Can Mitochondria and Synaptosomes of Guinea-Pig Brain Synthesize Phospholipids?

By E. K. MILLER and R. M. C. DAWSON

*Department of Biochemistry, Agricultural Research Council
Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.*

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1. The use of 'marker' enzymes for investigating the contamination by endoplasmic reticulum of mitochondrial and synaptosomal (nerve-ending) fractions isolated from guinea-pig brain was examined. NADPH-cytochrome *c* reductase appeared to be satisfactory. With the synaptosomal preparation there was a non-occluded enzymic activity believed to arise from contaminating microsomes and an occluded form released by detergent, which probably was derived from some type of intraterminal smooth endoplasmic reticulum. 2. Isolated brain mitochondria, both intact and osmotically shocked, could not synthesize more labelled phosphatidylcholine from CDP-[*Me*-¹⁴C]choline or phosphoryl[*Me*-¹⁴C]choline than could be accounted for by microsomal contamination. They could synthesize only phosphatidic acid and diphosphatidylglycerol from a [³²P]P_i precursor and not nitrogen-containing phosphoglycerides or phosphatidylinositol. 3. The synaptosomal outer membrane and the intraterminal mitochondria could not synthesize phosphatidylcholine from CDP-[*Me*-¹⁴C]choline but the synaptic vesicles and probably the intraterminal 'endoplasmic reticulum' appeared to be capable of catalysing the incorporation of label from this substrate into their phospholipids. 4. Microsomal fractions and synaptosomes from guinea-pig brain could incorporate [*Me*-¹⁴C]choline into their phospholipids by a non-energy-requiring exchange process, which was catalysed by Ca²⁺. Fractionation of the synaptosomes after such an exchange had taken place revealed that the label was predominantly in the intraterminal mitochondria and not associated with membranes containing NADPH-cytochrome *c* reductase. 5. On the intraperitoneal injection of [³²P]P_i into guinea pigs, incorporation of radioactivity into phosphatidylinositol and phosphatidic acid was much faster than into the nitrogen-containing phosphoglycerides. Mitochondria and microsomal fractions showed a roughly equivalent incorporation into individual phospholipids, and that into synaptosomes was appreciably less, whereas the phospholipids of myelin showed little ³²P incorporation up to 10h.

Present evidence suggests that during dynamic turnover of membrane components in the liver the newly synthesized phospholipids are formed in the endoplasmic reticulum and transferred by some process of exchange to the other parts of the cell. Although isolated liver mitochondria can incorporate labelled precursors into phospholipids this synthesis can all be accounted for by microsomal contamination, apart from phosphatidic acid which can be formed independently in both membranes (McMurray & Dawson, 1969; Jungalwala & Dawson, 1970; Williams & Bygrave, 1970).

However, it cannot be assumed that the intracellular localization of enzymes synthesizing phospholipid remains invariant from tissue to tissue. The present paper reports studies to ascertain whether the mitochondria present in the brain are capable of the independent synthesis of phospholipid. At the same time attention has been paid to the synaptosomal fraction, since it is not known to what extent

phospholipids present in the membranes of nerve terminals turn over by local synthesis and exchange or by transport of phospholipids synthesized in the nerve cell body down the axon (Miani, 1964). There have been a number of reports that mitochondrial and synaptosomal fractions isolated from brain can synthesize phospholipids from labelled precursors (e.g. McMurray *et al.*, 1957; McCaman & Cook, 1966; Abdel-Latif *et al.*, 1968; Bosmann & Hemsworth, 1970a; Gomez *et al.*, 1970; Lunt & Lapetina, 1970), but in these the extent and contribution to the synthesis resulting from contamination by other membranes, particularly the endoplasmic reticulum, was not assessed. It seemed likely, because of the extreme cellular heterogeneity of nervous tissues compared with liver, that such contamination would be even more extensive than in subcellular fractions prepared from liver. Methods of assessing the contaminating microsomal components of the mitochondrial and synaptosomal fractions isolated

from brain tissue have been examined and such measurements have been used to calculate the contribution of the contamination to phospholipid synthesis. In particular, use has been made of a technique whereby graded amounts of a microsomal fraction have been added to a constant concentration of the organelles and incorporation into the phospholipids extrapolated to zero microsomal (contaminating+added) concentration (McMurray & Dawson, 1969). The results suggest that cerebral mitochondria have negligible ability to synthesize phospholipids *de novo* apart from phosphatidic acid, whereas synaptosomes may possess some, but very limited, capacity for synthesis. At the same time experiments have been carried out to try to assess the intracellular site of phospholipid synthesis in brain cells by administering radioactive precursors of phospholipids and measuring their incorporation into brain subcellular fractions.

Experimental

Methods

³²P-exchange experiments *in vivo*. [³²P]P_i (2.86 mCi, 28.6 μg of P) was injected intraperitoneally into adult male guinea pigs, which were allowed food *ad lib* during the experimental period. Subcellular fractions were derived from the forebrain rostral to the inferior colliculi. Small pieces of forebrain (0.2–0.3 g) were removed before fractionation and homogenized in 20 vol. of 10% (w/v) trichloroacetic acid at 0°C. The specific radioactivity of the phosphorus in the acid-soluble extract was measured so that the specific radioactivities of the phospholipids could be expressed relative to this (Dawson & Richter, 1950).

Subcellular fractionation. Brain tissue was rapidly removed from the guinea pigs after killing, weighed and homogenized (1g/10ml) in ice-cold 0.32M-sucrose. The whole forebrain gave a high yield of the subcellular fractions, but to minimize microsomal contamination of non-microsomal fractions it was found better to use only the cerebral cortex scraped free of white matter. A glass homogenizer with a Perspex piston giving a clearance of 0.025 cm on the diameter was used to break up the tissues (12 passages with cooling at 840 rev./min). The brain subcellular fractions and the synaptosomal subfractions obtained by osmotic shocking were isolated by differential and density-gradient centrifugation as described by Gray & Whittaker (1962), Whittaker *et al.* (1964) and Eichberg *et al.* (1964). Once isolated, the subcellular fractions were used immediately (phospholipid analysis and experiments *in vitro*), stored at 0°C (all enzyme determinations) or deep-frozen (protein and RNA analyses).

Incubation conditions. The samples were incubated in media designed by McMurray & Dawson (1969).

Incubations were carried out at 37°C generally for 1 h with mechanical shaking. In experiments with [³²P]P_i a phosphate-buffered medium was used containing: 20 mM-KH₂PO₄-K₂HPO₄ buffer, pH 7.4, 20 mM-sodium pyruvate, 1.3 mM-sodium malate, 5 mM-MgCl₂, 1.7 mM-ATP, 0.25 mM-CTP, 0.1 mM-CoA, 0.17 M-sucrose, 1.7 mM-choline, 1.7 mM-ethanolamine, 1.7 mM-inositol, 2 mM-NaF and 2 mM-EDTA; total volume 1.5 ml. In experiments with [Me-¹⁴C]-choline and phosphoryl[Me-¹⁴C]choline, the phosphate-buffered medium contained in addition 1.7 mM-CMP, and the choline, ethanolamine, inositol, NaF and EDTA were omitted. In experiments with CDP-[Me-¹⁴C]choline, a tris-buffered medium was used containing: 37.5 mM-tris-HCl buffer, pH 7.4, 10 mM-cysteine hydrochloride adjusted to pH 7.4, 20 mM-MgCl₂, and 0.17 M-sucrose; total volume 0.5 ml.

Phospholipids: extraction and analysis. Phospholipids in particulate fractions were extracted overnight at 0°C with 10 vol. of chloroform-methanol (11:9, v/v). The pellet was re-extracted with 5 vol. of chloroform and the combined extracts washed as described by McMurray & Dawson (1969). The phospholipids of the soluble fraction were extracted as described by Eichberg *et al.* (1964). Individual phospholipids were assessed by the method of Dawson *et al.* (1962), the phosphorus spots on the paper being digested and assayed for ³¹P (Bartlett, 1959) and ³²P where applicable.

Radioactivity counting. In some studies (turnover *in vivo*) ³²P was determined in HClO₄ digests by using an N-6 Liquid Geiger-Müller counter, but in later work Čerenkov radiation was measured in a Packard automatic liquid-scintillation spectrometer (Läuchli, 1969). ¹⁴C in lipid extracts was also assessed by liquid-scintillation counting in toluene scintillation fluid (McMurray & Dawson, 1969). Spots of ¹⁴C-labelled water-soluble degradation products of individual phospholipids (e.g. glycerylphosphorylcholine) were located on chromatograms by radioautography, cut out and assayed directly in a toluene scintillation fluid (McMurray & Dawson, 1969); this procedure did not decrease the efficiency of counting significantly.

Enzyme determinations. Acyl transferase (EC 2.3.1.) was assayed by the release of the thiol group of CoA determined photometrically on incubating oleoyl-CoA (100 μM) and lysophosphatidylcholine (170 μM) with the fraction at 25°C as described by Eibl *et al.* (1969). Deacylase activity was suppressed with 1% serum albumin. Arylsulphatase C (EC 3.1.6.1) was determined as described by Dodgson *et al.* (1953). Adenosine triphosphatase (ATPase, EC 3.6.1.4) was assayed as described by Hosie (1965) with the released P_i being determined by the procedure of Post & Sen (1967); cytidine triphosphatase (CTPase) was determined likewise. Cytochrome *c* oxidase (EC 1.9.3.1) and NADPH-cytochrome *c* reductase (EC

1.6.2.3) were assayed as described by McMurray & Dawson (1969), 'occluded' lactate dehydrogenase (EC 1.1.1.27) by the procedure of Marchbanks (1967), NAD nucleosidase (EC 3.2.2.6) by the method of Jacobson & Kaplan (1957) with the appropriate correction being made for variation of the pH optimum with cell fraction (mitochondria pH 6.5, microsomal fractions pH 7.5). 2':3'-Cyclic nucleotide 3'-phosphohydrolase was assayed by the procedure of Olafson *et al.* (1969) after treatment of the fraction with 0.5% deoxycholate, and glucose 6-phosphatase (EC 3.1.3.9) by the method of Swanson (1955).

Protein and RNA determinations. Protein was determined by the method of Lowry *et al.* (1951) with serum albumin as standard. RNA was determined as described by Fleck & Munro (1962); the extracted ribonucleotides of a brain homogenate and the mitochondria and microsomal fractions gave qualitatively identical u.v. spectra.

Materials

Potassium *p*-acetylphenylsulphate was kindly given by Professor K. S. Dodgson. [^{32}P]P_i (50 Ci/mg of P) and [$\text{Me-}^{14}\text{C}$]choline (54 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Phosphoryl[$\text{Me-}^{14}\text{C}$]choline and CDP-[$\text{Me-}^{14}\text{C}$]choline were prepared enzymically by the method of Schneider *et al.* (1966); however, the reaction products were separated by preparative band paper ionophoresis. Choline and phosphorylcholine were separated by ionophoresis (40 V/cm) for 45 min at pH 3.6 (pyridine-acetic acid-water, 1:10:89, by vol.); for separation of phosphorylcholine and CDP-choline the ionophoresis was carried on for 5 h. After ionophoresis the bands were located by radioautography and eluted. L-1,2-Diglyceride was prepared from ovophosphatidylcholine by using phospholipase C from *Clostridium perfringens*. Early preparations of diglyceride were found to be inhibitory owing to their content of Ca^{2+} . This was removed by shaking in a water-diethyl ether system containing cyclohexane-1,2-diaminetetra-acetate in the water phase to chelate calcium.

Results

Cross-contamination of subcellular fractions obtained from brain

Perhaps the most important contamination to be considered in the present investigation is that of the mitochondria, synaptosomes and myelin with microsomal fractions (hereafter termed microsomes) since it is known that in the liver the endoplasmic reticulum is the main, if not the only, site of phospholipid

synthesis (McMurray & Dawson, 1969; Jungalwala & Dawson, 1970). Glucose 6-phosphatase was found not to be a satisfactory marker for brain microsomes, showing very variable activity and a specific activity in the synaptosomes many times higher than the microsomes themselves. Arylsulphatase C, considered to be located in the endoplasmic reticulum in liver (Milsom *et al.*, 1968), was present at too low a concentration in the brain fractions to be employed as a marker. Acyl-CoA-lysophospholipid transferase, which was reported as a microsomal marker for liver by Eibl *et al.* (1969), although this was subsequently disputed by others (Sarzala *et al.*, 1970; Waite *et al.*, 1970), had a higher specific activity in myelin and synaptosomes than in the microsomes and was therefore unsuitable.

The most useful markers found for assessing microsomal contamination were NADPH-cytochrome *c* reductase (Sottocasa *et al.*, 1967), NAD nucleosidase (Jacobson & Kaplan, 1957) and RNA concentration as an approximate indicator of rough endoplasmic reticulum. Such markers suggested that approx. 15–20% of the protein occurring in the mitochondrial fractions was due to the presence of microsomal material, with a rather higher contamination of the synaptosomes (25–30%, Table 1). The small myelin fractions obtained were badly contaminated with microsomes, giving values of 65, 60 and 50% protein contamination as judged by NADPH-cytochrome *c* reductase, NAD nucleosidase and RNA determinations respectively. The values for the large myelin fraction are all approx. 20% less. Although the low protein/lipid ratio in myelin would tend to emphasize the percentage contamination, it is known that these comparatively crude myelin preparations contain cytoplasmic inclusions (Whittaker, 1966).

The microsomal preparation was not appreciably contaminated with mitochondria or mitochondrial fragments, since it contained little cytochrome *c* oxidase and negligible diphosphatidylglycerol (cardiolipin). The mitochondria themselves contained $9.5 \pm 1.1\%$ of their phospholipid phosphorus as cardiolipin whereas the synaptosomes contained $2.2 \pm 0.2\%$ (presumably present in synaptosomal mitochondria). On the other hand, assays of the myelin marker enzyme 2':3'-cyclic nucleotide 3'-phosphohydrolase (Olafson *et al.*, 1969) indicated a specific activity in microsomes which was 25% of that of the myelin fraction. Attempts to purify the microsomal fraction by density-gradient centrifugation resulted in a very incomplete resolution of NADPH-cytochrome *c* reductase and 2':3'-cyclic nucleotide 3'-phosphohydrolase activities. However, it is now known that the latter enzyme is not exclusively located in myelin (Morgan *et al.*, 1971).

Repeated washing of the mitochondrial fraction with 0.32M-sucrose resulted in some loss of microsomal contamination based on NADPH-cytochrome

Table 1. *Microsomal contamination of forebrain mitochondrial and synaptosomal fractions*

The specific activities of NADPH-cytochrome *c* reductase, RNA and NAD nucleosidase were measured and were assumed to be exclusively microsomal for the purposes of the calculation. The percentage contaminations of the mitochondrial and synaptosomal fractions from the total homogenate are based on the specific activities of the enzyme (or RNA) in the microsomal fraction from the total homogenate whereas the percentage contaminations of the mitochondria prepared from synaptosomes and the partially disrupted synaptosomes are based on the specific activities of the microsomal fraction prepared from synaptosomes by osmotic disruption.

Fraction	Specific NADPH-cytochrome <i>c</i> reductase activity ($10^2 \times \Delta E_{550}/\text{min per}$ mg of protein)	RNA ($\Delta E_{260}/\text{mg of}$ protein)	Specific NAD nucleosidase activity (corrected) [†] ($\Delta E_{325}/\text{h per mg}$ of protein)
Microsomal fraction from total homogenate	24.5	5.2	1.23
Microsomal fraction from synaptosomes	35.0	10.8	1.91
Mitochondrial fraction from total homogenate	3.5	1.0	0.31
Mitochondrial fraction from synaptosomes	5.5	1.7	0.35
Synaptosomes	7.5*	1.2	0.37
Partially disrupted synaptosomes	9.3	3.2	0.59
% contamination by microsomes based on assays above			
Mitochondrial fraction from total homogenate	14.4	19.2	25.2
Mitochondrial fraction from synaptosomes	15.7	15.8	18.4
Synaptosomes	30.6	23.0	30.0
Partially disrupted synaptosomes	26.5	29.5	30.9

* Value based on unoccluded synaptosomal activity.

[†] Corrected for pH optimum variation.

c reductase assays. In one experiment, microsomal contamination was lowered from 17% to 9% by five washes while the specific activity of cytochrome *c* oxidase increased slightly, suggesting the removal of non-mitochondrial protein (Fig. 1*a*). The washing did not impair the respiration of the mitochondria or the response of this to added ADP (kindly performed by L. Packer).

'Endoplasmic reticulum' within the synaptosome

The synaptosome fraction as well as being contaminated with microsomes probably contains intrinsic membrane similar in function to endoplasmic reticulum. Therefore, the effects of two disruptive treatments were examined, namely hypo-osmotic shock with mechanical agitation and non-ionic detergent (Triton X-100) on the activities of NADPH-cytochrome *c* reductase and cytochrome *c* oxidase (Table 2). The effects on the mitochondrial fraction are included for comparison. Although neither treatment increased the microsomal or mitochondrial NADPH-cytochrome *c* reductase activities, that of the synaptosomal activity was increased by 30% on osmotic shocking and by 80% on addition of

detergent. That the former treatment does not result in complete disintegration of the synaptosome is clear from the retention of synaptosomal inclusions, e.g. vesicles (marked by acetylcholine) and mitochondria (marked by succinate dehydrogenase in the H band of disrupted synaptosomes; Whittaker, 1965). An increase in activity of 80% was also noted for the cytochrome *c* oxidase of synaptosomes on detergent treatment, whereas Triton X-100 did not affect the mitochondrial enzyme. The effect of osmotic shock on synaptosomal cytochrome *c* oxidase is difficult to assess, since this treatment increases by 40% the activity of the mitochondrial fraction. This latency of mitochondrial cytochrome *c* oxidase may be due to a permeability barrier for cytochrome *c* at the inner face of the mitochondrial membrane (Muscatello & Carafoli, 1969). Washing the synaptosome sample with 0.4M-sucrose causes considerable loss of the cytoplasmic marker lactate dehydrogenase, so presumably the nerve endings tend to fragment during this procedure. This is in agreement with Autilio *et al.* (1968), who reported that re-centrifugation of synaptosomes decreased their yield with no enhancement of purity. The ratio of free NADPH-cytochrome *c* reductase to occluded lactate

Fig. 1. Effect of washing with sucrose solution on microsomal contamination of brain mitochondrial and synaptosomal fractions

The appropriate pellets obtained from density-gradient centrifugation were suspended in the sucrose solution. The mitochondria were spun for 10min at 4000g followed by 10300g for 2min. During subsequent washes the preparation was only spun at 4000g for 10min. The synaptosomes were spun at 12000g for 20min after each wash. (a) Mitochondria washed with 0.32M-sucrose: ■, NADPH-cytochrome *c* reductase; ○, cytochrome *c* oxidase. (b) Synaptosomes washed with 0.4M-sucrose [results expressed in relation to occluded lactate dehydrogenase activity, which is a measure of the intactness of the synaptosomal membrane (Marchbanks, 1967)]: ■, labile NADPH-cytochrome *c* reductase; □, occluded NADPH-cytochrome *c* reductase; △, RNA (ΔE_{260} for extracted ribonucleotides/mg of protein).

Table 2. Effect of detergent treatment and osmotic shock on enzymic activities of synaptosomes and mitochondria

Results are expressed as specific activities ($10^2 \times \Delta E_{550}$ /min per mg of protein). Control activities were linear for at least 10min.

Enzyme	Fraction	Enzyme activity		
		Control	+1% Triton X-100	Osmotic shock (mechanical agitation with 9vol. of water)
NADPH-cytochrome <i>c</i> reductase	Microsomes	17.0	17.0	16.0
	Mitochondria	5.5	3.5	5.5
	Synaptosomes	5.0	9.0	6.6
Cytochrome <i>c</i> oxidase	Microsomes	0.1	0.1	0.15
	Mitochondria	2.0	2.0	2.8
	Synaptosomes	0.5	0.9	1.2

dehydrogenase activity fell during washing (Fig. 1b) whereas the occluded NADPH-cytochrome *c* reductase activity (liberated by detergent) remained the same and may have even increased somewhat.

This suggests that the occluded form of the enzyme is contained in intact synaptosomes. Rather surprisingly the RNA associated with the synaptosomes did not decrease on washing: presumably some RNA

Fig. 2. Incorporation of ^{32}P into the phospholipids of guinea-pig forebrain subcellular components

Adult male guinea pigs were injected intraperitoneally with 2.86 mCi of $[\text{}^{32}\text{P}]\text{P}_i$ (28.6 μg of P). Results express the specific radioactivities of the total phospholipid fractions relative to that of acid-soluble P. $\circ$, Microsomes; $\bullet$, mitochondria (lines not drawn because of the appreciable scatter of points); Δ , synaptosomes; $\square$, myelin. (a), Uncorrected; (b) corrected for contamination with microsomes based on NADPH-cytochrome *c* reductase assays.

must be an integral part of the nerve terminal, which is consistent with the distinct, albeit limited, synthesis of protein by the nerve terminal (Morgan & Austin, 1968).

Turnover of brain phospholipids *in vivo*

Fig. 2 shows the accumulation of ^{32}P in the phospholipids of the main subcellular fractions of forebrain after the intraperitoneal injection of $[\text{}^{32}\text{P}]\text{P}_i$. The results expressed as specific radioactivities relative to the specific radioactivity of the forebrain acid-soluble phosphorus are plotted both in their original form (Fig. 2a) and corrected for microsomal contamination based on NADPH-cytochrome *c* reductase assays (Fig. 2b). The turnover of the total phospholipid seems to be approximately equal in the microsomal and mitochondrial fractions with that of the synaptosomes appreciably less. The influence of contaminating microsomes on the apparent turnover is illustrated most markedly with the myelin

fraction, where the appreciable uncorrected turnover was decreased to a very low value when the correction was applied.

The increase in the specific radioactivities of the individual phosphoglycerides was always less in the synaptosomes than the mitochondrial or microsomal fractions (Fig. 3). The turnover of phosphatidylinositol (Fig. 3a) was much greater than that of the other phospholipids and its radioactivity was comparable with that of phosphatidic acid. Apart from phosphatidylethanolamine (Fig. 3c) at longer time-periods the relative specific radioactivity of the mitochondria was generally somewhat above that of the microsomal fraction. An unexpected result was that incorporation into the synaptosomal diphosphatidylglycerol (cardiolipin) was initially higher than that in the non-synaptosomal mitochondria (Fig. 3b). Since this phospholipid is rather specifically localized in the inner mitochondrial membrane this suggests that this is either being renewed more readily in the synaptosomal mito-

Fig. 3. Incorporation of ^{32}P into the individual phospholipids of guinea-pig forebrain subcellular fractions

Experimental details are as for Fig. 2. Specific radioactivities of individual phospholipids are expressed relative to that of acid-soluble P. (a), Phosphatidylinositol; (b) diphosphatidylglycerol; (c), phosphatidylethanolamine; (d), phosphatidylcholine; (e), ethanolamine plasmalogen. $\circ$, Microsomes; $\bullet$, mitochondria (in c line not drawn because of the wide scatter of points); Δ , synaptosomes.

chondria or that supplies of precursor ^{32}P are reaching the latter more readily.

Synthesis of phospholipids from $[^{32}\text{P}]\text{P}_i$

Table 3 shows the synthesis of phospholipids in a washed mitochondrial fraction either with or without the addition of a supernatant or microsomal fraction.

In the mitochondrial fraction alone, only phosphatidylinositol and phosphatidic acid were labelled to any appreciable extent and incorporation into the nitrogen-containing phospholipids, which comprise the bulk of those present, was extremely limited. Addition of microsomes and microsomes plus supernatant produced a stimulation of incorporation into all the phospholipids, but again the incorporation

Table 3. Incorporation of [32 P] P_i in vitro into the individual phospholipids of washed brain mitochondria and microsomes

Mitochondria (equivalent of 2g of forebrain, washed 5 times, microsomal contamination = 6%), microsomes (equivalent to 1g of forebrain) and supernatant (equivalent to 0.06g of forebrain) were incubated for 60min at 37°C in phosphate buffer medium (see the Experimental section) with 50 μ Ci of [32 P] P_i . Results are given as total radioactivity (c.p.m.), with specific radioactivity (c.p.m./ μ g of P) in parentheses.

Phospholipid	Radioactivity incorporated			
	Mitochondria	Mitochondria + supernatant	Mitochondria + microsomes	Mitochondria + microsomes + supernatant
Total (recovered)	259 (26.6)	279 (29.5)	2396 (28.5)	2109 (17.7)
Phosphatidylcholine	4 (0.4)	30 (2.7)	10 (0.3)	25 (0.6)
Phosphatidylethanolamine	0 (0)	2 (0.3)	15 (0.8)	13 (0.7)
Phosphatidylserine	8 (5.9)	11 (7.3)	15 (1.3)	18 (1.4)
Phosphatidylinositol	92 (142.0)	106 (77.2)	1420 (409.0)	1500 (366.0)
Phosphatidic acid	116 (290.0)	77 (176.0)	843 (770.0)	508 (407.0)
Cardiolipin	11 (5.2)	36 (28.1)	*	*

* Chromatographic separation incomplete.

Fig. 4. Synthesis of phospholipid from [32 P] P_i by brain mitochondria in the presence of various amounts of added microsomal protein

Mitochondria equivalent to 0.75g of cerebral cortex and washed 3 times, and supernatant equivalent to 0.5g of cerebral cortex, were used. Incubation was at 37°C for 1h. (a) $\blacktriangle$, Phosphatidic acid; $\blacksquare$, phosphatidylcholine; $\bullet$, phosphatidylinositol; (b) $\circ$, diphosphatidylglycerol; $\square$, phosphatidylethanolamine. The first point represents the microsomal contamination of the mitochondria computed from NADPH-cytochrome *c* reductase assays.

into phosphatidylcholine and phosphatidylethanolamine was very small compared with the acidic phosphoglycerides. This contrasts markedly with similar experiments with the same subcellular fractions from liver where the incorporation into the nitrogen-containing phospholipids, in particular phosphatidylethanolamine, was predominant (McMurray & Dawson, 1969).

In further experiments, isolated mitochondria together with a brain supernatant fraction and various amounts of microsomes were incubated with [32 P] P_i , and the total incorporation into phospholipids was plotted as a function of microsomal complement (Fig. 4). The latter represents the sum of the added microsomes and those contaminating the mitochondrial preparation as assessed by NADPH-cytochrome *c* reductase assays. The incorporation into phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine was approximately linearly related to the microsomal component, and as far as can be judged each curve cuts the origin region when extrapolated back to 'zero' microsomal concentration. This result indicates that the small 32 P-labelling of these three phospholipids by isolated brain mitochondria can probably be attributed to the residual microsomal enzyme systems contaminating the mitochondrial preparation. In contrast, as with liver (McMurray & Dawson, 1969), there is evidence that mitochondria can independently synthesize phosphatidic acid and diphosphatidylglycerol. The addition of microsomes depresses the formation of diphosphatidylglycerol presumably by competing for some substrate or cofactor. In other tissues the enzyme systems for

synthesizing diphosphatidylglycerol are known to be located in mitochondria (Kiyasu *et al.*, 1963; Stanacev *et al.*, 1969).

When [32 P]P_i was incubated with synaptosome preparations under similar conditions the amount of incorporation into the phospholipids was extremely limited and the addition of microsomes and supernatant only produced a small stimulation. Most of the incorporation was into phosphatidylinositol and when microsomes were added to synaptosomes in various amounts and the incorporation was plotted against the total microsomal complement (added+contaminating) the curve for phosphatidylinositol synthesis extrapolated to the origin at 'zero' microsomal concentration. This indicated that the intact synaptosomes were incapable of independent phosphatidylinositol synthesis. Incorporation into other phospholipids was too low for their site of synthesis to be assessed satisfactorily and variations in the medium or conducting the incubation at 25°C in an attempt to maintain the structural integrity of these organelles (Potter, 1968) resulted in no worthwhile improvement. The synaptosomes are perhaps incapable of producing sufficient [γ - 32 P]ATP for external phospholipid synthesis by the added and contaminating microsomes (although added mitochondria did not stimulate incorporation). Moreover other experiments showed that microsomal fractions contain an active system for degrading CTP.

Synthesis of phospholipids from [14 C-Me]choline

Table 4 shows the amount of choline incorporated when washed mitochondria, microsomes and supernatant are incubated in various combinations with labelled choline. It is clear that although the mitochondrial preparation can incorporate some choline into its phospholipids, the microsomal fraction from an equivalent weight of forebrain can incorporate

far more. The results are different from those obtained for liver by Kaiser (1969) who failed to find any increase in choline incorporation when microsomes were added to mitochondria. The present incorporation was largely into the phosphatidylcholine component of the subcellular fractions, but it was not stimulated by the addition of L-1,2-diglyceride solubilized in detergent (0.1% Tween 20); rather, a complete inhibition of the microsomal stimulation of mitochondrial incorporation was obtained.

Liver microsomes are known to incorporate choline into phosphatidylcholine by a type of base-exchange reaction catalysed by Ca²⁺ and not requiring metabolic energy (Dils & Hübscher, 1959), although this has not been detected in microsomes from chicken brain (Arienti *et al.*, 1970). Fig. 5 shows experiments in which labelled choline (3.3 mM concentration) was incubated with both mitochondria and synaptosomes and where graded increments of microsomes have been added to each system. In a medium containing 3.3 mM-Ca²⁺ and devoid of substrate and cofactors uptake of choline into phosphatidylcholine was greatly stimulated above that obtained in the normal medium and this increased incorporation was related to the amount of microsomes added to the system. However, whereas no evidence was found that mitochondria could incorporate choline into their phospholipid by the Ca²⁺-stimulated reaction, the synaptosomes clearly did, since the curve relating incorporation to microsomal protein when extrapolated indicated some synthesis at zero microsomal concentration. When synaptosomes were osmotically disrupted after the Ca²⁺-stimulated incorporation had taken place fractionation of the various components indicated that the synaptosomal mitochondria had become the most heavily labelled (Table 5). There was no correlation between the specific activity of the microsomal marker enzyme NADPH-cytochrome *c* reductase in the various fractions and the specific radioactivity of their

Table 4. *Incorporation of [14 C]choline into brain mitochondria and microsomes*

Thrice washed mitochondria (≈ 1 g of brain), microsomes (≈ 1 g of brain) and supernatant (≈ 0.025 g of brain) were incubated in various combinations in phosphate-buffered medium (see the Experimental section) containing 3.3 mM-[14 C]choline (0.5 μ Ci/1.5 ml of medium) for 60 min at 37°C.

Fraction	Choline incorporated (nmol)	Sp. radioactivity (c.p.m./ μ g of P)
Mitochondria	0.61	22.2
Mitochondria + supernatant	0.78	41.0
Mitochondria + microsomes	2.90	43.0
Mitochondria + microsomes + supernatant	2.50	40.0
Microsomes	1.92	30.8
Microsomes + supernatant	1.58	30.2

Fig. 5. Incorporation of $[Me-^{14}C]$ choline into the phospholipids of mitochondrial (a) and synaptosomal preparations (b) containing various amounts of added microsomes

The first point plotted on each curve represents no added microsomes, the microsomal contamination having been assessed by NADPH-cytochrome *c* reductase assays. The choline concentration was 3.3 mM ; incubation was for 1 h at 37°C . ●, Normal incubation medium; ○, medium with substrates and ATP omitted and $3.3\text{ mM-}Ca^{2+}$ added.

Table 5. Distribution of $[Me-^{14}C]$ choline in phospholipids (labelled via the Ca^{2+} -stimulated base-exchange reaction) in synaptosomal subfractions

Synaptosomal and supernatant fractions were incubated in phosphate-buffered medium containing $0.5\text{ }\mu\text{Ci}$ of $[Me-^{14}C]$ choline (3.3 mM) and $3.3\text{ mM } Ca^{2+}$, but with no substrate or cofactors. After incubation for 1 h at 37°C , the material was sedimented, osmotically shocked and subjected to density-gradient centrifugation.

Subfraction with probable main component (Whittaker <i>et al.</i> , 1964)	$[Me-^{14}C]$ Choline incorporated (nmol/mg of protein)	Specific radioactivity of phospholipid extract (c.p.m./ μg of P)	Specific NADPH-cytochrome <i>c</i> reductase activity [$10^2 \times \Delta E_{550}/\text{min per mg of protein}$]
O Soluble	0.06	20	8
D Synaptic vesicles	0.09	30	11
E Microsomes	0.22	50	33
F Unidentified membranes	3.00	65	7
G 'Ghosts'	5.01	90	16
H Partially disrupted synaptosomes	6.01	125	14
I Terminal mitochondria	23.5	320	10

labelled phospholipids. Abdel-Latif (1971) has reported that the uptake of choline into the phospholipids of a rat brain synaptosomal fraction is enhanced by $0.85\text{ mM-}Ca^{2+}$ but markedly inhibited by $5\text{ mM-}Ca^{2+}$.

Since the contaminated mitochondrial fraction and synaptosomes appeared to be able to incorporate choline into their phospholipids by the base-exchange reaction when Ca^{2+} was added, the possibility occurred that some of the incorporation observed

in the normal medium containing no added Ca^{2+} was due to a similar reaction caused by small amounts of Ca^{2+} present in the medium materials and sub-cellular fractions. Incubation in the absence of substrate and nucleotide cofactors and with the addition of KCN to eliminate any energy-dependent pathway for choline incorporation mediated by cytidine, decreased the uptake obtained on addition of various amounts of microsomes to a mitochondrial preparation (Fig. 6a). Both the energy-dependent incorpora-

Fig. 6. Incorporation of $[Me-^{14}C]$ choline into the phosphatidylcholine of mitochondrial (a) and synaptosomal preparations (b) to which were added various amounts of a microsomal fraction

The choline concentration was 0.2 mM. Other details as in Fig. 5. ○, Complete medium; Δ, medium devoid of substrates and nucleotide cofactors and containing 0.3 mM-KCN; ●, difference (energy-dependent incorporation).

tion and the base-exchange pathway of incorporation extrapolated to the origin at zero microsomal concentration, implying that both types of uptake were dependent on microsomes. The concentration of choline used in these experiments was 0.2 mM, which approximates to the physiological value reported for rat brain (Ansell & Spanner, 1968). At this concentration of choline, uptake into synaptosomal phospholipids was mainly by the base-exchange mechanism (Fig. 6b) and this seemed to be largely dependent on the microsomal complement although the extrapolation is not certain enough to eliminate extramicrosomal activity. The small energy-dependent incorporation appeared not to increase on the

Fig. 7. Incorporation of label from phosphoryl- $[Me-^{14}C]$ choline into synaptosomal or mitochondrial preparations with microsomes added

(a) Mitochondria; (b) synaptosomes. The phosphate-buffered medium (see the Experimental section) contained 0.5 μ Ci of phosphoryl $[Me-^{14}C]$ choline (6.6 μ mol)/1.5 ml. The first point plotted represents the microsomal contamination computed from NADPH-cytochrome *c* reductase assays.

addition of microsomes and presumably, unlike the mitochondria, the synaptosomes may not be able to regenerate sufficient ATP or CTP for the additional synthesis by the added microsomes to take place.

Synthesis of phospholipids from phosphoryl $[Me-^{14}C]$ choline

When phosphoryl $[Me-^{14}C]$ choline was used as a substrate for phospholipid synthesis both mitochondria and synaptosomal preparations showed a response to added microsomes (Fig. 7). The incorporation by the mitochondria themselves could largely be accounted for by microsomal contamination (NADPH-cytochrome *c* reductase assays), since the curve relating incorporation to microsomal concentration extrapolated to the region of the origin (Fig. 7a). However, a similar curve for synaptosomes

Fig. 8. Incorporation of CDP-[Me-¹⁴C]choline into the phosphatidylcholine of mitochondrial and synaptosomal fractions alone and with the addition of microsomes

The incubation medium contained 0.028 μCi of CDP-[Me-¹⁴C]choline (0.2 $\mu\text{mol}/0.5\text{ml}$) as described in the Experimental section. Incubation was for 1 h at 37°C. (a) Mitochondria; (b) synaptosomes; $\blacktriangle$, tris-buffered medium; $\triangle$, tris-buffered medium with L-1,2-diglyceride solubilized in 0.1% Tween 20; $\bullet$, intact synaptosomes; $\circ$, osmotically disrupted synaptosomes. The first point plotted represents the microsomal contamination of either mitochondrial or synaptosomal fractions calculated from NADPH-cytochrome *c* reductase assays.

suggested that a small part of their intrinsic incorporation of labelled choline was not due to contaminating microsomes (Fig. 7b). Since it is possible that phosphorylcholine cannot readily penetrate the intact synaptosome, experiments were carried out with the latter preparation disrupted osmotically. Incorporation of labelled choline into phospholipid increased by 25% but since the NADPH-cytochrome *c* reductase activity of the preparation also increased by 30% this would suggest that the increase may be largely due to the microsomal membranes occluded in the synaptosome.

Synthesis of phospholipids from CDP-[Me-¹⁴C]choline

Label from CDP-[Me-¹⁴C]choline was incorporated into the phospholipids of a brain mitochondrial preparation and the incorporation was greatly enhanced by the addition of microsomes (Fig. 8a). The presence of L-1,2-diglyceride markedly stimulated the incorporation. Extrapolation of the curves relating the degree of incorporation to the microsomal concentration indicated that contaminating microsomes accounted for all the incorporation into the mitochondrial preparation.

As had been observed with liver (McMurray & Dawson, 1969) ultrasonic treatment of the mitochondria caused a decrease in the ability to incorporate label from the CDP-[Me-¹⁴C]choline into phos-

pholipids. However, this treatment also decreased the ability of the microsomes to synthesize phospholipid (Table 6). Osmotic disruption on the other hand, which produced enhancement of cytochrome *c* oxidase activity (Table 2), caused no change in the ability of the mitochondria to incorporate label, nor did the same treatment alter the synthetic ability of the microsomes. Although brain mitochondria are rather insensitive to hypo-osmotic conditions (Whittaker *et al.*, 1964) it is likely that they would become swollen and their outer membranes ruptured. This therefore suggests that the inability of mitochondria to incorporate label from CDP-[Me-¹⁴C]choline into phospholipids is not due to permeability barriers.

The incorporation of label from CDP-[Me-¹⁴C]choline into synaptosomes could be largely accounted for by the microsomal component in both the intact and the osmotically disrupted organelles (Fig. 8b), but because of high microsomal contamination the extrapolation to zero microsomal content is too indefinite to say whether or not there is a small extra microsomal synthesis of choline phospholipid. The distribution of [Me-¹⁴C]choline labelling among the phospholipids of the various subfractions of the synaptosome after incubation of the intact preparation with CDP-[Me-¹⁴C]choline was examined in conjunction with the distribution of the microsomal marker activity. This may be compared with the incorporation of CDP-[Me-¹⁴C]choline into the

Table 6. *Incorporation of radioactivity from CDP-[Me-¹⁴C]choline into the phospholipids of brain mitochondrial and microsomal fractions treated by various disruptive procedures*

Isolated microsomal fractions were incubated (1 h, 37°C), either directly or after ultrasonication or hypo-osmotic conditions, with CDP-[Me-¹⁴C]choline (0.028 μ Ci, 0.4 mM). The incubation medium was a Mg²⁺-tris buffer of 0.5 ml total volume as described in the Experimental section.

Treatment	Fraction	Intact		Disrupted	
		Amount incorporated (nmol)	Sp. radioactivity (c.p.m./ μ g of P)	Amount incorporated (nmol)	Sp. radioactivity (c.p.m./ μ g of P)
Ultrasonication (0°C, 5 periods of 30 s in Mullard-MSE 60 W instrument)	Microsomes ($\equiv$ 0.5 g of brain)	34	102	10.6	23.3
Hypo-osmoticity (10 vol. of water added to a suspension in 0.32 M-sucrose and mechanically agitated)	Mitochondria ($\equiv$ 2 g of brain)	2.8	48.6	1.9	29.8
	Microsomes ($\equiv$ 0.1 g of brain)	5.5	95.0	5.0	96.0
	Mitochondria ($\equiv$ 2 g of brain)	2.8	22.3	2.7	22.2

Table 7. *Incorporation of CDP-[Me-¹⁴C]choline into the phospholipids of synaptosomal subfractions isolated before and after incubation*

Intact synaptosomes (A) or synaptosomal subfractions (B) were incubated with 0.028 μ Ci of CDP-[Me-¹⁴C]choline (0.4 mM) and L-1,2-diglyceride (1.25 mM) in tris-buffered medium for 1 h at 37°C.

Fraction (Whittaker <i>et al.</i> , 1964)	Specific radioactivity (c.p.m./ μ g of P)		Specific NADPH-cytochrome <i>c</i> reductase activity ($10 \times \Delta E_{550}$ /min per mg of protein)
	A	B	
O Soluble	12.3	1.3	0.8
D Synaptic vesicles	5.4	15.2	1.08
E Microsomes	3.0	10.8	3.3
F 'Membranes'	2.8	4.2	0.7
G 'Ghosts'	2.7	5.3	1.7
H Partially-disrupted synaptosomes	3.4	3.7	1.4
I Mitochondria	1.6	2.5	1.0

isolated subfractions, incubated separately with the precursor (Table 7). In neither case does a correlation exist between CDP-[Me-¹⁴C]choline incorporation and NADPH-cytochrome *c* reductase activity among the synaptosomal subfractions. Of the isolated fractions incubated with precursor the synaptic vesicles appear to be most active with respect to [¹⁴C]choline incorporation, whereas of the fractions isolated from the intact synaptosome incubated with precursor, the soluble fraction is most highly labelled (on a specific-radioactivity basis), the vesicles being

more labelled than the remaining membranous fractions. These results are not compatible with the concept of an absolutely specific localization of the enzyme CDP-choline-1,2-diglyceride choline phosphotransferase in the endoplasmic reticulum of nervous tissue. The subsynaptosomal distribution of this enzyme was clarified as a result of incubation of the isolated synaptosomal fractions with increments of a microsomal preparation (isolated from whole cortex) by using CDP-[Me-¹⁴C]choline as precursor. With all subfractions of the synaptosomes

Fig. 9. Incorporation of radioactivity from CDP-[Me- ^{14}C]choline into synaptosomal subfractions alone or with the addition of microsomes

Experimental details are as given in Table 7. Designation of subfractions is as given by Whittaker *et al.* (1964). (a) Soluble fraction (O); (b) synaptic vesicles (D); (c) microsomes (E); (d) 'membranes' (F); (e) synaptosome 'ghosts' (G); (f) partially disrupted synaptosomes (H); (g) synaptosomal mitochondria (I). The first point plotted represents the microsomal protein in the sub-synaptosomal fraction calculated from NADPH-cytochrome *c* reductase assays.

the relationship between choline incorporated into the total phospholipid extract and the added microsomes was always approximately linear (Fig. 9). Extrapolation to zero microsomal protein suggested that no appreciable incorporation occurs in any fraction except the synaptic vesicles. However, it is likely that the synaptosomal 'microsomes' can also synthesize phosphatidylcholine, since the computation of the first point in Fig. 9(c) will depend on total NADPH-cytochrome *c* reductase activity rather than that due to contaminating microsomes. The results given in Fig. 9 and Table 7 (columns A and B) suggest that the appreciable labelling of the phospholipids of the supernatant fraction found after incubation of intact synaptosomes with CDP-[Me- ^{14}C]choline is not derived by synthesis within this fraction but possibly by transfer of labelled material from a membrane fraction.

Discussion

The cellular heterogeneity of nervous tissue has always made it especially difficult to obtain subfractions of brain cells in any state of purity. Although the technique of density-gradient centrifugation superimposed on differential centrifugation

has enormously improved separations, it is very apparent that the isolated organelles are heavily contaminated with other membrane fractions. This can be seen on a qualitative basis by electron microscopy (carried out on our own fractions through the kindness of Dr. E. A. Munn) and from the reported examinations of subcellular fractions by this technique by other workers. However, as a method of measuring cross-contamination electron microscopy has severe limitations, e.g. it is possible to show bias in the selection of fields; identification of the contaminating membrane from other similar membranes in the field is often difficult and it is almost impossible to measure the concentration of the contaminant in anything but indefinite quantitative terms.

It is probable that the main site of synthesis of phospholipids in the brain cells is in the endoplasmic reticulum, and this is borne out by all the investigations *in vitro* reported here. Consequently, the contamination of main concern in the present work is that of the organelles by microsomes. Of six enzymes or substances reported previously as markers of endoplasmic reticulum in liver only three seemed to be at all suitable for examining brain subcellular fractions, namely NADPH-cytochrome *c* reductase, NAD nucleosidase and RNA. In theory, for an

enzyme or substance to act as a satisfactory marker it is best for it to be exclusively located in the membrane fraction concerned. This means that for absolute certainty in defining the location it is necessary to isolate the completely pure membrane and organelle fractions. Even in liver this has not been possible, even less so with brain tissue. Certainly RNA can only act as an approximate marker of rough endoplasmic reticulum, since limited amounts will also be present inside the mitochondria, both intra- and extra-neural. It is clear that as far as NADPH-cytochrome *c* reductase is concerned washing of mitochondria and synaptosomes removes some of the enzyme, presumably associated with contaminating microsomes. At the same time, the mitochondrial inner-membrane marker cytochrome *c* oxidase remains and so does the occluded NADPH-cytochrome *c* reductase in the synaptosomes. It could of course be argued that the washing of mitochondria is selectively removing the outer membrane containing the enzyme, and Brunner & Bygrave (1969) have suggested that in liver the enzyme may be a component of the outer mitochondrial membrane. However, this work can be criticized on the grounds that cytochrome *P*-450, which was used to mark the 'absence' of microsomes from mitochondria, is very difficult to determine in low concentrations. The reasonably close agreement between the microsomal contamination of mitochondria and synaptosomes calculated by using the three markers, and especially that of the intraterminal mitochondria and partially disrupted synaptosomes, suggests that broadly speaking the markers are satisfactory. Certainly, electron microscopy shows that the subcellular fractions are far from homogeneous and in studies on phospholipid synthesis it seems better to correct for such contamination of the organelles rather than ignore it.

The effect of such a correction can clearly be seen in the experiments recording the turnover of the whole phospholipid fraction of brain subcellular fractions after the injection of [32 P]P_i *in vivo*. The turnover of the myelin fraction is decreased virtually to zero (Fig. 2b) compared with the fairly substantial incorporation recorded when the turnover is uncorrected. It has been known for some time that the turnover of most phospholipid components in myelin is very slow after intraperitoneal injection of labelled precursors (see, e.g., Cuzner *et al.*, 1966).

Apart from the lack of phospholipid synthesis in myelin the turnover *in vivo* of the individual phospholipids gives little indication of the site of synthesis within the cell. The incorporation into the synaptosomal phospholipid is invariably slower than that into the microsomes and mitochondria but this could merely result from the precursor [32 P]P_i reaching the nerve endings less readily than other parts of the brain, although neurones as a whole seem to have a more rapid turnover of phospholipids than glial

cells (Freysz *et al.*, 1969). Lapetina *et al.* (1969) state that the 32 P turnover of synaptosomal phospholipids *in vivo* was no less than that of microsomal phospholipids when the label was injected directly into the brain ventricles. Turnover of the microsomal and mitochondrial phospholipids in the present experiments seems to be roughly equivalent and the rate-limiting step in the incorporation is probably the impermeability of the blood-brain barrier to the radioisotope, rather than the synthesis by one fraction and transfer of labelled phospholipid molecules to another.

In some instances the amount of labelling in the mitochondrial fraction appears to be higher than that in the microsomes. It cannot, of course, be concluded from this that phospholipid synthesis necessarily occurs in mitochondria, since the isolated microsomal fraction is itself heterogeneous and almost certainly contains phospholipid pools of differing turnover time. For example, 2':3'-cyclic nucleotide 3'-phosphohydrolase assays suggest the possibility of the microsomal fraction containing some small myelin particles, although the specific location of this enzyme in myelin is now open to question (Morgan *et al.*, 1971). The predominance of phosphatidylinositol labelling from [32 P]P_i in brain subcellular fractions compared with the nitrogen-containing phosphoglycerides is different from that found with the equivalent subcellular fractions from liver (McMurray & Dawson, 1969). However, the result probably reflects the situation *in vivo*. In brain (Fig. 3) phosphatidylinositol is much more rapidly labelled than both phosphatidylcholine and the ethanolamine phosphoglycerides, whereas in the intact rat liver phosphatidylethanolamine is labelled at a faster rate than phosphatidylinositol, with phosphatidylcholine only slightly slower (Dawson, 1955).

The curve relating 32 P incorporation into phosphatidylinositol against microsomal complement (added and contaminating) in a mitochondrial system extrapolates to zero. Since the mitochondria can probably synthesize phosphatidic acid (Fig. 4), this would suggest that the mitochondria do not contain the necessary enzymic apparatus for converting this into phosphatidylinositol via CDP-diglyceride. Nor is there any evidence that the mitochondria can synthesize phosphatidylethanolamine from [32 P]P_i or phosphatidylcholine from [32 P]P_i, [Me - 14 C]-choline, phosphoryl[Me - 14 C]choline or CDP-[Me - 14 C]choline, when due correction is made for the presence of microsomal contamination based on NADPH-cytochrome *c* reductase assays. The extrapolation of the curves relating incorporation to microsomal complement to the origin gives confidence to the assumption that this latter enzyme can act as a satisfactory marker for endoplasmic reticulum. It seems unlikely that the results can be ascribed to the inability of the precursors to

penetrate into the organelle, because they can synthesize phosphatidic acid and cardiolipin from [^{32}P]P_i. Further, osmotic disruption of the mitochondria did not allow them to incorporate label from CDP-[Me- ^{14}C]choline above that which could be predicted from the amount of contaminating endoplasmic reticulum. It could be argued that the mitochondria or their fragments were being incubated in a medium that was not conducive to phosphatidylcholine synthesis. If this is so, then the properties of CDP-choline-1,2-diglyceride choline phosphotransferase must be quite different from those of the enzyme present in microsomes. The mitochondria prepared and purified by washing showed an ADP-coupled respiratory phosphorylation, which suggests that their biochemical integrity is substantially retained during the fractionation procedure.

The evidence therefore indicates that brain mitochondria do not possess the ability to carry out the complete *de novo* regeneration of the bulk of the phospholipids which constitute their membranes. This implies that *in vivo* they rely on some process involving a transfer of whole phospholipid molecules from the endoplasmic reticulum, as is now assumed to exist in liver. Some evidence for such a process in brain is given in the accompanying paper (Miller & Dawson, 1972). It must be emphasized that these statements do not preclude the possibility of the exchange or replacement of individual molecular moieties in the phospholipids, e.g. as might occur with the fatty acids.

The situation concerning the phospholipid-synthesizing ability of synaptosomes is not so clear cut. With [^{32}P]P_i as precursor, the synthesis of the nitrogen-containing phospholipids is very limited, which probably results from the inability of the intraterminal mitochondria to form adequate supplies of ATP and CTP in face of the competition by the active ATPase and CTPase present. Likewise, the energy-dependent incorporation of [Me- ^{14}C]choline in response to added microsomes is negligible, in contrast with the situation where isolated mitochondria are present (Fig. 6). The [^{32}P]P_i incorporation into phosphatidylinositol, and also the incorporation of ^{14}C into phosphatidylcholine from phosphoryl[Me- ^{14}C]choline and CDP-[Me- ^{14}C]choline, appear to extrapolate approximately to the origin at 'zero' microsomal contamination, but the comparatively high activity of 'non-occluded' NADPH-cytochrome *c* reductase in the synaptosomal preparation makes it possible that a small phospholipid synthesis by the synaptosome could be missed. The decrease in energy requirement for choline incorporation brought about by the use of phosphorylated precursors brings about a response of total synthesis to the added microsomes, which the more energy-dependent [Me- ^{14}C]choline incorporation does not show. However, the clearest

picture regarding phosphatidylcholine synthesis within the synaptosomes is probably shown by the experiments in which the synaptosomes were fractionated after the incubation of intact synaptosomes with CDP-[Me- ^{14}C]choline followed by subfractionation of the organelles and where CDP-[Me- ^{14}C]choline was incubated with the isolated subfractions. In both instances, of all the membrane fractions, the synaptic vesicles showed the highest degree of labelling of the phospholipids whereas there was no correlation of the incorporation with the NADPH-cytochrome *c* reductase distribution in the synaptosomal subfractions. The response to added microsomes (Fig. 9c) again clearly indicates that the synaptic vesicles are capable of independent choline incorporation from CDP-choline whereas the synaptosomal outer membranes and intraterminal mitochondria are not. Again, the same experiments suggest that if the occluded NADPH-cytochrome *c* reductase of the synaptosomal preparation does truly represent an intraterminal smooth endoplasmic reticulum, then this again must be capable of phosphatidylcholine biosynthesis as are the contaminating extraneural microsomes. The nerve ending seems therefore capable of *de novo* phospholipid synthesis although this does not, of course, exclude the possibility that whole phospholipid molecules from the nerve cell body can pass down the axon and play a part in the repair and turnover of the nerve-ending membranes (Miani, 1964).

The results indicate that guinea-pig brain contains a Ca^{2+} -catalysed choline exchange system for forming phosphatidylcholine, which was originally described by Dils & Hübscher (1959) in liver microsomes. Arienti *et al.* (1970) found no evidence for this in chick brain, although an equivalent ethanolamine exchange reaction occurred as had been suggested by the work of Ansell & Spanner (1966). The choline exchange reaction is largely confined to the microsomes, although there is some evidence that at high choline substrate concentrations (3.3 mM) intact synaptosomes can carry out the reaction at a rate above that which can be accounted for by the contaminating microsomes. It is most surprising that this extra choline-exchange ability of the synaptosomes appears on subfractionation to lie not in the intraterminal 'endoplasmic reticulum' but in the terminal mitochondria. Without knowing the precise lipid acceptor for the choline exchange and its location within the nerve ending, it is difficult to conclude with certainty that the exchange enzyme is also located in intraterminal mitochondria.

Lunt & Lapetina (1970) found that when brain slices were incubated with [Me- ^{14}C]choline the incorporation of choline was faster into the phosphatidylcholine of the synaptic vesicles than the other membranes of the nerve ending. Bosmann & Hemsworth (1970b) also reported a high amount of

choline incorporation into synaptic-vesicle lipid. Lunt & Lapetina (1970) attributed this incorporation to base-exchange reaction with the phosphatidylcholine contained in the membranes, but our present results would suggest that the incorporation in the synaptic vesicles may be largely through a cytidine-mediated pathway.

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