

## Exchange of Phospholipids Between Brain Membranes *in vitro*

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1. When unlabelled mitochondria from guinea-pig brain were incubated with a  $^{32}\text{P}$ -labelled microsomal fraction from brain there was a transfer of phospholipid to the mitochondria, which could not be accounted for by an aggregation of microsomes and mitochondria or an exchange with microsomes contaminating the mitochondria. Under similar circumstances there was a transfer of phospholipid from  $^{32}\text{P}$ -labelled mitochondria to microsomes, indicating that the process was one of exchange. 2. The transfer from microsomes was greatly stimulated by a non-dialysable heat-labile macromolecular component in the brain supernatant fraction but not by the concentration of the particulate fractions. 3. Phospholipid-exchange processes occurred most readily between pH 7 and 7.5 and were inhibited by the presence of myelin and on the addition of lysophosphatidylcholine. 4. The rates of transfer of individual phospholipids from brain microsomes to mitochondria were similar. 5.  $^{32}\text{P}$ -labelled microsomes could slowly donate phospholipid to the isolated synaptosomal (nerve-ending) fraction but the phospholipids of the myelin fraction did not exchange. 6. Subfractionation of the synaptosomal fraction after [ $^{32}\text{P}$ ]phospholipid transfer showed that the mitochondria were most actively labelled during the incubation. All of the isolated individual synaptosomal membranes were capable of acquiring phospholipid on incubation with a  $^{32}\text{P}$ -labelled brain supernatant fraction although a greater percentage was again exchanged by the mitochondrial fraction.

In the accompanying paper (Miller & Dawson, 1972) it has been shown that mitochondria isolated from guinea-pig brain and many of the membranes of synaptosomes have no capacity for synthesizing *de novo* the main nitrogen-containing phosphoglycerides such as phosphatidylcholine, which constitute the bulk of the phospholipid present. Yet it is known that when labelled precursors of the phospholipids are injected into intact animals, either into the blood circulation or directly into the brain ventricles, there is an active turnover of these phospholipids in both organelles (see, e.g., Mandel & Nussbaum, 1966; Lapetina *et al.*, 1969; Miller & Dawson, 1972). Although it could be argued that the failure to obtain phospholipid synthesis *in vitro* is due to a deficiency in the incubation conditions, it seems more likely that phospholipid synthesis (apart from phosphatidic acid and diphosphatidylglycerol) is confined to the endoplasmic reticulum, and that there is a relatively free exchange of phospholipid molecules between the various cellular membranes so that these eventually come into isotopic equilibrium.

Evidence is accumulating from studies both *in vitro* and *in vivo* that such an exchange is responsible for the redistribution of newly synthesized phospholipid within the liver cell. Thus Wirtz & Zilversmit (1968), McMurray & Dawson (1969) and Akiyama & Sakagami (1969) showed in independent investi-

gations that rat liver microsomes could exchange phospholipids with liver mitochondria when incubated together provided that a 'soluble' macromolecular component of the supernatant fraction was present. That a similar exchange occurs in the intact liver cell is suggested by radioautographic studies of Stein & Stein (1969) using [ $\text{Me-}^3\text{H}$ ]choline, and 'pulse-chase' experiments with labelled phospholipid precursors in isolated hepatic cells (Jungalwala & Dawson, 1970). Wirtz & Zilversmit (1969) have used the parallelism in the changes in  $^{32}\text{P}$  specific radioactivities of liver mitochondrial and microsomal phospholipids on treatment with phenobarbital and carbon tetrachloride as an argument for the exchange in intact rats, since these agents act specifically on the endoplasmic reticulum. However, it cannot be assumed that similar exchange processes occur in all tissues and between different types of membranes, with the synthesis of phosphoglycerides occurring only in the endoplasmic reticulum. For example, it is reported that phosphatidylcholine synthesis in *Tetrahymena pyriformis* cells occurs predominantly in the mitochondrial fraction (Smith & Law, 1970).

The present investigation has shown that there is a well-defined exchange of  $^{32}\text{P}$ -labelled phospholipids between the isolated microsomal and mitochondrial fractions of guinea-pig brain when these are incubated together under suitable conditions. On the other

hand, there is little evidence of any appreciable phospholipid exchange between  $^{32}\text{P}$ -labelled microsomes and intact synaptosomes (other than their mitochondrial complement) or myelin.

## Experimental

### *Preparation of donor and receptor subcellular fractions*

Adult male guinea pigs received an intraperitoneal dose (about 2.5 mCi) of [ $^{32}\text{P}$ ]P<sub>1</sub> and were killed after 5 days. The subcellular fractions from these and control guinea pigs were isolated as described before (Miller & Dawson, 1972), 0.1 mM-EDTA (sodium salt), pH 7.4, being added to all the sucrose solutions. The microsomal fraction was normally prepared from cerebral cortex to minimize contamination with myelin, and the mitochondria were prepared from forebrain to obtain maximum amounts of material. The latter were washed twice to remove contamination by endoplasmic reticulum (Miller & Dawson, 1972). The phospholipid composition of the brain microsomal fraction and the distribution of  $^{32}\text{P}$  labelling after 5 days are given in Table 1.

### *Incubation conditions*

All incubations, in a final volume of 2 ml of 0.32M-sucrose [or a supernatant from a 10% (w/v) brain homogenate in 0.32M-sucrose where stated], were performed at 37°C with mechanical shaking. Where stated the pH of the mixture was adjusted to 7.4 by adding 0.1 vol. of 0.1M-KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> buffer. It was not possible to replicate the quantities of particulate fraction added in each experiment, but usually the microsomal fraction added contained 5–20 mg of protein and the mitochondrial 4–8 mg of protein. At the end of the incubation period ice-cold 0.32M-sucrose (containing 0.1 mM-EDTA) was added, to give a final volume of 10 ml.

### *Re-isolation procedures*

Depending on the fractions involved, the diluted mixture was centrifuged at a speed equivalent to that used in the original fractionation scheme. Thus mitochondria were sedimented at 4000g<sub>av.</sub> for 10 min, followed by 10000g<sub>av.</sub> for 2 min. Both myelin and synaptosomes were sedimented at 12000g<sub>av.</sub> for 20 min. The synaptosomal subfractions were sedimented at 100000g<sub>av.</sub> for 30 min, and the supernatant was retained. These pellets were resuspended and repelleted once. The first supernatant was centrifuged at 100000g<sub>av.</sub> for 30 min to sediment microsomes. Unless otherwise stated the final supernatant fraction was discarded.

### *Phospholipids*

Portions of the pellets (resuspended in a small volume of 0.32M-sucrose) were extracted overnight at 4°C with 8 vol. of chloroform-methanol (1:1, v/v). Samples of the supernatant were extracted with 2 vol. of chloroform-methanol (1:2, v/v). The extracts were subsequently treated as described previously (Miller & Dawson, 1972). The total  $^{32}\text{P}$  radioactivity of the extract was determined by measuring the Čerenkov radiation in a Packard automatic liquid-scintillation spectrometer (model 2311). The efficiency of counting by this method was 30%. Phosphorus ( $^{31}\text{P}$ ) was measured on the same sample (Miller & Dawson, 1972). Radioactivity contained in individual phospholipids was determined as previously described (Miller & Dawson, 1972).

### *'Marker enzyme activities'*

The enzymes NADPH-cytochrome *c* reductase (EC 1.6.2.3), cytochrome *c* oxidase (EC 1.9.3.1) and 2':3'-cyclic nucleotide 3'-phosphohydrolase and

Table 1. *Distribution of  $^{32}\text{P}$ -labelled phospholipids in microsomal fraction isolated from guinea-pig brain cortex*

A guinea pig was injected intraperitoneally with 3.0 mCi of [ $^{32}\text{P}$ ]P<sub>1</sub> 5 days before killing. The microsomal fraction was isolated from the brain cortex, and the phospholipids were extracted and assayed for  $^{31}\text{P}$  and  $^{32}\text{P}$  as described by Miller & Dawson (1972).

Phospholipid	Total radioactivity (c.p.m.)	Percentage distribution of radioactivity	Total phospholipid (μg of P)	Percentage of P	Sp. radioactivity (c.p.m./μg of P)
Total	13 200		79.0		166
Phosphatidylcholine	6940	54	38.0	48	182
Phosphatidylethanolamine	1960	15	17.7	22	111
Phosphatidylserine	830	6	7.0	9	118
Phosphatidylinositol	800	6	2.9	4	275
Phosphatidic acid	157	1	0.6	1	262
Ethanolamine plasmalogen + sphingomyelin	2350	18	12.5	16	188

protein were determined by methods given previously (Miller & Dawson, 1972).

#### Expression of results

The results are expressed either as total c.p.m. or specific radioactivities of the phospholipid fraction. Corrections were made for any loss of phospholipid that occurred during the incubation or re-isolation procedures so that the total c.p.m. is referred to the original particulate fraction, assuming this would be labelled in the same way as the isolated sample. This correction was not necessary when specific radioactivity was being determined. The radioactivities in the recovered organelle fractions were corrected for contamination by endoplasmic reticulum by using NADPH-cytochrome *c* reductase assays and assuming that the contaminating material had the same specific activity as the microsomal fraction isolated at the end of the incubation. The non-occluded NADPH-cytochrome *c* reductase activity (Miller & Dawson, 1972) was used when assessing microsomal contamination of the intact synaptosomal fraction (Miller & Dawson, 1972).

## Results

### Inhibitory effects of myelin

Preliminary experiments showed that there was a slow exchange of phospholipids between microsomes and a crude mitochondrial fraction prepared from predominantly grey matter from cerebral cortex and contaminated with about 20% myelin phospholipid. No exchange occurred with a mitochondrial fraction prepared from whole forebrain and contaminated with 45% myelin phospholipid. This suggested that exchange processes might be inhibited by myelin.

To test this supposition use was made of a liver exchange system in which labelled liver microsomes were incubated with unlabelled liver mitochondria (McMurray & Dawson, 1969). The exchange process appeared to be completely inhibited by the presence of added brain myelin (Fig. 1). On re-isolation the myelin tended to sediment with the microsomes, the amount of phospholipid in the microsomal fraction increased and consequently its specific radioactivity decreased even at zero time. Since this specific radioactivity was used to correct the mitochondrial fraction for contaminating endoplasmic reticulum, the mitochondria in the presence of myelin thus appear to have associated radioactivity at zero time which does not change during the experiment.

In further experiments it was shown that brain microsomes labelled with  $^{32}\text{P}$  could readily transfer phospholipid to liver mitochondria (in the presence of liver supernatant) but  $^{32}\text{P}$ -labelled microsomes from liver could not transfer phospholipids to brain

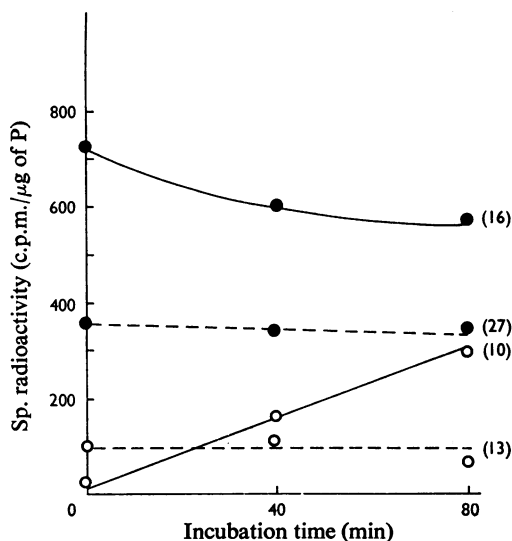


Fig. 1. Effect of brain myelin on phospholipid exchange between  $^{32}\text{P}$ -labelled liver microsomes and unlabelled mitochondria

$^{32}\text{P}$ -labelled liver microsomes (●) and unlabelled mitochondria (○) from guinea pigs were incubated in the presence (----) and absence (—) of brain myelin ( $12.5\mu\text{g}$  of phospholipid P). Liver supernatant fraction ( $2\text{ml}$ ) was present in both. After incubation the microsomes and mitochondria were re-isolated and their phospholipids extracted. The mitochondrial labelling was corrected for microsomal contamination by NADPH-cytochrome *c* reductase assays. Amounts of phospholipid P isolated ( $\mu\text{g}$ ) are given in parentheses.

mitochondria, possibly because of the myelin contaminating the latter preparation.

### Transfer of phospholipids between brain microsomal fraction and mitochondria

In subsequent experiments brain microsomal fractions were prepared from cerebral cortex grey matter to minimize contamination with myelin. Fig. 2 shows the results of a typical experiment illustrating the transfer of phospholipid from  $^{32}\text{P}$ -labelled microsomes to unlabelled brain mitochondria expressed in total counts (*c*) and specific radioactivities (*b*). On re-isolation of the mixed microsomes and mitochondria after no incubation there was a loss of phospholipid material from both particulate fractions compared with that originally added (Fig. 2*a*). A considerable amount was lost from the mitochondrial fraction and less from the microsomes. The loss from both fractions tends to increase

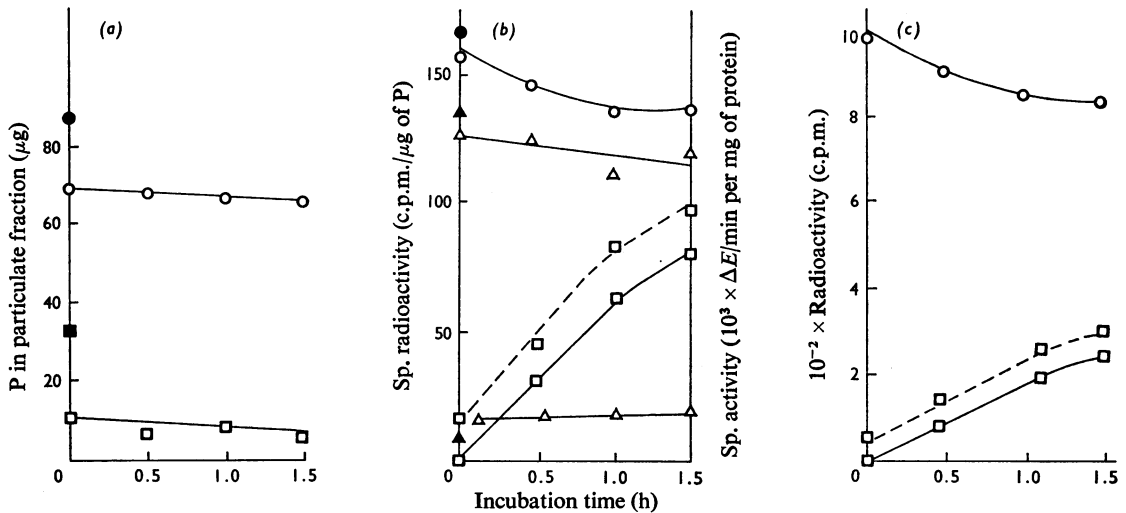


Fig. 2. Transfer of labelled phospholipids from  $^{32}\text{P}$ -labelled brain microsomes to unlabelled brain mitochondria

Brain microsomes labelled with  $^{32}\text{P}$  (○) were incubated for various times with brain mitochondria (□) in the presence of brain supernatant fraction (2 ml) and the particulate fractions were then re-isolated. ●, ■, Values for microsomes and mitochondria respectively before mixing, incubation and re-isolation. (a) Phospholipid content of particulate fraction. (b) Specific radioactivities of microsomes (○) and mitochondria (□). With the broken line (----) the mitochondrial specific radioactivity has not been corrected for microsomal contamination. The specific activity of NADPH-cytochrome *c* reductase (Δ) is shown for microsomes (upper) and mitochondria (lower), with ▲ indicating the original values. (c) Radioactivity (c.p.m.) in microsomes (○) and mitochondria (□) corrected for phosphorus loss (a). With the broken line (----) the mitochondrial radioactivity has not been corrected for microsomal contamination.

slightly on incubation (Fig. 2a). This loss has been corrected for in the results given in Fig. 2(c).

There appeared to be no transfer of NADPH-cytochrome *c* reductase activity from the microsomes to mitochondria on incubation sufficient to account for the transfer of phospholipids (Fig. 2b). On the other hand the NADPH-cytochrome *c* reductase activity of the mitochondria recovered from a microsome-mitochondria mix at zero time was appreciably above that of the original mitochondria (Fig. 2b). This suggested that some aggregation of the two particles could occur, which was not dependent on incubation. The curve relating mitochondrial radioactivity (c.p.m.) and specific radioactivity to the time of incubation, when corrected for contamination by endoplasmic reticulum, extrapolated to zero at zero time, suggesting that the microsomal phospholipids associating with the mitochondria had come into rapid equilibrium with those in the added microsomal fraction.

The transfer appears to be a two-way process since unlabelled microsomes accumulated labelled phospholipids when incubated with  $^{32}\text{P}$ -labelled brain mitochondria (Fig. 3). In these experiments the total amount of mitochondrial phospholipid was low in

comparison with the total microsomal phospholipid. This has the effect of decreasing the specific-radioactivity change of the microsomes compared with the mitochondria in the course of the phospholipid exchange. The yield of mitochondria obtained from brain tissue was small under the conditions of isolation employed, since the nerve-terminal population of mitochondria was not included.

A comparison was made between the transfer of phospholipid from  $^{32}\text{P}$ -labelled microsomes to the ordinary mitochondrial fraction and also to intraterminal mitochondria obtained by osmotic shocking of the synaptosomes for 30 min followed by centrifugation on a sucrose density gradient (Whittaker *et al.*, 1964). Although the yield of intraterminal mitochondria was small the specific radioactivity of their phospholipids rose to approximately the same value on incubation with  $^{32}\text{P}$ -labelled microsomes as non-terminal mitochondria (Fig. 4).

#### Effect of pH on transfer of phospholipids

Preliminary experiments suggested that when the brain supernatant fraction in which the exchange was carried out was adjusted to pH 7.4 with  $\text{K}_2\text{HPO}_4$ -

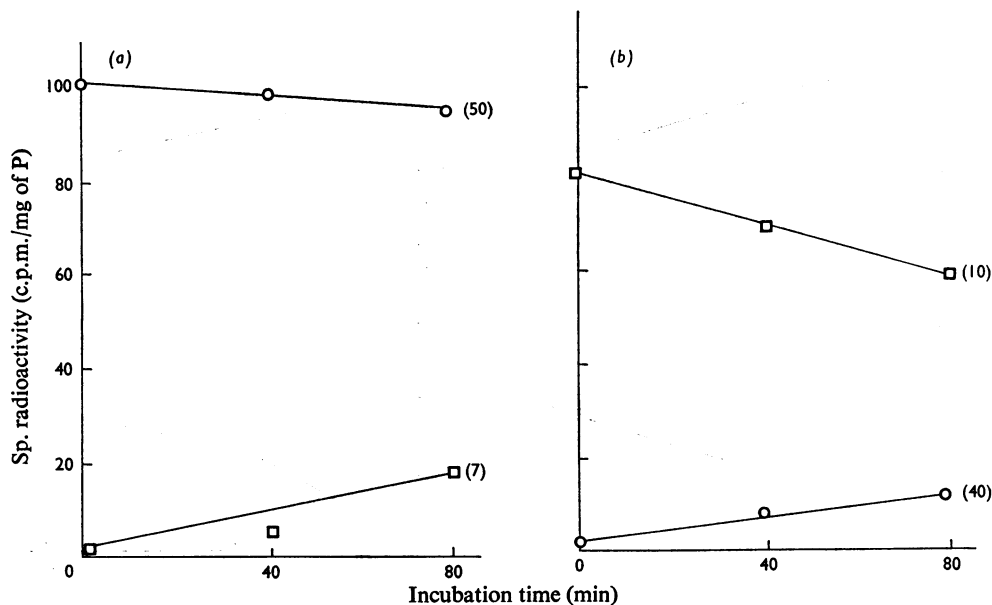


Fig. 3. Phospholipid exchange between <sup>32</sup>P-labelled microsomes and unlabelled mitochondria (a) and between <sup>32</sup>P-labelled mitochondria and unlabelled microsomes (b)

○, Microsomes; □, mitochondria. The particulate fractions were incubated together in the presence of 2ml of brain supernatant at pH 6.5. On re-isolation the mitochondrial values in (a) were corrected for microsomal contamination (NADPH-cytochrome *c* reductase assays) and the values for microsomes in (b) were corrected for mitochondrial contamination (cytochrome *c* oxidase assays). Amounts ( $\mu$ g) of lipid P isolated are given in parentheses.

KH<sub>2</sub>PO<sub>4</sub> buffer there was a more rapid transfer of [<sup>32</sup>P]phospholipid from microsomes to mitochondria. In further experiments the pH was systematically changed between pH 6.0 and 8.0 by the use of phosphate buffers and the transfer of phospholipid radioactivity was then measured after 60min incubation. Unfortunately the results are not clear-cut because the zero-time microsomal contamination of mitochondria increased at the higher pH values. However, when the increase in the mitochondrial specific radioactivity on incubation with <sup>32</sup>P-labelled microsomes was compared with their increase in NADPH-cytochrome *c* reductase activity (contamination) there appeared to be a far higher real transfer of phospholipids at pH 7.5 (ratio *A/B*, Table 2).

In an endeavour to clarify the effect of pH on mitochondrial phospholipid exchange <sup>32</sup>P-labelled brain supernatant and unlabelled mitochondria were incubated together and subsequently separated. The loss of total radioactivity from the supernatant and gain by the mitochondria was maximal between pH 7 and 7.5 (Fig. 5). In these latter experiments

complications due to aggregation between soluble lipoprotein and the mitochondrial membrane are excluded since the total phospholipid values of the former did not decrease and those of the latter did not increase over the pH range studied.

#### Effect of brain supernatant on transfer

Fig. 6 shows the effect of brain supernatant fraction on the transfer of phospholipids from <sup>32</sup>P-labelled microsomes to mitochondria during a 30min incubation. The addition of the supernatant caused a marked increase in the specific radioactivity of the mitochondrial phospholipids whereas the microsomal contamination of the mitochondria showed no increase on incubation. Dialysis of the supernatant caused no decrease in the transfer, suggesting that the active component in the supernatant fraction was macromolecular. Heating the supernatant for 5min at 100°C and centrifuging to remove denatured material caused a decrease to the low extent of phospholipid transfer observed in the absence of supernatant.

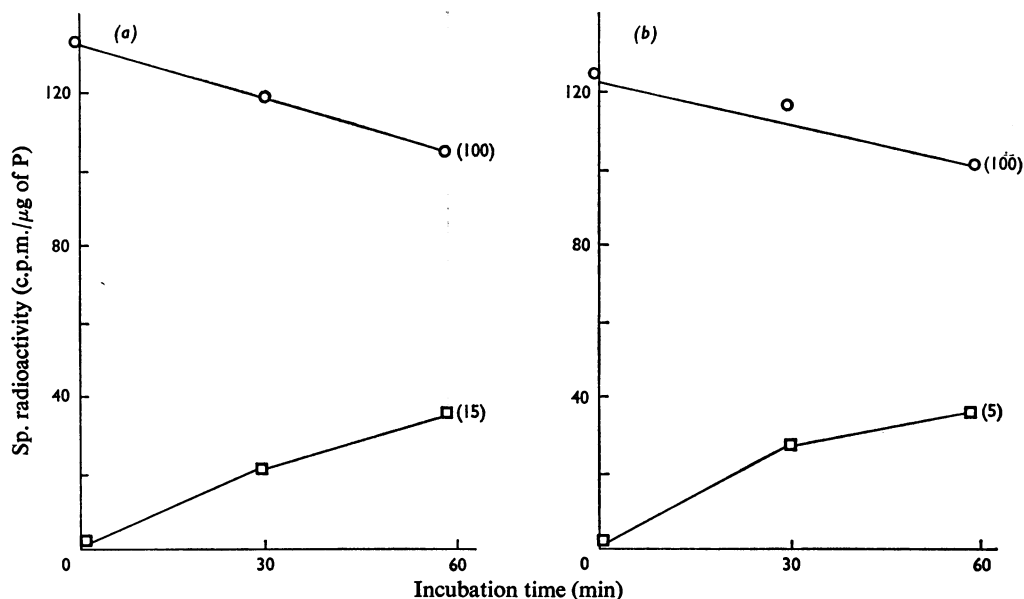


Fig. 4. Phospholipid transfer from  $^{32}\text{P}$ -labelled microsomes to unlabelled mitochondria obtained from (a) non-nerve terminal cytoplasm, and (b) the nerve terminal

$^{32}\text{P}$ -labelled brain microsomes ( $\circ$ ) were incubated with unlabelled mitochondria ( $\square$ ) in the presence of brain supernatant (2ml) at pH 7.4. The particulate fractions were re-isolated and their phospholipids extracted. Amounts ( $\mu\text{g}$ ) of phospholipid P isolated are given in parentheses. Specific radioactivities of mitochondrial phospholipids are corrected for microsomal contamination based on NADPH-cytochrome *c* reductase assays.

Table 2. Effect of pH of incubation medium on transfer of phospholipids from  $^{32}\text{P}$ -labelled microsomes to unlabelled mitochondria

$^{32}\text{P}$ -labelled microsomes isolated from guinea-pig brain cortex after labelling *in vivo* were incubated with unlabelled mitochondria for 1 h at  $37^\circ\text{C}$ . The incubation medium consisted of brain supernatant made up to a final concentration of 10mM with  $\text{K}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  buffer (see the Experimental section) (0.2 vol). After incubation the mitochondria were re-isolated.

pH of buffer	Increase in sp. radioactivity of brain mitochondria on incubation for 60 min (A) (c.p.m./ $\mu\text{g}$ of P)	Increase in sp. activity of NADPH-cytochrome <i>c</i> reductase in brain mitochondria on incubation for 60 min (B) ( $\Delta E_{550}/\text{min}$ per mg of protein)	Ratio A/B
6.0	14.5	0.104	138
6.5	11.4	0.109	104
7.0	18.4	0.074	247
7.5	18.4	0.044	420
8.0	16.2	0.065	250

#### Effect of the concentration of the particulate fractions on transfer

To determine whether the concentration of microsomes and mitochondria in the system had any effect on phospholipid transfer, experiments were carried out in which the volume of the incubation

medium was decreased. In the presence of neither 0.32M-sucrose alone nor of a brain supernatant fraction did the transfer of phospholipids from  $^{32}\text{P}$ -labelled microsomes to unlabelled mitochondria increase in the more concentrated suspensions (Table 3); rather, a slight decrease occurred. The

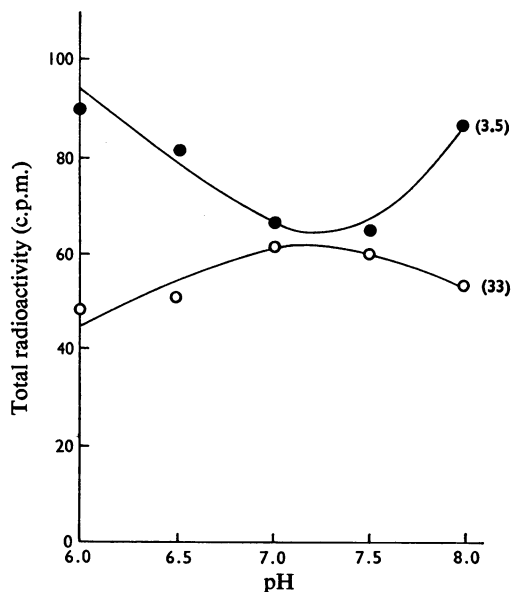


Fig. 5. Effect of pH on the transfer of phospholipids from  $^{32}\text{P}$ -labelled brain supernatant to unlabelled mitochondria

$^{32}\text{P}$ -labelled brain supernatant (prepared from a 10% homogenate of  $^{32}\text{P}$ -labelled brain in 0.32M-sucrose) was adjusted to each pH by the addition of 0.1 vol. of  $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  buffer (0.1M). After incubation with unlabelled mitochondria the latter were re-isolated and their radioactivity was determined (○) and compared with that remaining in the supernatant (●). Numbers in parentheses indicate the total phospholipid P ( $\mu\text{g}$ ) recovered, which did not change throughout the pH range studied.

apparent increase in the mitochondrial specific radioactivity at low volumes could be entirely ascribed to the considerably increased microsomal contamination revealed by NADPH-cytochrome *c* reductase assays.

#### Individual phospholipids involved in transfer

The transfer of individual labelled phospholipids from  $^{32}\text{P}$ -labelled microsomes to unlabelled mitochondria after 1 h is shown in Table 4. In terms of total phospholipid transfer 64% is represented by phosphatidylcholine and 20% by phosphatidylethanolamine. If the results are expressed as the percentage of total mitochondrial phospholipid exchanged per h, it appears that the rates for the individual phospholipids do not differ to any great extent. Thus under these conditions *in vitro*, it seems that the relative amounts of each phospholipid

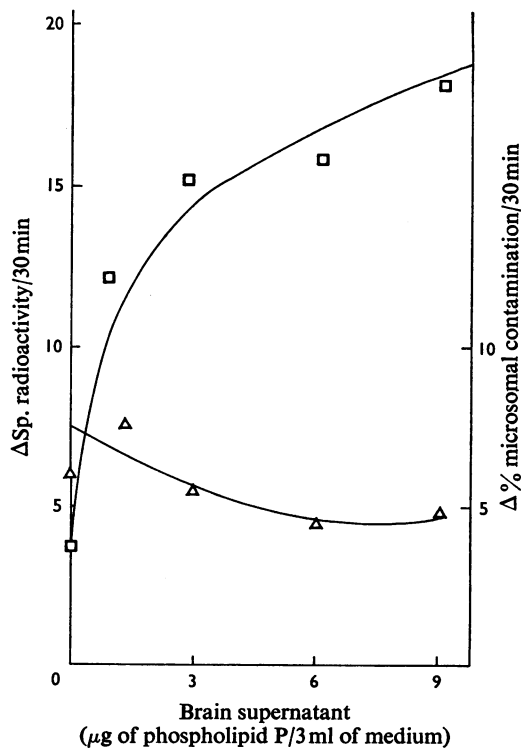


Fig. 6. Effect of brain supernatant concentration on transfer of phospholipid from  $^{32}\text{P}$ -labelled microsomes to unlabelled mitochondria

The  $^{32}\text{P}$ -labelled microsomes (80  $\mu\text{g}$  of phospholipid P) were incubated at pH 7.4 for 30 min with unlabelled mitochondria (20  $\mu\text{g}$  of phospholipid P) in the presence of various amounts of a brain supernatant fraction. □, Increase in specific radioactivity of the mitochondria isolated after incubation; Δ, change in their NADPH-cytochrome *c* reductase complement (microsomal contamination).

exchanged in the mitochondria are equivalent to the relative pool sizes of each phospholipid.

#### Effect of KCl concentration and lysophosphatidylcholine on transfer

The addition of KCl up to a concentration of 150 mM caused a linear increase in the specific radioactivity of the phospholipids in mitochondria incubated for 30 min with  $^{32}\text{P}$ -labelled microsomes in the presence of supernatant. However, NADPH-cytochrome *c* reductase assays of the mitochondria indicated that this increase could all be accounted for by increased microsomal contamination: if anything

Table 3. *Effect of medium volume on phospholipid transfer from <sup>32</sup>P-labelled microsomes to unlabelled mitochondria*

<sup>32</sup>P-labelled microsomes isolated from guinea-pig brain were incubated (30 min) with unlabelled brain mitochondria in a medium consisting of either 0.32M-sucrose or a brain supernatant fraction, both of which had been adjusted to pH 7.4 with phosphate buffer (see the Experimental section). The volume of the medium was varied but the quantity of microsomes and mitochondria was kept constant. After incubation the mitochondria were re-isolated.

Composition of medium	Volume of medium (ml)	Sp. radioactivity of mitochondria (A) (c.p.m./ $\mu$ g of lipid P)	Microsomal contamination of mitochondria* (B) (%)	Ratio A/B
0.32M-Sucrose	0.2	27	48	0.56
0.32M-Sucrose	0.5	19	31	0.61
0.32M-Sucrose	2.0	18	25	0.72
Brain supernatant fraction	0.5	28	23	1.2
Brain supernatant fraction	1.0	25	18	1.4
Brain supernatant fraction	2.0	23	17	1.4

\* Computed from the relative specific NADPH-cytochrome *c* reductase activities of the isolated microsomes and mitochondria.

the actual transfer of labelled phospholipid appeared to be slightly decreased at higher KCl concentrations.

Lysophosphatidylcholine (0.7mM) added to the system (containing brain supernatant) completely inhibited the transfer of phospholipid from <sup>32</sup>P-labelled microsomes, although it may have induced disintegration of the receptor mitochondria.

#### *Comparison of transfer of phospholipids from <sup>32</sup>P-labelled microsomes to mitochondria, myelin and synaptosomes*

When <sup>32</sup>P-labelled brain microsomes were incubated in the presence of the supernatant fraction with mitochondria, synaptosomes or myelin, considerable differences in the phospholipid transfer rates were observed (Fig. 7). Thus calculation of the percentage of phospholipid exchanged in each fraction by the method given in Table 4 showed that 40% of the mitochondrial phospholipids, 15% of the synaptosomal phospholipids and none of the myelin phospholipid were exchanged. Although a small net transfer of NADPH-cytochrome *c* reductase from the microsomes to the various fractions took place (increase in contamination after 90 min, <15%), none of the myelin marker activity (2':3'-cyclic nucleotide 3'-phosphohydrolase) present in the microsomal fraction was transferred. The transfer of <sup>32</sup>P label was thus not due to the main non-endoplasmic reticulum component of the <sup>32</sup>P-labelled microsomes, i.e. contaminating myelin becoming bound to the mitochondria or synaptosomes.

The correction for microsomal contamination decreased the value for mitochondria and synaptosomal (using non-occluded NADPH-cytochrome *c* reductase activity; Miller & Dawson, 1972) radioactivity to zero at zero incubation time. The theoretic

cal radioactivity due to the microsomal contamination of the re-isolated myelin (judged by NADPH-cytochrome *c* reductase assays) was more than twice the actual radioactivity. It was concluded that, unlike in the other fractions, equilibrium between added microsomes and contaminating microsomes was not achieved. Electron microscopy of the myelin fraction shows that there is some endoplasmic reticulum trapped within the myelin 'figures'. It was also clear from these experiments that some radioactivity was transferred from the <sup>32</sup>P-labelled microsomes to the phospholipids contained in the brain supernatant fraction.

#### *Transfer of phospholipids from <sup>32</sup>P-labelled microsomes to synaptosomal subfractions*

Although the exchange of phospholipids between microsomes and synaptosomes was not rapid it was decided to determine the distribution of the <sup>32</sup>P label transferred to intact synaptosomes among the various synaptosome membrane fractions. The labelling of the phospholipids in the individual synaptosomal subfractions after incubation of the intact synaptosomes with <sup>32</sup>P-labelled microsomes is shown in Fig. 8. The results are expressed as specific radioactivities to emphasize the fact that certain of the re-isolated fractions are heavily contaminated with the added microsomes at zero incubation time. The membranous fractions D and F are contaminated with microsomes, presumably intra- and extra-synaptosomal, to between 60 and 75% on the basis of the relative NADPH-cytochrome *c* reductase activities. Thus, although it appears that none of these fractions exchanges its phospholipid on incubation with <sup>32</sup>P-labelled microsomes (since the specific radioactivity



Table 4. *Transfer of individual phospholipids from <sup>32</sup>P-labelled brain microsomes to unlabelled mitochondria*

The mitochondria were incubated for 60min with the <sup>32</sup>P-labelled microsomes in the presence of brain supernatant at pH7.4. The total-radioactivity values are corrected for any phospholipid loss during incubation and the mitochondrial values for microsomal contamination. The amount ( $\mu\text{g}$ ) of mitochondrial phospholipid exchanged is calculated approximately from

$$\Delta(\text{c.p.m. in mitochondria during incubation})$$

Sp. radioactivity of microsomal phospholipids at zero time

Thus the % total phospholipid exchanged during incubation can be computed from this value and the total mitochondrial phospholipid.

Phospholipid	Distribution of individual [ <sup>32</sup> P]phospholipids				Mitochondrial phospholipid ( $\mu\text{g}$ of P)	% of mitochondrial phospholipid exchanged in 1 h
	Microsomes		Mitochondria			
	(c.p.m./ $\mu\text{g}$ of P) (at 0min)	(Total c.p.m.) (at 60min)	(c.p.m.)	% distribution of counts at 60min		
Total	166	13200	2100		23.0	52
Phosphatidylcholine	182	6940	1240	64	10.6	64
Phosphatidylethanolamine	111	1960	390	20	6.0	58
Phosphatidylserine	118	830	68	3	1.3	44
Phosphatidylinositol	275	800	60	3	0.4	53
Phosphatidic acid	262	157	130	1	0.15	53
Cardiolipin	0	0	0	0	2.9	0
Alkali-stable phospholipids; mainly ethanolamine plasmalogen and sphingomyelin	188	2350	170	9	1.5	60

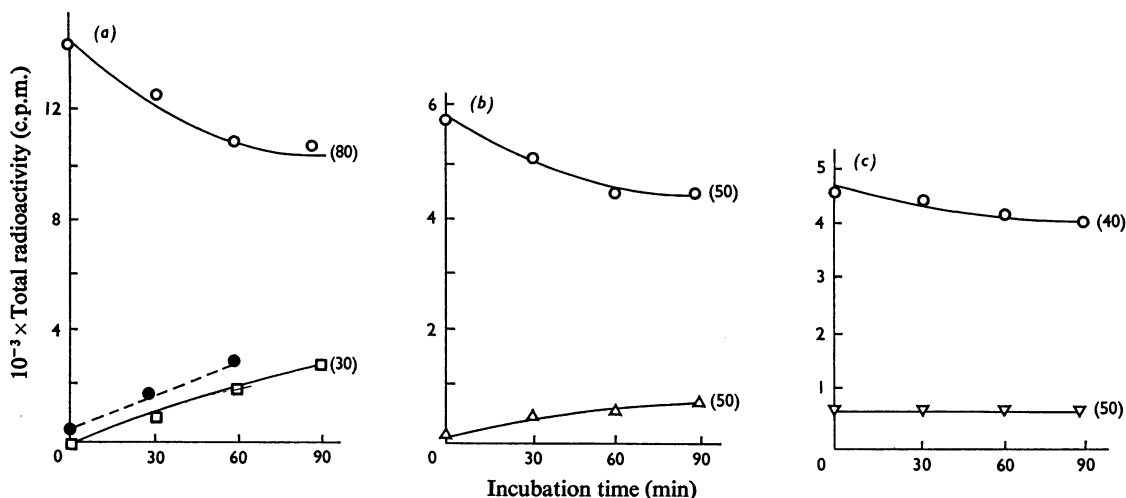


Fig. 7. Phospholipid transfer from  $^{32}\text{P}$ -labelled microsomes to unlabelled mitochondria (a), synaptosomes (b) and myelin (c)

$^{32}\text{P}$ -labelled microsomes (○) were incubated with mitochondria (□) synaptosomes (Δ) or myelin (∇) in the presence of brain supernatant (2 ml) adjusted to pH 7.5 and the total radioactivities corrected for loss of P occurring on re-isolation of the fractions. NADPH-cytochrome *c* reductase assays (non-occluded; Miller & Dawson, 1972) were used to correct the mitochondrial and synaptosomal phospholipids for microsomal contamination. The myelin phospholipid values are uncorrected; ●, radioactivity in the supernatant. Numbers in parentheses indicate the phospholipid P recovered ( $\mu\text{g}$ ) after 90 min.

does not rise), the gross contamination may obscure any small increase in labelling.

The fraction most rapidly labelled during incubation was the mitochondrial (I) with smaller increases in the labelling of the partially disrupted synaptosomes (H), 'ghosts' (G) and soluble supernatant (O). To investigate whether the individual isolated synaptosomal subfractions were capable of phospholipid exchange it was not possible to use  $^{32}\text{P}$ -labelled microsomes because of the difficulties of re-isolation; instead a  $^{32}\text{P}$ -labelled brain supernatant fraction was used. Exchange of phospholipid between the 'soluble' lipoprotein of the supernatant fraction and the various nerve-terminal membranes appeared to occur with each fraction (Fig. 9). Although the re-isolated fractions were washed they retained some of the  $^{32}\text{P}$ -labelled phospholipid as a contaminant at zero incubation time. However, the total phospholipid values for the re-isolated particulate fractions did not increase on incubation, nor did the values for the re-isolated 'soluble' fraction decrease. A time-dependent aggregation between 'soluble' and membrane lipoprotein is therefore excluded. On calculation of the rates of phospholipid exchange at the single concentration of supernatant used it was found that although the quantity of phospholipid exchanged is almost identical for all

the fractions, the percentages of the particulate phospholipid exchanged vary. Thus the phospholipids of the membrane fractions D, E, F and G were exchanged to the same extent, whereas more of the mitochondrial phospholipid was replaced since it contained less phospholipid than the other fractions. Since the mitochondrial pool was much smaller than that of the other fractions this suggests that the rate of transfer of phospholipid from the supernatant fraction to each synaptosomal subfraction may not depend on the quantity of phospholipid in the recipient membrane.

## Discussion

There seems little doubt from the present observations that the exchange of phospholipids previously observed when liver mitochondria and microsomes are incubated together also occurs with the corresponding subcellular fractions isolated from brain. The use of  $^{32}\text{P}$  label attached by two ester bonds to the phosphoglyceride structure and the incubation conditions employed make it extremely unlikely that the isotope exchange observed could be due to a breakdown of phospholipids in the labelled particulate fraction into water-soluble products and a

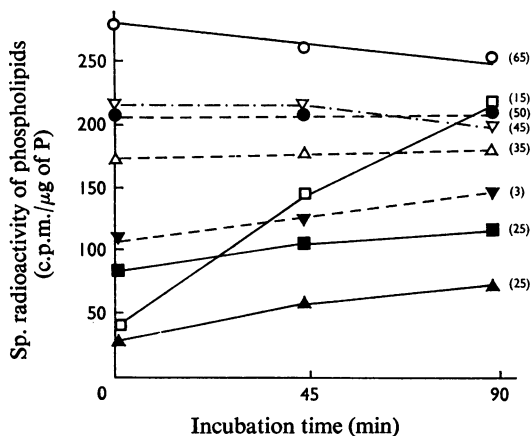


Fig. 8. Transfer of phospholipid from  $^{32}\text{P}$ -labelled microsomes to intact synaptosomes with subsequent subfractionation of the latter structures

$^{32}\text{P}$ -labelled microsomes (○) were incubated with synaptosomes in the presence of 2 ml of brain supernatant adjusted to pH 7.5 and the various subfractions isolated and designated as described by Whittaker *et al.* (1964). ▼, Supernatant (O); ▽ synaptic vesicles (D); ●, synaptosomal 'microsomes' (E); △, membranes (F); ■, synaptosomal 'ghosts' (G); ▲, partially disrupted synaptosomes (H); □, intraterminal mitochondria (I). Numbers in parentheses, total phospholipid isolated at 90 min ( $\mu\text{g}$ ).

resynthesis of these into phospholipids by the other unlabelled fraction.

Because of the far greater complexity of the brain with its comparative cellular heterogeneity the sub-cellular fractions obtained are not as pure as those obtained in liver. Nevertheless, on the basis of NADPH-cytochrome *c* reductase assays as a marker for endoplasmic reticulum the transfer of phospholipid cannot be solely ascribed to a simple aggregation of the two particles nor to an exchange with the endoplasmic reticulum contaminating the mitochondrial preparation. Such assays suggest that limited aggregation can occur, of a magnitude depending on the incubation medium used. However, it appears that the NADPH-cytochrome *c* reductase determination can be satisfactorily used for correcting for both this aggregation and intrinsic contamination of the mitochondria, since at zero time the corrected specific radioactivity of the mitochondrial phospholipids is zero (Figs. 2, 3 and 7a). This observation also suggests that the added  $^{32}\text{P}$ -labelled brain microsomes rapidly equilibrate with the microsomes contaminating the mitochondrial fraction, which appears to be

the same as in a liver system (McMurray & Dawson, 1969). The same conclusion applies to the synaptosomal fraction when the external (labile) NADPH-cytochrome *c* reductase activity is used to correct for contamination, but Fig. 7(c) shows that the same is not true for the myelin fraction. Here the contaminating endoplasmic reticulum may be trapped within myelin particles and unable to equilibrate with the added microsomes. It was necessary with myelin to perform the NADPH-cytochrome *c* reductase assay in the presence of detergent (Triton X-100) and it is therefore likely that the trapped endoplasmic reticulum would be exposed to substrate. In addition, the labelling of mitochondrial phospholipids by  $^{32}\text{P}$ -labelled microsomes cannot be due to a transfer of myelin fragments contaminating the latter preparation, since there is no transfer of cyclic nucleotide 3'-phosphohydrolase to the mitochondria. This enzyme has been suggested as a myelin marker (Kurihara & Tsukada, 1967, 1968; Kurihara *et al.*, 1970) although recent studies have suggested that it may be present in other membranes (Morgan *et al.*, 1971).

As in the liver system, the transfer of phospholipid between the two particulate brain fractions is greatly accelerated by the presence of a macromolecular heat-labile fraction present in the brain supernatant fraction. The concentration of this factor seems to be the rate-limiting determinant of the exchange *in vitro*. Its intracellular concentration must be many times that which it has been possible to achieve in the present work, owing to the dilution attendant on the preparation of the brain supernatant fraction. Since the rate of phospholipid exchange between microsomes and mitochondria does not increase when the concentration density of the particulate preparations is increased while the concentration of the supernatant fraction is maintained constant, the exchange appears not to depend on the thermal collisions between the two particles, which would be much more numerous in the concentrated suspensions. It was noticed that any change in the incubation medium that increased aggregation of the two particles at zero time or on incubation, e.g. pH changes (Table 2), increased  $\text{K}^+$  concentration, tended to decrease the transfer of phospholipid. These results favour the idea that the supernatant factor acts as a carrier for the phospholipid molecules. It has been shown that the phospholipids in the brain supernatant fraction do become actively labelled on incubation with  $^{32}\text{P}$ -labelled microsomes (Fig. 7a) and that labelled phospholipids in the supernatant can exchange with those in the mitochondria (Fig. 5) by a process with approximately the same pH-activity profile as between microsomes and mitochondria.

The work of Wirtz & Zilversmit (1970) has suggested that in liver there is a specific protein factor

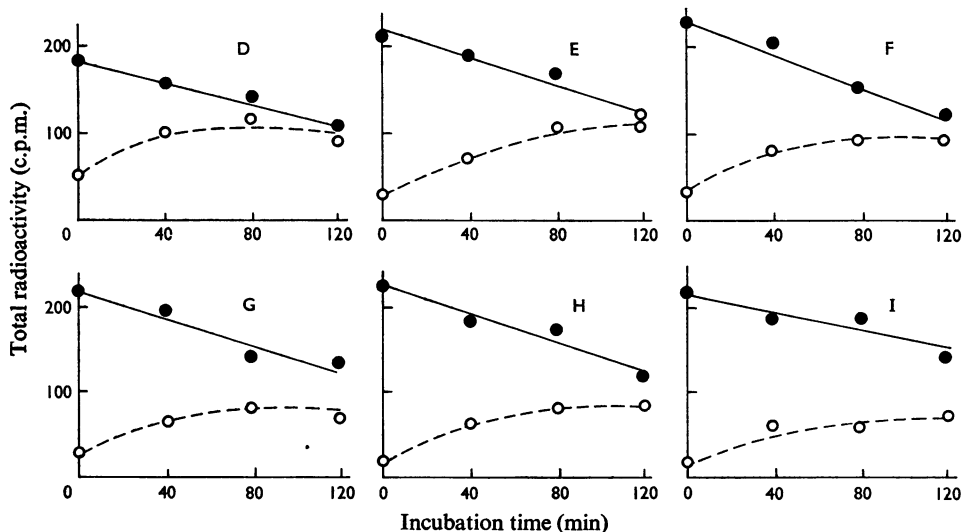


Fig. 9. Transfer of phospholipid from  $^{32}\text{P}$ -labelled brain supernatant (●) to unlabelled synaptosomal subfractions (○)

The particulate synaptosomal subfractions isolated from 0.6g of brain tissue were each incubated with  $^{32}\text{P}$ -labelled brain supernatant (equivalent to 0.28g of brain tissue and containing 3–4 μg of lipid P). The particulate fractions are designated as described by Whittaker *et al.* (1964). D, Synaptic vesicles (15.3); E, synaptosomal microsomes (15.5); F, membranes (12.9); G, 'ghosts' (9.7); H, partially disrupted synaptosomes (7.6); I, intraterminal mitochondria (3.1); the number in parentheses indicates the amount (μg) of phospholipid P contained in each fraction isolated after 2h incubation. Incubation was carried out at pH 7.5 and the subfractions were washed with 0.4M-sucrose after re-isolation, following which their phospholipids were extracted.

that is responsible for the transfer of phosphatidylcholine and phosphatidylinositol between membranes. However, it remains to be seen whether this protein has a specific affinity for these phospholipids and can thus form a carrier lipoprotein complex. The finding that a macromolecular supernatant factor is required for phospholipid-exchange processes between brain particles suggests that the sole function of such a factor in liver is not connected with the secretory function of the latter organ. Liver secretes the bile and plasma phospholipid whereas brain is not known to act as a secretory organ, phospholipid synthesis being required solely for membrane turnover. The brain system tends to differ from that of rat liver, as in the latter the exchange of phosphatidylcholine and phosphatidylinositol is rapid compared with that of phosphatidylethanolamine (McMurray & Dawson, 1969; Akiyama & Sagakami, 1969) whereas in the former there is little difference in the exchange rates (Table 4).

Although the slower transfer of phospholipids from  $^{32}\text{P}$ -labelled microsomes to intact synaptosomes compared with that to mitochondria fits in with the labelling pattern *in vivo* (Miller & Dawson, 1972), the physiological validity of this comparison must

remain in doubt. One is presenting to the microsomes what is tantamount to the external membrane of a pinched-off part of the neurone which has resealed. This situation would only occur *in vivo* if phospholipid synthesized in the endoplasmic reticulum of glial cells, e.g. oligodendria, could be transferred through the external membrane of these cells to the external membrane of the nerve ending. In fact the latter membrane (synaptosome 'ghosts') does not appear to be labelled appreciably under the incubation condition used. It is perhaps surprising that the synaptosomal subfraction showing most labelling after incubation of intact synaptosomes with  $^{32}\text{P}$ -labelled microsomes was the mitochondrial fraction. It is to be expected that although some of these mitochondria might be contaminating the synaptosomal preparation most of them would be intraterminal (Whittaker, 1968). Since the phospholipids of the mitochondrial fraction almost reach isotopic equilibrium with the added  $^{32}\text{P}$ -labelled microsomes, this would suggest that labelling of the intraterminal mitochondria occurs, and it has been shown that on isolation the phospholipids of these can exchange as readily as those of the cytoplasmic mitochondria (Fig. 4). If this is so it must mean that phospholipids

can pass through the synaptosomal external membrane without coming into equilibrium with its phospholipids. It is not possible to assess from the results whether the heavy labelling of the microsomal and vesicular fractions of the synaptosomes at zero time of incubation is due to a very rapid transfer of phospholipids from the added  $^{32}\text{P}$ -labelled microsomes before re-separation is achieved, or to excessive contamination with external microsomes.

It is apparent that phospholipid renewal of the synaptosome membranes could also occur through biosynthesis in the 'endoplasmic reticulum' or synaptic vesicles present in this part of the neurone (Miller & Dawson, 1972) followed by exchange of this newly synthesized material with the phospholipids of the other synaptosomal membranes. It would seem from the results shown in Fig. 9 that the phospholipids of all synaptosomal membranes are capable of undergoing phospholipid exchange in appropriate circumstances. Further, it is possible that phospholipids, as has now been abundantly demonstrated for protein, can be synthesized in the neurone body and transported down the fibre to the nerve ending (Miani, 1964).

Although there is good evidence that in the intact animal myelin phospholipids take up label from labelled precursors present in the bloodstream rather slowly (Davison, 1970), the turnover can be appreciable in both rate and extent when related to the microsomal phospholipids (Jungalwala & Dawson, 1971). It has been concluded by the latter authors that a substantial part of myelin phospholipids are readily exchangeable, although a small pool of slowly exchangeable material also exists. Although it has not been possible to demonstrate phospholipid exchange between  $^{32}\text{P}$ -labelled microsomes and myelin particles, it seems necessary for some type of exchange of phospholipid molecules to occur *in vivo*, since the myelin membrane is devoid of most enzymes and particularly those involved in lipid synthesis. It is apparent that the conformation of the macromolecules of the rolled-up myelin membrane in the myelin fragment may be different from the linear form existing *in vivo* in the intact myelin sheath. The inhibitory effect of such myelin particles on phospholipid exchange process has been demonstrated in the present work (Fig. 1), although its mechanism is unknown. It cannot be due to isotopic dilution, since the myelin phospholipids are not themselves labelled, but it is possible that the myelin, being

excessively rich in lipid material, could bind the supernatant protein factor and thus prevent it fulfilling its essential role in the exchange. Banik & Davison (1971) report a small transfer of phospholipids from a  $^{14}\text{C}$ -labelled myelin preparation ( $[1\text{-}^{14}\text{C}]\text{acetate}$ ) to the microsomes present in a young rat brain homogenate. It will be of great interest to know whether this represents a true exchange of whole phospholipid molecules of the myelin sheath rather than of other membranes, which might contaminate the myelin preparation.

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