in the rat liver cell. Five minutes after injection of labeled choline, glycerol or linoleate the ratio of microsomal to mitochondrial PE specific activity was 5.3, however the ratio of microsomal to mitochondrial PC was 1.5. This suggested to these workers that the rates of intermembrane transfer of these two phosphatide families differed.

The protein-like nature of the supernatant transfer factor in liver has been explored principally by K.W.A. Wirtz and co-workers (1969). Precipitation of the 100,000 xg. supernatant of rat liver homogenate by pH adjustment to 5.1 renders the preparation free from phospholipid, and the phospholipid transferring factor was not precipitated. Furthermore, the factor was heat-labile and trypsin-digestible. Lately, Wirtz and Zilversmit (1970) have purified a protein from beef-heart which stimulates the exchange of the phospholipids PC and PI.

Evidence is accumulating that in brain cell and liver cell mitochondria there is a deficiency of enzymes which are required for de novo synthesis of phospholipids (supra vide). The metabolism of fatty acyl groups is of interest since it is in the fatty acyl composition

that phosphoglycerides even in the same class (i.e. with the same polar head group) obtain diversity. Fatty acid metabolic pathways and phosphatide pathways join at the point of acyl-transferase activity. H. Eibl and co-workers (1969) are apparently convinced that the outer membrane of rat liver mitochondria does not contain significant acyl transferase activity. Furthermore, fatty-acyl elongation and desaturase activities of rat liver microsomes have been investigated in depth (Stumpf, 1969) and the main arena of fatty acid metabolism in the liver cell seems to be in the endoplasmic reticulum as well. Liver microsomes have 7 times the capacity of mitochondria for the elongation of fatty acids (Mohrauer et al., 1967). The pathways of linoleic and linolenic elongation yield principally arachdonic and docosahexaenoic acids by the following sequence of metabolic events:

$$18{:}2\omega 6 \rightarrow 18{:}3\omega 6 \rightarrow 20{:}3\omega 6 \rightarrow 20{:}4\omega 6$$

$$18{:}3\omega 3 \rightarrow 18{:}4\omega 3 \rightarrow 20{:}4\omega 3 \rightarrow 20{:}5\omega 3 \rightarrow 22{:}5\omega 3 \rightarrow 22{:}6\omega 3$$

(Wakil, 1970)

It would seem then that intermembrane transfer of fatty acyl groups must occur via whole phosphoglyceride species which are composed in part of elongated and/or desaturated fatty acyl moieties. Wirtz and co-workers (1970) observed that the in vitro intermembrane transfer of 20:4ω6 or arachidonic acid and/or 20:3ω9 recover to a

greater extent than other fatty-acyl species upon addition of supernatant to an incubation containing ^{32}Pi labeled microsomes and unlabeled mitochondria. K.W.A. Wirtz (1972) has approached the problem of specificity of intermembrane transfer using liposome species (instead of "donor microsomes") of known surface charge (as measured by electrophoretic mobility). Addition of negatively charged species such as phosphatidic acid to the liposome mixture affected the Zeta potential of the liposomes and also the rate of transfer of phosphatidyl choline to mitochondria from the liposomes.

Intermembrane exchange of phospholipids may be relevant to the development of the central nervous system, since nervous tissue is characterized by a wide structural and functional diversity of membranous structures. We, therefore undertook the study of the in vitro transfer of phosphatides between microsomal and mitochondrial preparations from developing rat brain. In addition, since membrane composition studies reveal the extent of diversity and differentiation among fatty-acyl group and group combinations of the phosphatides, we were interested in studying in vitro intermembrane transfer of 1-C^{14} fatty-acyl labeled phosphatides. The likelihood of whole phosphatide transfer was so high our use of a ^{14}C label of

donor microsomes was felt justified. One-^{14}C fatty-acyl compounds of known structure are readily available. Thus, any specificity of in vitro intermembrane transfer with respect to phosphatide species containing a preponderance of omega 6 or omega 3 fatty acids could be studied directly.

This is the first such study using nervous tissue and 1-^{14}C labelled fatty acyl labeled phosphatides; brain mitochondria are difficult to purify. We also present the first direct evidence of simultaneous transfer of fatty-acyl and phosphate moieties of the phosphoglyceride.

Central nervous system lipid analyses reveal the presence of the omega 6 and omega 3 families of fatty acids (Bernsohn and Stephanides, 1967). It would seem an inter-membrane transport mechanism would play a role in providing the mitochondrial membrane with its full complement of fatty acyl groups. The biological membrane may after all be the focal point for the functional expression of essentiality of the omega 6 and omega 3 families of fatty acids via the structural characteristics lent to the membrane by their presence and the presence of their elongation products (Bernsohn and Liepkalns, in press). The fatty acyl composition of lipid co-factors for some lipid dependent (and membrane bound) enzymes has an effect on the rates of these enzymes (Porcellati and Arienti, 1970).

MATERIALS AND METHODS

PREPARATION AND IN VIVO INJECTION OF RADIOACTIVE PRECURSORS

Each of 24, 10-day old rats was injected intracerebrally with the following preparation: 1-C^{14}18:2ω6 or 1-C^{14}18:3ω3 (50uc/umole, Applied Science, State College, Pa.), 0.25uc, BSA Fraction V 1mg. (Sigma Chemical Co., St. Louis, Mo.), 0.02umoles NaOH all in 10ul.

In double label experiments, 1uc of P^{32}-orthophosphate (500 uc/umole disodium salt, Abbott Laboratories, North Chicago, Illinois) was injected into each rat in the same mixture as the 1-C^{14} fatty-acyl compound.

Rats were allowed 2 to 4 hours of life after injection and the animals were killed by one blow to the neck. The cerebral hemispheres were excised and bathed in medium A prior to homogenization. Excision was completed within 20 seconds following sacrifice of the animal.

FRACTIONATION PROCEDURE. The Isolation of Subcellular Fractions.

What follows is a diagram of the general procedure of initial isolation of mitochondria, microsomes and 110,000 xg supernatant from 10-day old rat brain (Gabay, et al., 1971, Koenig, et al., 1964, Stahl, et al., 1963).

24 cerebral hemispheres

2 mls. medium A/gm. wet weight of tissue

↓

homogenate (Dounce A 10 strokes, Dounce B 10 strokes)

↓

squeeze through cheese cloth

↓

Dilute to 10 mls. medium A/gram wet weight tissue

↓

Centrifuge 2000xg/20 min.

Supernatant → Centrifuge 12,000xg/15 min.

Pellet

Supernatant → Centrifuge 20,000xg/20 min.

Pellet (discard)

Supernatant → Centrifuge 110,000xg/2 hrs.

Supernatant

Pellet

"Microsomes"

Pellet (Tan, with creamy layer) Discard creamy layer

↓

Homogenate (6ml. medium F/gm. wet wt. tissue)

↓

12,000xg/20 min.

Supernatant

Pellet

↓

Wash (with 4 ml. Medium A/gm. wet wt. tissue

↓

Layer in 5 ml. on sucrose gradient 50,000xg/2 hrs. Head 23,500 RPM/IEC SB-100

Medium A =
.32M Sucrose
.001M EDTA
(Disodium Salt)

adjusted to pH 7.4
with Tris Base (1M)

Medium F =
6% Ficoll in
Medium A

5 ml. .32M Sucrose (.001M Na_2EDTA, adjusted to pH 7.4 with 2 M Tris-Base)
" .8M " "
" 1.0M " "
" 1.2M " "
" 1.4M " "

↓

Fraction between 1.2M and 1.4M is "mitochondria"

↓

Cilute 3 fold centrifuge 15,000xg/20 min.

PREPARATION OF DIALYZED SUPERNATANT

Routinely the unlabeled post-microsomal supernatant was adjusted to pH 5.1 by drop by drop addition of 2M HCl. The cloudy suspension was allowed to stand on ice for 30 minutes and was then centrifuged 12,000 xg/20 min. The supernatant from that centrifugation was re-adjusted to pH 7.5 with 2M Tris-Base. This clear solution was then passed by pasteur pipette into a 60 ml. capacity Diaflo cell (the Amicon Corp. Lexington, Mass.) which had been supplied with the desired membrane (UM 2 or UM 10). A 45 psi pressure was applied to the mixing solution until the original volume had passed through the membrane (usually 40-60 mls). Medium A was then added to the dialyzed supernatant to obtain the desired concentration of protein. Protein determinations were performed according to Lowry et al. (1951).

INCUBATION AND RE-ISOLATION OF SUBCELLULAR FRACTIONS

The final, washed, labeled microsomal pellet from rats which had been injected with a radioactive precursor was re-suspended in 2 to 8 mls. of medium A (1ml/incubation). Unlabeled mitochondria from 24 to 96 rats were also re-suspended in Medium A (1ml/incubation). There was a requirement for the relatively large number of rats in

order to obtain a sufficient amount of pure mitochondria. The unlabeled post-microsomal supernatant was obtained from the same homogenate as the unlabeled mitochondria.

Each incubation was initiated by the addition of 1 ml of labeled microsomal suspension to a suspension of unlabeled mitochondria in cold dialyzed supernatant in a 37° water bath, for the indicated amount of incubation time.

Incubations were terminated by the addition of the equivalent of the incubation volume of ice-cold medium A and kept on ice until re-isolation.

RE-ISOLATION OF SUBCELLULAR FRACTIONS AFTER INCUBATION

Each chilled incubation was then centrifuged at 11,500xg and the pellet was re-suspended by gentle but thorough homogenization in 5 mls of Medium A and layered on the sucrose gradient outlined above. The fraction between 1.4 and 1.2 M sucrose (0.001M EDTA - adjusted to pH 7.5) was pelleted by 3x dilution and centrifugation at 15,000 xg. After washing with Medium A the preparation was lyophilized prior to lipid extraction.

Microsomes and supernatant were re-isolated by centrifugation of the 11,500 xg supernatant at 110,000 xg for 90 minutes. The re-isolated supernatant was also lyophilized prior to lipid extraction.

EXTRACTION OF PHOSPHOLIPIDS (Folch et al., 1957)

Each lyophilyzate was re-suspended in 12 ml. methanol (+0.1% IONOL, Shell Chemical, N.Y., N.Y.) and the suspension was transferred to a 50 ml conical centrifuge tube to which was added 24 ml $CHCl_3$ (+.1% IONOL) and 9 mls. 0.9% NaCl. The extractions were allowed to stand refrigerated overnight and the lower organic phase was washed with theoretical upper phase ($CHCl_3$:CH_3OH:0.9% NaCl, 3:48:47). The washed organic phases were transferred to round-bottom flasks for flask vaporation on a Büchi Rotavapor (Deluxe Model VE 50 GO Rinco Instrument Company, Inc., Greenville, Illinois 62246) equipped with an inlet for N_2 gas. The residue was picked up in 3 mls $CHCl_3$ and applied to a 0.5 x 13 cm., 1 gm. SiO_2 (100-200 mesh Bio-Rad Laboratories, Richmond, California) column. The column was washed first with 50 mls. $CHCl_3$ (contg. 1% IONOL) 20 mls. acetone (containing .1% IONOL) and finally 50 mls. CH_3OH (containing 0.1% IONOL). Monitoring of "counts" showed that these volumes of eluents were sufficient to avoid cross-contamination of column fractions. The CH_3OH fraction was termed the phosphatide fraction after Rouser and co-workers (1970) and was evaporated on the Rotavapor and dissolved in 2 to 3 mls. of $CHCl_3/CH_3OH$(4/1).

DETERMINATION OF SPECIFIC ACTIVITIES

One aliquot of the phosphatide extract was transferred to a scintillation vial which was brought to dryness and to which was added 15 mls. of a solution of pre-mix P (5 gms/liter toluene (Packard Co., Des Plaines, Illinois). The amount of radioactivity in counts per min. (CPMS) in the vials was determined with a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3003. Another aliquot was used for the determination of organic phosphorus by the method of Bartlett (1959). All test tubes used for phosphorus assays had been soaked in concentrated HCl for at least 24 hours.

ISOLATION OF INDIVIDUAL PHOSPHATIDES

If a sufficient amount of radioactivity was available, aliquots of the phosphatide fractions were streaked on thin-layer plates for chromatography. Standards, PS, PE, PC, lysophosphatidyl choline (LPC), sphingomyelin (Supelco, Inc., Bellefonte, Pa.) were spotted on the plate as well. After 2-1/2 to 3 hrs. of chromatography in a dehumidified atmosphere, the plate was evaporated overnight. Only the lane traversed by the standards was sprayed with phospho-molybdate (Ditmer and Lester, 1964) to determine the location of individual phosphatide spots. The silica gel areas corresponding to the identifying spots were scraped off

and transferred to test tubes. Phosphatides were eluted according to a modified method of Skipski (1964) which employs 5 mls. of the heated solvent system, and 4 mls. of heated CH_3OH/H_2O/acetic acid (94/1/5). Specific activities of the eluted phosphatides were determined in like manner to the total phosphatide specific activity determinations.

METHYLATION AND GAS-LIQUID CHROMATOGRAPHY (GLC)
(Metcalf, Schmitz and Pelka, 1966)

Methylation was accomplished by BF_3-methanol and GLC was done on a 1/4"x6' column with 10% DEGS (Diethylene Glycol Succinate, Applied Science, Corp., State College, Pa.) as the liquid phase and Gas-Chrom Q (100 mesh, Applied Science) as the solid support with helium as the carrier gas at an input pressure of 45 psi at 180° on a Varian 90P. Recovery of individual methyl esters from the gas chromatograph has been described by R. Wood and R. Reiser (1965). All lipid samples were stored when necessary at -60° under N_2.

ELECTRON MICROSCOPY

Samples of pellets for electron microscopy were fixed in 0.1M phosphate buffered glutaraldehyde (3.125%). After overnight washing in 0.1M phosphate the sample was post-osmicated for 1 hr. in 1% buffered osmium tetroxide in the dark. This was followed by dehydration

in 35%, 50%, 70%, 80%, 95% and absolute ethanol (10 min. each) at approx. 4°C. Then the sample was allowed to stand in propylene oxide for 20 min., 1:1 propylene oxide to Epon (45 min) at 1:3 propylene to Epon (45 min) and finally Epon for 1 hr. and Epon for 8 hrs. The tissue was then embedded in fresh Epon in Electron Microscopy capsules and heated at 65° for 48-72 hrs. Electron Microscopy was kindly performed by the electron Microscopy Laboratory of the V.A. Hospital, Hines, Illinois (Dr. Tonaki, Director) with the help of Drs. Paul Bramson and Andrew Payer of the Department of Anatomy, the Electron Microscope Laboratory, Loyola Stritch School of Medicine.

INCUBATION WITH PRE-INCUBATED MICROSOMES

In some experiments instead of using microsomes which had been pre-labeled *in vivo*, microsomes which had been pre-incubated with co-factors and radioactive precursors were used.

Aerobic pre-incubation conditions for microsomes were similar to those described by Aeberhard and Menkes (1968) except that 1-^{14}C 18:2ω6 or 1-^{14}C 18:3ω3 (Na salts) and CoA (1 mM) were used instead of 1-^{14}C-acetyl CoA or 1-^{14}C malonyl CoA. Microsomes were washed with medium A and incubations with unlabelled mitochondria and re-isolation of organelles were performed as described above for microsomes labeled by *in vivo* injection. Each incubation contained

(in umoles) acetyl CoA (0.5) (Sigma), NADH (3) (Sigma), NADPH (3) (Sigma), ATP (16) (P-L Bio-chemicals), K_2HPO_4 (100), Antimycin A (5 ug) (Sigma) and 1 uc of 1-^{14}C 18:2ω6 or 1-^{14}C 18:3ω3 and the desired amount of microsomal or mitochondrial protein all in 1 ml.

ENZYME ASSAYS

Glucose-6-phosphatase was assayed according to Swanson (1955). Succinic acid dehydrogenase was assayed according to Schnaitman and Greenawalt (1968) with INT (Sigma) as the electron acceptor. Monoamine oxidase was assayed according to Gabay (1971) with benzyl amine as control substrate.

RESULTS

Electron micrographs of the 10-day old rat mitochondria preparations used in the experiments are shown in Figures A and B. It appears that the organelles are relatively intact but with some distortion, no doubt due to the rather rigorous purification procedure outlined in MATERIALS AND METHODS. The appearance in a micrograph field of particles of membranous elements and veiscles of other than mitochondrial origin was sufficiently rare to merit routine use of this purification procedure to obtain rat brain mitochondria for the studies described in this dissertation. A typical discontinuous sucrose gradient is shown in Figure D. The layer between 1.4 and 1.2 M sucrose contains the mitochondria shown in Figures A and B. These mitochondria had succinic acid dehydrogenase activity and monoamine oxidase activity but no glucose-6-phosphatase activity. A microsome preparation is shown in Figure C.

Initial experiments were designed to test the dependency of the transfer of labelled phospholipid on a soluble factor in developing rat brain since such a factor had been reported to be present in other tissues. Microsomes which had been labelled in vivo with 1-^{14}C 18:2ω6 or 1-^{14}C18:3ω3 were incubated with unlabelled mitochondria in the presence of the post-

microsomal supernatant which had been variously treated as shown in Table I. Mitochondria re-isolated following incubation with "live" supernatant and labeled microsomes showed an increased phosphatide specific activity relative to mitochondria re-isolated following incubation with boiled (3 min.) supernatant and labeled microsomes. Apparently, the factor which facilitates transfer (PTF)[1] is also pH 5.1 non-precipitable. Wirtz and Zilversmit (1969b) had reported that pH 5.1 precipitation in their hands renders the supernatant devoid of lipid phosphorus. Even after dialysis for 17 hours against 30 liter, of medium A 15 mls. of supernatant (4 mg protein/ml) still retained some transfer activity.

In order to facilitate the assay of phospholipid transfer, a more rapid method of dialysis was embraced. An Amicon Diaflo cell under N_2 pressure (40-50psi) was employed and supernatant preparations were forced through membranes of various pore sizes. Results of two such experiments are shown in Table II. These figures suggest that the PTF is greater than 10,000 M.W. since dialyzates under 10,000 failed to stimulate transfer of phospholipids into mitochondria, as measured by the phosphatide specific activity above the values of heated supernatants. Furthermore, a dialyzate under 1,000 M.W., when added back to its molecular-

[1] PTF (=Phospholipid Transferring Factor)

sized counterpart retained by the membrane, diluted transferring activity proportionately. We conclude from the data in Tables I and II that PTF behaves like a protein.

The mitochondrial phosphatide specific activity achieved via incubation with labeled microsomes is a function of the soluble dialyzed protein concentration (Fig. 1). Moreover, it appears to be a semi-logarithmic function. This and the rest of the transfer experiments were performed using pH 5.1 precipitated and Diaflo UM 10 forced-flow dialyzed material. Note that the log curve "breaks" at a value of just above 2.0 mg/ml protein. We think that this is a reflection of exchange as well as one-directional transfer since the donor microsome starting material specific activity was 1.7 CPM phospholipid-phosphorus x 750. Thus, the leveling of the curve suggests the more rapid onset of isotopic equilibrium "catalyzed" by the PTF.

Acceptor mitochondrial phosphatide specific activity is also a function of incubation time at 37°C (Figure II). Note that the "O" time incubation contained live PTF and was merely kept on ice during the time experimental tubes were incubated. Either we were not able to detect neutral count incorporation or mitochondria were unable to incorporate free fatty acids from in vivo labeled microsomes or the medium. In any case, the neutral fraction which would include labeled fatty acid incorporation curve in Figure II indicates that the source of

radioactive phosphatides in the mitochondria is not through contamination. We would expect parallel curves in Figure II if the source of increasing phospholipids and neutral counts as a function of time was contamination from labeled microsomes.

Relationships of the three sub-cellular members of the incubation are indicated in Figure III. With increasing dialyzed supernatant concentration, the supernatant was observed to have a lower phosphatide specific activity value than the 0.5 mg/ml value. No phosphorus was detectable in the re-isolated supernatant after incubation with labeled microsomes, unlabeled mitochondria and buffer alone ("O" mg/ml), conditions under which microsomes lowered in specific activity considerably. At dialyzed supernatant protein concentrations greater than zero, microsomes retained more or less a steady phosphatide specific activity (Figure III). Mitochondria increased in phosphatide specific activity in response to increases in dialyzed supernatant concentration, but the curve has a log phase at lower concentrations, and it follows that the mitochondria points in Figure III fit the semi-log plot in Figure I.

From Figure IV, the response of mitochondrial phosphatide specific activity to incubation time at 37°C was answered by decreases in microsomal phosphatide specific activity. Thus, either the microsomal phosphatide pool was being "diluted" by a cold pool

(e.g., via exchange) or a specific microsomal exchangeable pool exists. The supernatant maintained a steady-state phosphatide specific activity, for most time points, somewhat less than or approximately equal to the value for microsomes.

In order to study mitochondrial phosphatides, the lipid extracts of each of the sub-cellular fractions participating in the incubation mixture was applied to silicic acid columns and the polar (CH_3OH) fractions from column chromatography were chromatographed on Quantogram thin-layer plates. Phosphorus spray sensitive spots were eluted from the plates by the method of Skipski (1964) and CPM's and phosphorus determinations were performed to obtain the specific activity values on each of the phosphatides listed in Table III. Note the decreased specific activity value of each of the major phosphatides of microsomes after incubation relative to the values for microsomes prior to incubation. Unlabeled mitochondria failed to incorporate significant amounts of radioactivity into any of the phosphatides analyzed when incubated in the presence of labeled microsomes and a heated (boiled) dialyzed supernatant preparation. Table III was obtained by using 1-^{14}C 18:2ω6 as a radioactive precursor for the labeling of donor microsomal material. In some experiments, 1-^{14}C 18:3ω3 was used to label the microsomes. For comparison purposes, Table IV lists the specific activity values

relative to phosphatidyl choline of each individual phosphatide of labeled microsomes, mitochondria which obtained their labeled phosphatides *via in vitro* incubation, and mitochondria labeled *in vivo* (obtained from the same brains as the labeled microsomes). Mitochondria labeled *in vitro* in the presence of microsomes pre-labeled with 1-^{14}C 18:3ω3 had higher specific activity values for phosphatidyl ethanolamine (PE) than mitochondria incubated in the presence of microsomes pre-labeled with 1-^{14}C 18:2ω6. Sphingomyelin and lysophosphatides of pre-labeled microsomes did not incorporate significant amounts *via* the *in vivo* route in the case of either 1-^{14}C 18:3ω3 or 1-^{14}C 18:2ω6.

In order to initiate studies on the specificity or non-specificity of intermembrane transport, we studied the degree of elongation of the mitochondrial phosphatides after incubation with dialyzed supernatant, microsomes pre-labeled *in vivo* with 1-^{14}C 18:2ω6 or 1-^{14}C 18:3ω3. Mitochondria which had been incubated with dialyzed supernatant and 1-^{14}C 18:3ω3 had an almost two-fold greater percentage of ^{14}C in elongated fatty acids in the phosphatide fraction (Table V), than mitochondria incubated with 1-^{14}C 18:2ω6.

To further explore the specificity problem, mitochondria were incubated with microsomes labeled with either fatty acid in separate incubation time studies. Differences in the transfer rates

between $^{14}C\omega6$ and $^{14}C\omega3$ labeled phosphatides would be consistent with some recognition of the transferred moiety. Apparently, dialyzed supernatant was able to donate phosphatides from microsomes pre-labeled *in vivo* with 1-^{14}C 18:2ω6 at a greater rate (Figure V), assuming saturation of PTF(s) with respect to all transferable phosphatides.

A possible criticism of these *in vitro* phospholipid transfer studies is that a fatty acyl label is not an accurate tracer of the whole phospholipid species since other moieties of the molecule such as the glycerol backbone phosphodiester and bases are not accounted for. This is a fair criticism in view of the existence of phospholipases A_1 and A_2 (Hill and Lands, 1970), which cleave the fatty acyl group in the and positions of phosphoglycerides, phospholipase C (Hill and Lands, 1970) which is a phosphodiesterase and produces a monophosphoryl alcohol and diglyceride from a phosphatide; and phospholipase D (Hill and Lands, 1970) which produces phosphatidic acid and the base (e.g., ethanolamine, choline). Thus, in an effort to justify the use of the ^{14}C fatty acyl label and to support whole molecule transfer of the phosphatide, a double label experiment was performed. Microsomes were pre-labeled coincidentally with 1-^{14}C 18:3ω3 and $P^{32}i$ *in vivo*. Unlabeled mitochondria were then incubated with dialyzed supernatant and double labeled microsomes and the specific

activity of the "acceptor" mitochondria as a function of 37°C incubation time was determined (Figure VI).

The ratio of $^{32}P/^{14}C$ specific activities shows a slight but significant decrease as a function of incubation time. But, the decrease in the ratio of only 20% after up to one hour's incubation time indicates that, by and large, the transfer of the whole phosphatide molecule occurs. The ratio of P^{32}/C^{14} specific activities of "donor microsomes" remains constant as a function of time of incubation (Fig. VII).

TOTALLY IN VITRO STUDIES

Mitochondria incorporate ^{14}C fatty acids in the presence of fortified buffer (see MATERIALS AND METHODS), 110,000 xg supernatant plus microsomes. The greatest amount of incorporation into mitochondria was observed when microsomes were added along with the supernatant (Table VI).

Mitochondria were incubated with 110,000 xg supernatant and microsomes which had been labeled by pre-incubation *in vitro*. Microsomes which have been previously pre-labeled with 1-^{14}C 18:2ω6 decreased in specific activity from 143 to 54 ^{14}C cpm/ug phospholipid-P while mitochondria increased in phosphatide specific activity after an hour's incubation to 53 phosphatide ^{14}C/ug phospholipid-P (Table VII). Microsomes which had been pre-incubated *in vitro* with

1-^{14}C 18:3ω3 decreased in specific activity from 73 to 25 ^{14}C phosphatide cpm/ug phospholipid-P after incubation while mitochondria increased to 26 ^{14}C cpm/ug phospholipid-P. Table VIII summarizes the data concerning the individual phosphatides after incubation of mitochondria with pre-incubated microsomes and supernatant. Note the much higher proportion of radioactivity in PC of mitochondria when compared to other individual phosphatides. Contrast this data with that of Table III where PE and PC have closer specific activity values. It must be kept in mind that the source of label for mitochondria in the experimental results described in Table III was microsomes labeled in vivo.

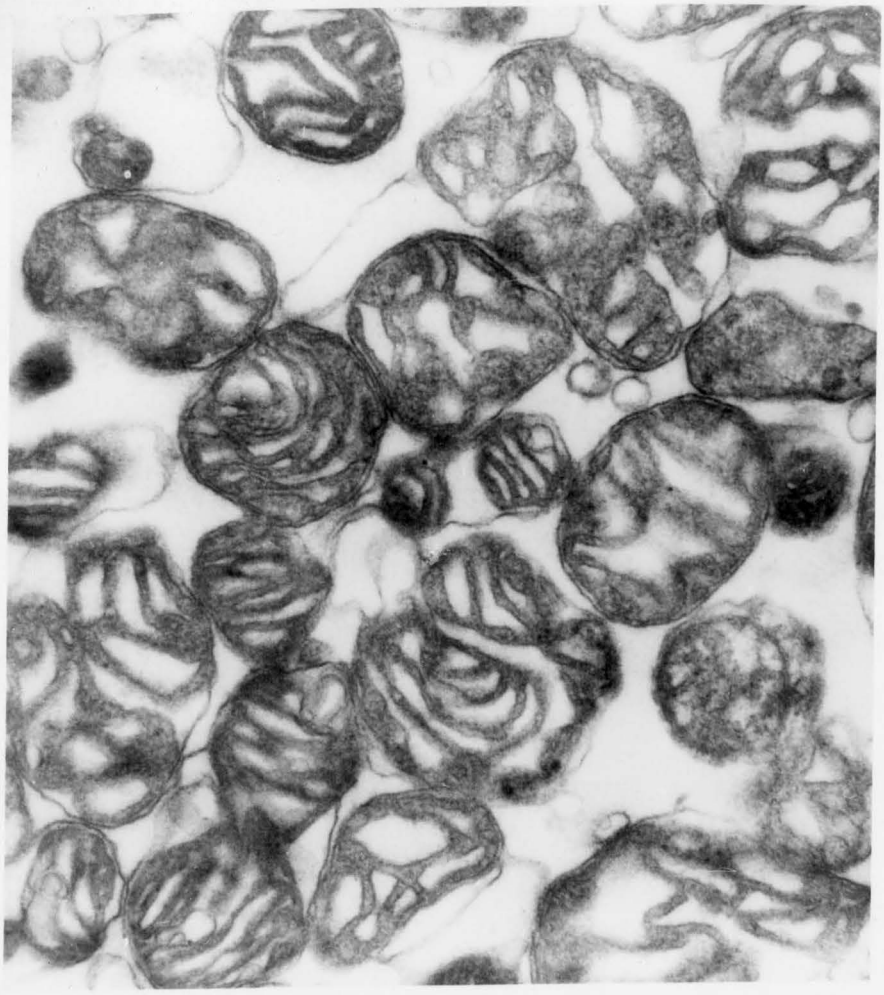

FIGURE A

Electron micrograph of 10-day old rat brain mitochondrial preparation . 36, 300x magnification.

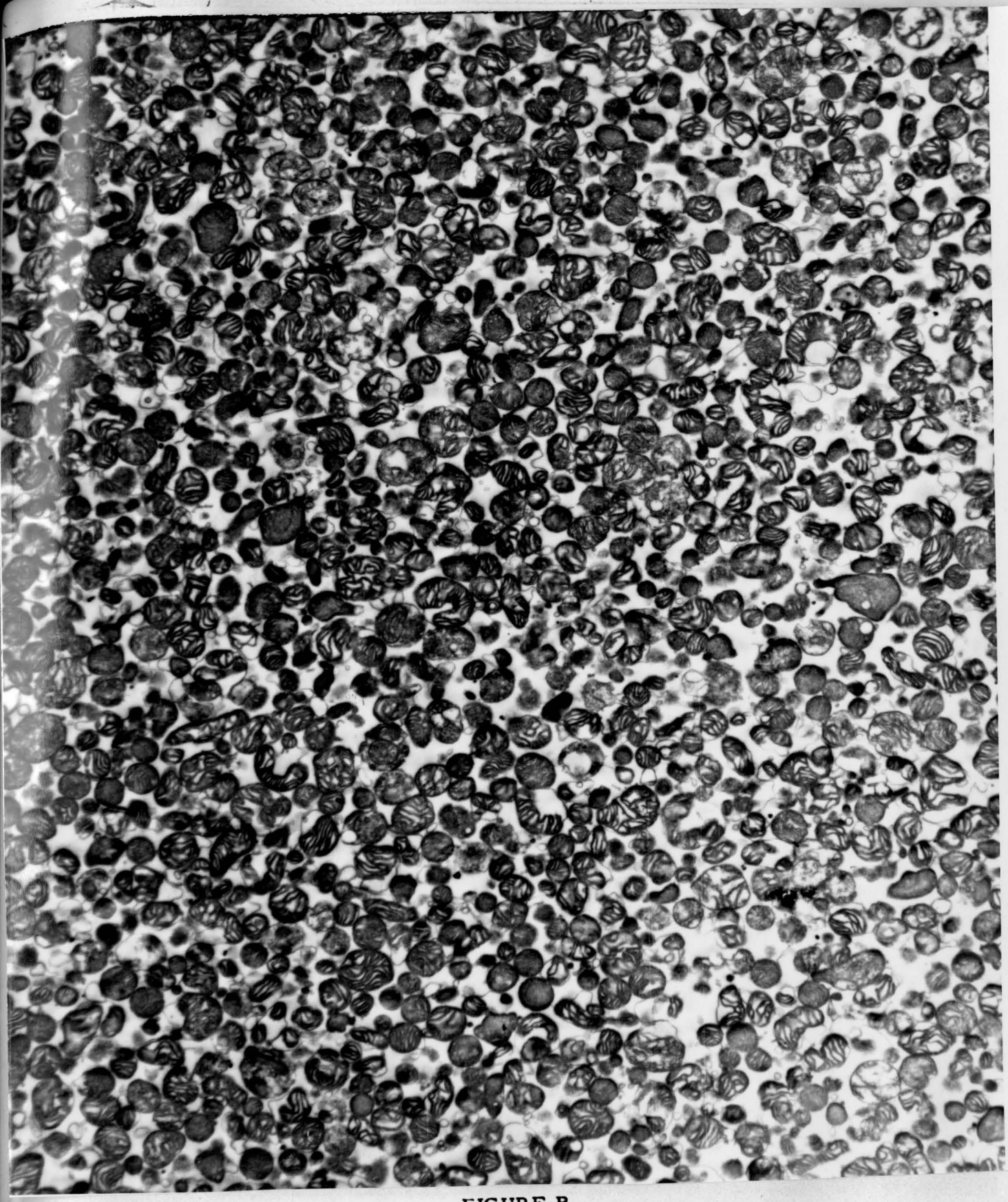

FIGURE B

Electron micrograph of mitochondrial preparation from 10-day old rat brain. 12,000 x magnification

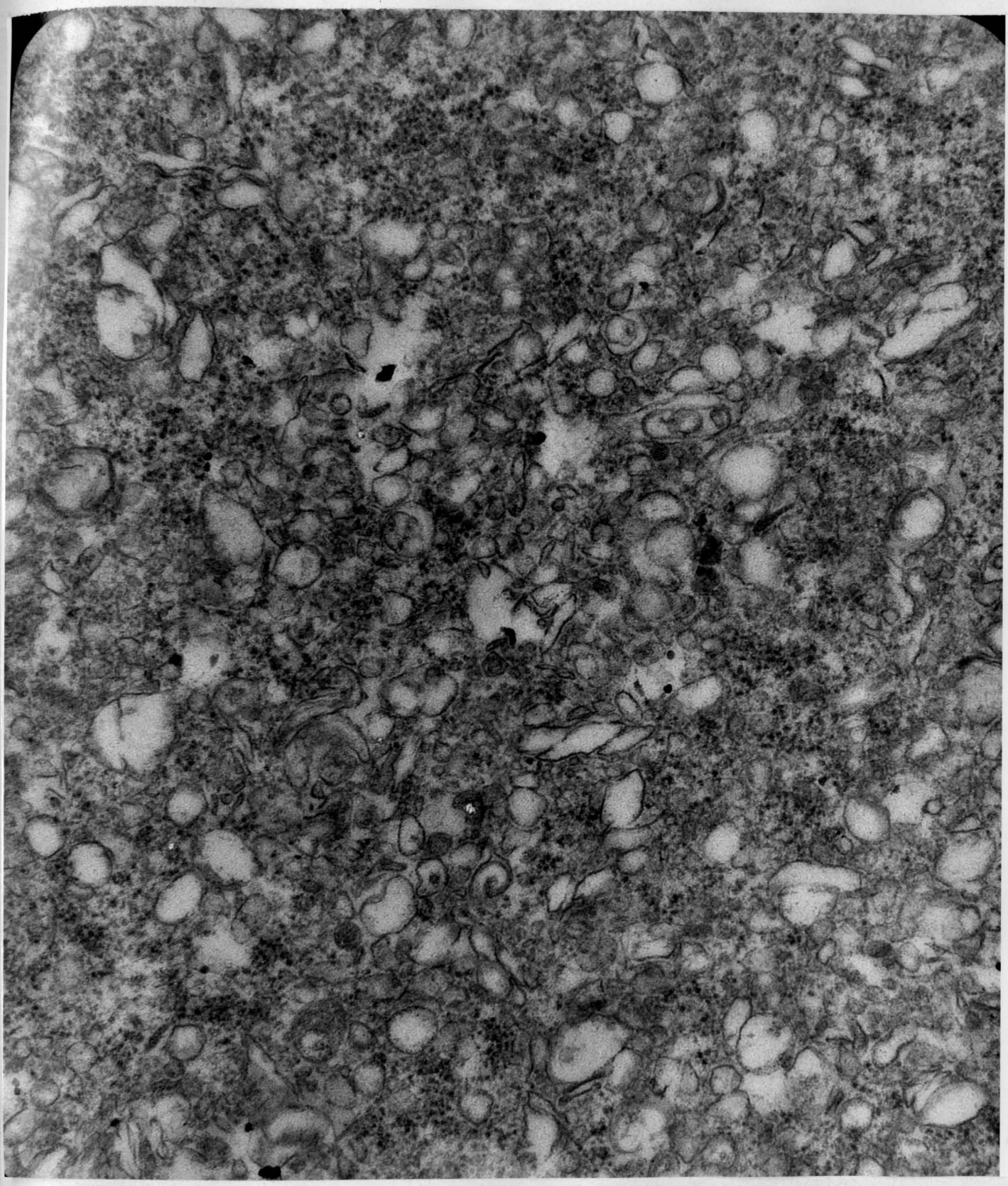

FIGURE C

Electron micrograph of microsomal preparation from 10-day old rat brain. 59,000x magnification.

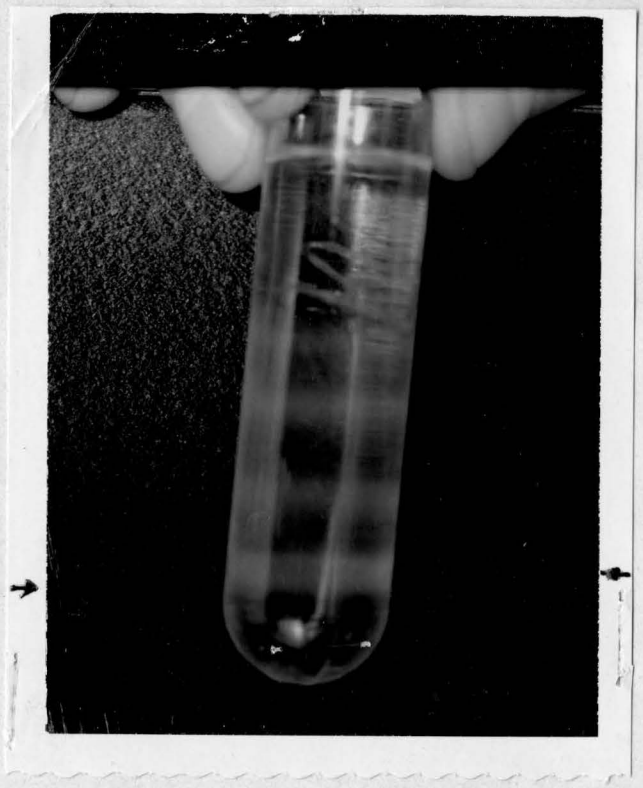

FIGURE D

Final stage of purification of 10-day old rat brain mitochondria: the discontinuous gradient. Arrows point to mitochondrial sediment at the junction of 1.2 and 1.4M sucrose. Method is described in Materials and Methods section.

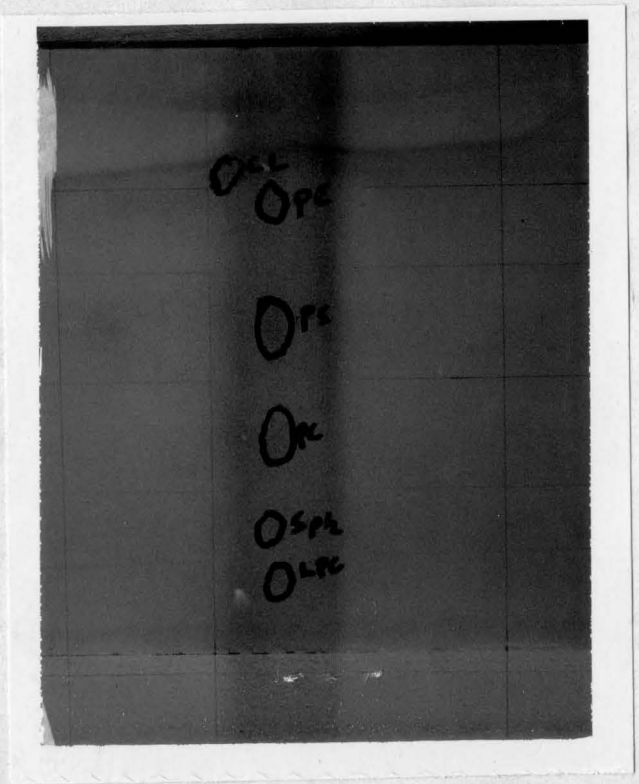

FIGURE E

Typical thin-layer chromatograms of mitochondrial phosphatides. After incubation, mitochondria were re-isolated as described in Materials and Methods. Lipid extracts were obtained and chromatographed on SiO_2 columns to obtain the CH_3OH or phosphatide fractions of the mitochondrial preparations. The solvent system was $CHCl_3$/CH_3OH/AcOH/H_2O:25/15/4/2 on PQ1 Quantograms (Quantum Industries, Fairfield, N. J.). The plates were sprayed with molybdenum blue reagent to detect phospholipids as described by Dittmer and Lester (1964).

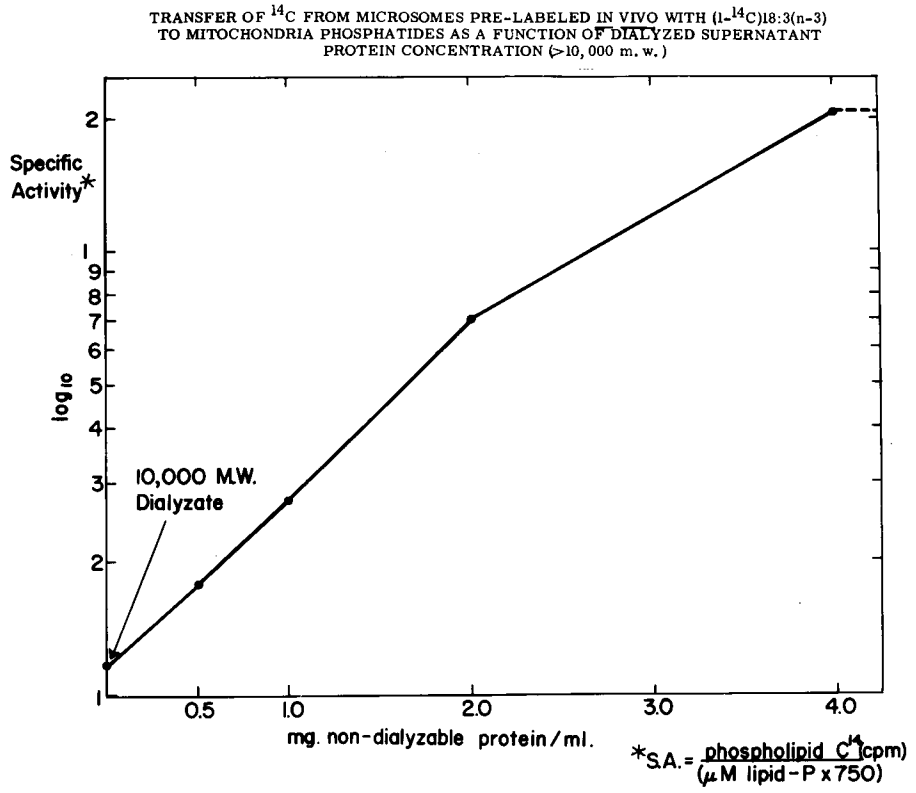

FIGURE I

In vitro transfer of phospholipid C^{14} from microsomes (pre-labeled in vivo with 1-C^{14} 18:3ω3) to unlabeled "acceptor mitochondria" as a function of dialyzed supernatant protein concentration (> 10,000 M.W. dialyzate). Incubations were for 1 hr at 37° and each vessel contained 3 mg. mitochondrial protein 9 mg. microsomal protein from 10-day old rat brain and the indicated amount of supernatant protein which was pH 5.1 precipitated and force-flow dialyzed as described in Materials and Methods. Total and specific activity of starting material microsomes was 4419 cpm/3.5 umoles phospholipid phosphorus.

SPECIFIC ACTIVITY OF MITOCHONDRIAL PHOSPHATIDES AFTER INCUBATION WITH MICROSOMES PRE-LABELED IN VIVO WITH (1-^{14}C)18:3(n-3)

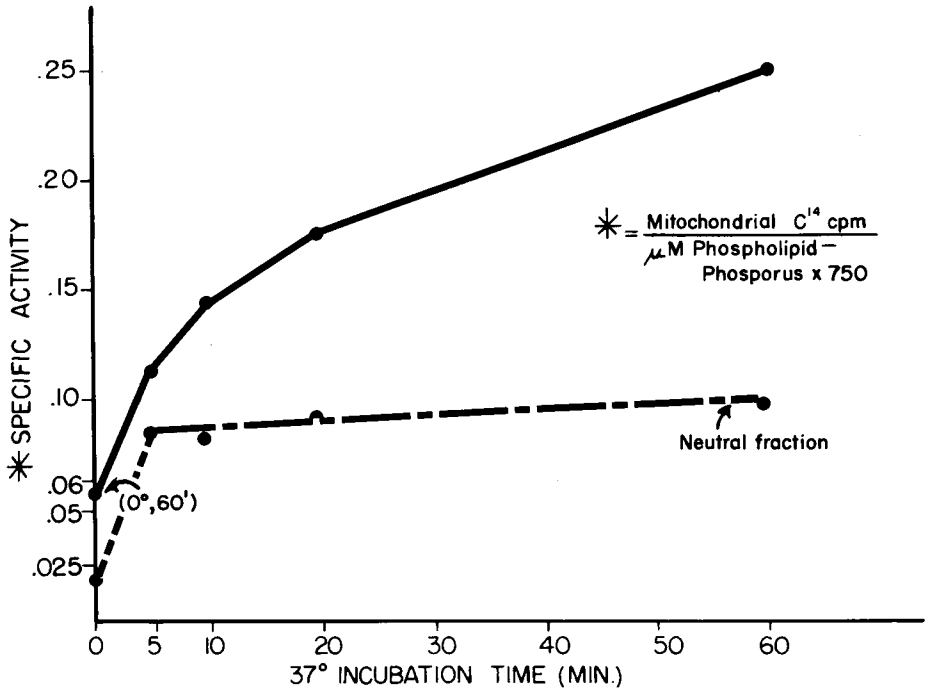

FIGURE II

In Vitro transfer of phospholipid C^{14} to mitochondria from microsomes as a function of 37° incubation time. Each vessel contained 12 mg. mitochondrial protein, 15 mg. microsomal protein and 36 mg. supernatant protein which had been pH 5.1 precipitated and force-flow dialyzed as described in Materials and Methods. Total and specific activity of starting material microsomes ("donor" microsomes) was 4024 cpm/6.1uM phospholipid phosphorus.

RELATIONSHIP OF AMOUNT OF NON-DIALYZABLE SUPERNATANT PROTEIN TO INCORPORATION OF RADIOACTIVITY INTO MITOCHONDRIA AFTER INCUBATION WITH MICROSOMES PRE-LABELED IN VIVO WITH (1-^{14}C)18:3(n-3)

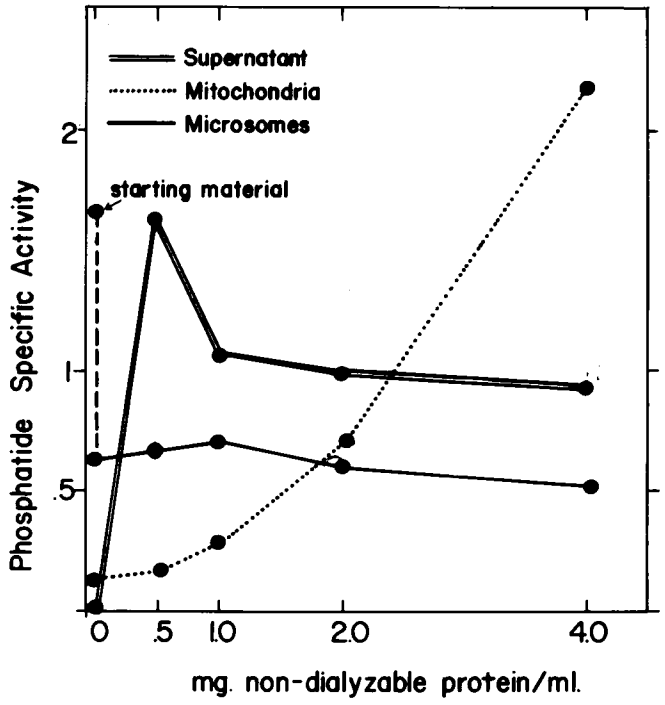

FIGURE III

Conditions were the same as described for Figure I.

PHOSPHATIDE SPECIFIC ACTIVITIES OF MITOCHONDRIA, MICROSOMES AND SUPERNATANT AFTER INCUBATION WITH MICROSOMES PRE-LABELED IN VIVO WITH (1-^{14}C)18:3(n-3)

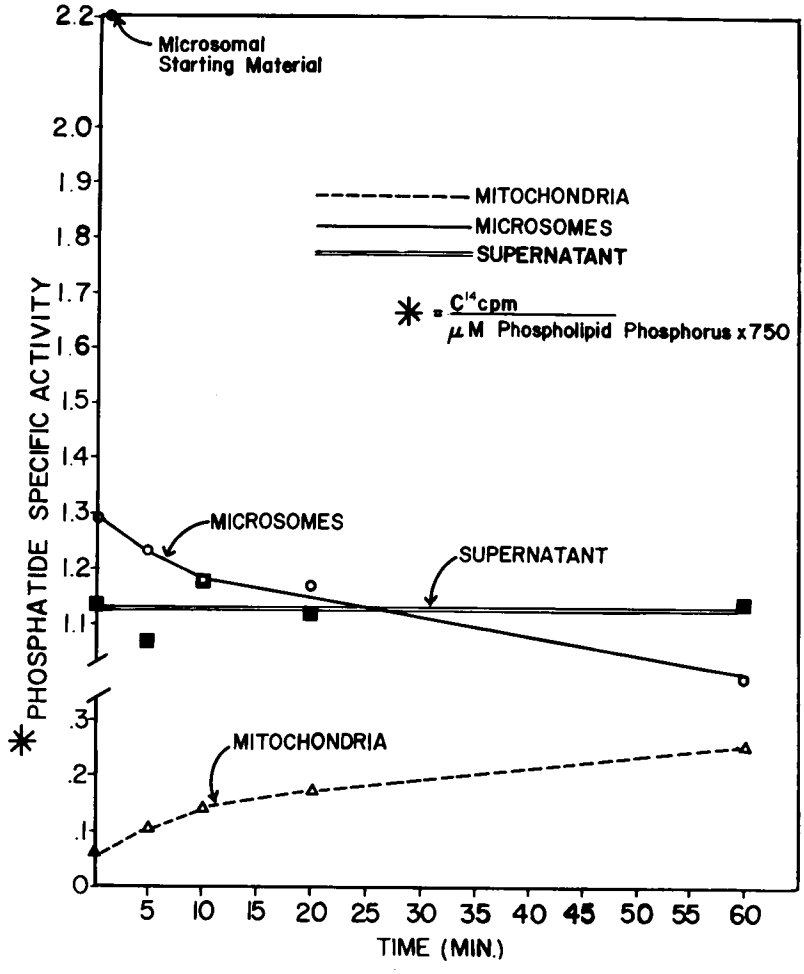

FIGURE IV

Conditions were the same as described for Figure II

RATES OF INCORPORATION OF RADIOACTIVITY INTO MITOCHONDRIAL PHOSPHATIDES AFTER INCUBATION WITH MICROSOMES PRE-LABELED IN VITRO WITH (1-^{14}C)18:2(n-6) or (1-^{14}C)18:3(n-3)

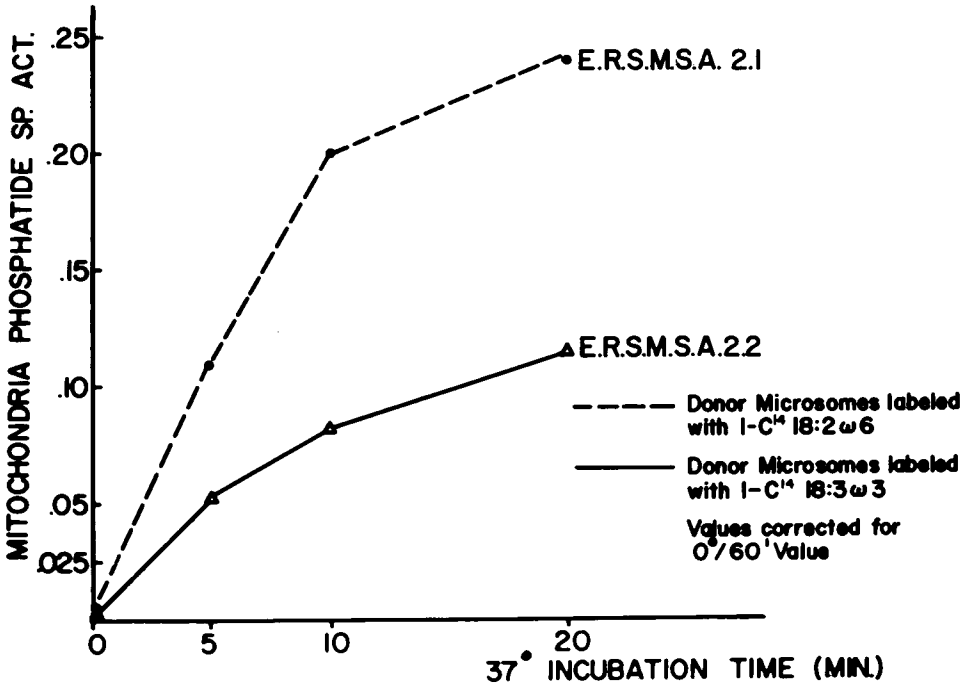

FIGURE V

Conditions of incubation were identical to those described for Figure II. E.R.M.S.A (=microsomal specific activity prior to incubation with the unlabelled mitochondria).

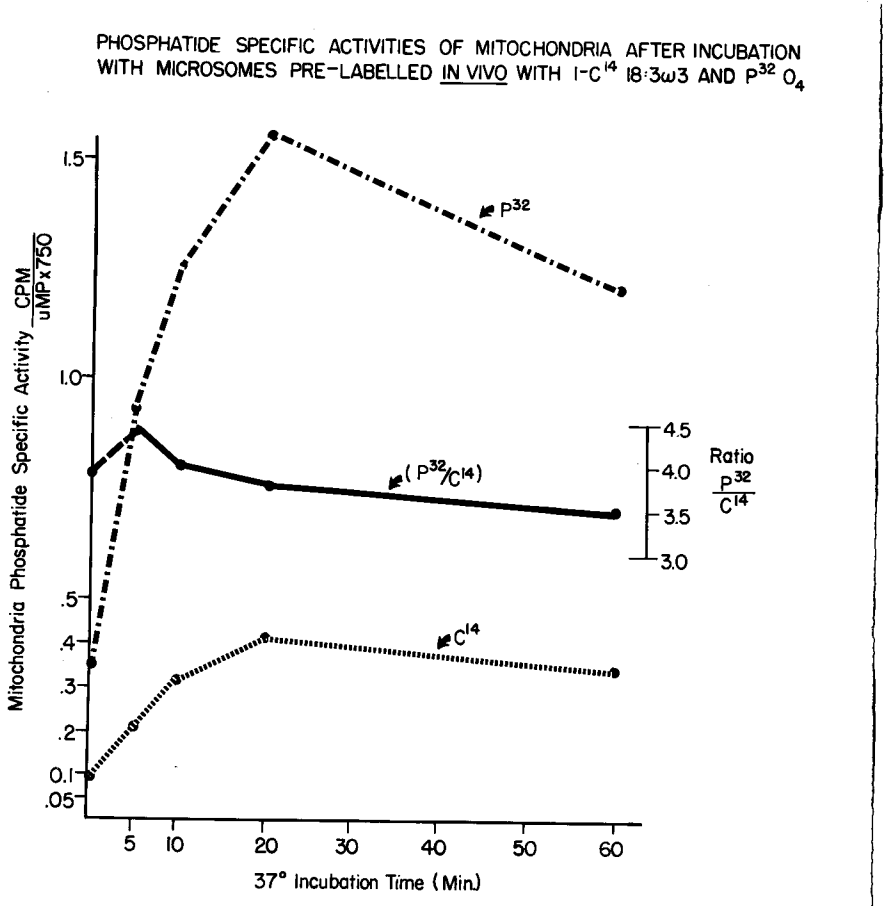

FIGURE VI

Each vessel contained 24 mg. of microsomal protein, 6.5 mg. of mitochondrial protein and 52 mg. of supernatant protein. Total volume of each incubation was 8 mls. Specific activity of microsomal ("donor") starting material was 2.0 C^{14}cpm/uM phospholipid-phosphorus x 750 and 6.2 P^{32}cpm/uM Phospholipid-phosphorus x 750.

PHOSPHATIDE SPECIFIC ACTIVITIES OF MICROSOMES AND SUPERNATANT. AFTER INCUBATION WITH MITOCHONDRIA. MICROSOMES PRE-LABELLED IN VIVO WITH 1-C^{14} 18:3ω3 AND $P^{32}O_4$

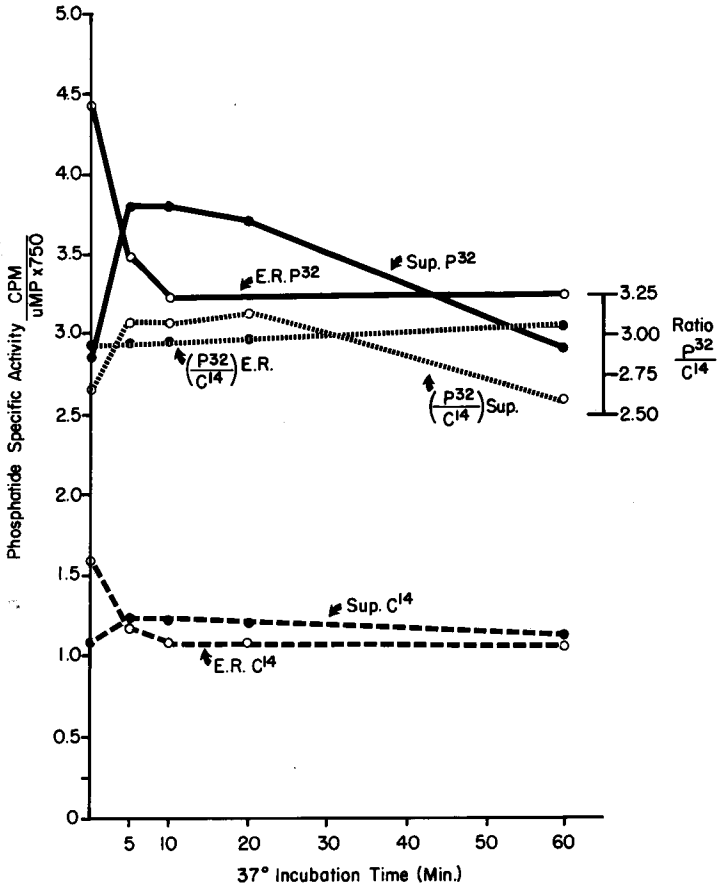

FIGURE VII

Conditions of incubation were identical to those described for Figure VI.

SPECIFIC ACTIVITY① OF ACCEPTOR MITOCHONDRIA AFTER INCUBATION WITH MICROSOMES PRELABELED WITH 1-^{14}C LINOLEATE OR 1-^{14}C LINOLENATE IN DEVELOPING RAT BRAIN

	18:3(n-3)			18:2(n-6)			
Exp. No.	1	2	3	4	5	6	7
Sample							
Supernatant Protein mg/ml	4.0	1.0	0.7	4.0	1.7	1.4	0.5
Microsomes Specific Activity	1.7	.60	.28	2.3	3.7	1.7	0.7
Acceptor Mitochondria +Supernatant		.34	.28	1.5			
+heated supernatant		.14	.14				
pH5.0 soluble					.43		.34
" " +heat					.11		.11
and dialysed 17 hrs.						.24	
" +heat						.11	
Mitochondria in vivo	2.1	1.0	.31	4.2	4.0	2.2	1.1

① Specific Activity=cpm/μM phospholipid phosphorus x750

TABLE I

SPECIFIC ACTIVITY① OF ACCEPTOR MITOCHONDRIA AFTER INCUBATION WITH PRELABELED MICROSOMES AS AFFECTED BY MOLECULAR SIZE OF SUPERNATANT FACTOR

	18:3(n-3)			18:2(n-6)			
Exp. No.	1	2	3	4	5	6	7
Sample							
Fraction m. w. >10,000	2.2			.98			
Fraction + heat				.32			
Sup. Fraction <10,000 m. w. dialysate	.13						
Sup. + <1,000 m. w. dialysate				1.5 1.2			

① Specific Activity=cpm/μM phospholipid phosphorus x750

TABLE II

SPECIFIC ACTIVITIES OF PHOSPHATIDE FRACTIONS OF MICROSOMES AND MITOCHONDRIA AFTER PRIOR LABELING OF MICROSOMES IN VIVO WITH (1-^{14}C) LINOLEATE

	Microsomes		Mitochondria	
Phosphatide	Before Incubation	After Incubation	+Supernatant	+Heated supernatant
Ethanolamine	.88	.50	.16	.04
Serine	1.24	.38	.22	.04
Choline	1.60	.73	.36	.03
Sphingomyelin	<.01	<.01	<.01	0
Lysophosphatidyl choline	<.01	<.01	<.01	0

TABLE III

After thin-layer chromatography and detection of standard spots. The unsprayed lanes of mitochondrial phosphatides were scraped and the silica gel was transferred to test tubes. The lipid absorbed to each silica gel sample was extracted with 5 mls. of the solvent system, 2 mls. of heated CH_3OH and finally 2 mls. of heated $CH_3OH/ACOH/H_2O$:94/1/5. Aliquots of the pooled eluants were analyzed for specific activity as described in the Methods section.

RELATIVE SPECIFIC ACTIVITIES OF PHOSPHATIDES (PC=1.0) AFTER INCUBATION WITH MICROSOMES LABELED WITH EITHER 18:3(n-3) or 18:2(n-6)

	(1-^{14}C) 18:3(n-3)			(1-^{14}C) 18:2(n-6)		
	ERSM	Mi	MIV	ERSM	Mi	MIV
PC	1.0	1.0	1.0	1.0	1.0	1.0
PE	0.9	3.0	0.8	0.6	0.4	0.7
PS	0.3	0.7	0.6	0.8	0.6	0.3
SPH	ns	ns	ns	ns	ns	ns
LPC	ns	ns	ns	ns	ns	ns

ERSM=E.R. starting material
Mi=Mitochondria labeled in vitro by transfer from microsomes
MIV=Mitochondria labeled in vivo
ns=not significant

TABLE IV.

DISTRIBUTION OF RADIOACTIVITY OF LONG-CHAIN FATTY ACIDS[1] IN MITOCHONDRIA AFTER INCUBATION WITH MICROSOMES LABELED WITH (1-^{14}C) LINOLEATE AND (1-^{14}C) LINOLENATE

	Microsomes		Mitochondria + supernatant
	Before Incubation	After Incubation	
		Long chain cpm. / Total cpm. x 100	
18:2(n-6)	30	30	36
18:3(n-3)	24	37	60

[1] Greater than 18 carbon atoms

TABLE V

Methyl esters of the mitochondrial phosphatides were obtained as described previously (Liepkalns and Bernsohn, 1972) and in the Methods section.

TABLE VI.

PER CENT OF INCORPORATION OF 1-C^{14} 18:2ω6 INTO PHOSPHATIDE BY RAT BRAIN MITOCHONDRIA

MITOCHONDRIA + CO-FACTORS (Aeberhard & Menkes, 1968)	8
MITOCHONDRIA + SUPERNATANT	29
MITOCHONDRIA + SUPERNATANT + MICROSOMES	39

Each incubation contained 12 mg. mitochondrial protein and/or 24 mg. supernatant and/or 12 mg microsomal protein, and 1 uC of 1-C^{14} 18:2ω6. Incubation time at 37° was 1 hr. and the total volume of each incubation was 3 ml. Phosphatides were isolated by SiO_2 column chromatography as described in the Methods section.

TABLE VII

SPECIFIC ACTIVITIES OF PHOSPHATIDE FRACTIONS OF SUB-CELLULAR ORGANELLES (C^{14}/uMP x 750)

	1-C^{14}18:2ω6	1-C^{14}18:3ω3
PRE-INCUBATED MICROSOMES	143	73
POST-EXCHANGE DATA		
MICROSOMES	54	25
MITOCHONDRIA	53	26
MITOCHONDRIA*	33	6

* +Boiled supernatant

Each incubation contained 12 mg. mitochondrial protein, 15 mg. microsomal protein and 24 mg. supernatant protein in a total volume of 3 mls. Mitochondrial phosphatides were isolated as described in the Methods section. Microsomes were pre-incubated with 1 uC of labelled fatty acid for 1 hr. at 37° as per Aeberhard and Menkes (1968) and then re-isolated and incubated with mitochondria (unlabeled) and supernatant.

TABLE VIII

SPECIFIC ACTIVITIES OF PHOSPHATIDE FRACTIONS ISOLATED BEFORE AND AFTER INCUBATION OF PRE-INCUBATED MICROSOMES WITH UNLABELED MITOCHONDRIA AND SUPERNATANT

	$1\text{-}C^{14}18{:}2\omega6$			$1\text{-}C^{14}18{:}3\omega3$		
	MITOCHONDRIA	MICROSOMES		MITOCHONDRIA	MICROSOMES	
		Pre-incubate	Post-exchange		Pre-incubate	Post-exchange
PE	6	122	15	3	32	5
PS	12	160	61	3	33	9
PC	73	210	61	12	61	24
SPH	1.4	5.4	ND	ND	2	ND
LPC	1.4	2	ND	ND	3	ND
ORIGIN	ND	ND	ND	ND	3	ND

ND = Not detectable

DISCUSSION

The rather extended and rigorous procedure for purification of mitochondria employed here yields relatively intact and uncontaminated organelles. Indeed, even the "bilayer" outer membrane is observable in some specimens. Mammalian brain mitochondria are difficult to purify due to a number of possible contaminating membranous elements. Among these are synaptosomes, lysosomes and myelin figures. We attempted to circumvent some of the purification problems by using 10-day old rats, in which myelin synthesis is in its earliest stages.

Some enzymatic assays also verified the purity of the mitochondria. Succinate dehydrogenase activity and monoamine oxidase activity were observed in the mitochondria but no glucose-6-phosphatase. Few vesicular-like elements (non-mitochondrial) were observable in the electron micrographs which were consistent with the enzymatic assay data which indicated that the contamination of the mitochondria by microsomal membranes (containing glucose-6-phosphatase) had to be something less than 10%. Two points should be kept in mind regarding the assay for contamination of the mitochondria before or during the incubation. First, although the assays were performed after incubation, it is far from axiomatic that the enzyme markers will distribute themselves during the incubation as the "contamination"

would. Secondly, most of the incubations were internally controlled.

There are a number of indications that PTF is a protein similar to transfer factors observed in other tissues. In order to lend more credence to the transfer system we observed, we tried first to address our experimental designs toward the literature precedent. Thus, Wirtz and Zilversmit (1969b), using ^{32}P labeled microsomes and "liposomes", described an inter-membrane transfer factor which is heat-labile, non-dialyzable and pH 5.1 non-precipitable. We find such properties to be indigenous to the brain PTF as well, although we followed the transfer of a fatty acyl label instead of a ^{32}P label. K.W.A. Wirtz and D. B. Zilversmit (1970) have been able to divorce the PC transfer from the PE transfer and report a molecular weight of 22,000 for the "PC transfer factor" (using labeled liposomes) from beef heart. In agreement, we would like to report a molecular weight of greater than 10,000 for the PTF of developing rat brain. This was concluded from experiments in which 10,000 M.W. dialyzates did not stimulate the transfer of labeled phosphatides greater than boiled controls and 1,000 M.W. dialyzates "diluted" the transfer activity when added to the complete system. Although Wirtz (1972) has observed a requirement for Mg^{+2} for the purified beef heart transfer protein, our data tends to contradict such an observation.

However, our dialysis conditions (under N_2, Diaflo) may not rid the supernatant of all the "protein bound" Mg^{+2}.

In disagreement with other workers (Zilversmit, 1971a) we obtained a semi-logarithmic relationship between the specific radioactivity of "acceptor mitochondria" phosphatides and dialyzed supernatant protein concentration. The reasons for this type of curve are, as yet, unclear. We believe it to be part of a sigmoid curve, which would tend to indicate some sort of "co-operative mechanism (see below). At the highest concentration of dialyzed protein concentration tested (4 mg/ml), the curve had broken from the log function, a point at which the specific activity of "acceptor mitochondria" is in the range of the "donor" labeled microsomal phosphatide specific activity (1.7 ^{14}C cpm/uM P x750) and the phosphatide specific activity of the in vivo labeled mitochondria (2.1 ^{14}C cpm/u M P x750) taken from the same animals as in vivo labeled microsomes used in the incubation of Figure 1. This is not to say that the whole phosphatide pool of microsomes are exchangeable with the phosphatide pool of mitochondria. McMurray and Dawson (1969) and Wirtz and Zilversmit (1969a) have reported data to suggest that a specific exchangeable pool of microsomal phosphatides exists. Furthermore, in transfer experiments reported by other workers (Blok et al., 1971), outer membrane of mitochondria are labeled prior to inner membrane and cristae.

Not surprisingly, then, we were unable to achieve phosphatide isotopic equilibrium in the time curves described (Figure II), even though the exchangeable phosphatide pool was thought to have been saturating. Also, there may have been limiting factors (such as $[Mg^{+2}]$) imposed in our *in vitro* transfer experiments of which we were unaware. Since our basic premise is that the whole phosphatide moiety is transferred, we counted the $CHCl_3$ fraction (which would contain the 1-^{14}C fatty acyl starting material) as well as the CH_3OH fraction (see MATERIALS AND METHODS) from the SiO_2 column chromatography of the mitochondrial lipids for each time point. However, *in vivo* incorporation of 1-^{14}C 18:3ω3 into phosphatides of brain microsomes was virtually complete. Thus, any mitochondrial incorporation of a fatty acyl group *per se* and not as a fatty acyl moiety of a phosphatide would have to entail a deacylation-acylation cycle. We have evidence (see below and Figure V) that such a cycle is not operative under the conditions of our *in vitro* system. Note that the "zero time" point refers only to exposure to 37°C incubation time. Thus, this "incubation" contained *live* dialyzed supernatant and labeled microsomes and was kept on ice during the term of incubation of the other system. This was done in order to control for cross-contamination of particulate matter (i.e., labeled phosphatides) during the incubation time or during re-isolation. We think these time curves are unlike any predictable for

contamination vs. time since they do not go in parallel and that the incorporation of ^{14}C into mitochondria phosphatide cannot be explained by the ability of the PTF to stimulate contamination upon exposure of the organelles to 37°C.

Returning now to Figure III (Transfer of Labeled Phosphatides as a Function of Protein Concentration), mitochondria take up substantial amounts of labeled phosphatides at protein concentrations higher than those at which microsomes have dimidiated in phosphatide specific activity and supernatant at .5 mg/ml. protein obtains a phosphatide specific activity approximating that of the original "donor microsome" specific activity. This suggests that a minimum amount of dialyzed supernatant is required for microsomes to "release" labeled phosphatides or alternatively, it may mean that no supernatant protein results in no labeled non-particulate phosphatides. With dialyzed supernatant concentrations higher than 0.5 mg/ml, the supernatant lowers in specific activity. This suggest dilution of the "solubilized phosphatide pool" by an unlabeled pool which is more than likely of mitochondrial origin. Other workers have reported reversible inter-membrane exchange of phosphatides (McMurray and Magee, 1972) and we have observed, in pilot experiments, the transfer of labeled phosphatides from labeled mitochondria to unlabeled microsomes in vitro.

As "acceptor" mitochondria increase in phosphatide specific activity as a function of incubation time, "donor" microsomes decrease in specific activity (Figure IV) and re-isolated supernatant maintains a constant ("steady-state") specific activity slightly less than that of microsomes. Again, this suggests mutual exchange as well as transfer of phosphatides. The presence of the dialyzed soluble protein in the incubation results in the presence of donor microsome phosphatides as well as unlabeled mitochondrial phosphatide in the re-isolated supernatant. Furthermore, the increments of increases in mitochondrial specific activity are proportional to the decreases in specific activity in the microsomes after one hour of incubation is answered by a 77% increase over "0" time specific activity in the mitochondrial phosphatides. When normalized for the amount of particulate starting material (in mg. protein per incubation), the following numbers result:

$$\frac{\text{mitochondria sp. act.}}{\text{mg. protein}} \qquad \frac{0.2}{12} = 0.017$$

$$\frac{\text{microsomal decrease}}{\text{mg. protein}} \qquad \frac{0.3}{15} = 0.020$$

$$\frac{0.017}{0.020} = 0.85 \approx 1$$

All this suggests that whole phosphatide species are transferred and that the microsome to mitochondria transfer probably occurs without participation of the acylation-de-acylation cycle. Alternatively, one could postulate acyl-transferase activities working in synchrony in both particulate fractions. But, the presence of acyl-transferase in mitochondrial outer membrane has been seriously questioned (Eibl et al., 1969).

The pattern of incorporation into individual phosphatides (Table III) suggests that the PTF(s) present in dialyzed supernatant are heat-labile since PE, PS + PI and PC of mitochondria do not incorporate radioactivity in the presence of 1-^{14}C 18:2ω6 or 1-^{14}C 18:3ω3 labeled microsomes and heated supernatant. Also, the microsomes' individual phosphatides decrease in specific activities in the presence of "live" supernatant as the mitochondria's phosphatide's specific activities increase. One-^{14}C-linoleic acid is incorporated principally into the PC fraction of microsomes in vivo. Similarly, the acceptor mitochondria incorporated label from these microsomes more into PC than into PE after a one hour incubation in vitro. This is consistent with the existence of exchangeable pools present in the particulate members of the incubations. However, the pattern of incorporation into individual phosphatides of "acceptor mitochondria" in vitro is not evidence for the presence of more than one PTF specific for the protein and/or

base portion of the phosphatide. But, differences exist in the relative incorporation (PC vs. PE) into mitochondria in vitro depending on whether the donor microsomes had been labeled in vivo with 1-^{14}C 18:2ω6 or 1-^{14}C 18:3ω3. Whether this was governed by specificity of the PTF(s) or the "exchangeable" donor microsomal pool remains unanswered and still amenable to experimental investigation. A number of other workers have reported a predominance of the ω3 family in the PE fraction of the CNS (White et al., 1970). Note the low incorporation into sphingomyelin and lysophosphatides in vivo since sphingomyelin turnover is so slow in CNS (Dhopeshwarker et al., 1970) and the amounts of lyso compounds are so slow (Latetina et al., 1968). There should not be any sphingomyelin in purified brain mitochondria in any case (Skrbic and Cumings, 1970).

Quantitative gas chromatography of the phosphatides of acceptor mitochondria was performed, but the collected elongated fractions had to be pooled (Table IV). The low counts did not permit determination of incorporation into individual peaks. Again, the predominance of labeled elongated fatty acids of the ω3 family is evident when the microsomes were labeled in vivo with 1-^{14}C 18:3ω3 since 22:6ω3, the main product of 18:3ω3 elongation (Wakil, 1970) has a higher unsaturation to chain length ratio than 20:4ω6, the main product of 18:2ω6 elongation (Wakil, 1970). Furthermore, 18:3ω3 has

probably preferred access to the elongation-desaturation system of the endoplasmic reticulum (and consequently, acylation) relative to 18:2ω6. We have observed (above) that although virtually 100% of 1-^{14}C 18:3ω3 incorporated in the lipid extract of the microsome fraction was incorporated into phosphatides, 80% of 1-^{14}C 18:2ω6 incorporated into lipid extract of microsomes in vivo was incorporated into phosphatides and some of that was esterified 1-^{14}C 18:2ω6. On the other hand, the data in Table IV do not suggest specificity of transfer whether through availability of exchangeable phosphatides or the specificity of one or more PTF(s).

Differences in rates of incorporation of ω3 vs. ω6 containing species of phosphatides into mitochondria were apparently obtained (Fig. V). Since identical conditions could not be guaranteed, it is possible that phosphatides for some species of PTF(s) were limiting. Assuming that this was not the case, differences in the rates of transfer of some species of phosphatides is consistent with recognition of some moiety of the phosphatides. We labeled the fatty acyl portion of the phosphatides of microsomes with 1-^{14}C 18:2ω6 or 1-^{14}C 18:3ω3. Taking into account the predominance of the incorporation of the ω3 fatty acid into the PE fraction (White et al., 1971), the differences in rate observed in Figure V may reflect the recognition of the polar moiety of PE and PC. Wirtz and

Zilversmit (1970) have purified a PTF from rat liver homogenates which has an effect on the in vitro exchange of PC and PI but no effect on the inter-membrane exchange of PE. Stein and Stein (1969) reported that the ratio of specific activity of liver microsomal to mitochondrial lecithin (labelled with ^{14}C-choline) after 5 min. (as assayed by autoradiography) was 1.53 but the ratio of PE (labelled with ^{14}C-ethanolamine) was 5.3. Of interest is a report of a high level of PC species containing linoleic and arachidonate esters (Kanoh, 1969). Apparently, linoleate is taken up mostly by de novo synthesis of microsomal phosphatides while arachidonate is incorporated via acylation of pre-existing lysophosphatides (Kanoh, 1969). If this is true, this may have an effect on the species of labeled phosphatides "available" for transfer by the dialyzed supernatant from pre-labeled microsomes to unlabeled mitochondria.

Uni-molecular inter-membrane transfer of phosphatide species in the in vitro incubation was confirmed (Figure VI) when only a slight decrease in $^{32}P/^{14}C$ ratio was obtained during the transfer of microsomal phosphatides (coincidentally labelled with ^{32}P and 1-^{14}C 18:3ω3) to mitochondrial phosphatides. This "dip" in the ratio of $^{32}P^{14}C$ can be explained by the incorporation of mitochondria of some phosphatide species containing ^{32}P only, since ^{32}P will "scramble" throughout the microsomal phosphatide pool while 1-^{14}C 18:3ω3,

which will be elongated from the end (Wakil, 1970), will be channeled into the ω3 pathways. This will result in some microsomal phosphatides containing ^{32}P, but no $C^{14}\omega 3$ fatty acyl moieties. "Donor microsomes" maintain a constant specific activity ratio (P^{32}/C^{14}) as a function of time, contributing to the evidence for uni-molecular transfer (Fig. VII) from microsomes to mitochondria.

INCUBATIONS WITH PRE-INCUBATED MICROSOMES

The burden of evidence for the *in vitro* transfer of labeled phosphatides must lie in the above experiments in which only *in vivo* pre-labeled microsome fractions were employed.

Prior to this, however, our *initial* studies indicated that mitochondria incorporated ^{14}C fatty acid into phosphatides in the presence of fortified buffer, supernatant or supernatant plus microsomes (Table VI). The presence of microsomes stimulated the incorporation of ^{14}C-fatty acyl groups into mitochondria. We then turned to the incorporation of label into mitochondrial phosphatides in the presence of supernatant and microsomes which had been *pre-incubated* with the Na^+ salts of 1-^{14}C-fatty acids. Table VII gives some of the data so obtained, which is consistent with data obtained above with *in vivo* labeled microsomes. There is a decrease in specific activity of microsomal phosphatides commensurate with an

increase in mitochondrial phosphatide specific activity. Of the individual phosphatides, PC incorporates by far the greatest amount of radioactivity whether in pre-incubated microsomes or in acceptor mitochondria after incubation with supernatant and pre-incubated microsomes (TABLE VIII). If the acyl moiety of the PC fraction of both microsomes and mitochondria are not turning over synchronously, this is also evidence that the whole phosphatidyl choline molecule is transferred. Interestingly, LPC has a 50-fold greater specific activity in mitochondria, when compared to mitochondrial PC after incubation with supernatant and pre-incubated microsomes. This may indicate that the acylation of the glycerol backbone proceeds at a slower rate than the incorporation of the whole phosphatidyl choline molecule.

A Mechanism for the In Vitro Inter-Membrane Transfer of Phosphatides

A general mechanism of enzymatic catalysis proposes that the enzymatic surface provides for increased probability of collision between two or more substrates. May we suggest, then, that the PTF(s) functions similarly and renders the medium or the surfaces of the membrane fractions such as to increase the frequency of collision or "time of contiguity" between mitochondrial and microsomal surfaces.

In many electron micrographs of mammalian cells, the contiguity of the endoplasmic reticulum with mitochondrial inter-membrane is apparent (Peters et al., 1970). Thus, a gradient of differentiation may be occurring in this micro-environment, where the contiguity occurs. It is at this focal point that we suspect that the exchange of phosphatide species may well be occurring in the intact cell. If the endoplasmic reticulum is tubular in the intact cell, loosely bound proteins would be solubilized during the extraction procedure. We hypothesize that PTF(s) is (are) among these solubilized proteins which function in the intact cell in that micro-environment where membrane elements such as the ER become contiguous with and, indeed, may "become" the outer membrane of the mitochondrion. Schnaitman (1969) believed his data gave evidence that proteins of the outer membrane and "smooth" endoplasmic reticulum possess common precursors.

SUMMARY AND CONCLUSIONS

Owing to the dearth of enzymatic activities in mitochondria which are required for the *de novo* synthesis of phospholipids (Wilgram and Kennedy, 1963) some other mechanism of providing the mitochondria with its full complement of phosphatides, which help make up its functional membrane, must be involved (McMurray and Dawson, 1969). Such a mechanism could be the synthesis of phospholipids in the endoplasmic reticulum and their transport to the mitochondria. As a model system to study such a mechanism, we chose an *in vitro* system of the density-gradient purified mitochondria from 10-day old rat brain, a supernatant factor, and microsomes labeled *in vivo* with 1-^{14}C-fatty acyl compounds and/or ^{32}P. We conclude from our studies that the *in vitro* transfer of ^{14}C fatty acyl labeled phosphatides from labeled microsomes to mitochondria is facilitated by a heat-labile, pH 5.1 non-precipitable and non-dialyzable factor of greater than 10,000 M.W. An unusual logarithmic response of acceptor mitochondrial phosphatide specific activity to dialyzed supernatant protein concentration was observed.

That the whole phosphatide species were being transferred was evidenced by double label experiments in which both the fatty acyl moiety and the polar moiety of donor microsomes were pre-

labeled. Mitochondria incorporated phosphatides from these microsomes with 80% maintenance of $^{32}P/^{14}C$ ratio. Neutral fraction of mitochondria did not incorporate radioactivity under the conditions in which phosphatides were transferred. Decreases in microsomal specific activity were synchronous and commensurate with increases in mitochondrial phosphatide specific activity as a function of time.

Some specificity of the in vitro transfer mechanism was suggested in that the percentage of labeled, elongated fatty acyl groups incorporated into acceptor mitochondria differed according to whether the donor microsomes were labeled in vivo with 1-^{14}C 18:2ω6 or 1-^{14}C 18:3ω3. Mitochondrial PE to PC specific activity ratios were affected as well, according to which compound was used to label the donor microsomes. The rates of incorporation of ω3 and ω6 fatty acyl labeled phosphatides from pre-labeled microsomes into acceptor mitochondria differed. A mechanism for inter-membrane phosphatide transfer was suggested in which the presence of the protein in the medium facilitates the collision of the sub-cellular particles. A possible origin of the PTF(s) may be the micro-environment where the endoplasmic reticulum enters into contiguity with the outer mitochondrial membrane.

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APPROVAL SHEET

The dissertation submitted by Viesturs Argots Cernaks Liepkalns has been read and approved by a committee from the faculty of the Graduate School, Loyola University of Chicago.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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Date

Joseph Bernardin
Signature of Advisor