MEDIATION OF TRANSFER OF [1-^{14}C] POLYENOIC ACIDS BETWEEN MICROSOMES AND MITOCHONDRIA OF DEVELOPING RAT BRAIN BY A SOLUBLE MACROMOLECULAR FACTOR

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SUMMARY: a macromolecular factor in the supernatant fraction derived from rat brain appears to mediate *in vitro* incorporation of [1-^{14}C] phosphatide into brain mitochondria from a microsomal fraction labelled *in vivo* with [1-^{14}C] 18:2(n-6) or [1-^{14}C]18:3(n-3). The factor is non-precipitable at pH 5.1, heat-labile and non-dialyzable through a forced-flow dialysis system designed to extrude molecules of less than 10,000 molecular weight. The mitochondrial total phosphatide specific activity achieved is logarithmically related to the non-dialyzable supernatant protein concentration within limits.

Other workers have described the *in vitro* transfer and exchange of the p^{32}[1], [^{14}C] choline[2], [^{14}C] glycerol[3] and [^{14}C] palmitate[3] between liver microsomes and mitochondria. This exchange has been shown to be stimulated by a heat-labile, non-dialyzable soluble factor in rat liver. We report here the transfer of ^{14}C activity to brain mitochondria from brain microsomes pre-labelled with [1-^{14}C]18:2(n-6) or [1-^{14}C]18:3(n-3) which is mediated by a macromolecular factor in the supernatant fraction of rat brain homogenate. Evidence for the subcellular transport of fatty-acyl groups in nervous tissue is relevant to the wide functional and structural diversity of membranous structures which are characteristic of that tissue. The putative role of polyenoic acids in nervous tissue metabolism has been reported previously.[4]

MATERIALS AND METHODS

ISOLATION OF SUBCELLULAR ORGANELLES FROM RAT BRAIN Mitochondria were isolated at 0-4°C essentially as described by Gabay et al.[5] Microsomes were isolated as the pellet by centrifugation of the 20,000xg supernatant at 110,000xg for 2 hrs. The supernatant after this centrifugation was used for the transfer studies. The pH was adjusted at 0°C with 1M Tris Base or 1M HCL.

Conventional dialysis was performed at 4°C with 30ml of 1mg protein/ml supernatant against 15 liters standard buffer (.32M sucrose-.001M EDTA afjusted to pH 7.4 witn 1M Tris Base). Forced flow dialysis was performed through a UM 10,000 or UM[2] membrane in a Diaflo 50ml chamber (Amicon Co.) under N_2 pressure of 60 psi at 10°C.

IN VIVO LABELLING OF MICROSOMES AND INCUBATION In a typical experiment 250,000 cpm of [1-^{14}C]18:2(n-6) (50 mCi/mM) or [1-^{14}C]18:3(n-3) (22mCi/mM, Amersham-Searle) was injected intracerebrally into each of 24 rats, 10-days old, as the sodium salt in 15% bovine serum albumin (Pentex) solution in a volume of 10ul essentially as described by Sun and Horrocks[6]. An average of 2-1/2 hours after injection the rats were decapitated, the cerebral hemispheres removed and the labelled microsomes were incubated for 1 hr. at 37° with unlabelled mitochondria isolated from non-injected animals. The incubate was cooled then on ice and organelles and supernatant re-isolated at 0-4°C. Mitochondria were re-isolated by centrifugation at 11,500xg by density centrifugation as described above and microsomes and supernatant by centrifugation at 110,000xg as in the initial isolation procedure. It was determined by enzyme assays (succinic dehydrogenase[7], monoamine oxidase[8] and glucose-6-phosphatase[9] and electron microscopy that the mitochondria were viable and free of contamination before and after incubation. All isolation operations, incubation and re-isolations were in continuo and no freezing or storage of any viable components occurred, until the lipids had been extracted.

IN VITRO LABELLING OF MICROSOMES In some experiments cold microsomes were first isolated and incubated in a medium described by Aberhard and Menkes[10] except that 0.5μCi of [1-^{14}C] fatty acid was used in place of [^{14}C] acetate per incubation flask. Microsomes were re-isolated, washed twice, cold mitochondria and supernatant added, and incubation and subsequent re-isolations performed as described above. Because of the possibility of direct incorpora-

tion by acceptor mitochondria of unincorporated [^{14}C] acyl groups from microsomes labelled *in vitro*, labelled microsomes were analyzed before and after incubation.

ANALYTICAL PROCEDURES The organelles were extracted by the Folch procedure[11], washed twice and the extract which contained .3-1μM lipid phosphorus was applied in 1.0ml of $CHCl_3$ to a 0.5x13cm column containing 1gm of 100 mesh SiO_2 (Bio-Rad). The columns were washed successively with 100ml $ChCl_3$, 40ml acetone and 50ml CH_3OH[12]. Specific activity determinations on aliquots of the CH_3OH fractions were performed and the remaining sample was used for quantitative thin-layer chromatography on Quantograms (Quantum Co.). Methylation was accomplished by BF_3-methanol[13] and GLC was done on a 1/4"x6' column with 10% DEGS as the liquid phase and Gas-Chrom Q as the solid support with helium as the carrier gas at input pressure of 45 psi at 180° on a Varian 90P. Thin-layer chromatography and recovery of lipid spots was according to Skipski[14] and recovery of individual methyl esters from the gas chromatograph has been described by Wood[15]. Butylated hydroxy toluene (1%) was added to column eluents and chromatography solvents to retard oxidation. Authentic phospholipid standards were obtained from the Supelco Co. Phosphorus was performed according to Bartlett[16]. Radioactivity was measured using PreMix P (Packard Co.) in a Packard Liquid Scintillation Counter Model 3003. Protein was determined according to Folin and Ciocalteau[17]. All lipid samples were stored when necessary at -60°C under N_2.

RESULTS AND DISCUSSION Table I is a summary of brain mitochondrial specific activities obtained after incubation for 1 hr. with pre-labelled microsomes in the presence of the 110,000xg supernatant fraction or subfractions thereof obtained by pH precipitation and forced flow dialysis as described under Methods. In every case tested the transfer activity is heat-labile, and specific activity values of mitochondria obtained with heated supernatant range from .11-.32 compared with values of .24-1.5 obtained with unheated

TABLE I

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

Experiment No.	1	2	3	4	5	6	7
Sample	18:3(n-3)			18:2(n-6)			
Supernatant Protein mg/ml	4.0	1.0	0.7	4.0	1.7	1.4	0.5
Microsomes Specific Activity (1)	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	
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				1.2			
Mitochondria *in vivo*	2.1	1.0	.31	4.2	4.0	2.2	1.1

(1) Specific Activity=cpm/μM phospholipid phosphorus x750

supernatant. The supernatant activity appears to reside in a component with a molecular weight greater than 10,000. The <1,000 m.w. components of the supernatant failed to stimulate and in fact diluted transfer activity. The components of molecular weight <10,000 of rat brain supernatant had transfer values similar to control values obtained with supernatant heated for 3 min. at 100°. Microsomal starting material was able to act as a donor of lipids for transfer to mitochondria with variations in initial microsomal specific

activity from 0.7-3.7. With [1-^{14}C]18:2 as a precursor, the mitochondrial specific activity after incubation showed values of .24-1.5. The phosphatide specific activity of acceptor mitochondria approached the phosphatide specific activity of donor microsomes after a transfer incubation, when the soluble protein concentration was increased to 4.0 mg/ml.

The dependency of specific activity of mitochondrial phosphatide on dialyzed supernatant protein (>10,000 m.w.) concentration is shown in Fig. 1. There is an apparent semi-logarithmic response of the transfer activity to soluble protein concentration from 0.5 to 2.0 mg/ml under these experimental conditions. On the basis of isotopic equilibrium obtained in some *in vitro* transfer experiments and the similar values obtained for phosphatide specific

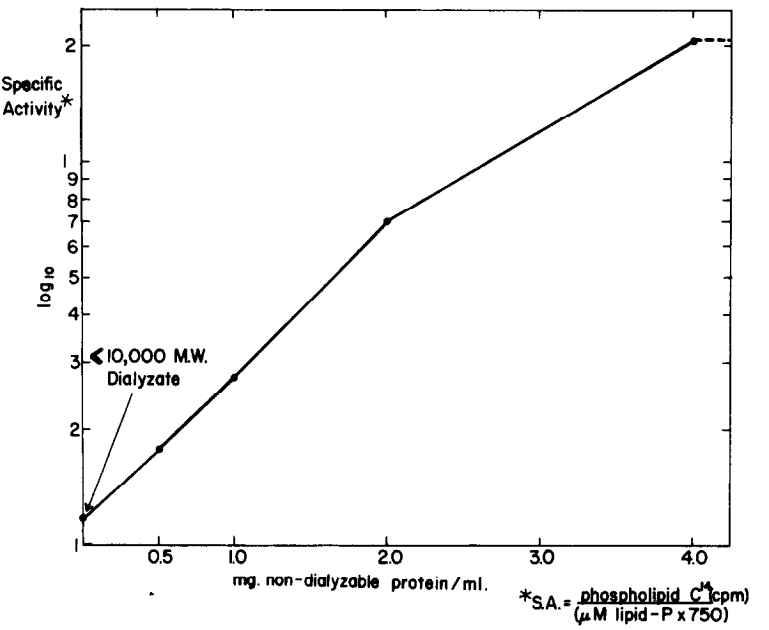

Fig. I *In vitro* transfer of ^{14}C from microsomes labelled *in vivo* with 1-^{14}C18:3(n-3) to acceptor mitochondria phosphatides as a function of dialyzed supernatant protein concentration (p > 10,000 m.w.). Incubations were for 1 hr. at 37°C and each vessel contained 3mg mitochondria, 9mg microsomes from 10-day old rat brain and the indicated amount of supernatant protein which was pH 5.1 precipitated and forced-flow dialyzed as described under Methods, t.v. = 16 ml. Total and specific activity of starting microsomes was 4419 cpm/3.5 μmoles phospholipid-lipid phosphorus.

activities of microsomes and mitochondria from rats injected with radioactive fatty acids, we would anticipate that concentrations of transfer factor greater than 4mg/ml would have produced a plateau of the concentration dependency curve shown in Fig. I. The significance of such a curve in the mechanism of transfer of lipids is the subject of a future publication.

The results of analysis of the phosphatide fractions indicate that the PE, PS and PC phosphatides of the microsomal donor material and mitochondrial acceptor material are all labelled after incubation if the pre-labelled (with 1-^{14}C18:2n-6) microsomal material was obtained by *in vivo* techniques. However, *in vitro* labelling (pre-incubation) of microsomes resulted in the transfer of PC principally until microsomes and mitochondria had come to isotopic equilibrium. In either case the specific activity of individual phosphatides was dependent on concentration of supernatant protein.

Phosphatide fatty-acyl analysis of the pre-labelled microsomes and acceptor mitochondria indicated that much of the label was retained in the original [^{14}C] fatty acid if the microsomes are labelled by *in vitro* techniques. A greater degree of elongated radioactive products obtain if [^{14}C] fatty acids are injected *in vivo* and the subcellular fractions then isolated. One such study is shown in Table II, and indicates that a greater percentage

TABLE II

DISTRIBUTION OF RADIOACTIVITY OF LONG-CHAIN FATTY ACIDS① IN MITOCHONDRIA AFTER INCUBATION WITH MICROSOMES LABELLED 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

of elongated ^{14}C fatty acyl groups is transferred when [1-^{14}C]18:3(n-3) than when [1-^{14}C]18:2(n-6) has been employed as a precursor to pre-label the microsomal fraction. Whether this indicated specificity (or lack of) of the transfer factor, availability of transferable microsomal phosphatides or even the existence of more than one type of transfer factor is currently under investigation. At this time we would like to suggest at least a 2-step mechanism for transfer: first the release of the ^{14}C lipid from microsomes into the medium and secondly, incorporation by mitochondria from the medium.

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