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Regulation by Cytidine Nucleotides of the Acylation of *sn*-[^{14}C]Glycerol 3-Phosphate

REGIONAL AND SUBCELLULAR DISTRIBUTION OF THE ENZYMES RESPONSIBLE FOR PHOSPHATIDIC ACID SYNTHESIS *DE NOVO* IN THE CENTRAL NERVOUS SYSTEM OF THE RAT

By F. POSSMAYER, B. MEINERS and J. B. MUDD
Department of Biochemistry and Air Pollution Research Center, University of California, Riverside, Calif. 92502, *U.S.A., and Department of Obstetrics and Gynaecology, University of Western Ontario, London* 72, *Ont., Canada*

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1. The regional and subcellular distribution of the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into rat brain lipids *in vitro* was investigated and compared with the relative specific activity of various chemical and enzyme markers. The similarity between the subcellular distribution of this incorporation and of NADPH-cytochrome *c* reductase activity indicated that the synthesis of phosphatidic acid via this route correlated with the presence of endoplasmic reticulum. 2. Experiments in which various amounts of the microsomal fraction were added to fixed amounts of nuclear, myelin, nerve-ending and mitochondrial preparations clearly demonstrated that the endoplasmic-reticulum contamination of these fractions was entirely responsible for the incorporation of *sn*-[^{14}C]glycerol 3-phosphate. 3. The presence of CMP or CTP inhibited the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into the whole homogenate. Similar effects were observed with individual fractions, except for the mitochondria. With the mitochondrial fraction the effect of these cytidine nucleotides varied with the preparation, stimulating in some preparations and inhibiting with other preparations. The presence of CDP-choline stimulated the incorporation into the whole homogenate and to a lesser extent into the subcellular fractions. 4. These results indicate that the various organelles of the central nervous system are more dependent on endoplasmic reticulum for the production of glycerolipids *de novo* than has previously been appreciated.

Phosphatidic acid is a key intermediate in the biosynthesis of the neutral glycerides and the complex phospholipids (Kennedy, 1961). Although there are several routes by which this lipid can arise in brain, it is generally accepted that the acylation of *sn*-[^{14}C]-glycerol 3-phosphate represents the major source *de novo* (Strickland, 1967; Rossiter & Strickland, 1970). Factors affecting the rate of phosphatidic acid synthesis from *sn*-[^{14}C]glycerol 3-phosphate would markedly alter the flow of carbon atoms from glycolysis into brain glycerophospholipids. Further, since phosphatidic acid lies at a critical branch point such an alteration could have a profound effect on the whole scheme of phospholipid metabolism. Evidence indicating that the formation of phosphatidic acid could be under metabolic control was first reported by McMurray *et al.* (1957), who observed that CTP inhibited the incorporation of *rac*-glycerol 3-[^{32}P]-phosphate into the lipids of rat brain preparations. Further studies by Possmayer & Strickland (1967*a*,*b*) clearly demonstrated that although the presence of low concentrations of CMP, CTP and CDP-glycerol markedly depressed the incorporation of *rac*-glycerol 3-[^{32}P]phosphate into rat brain lipids, the presence of CDP-choline or CDP-ethanolamine enhanced this uptake. Subsequent investigations with *sn*-[^{14}C]-glycerol 3-phosphate (Possmayer & Mudd, 1971) established that only small amounts of radioactive neutral lipids were derived from the synthesized phosphatidic acid under these conditions, indicating that cytidine nucleotides do not function by enhancing the degradation of phosphatidic acid.

To be able to assess more accurately the significance of these effects of the cytidine nucleotides *in vitro* on the production of phosphatidic acid for various cellular membranes *in vivo*, a study was conducted on the regional and subcellular distribution of the enzymes responsible for the acylation of *sn*-[^{14}C]glycerol 3-phosphate in rat brain. It was observed that under the conditions used, the acylation of *sn*-[^{14}C]glycerol 3-phosphate was confined to the microsomal fraction and the endoplasmic-reticulum contamination of the other fractions. These results indicate that nerve endings or brain mitochondria

cannot synthesize phosphatidic acid *de novo* from *sn*-[^{14}C]glycerol 3-phosphate. In view of previous reports, which demonstrated that brain synaptosomes could incorporate $^{32}P_i$ into lipids and liver mitochondria could acylate *sn*-[^{14}C]glycerol 3-phosphate, these results were not expected. A preliminary report has been given of some of this work (Possmayer & Mudd, 1972).

Methods

Preparation of subcellular fractions

Two adult rats of the Sprague–Dawley strain were decapitated and the brains were rapidly excised. Since preliminary studies (Tables 1–3) demonstrated that the cytidine nucleotide effect was evenly distributed throughout the central nervous system, the cerebrum and midbrain were dissected free by an incision behind the cerebral hemispheres through the inferior colliculi. This portion of the brain was rinsed with water to remove as much blood as possible, weighed and transferred to a glass homogenizer. The tissue was homogenized with 9vol. of 0.32M-sucrose–0.1mM-EDTA (pH7.0) with a Teflon pestle [A. H. Thomas Co., Philadelphia, Pa., U.S.A.; reported clearance 0.10–0.15mm]. Two consecutive sets of four strokes each at approximately 800rev./min with a Tri-Stir motor, indicator set at 2, were used for the homogenization. A portion of the whole homogenate was removed and the remainder was fractionated essentially by the method of Nyman & Whittaker (1963). The crude nuclear fraction, obtained by centrifuging at 1000*g* for 12min, was resuspended in 0.32M-sucrose–0.1mM-EDTA (pH7.0) (one-half the original volume), and re-centrifuged. The resulting pellet, P_1, which contains the nuclei, heavy myelin and cellular debris, was retained. The combined supernatants (S_1) were centrifuged for 60min at 17300*g* to yield a crude mitochondrial fraction and a supernatant S_2. The pellet was resuspended in 0.32M-sucrose–0.1mM-EDTA (pH7.0) and layered on to a discontinuous sucrose gradient in three tubes. Each tube contained 2.0ml of 1.2M-sucrose–0.1mM-EDTA (pH7.0), 2.0ml of 0.8M-sucrose–0.1mM-EDTA (pH7.0) and 0.2ml of 0.32M-sucrose–0.1mM-EDTA (pH7.0). The tubes were centrifuged at 100000*g* for 60min in a swinging-bucket rotor. This procedure separates the particles that do not pass through 0.8M-sucrose–0.1mM-EDTA (P_2; mainly myelin fragments), from those with a density between that of 0.8 and 1.2M-sucrose–0.1mM-EDTA (P_3; nerve-ending fraction), and from a fraction heavier than 1.2M-sucrose–0.1mM-EDTA (P_4; which contains the mitochondria). The isolated fractions were diluted to approximately 0.32M-sucrose and centrifuged at 145000*g* for 60min. Supernatant S_2 was also centrifuged in this manner to yield a microsomal fraction (P_5) containing cytoplasmic membranes and the cytosol, S_3. The pellets were redispersed in 0.32M-sucrose–0.1mM-EDTA (pH7.0) to give a final volume one-third that of the original homogenate used in the fractionation. The homogenization and successive operations were carried out at 0°C.

Assays for marker enzymes

*$Na^+ + K^+$-stimulated ATPase** (*EC* 3.6.1.3). The system used for measuring this enzyme activity was based on that described by Kai *et al.* (1966), and contained Tris–HCl buffer, pH7.4 (30mM), NaCl (120mM), KCl (30mM), $MgCl_2$ (3mM), Tris–ATP (5mM) and 0.1ml of enzyme preparation in a final volume of 1.0ml. The difference between the release of P_i in this system and that observed in the absence of NaCl and KCl, but with ouabain (1mM) added, was taken as the $Na^+ + K^+$-stimulated ATPase activity. These systems were incubated for 2min at 37°C and the reaction was stopped by adding an equal volume (1.0ml) of ice-cold 10% (w/v) trichloroacetic acid.

5′-Nucleotidase (*EC* 3.1.3.5). This plasma-membrane marker enzyme activity was assayed by the method of Michell & Hawthorne (1966). The basic system included Tris–HCl buffer, pH7.4 (50mM), KCl (100mM), $MgCl_2$ (10mM), sodium–potassium tartrate (10mM), 5′-AMP (5mM) and 0.1ml of the enzyme preparations in a total volume of 1.0ml. The system was incubated for 10min at 37°C and the reaction stopped with 1.0ml of 10% (w/v) trichloroacetic acid.

Succinate dehydrogenase (*EC* 1.3.99.1). This enzyme activity was assayed by measuring the succinate-dependent reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride essentially by the method of Pennington (1961) as modified by Porteous & Clark (1965). The incubation medium contained, in a final volume of 1.0ml: K_2HPO_4–KH_2PO_4 buffer, pH7.4 (50mM), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (0.1%, w/v), sucrose (25mM), EDTA (2mM) and 0.1ml of enzyme preparation. The systems were incubated at 37°C for 15min and the reaction was stopped by the addition of 1ml of 10% (w/v) trichloroacetic acid. The mixture was extracted with 4.0ml of ethyl acetate and the E_{490} of the organic layer was determined. A molar extinction coefficient of 20.1×10^3 litre·mol^{-1}·cm^{-1} was used to calculate the amount of formazan formed.

Lactate dehydrogenase (*EC* 1.1.1.27). This cytosol marker enzyme activity was assayed as described by Harwood & Hawthorne (1969). The incubation system (final volume 3.0ml) contained K_2HPO_4–KH_2PO_4 buffer, pH7.5 (100mM), NADH (0.25mM),

* Abbreviation: ATPase, adenosine triphosphatase.

0.02ml of a saturated ethanolic solution of rotenone, Triton X-100 (0.01%, v/v) and 50μl of diluted (1:10, v/v) enzyme preparation. The decrease in E_{340} produced by the addition of sodium pyruvate (0.2mM) to the system at room temperature was automatically recorded and the initial slope was used to calculate the rate of reaction.

NADPH-cytochrome c reductase (*EC* 1.6.2.3). This endoplasmic-reticulum marker enzyme activity was assayed under conditions similar to those reported by Sottocasa *et al.* (1967). The basic system was composed of K_2HPO_4–KH_2PO_4 buffer, pH7.5 (60mM), KCN (0.3mM), cytochrome *c* (0.075mM), Triton X-100 (0.01%, v/v) or Nonidet P40 (0.0025%, v/v) and 0.2ml of diluted (1:10, v/v) enzyme preparation (final vol. 2.7ml). This incubation was also carried out at room temperature. The reaction was initiated by the addition of enzymically reduced NADPH (0.1mM) and the E_{550} was recorded automatically for 10min. A molar extinction coefficient of 19.1×10^3 litre·mol^{-1}·cm^{-1} (Chance & Williams, 1956) was used to calculate the reduction of cytochrome *c*.

By using the whole homogenate, it was established that all assays responded linearly to changes of time or enzyme concentration under the conditions used in these studies.

Incubation procedures

Synthesis *de novo* of phospholipids was measured by the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into the lipid fraction. The basic system contained Tris–HCl buffer, pH7.2 (50mM), K_2HPO_4–KH_2PO_4 buffer, pH7.2 (10mM), $MgCl_2$ (10mM), NaF (10mM), CoA (0.1mM), EDTA (1mM), *sn*-[^{14}C]glycerol 3-phosphate (0.75mM) and 0.2ml of enzyme preparation in a final volume of 0.5ml. It has previously been demonstrated that this system is optimum for the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into the lipids of rat brain homogenates (Possmayer & Mudd, 1971). In some experiments dithiothreitol (1mM) was also present. Where indicated, cytidine nucleotides (0.2mM) were added. The system was incubated for 10min and the reaction stopped by adding 10ml of chloroform–methanol (1:1, v/v).

Determination of radioactivity in lipid extracts

The procedure used for the extraction, washing and isolation of the radioactive lipids and the subsequent measurement of the radioactivity were essentially the same as previously described (Possmayer & Mudd, 1971).

Analytical procedures

The P_i released in the enzymic assays described above or by digestion with $HClO_4$ was measured by the appropriate phosphorus methods of Bartlett (1959). Protein was determined by the biuret procedure described by Gornall *et al.* (1949). Sodium dodecyl sulphate (20μl, 0.1M) was added to the assay system (final volume 3.5ml) to dissolve the membranes. Standard curves with and without sucrose were established by using fatty acid-poor bovine serum albumin. RNA and DNA were extracted as described by Schneider (1957). RNA was measured as described by Schneider (1957) and DNA by the method of Burton (1956).

Electron microscopy

The fractions were fixed in Karnovsky's (1965) fluid or 4% (v/v) glutaraldehyde, post-fixed in 1% (w/v) osmium tetroxide and embedded in epoxy resin (Spurr, 1969). Sections were cut with a Porter Blum MT-1 or MT-2 ultramicrotome, stained for $\frac{1}{2}$h in aq. uranyl acetate and then for 2min in lead citrate (Reynolds, 1963) and viewed with a Philips EM 300 electron microscope. The fractions had an appearance similar to those described by Gray & Whittaker (1962) except that the mitochondria were sometimes somewhat swollen.

Materials

The radioactive precursor *sn*-[^{14}C]glycerol 3-phosphate was prepared enzymically from [^{14}C]glycerol (International Chemical and Nuclear Corp., Irvine, Calif., U.S.A.) as described previously (Possmayer & Mudd, 1971). The preparation of the lipid markers and the sources of the standard chemicals were also given in the previous paper. In addition 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride was purchased from Aldrich Chemical Co., San Leandro, Calif., U.S.A. NADH, NADPH and cytochrome *c* (type III, prepared from horse heart) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Nonidet P40 was supplied by Shell Chemical Co., London S.E.1, U.K.

Results

Regional distribution of the enzymes responsible for acylating sn-[^{14}C]glycerol 3-phosphate

Previous experiments investigating the effect of the cytidine nucleotides on the biosynthesis *de novo* of brain lipids have been conducted with preparations from rat cerebral hemispheres. To assess more accurately the significance of this effect *in vivo*, the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into homogenates prepared from different areas of the central nervous system was determined. The whole brain was dissected into four distinct areas: A, the cerebral hemispheres, including the corpus callosum;

Table 1. *Incorporation of sn-[^{14}C]glycerol 3-phosphate into total lipids by homogenates from different regions of rat central nervous system*

Homogenates prepared from the various areas of rat brain dissected as described in the text were incubated with *sn*-[^{14}C]glycerol 3-phosphate in the presence of $MgCl_2$, ATP and CoA as described in the Methods section.

	Incorporation of *sn*-[^{14}C]glycerol 3-phosphate into total lipids		
Region	Total activity (nmol incorporated/min per vessel)	Incorporation (nmol/min per mg of protein)	Incorporation relative to that in cerebral hemispheres
Cerebral hemispheres	5.40	0.246	1.00
Midbrain	3.40	0.190	0.77
Cerebellum	3.70	0.196	0.80
Medulla oblongata	3.10	0.172	0.70

Table 2. *Effect of cytidine nucleotides on the lipid synthesis de novo from different regions of rat brain*

Conditions were as described in Table 1, except that cytidine nucleotides (1 mM) were added as indicated.

	Incorporation of *sn*-[^{14}C]glycerol 3-phosphate							
	Cerebral hemispheres		Midbrain		Cerebellum		Medulla oblongata	
Addition	(nmol/min per mg of protein)	(% of control)	(nmol/min per mg of protein)	(% of control)	(nmol/min per mg of protein)	(% of control)	(nmol/min per mg of protein)	(% of control)
—	0.246	—	0.190	—	0.196	—	0.172	—
CMP	0.163	69	0.146	77	0.138	71	0.128	74
CTP	0.122	50	0.118	61	0.106	54	0.096	56
CDP-choline	0.260	106	0.206	109	0.211	109	0.186	110

B, the midbrain, defined as that portion of the brain stem lying ventral to the cerebral hemispheres and anterior to the inferior colliculi; C, the cerebellum; and D, the remainder of the brain stem including the pons and the medulla oblongata. The results of this experiment are listed in Table 1. Although the cerebral hemisphere preparation was most active on a total and per mg of protein basis, there was still a very good incorporation into the homogenates prepared from the other areas. The effect of the cytidine nucleotides on the incorporation of *sn*-[^{14}C]-glycerol 3-phosphate is shown in Table 2; although CMP and CTP markedly decreased the incorporation into the total lipids, CDP-choline produced a slight stimulation. Comparable effects were observed on the incorporation into the different homogenates, indicating that the cytidine nucleotides can play a role in regulating phosphatidic acid synthesis throughout the central nervous system. The incorporation into the individual radioactive lipids in these preparations is listed in Table 3. In the presence of CMP or CTP (1.0mM) the radioactivity incorporated into phosphatidic acid was markedly decreased. CDP-choline stimulated this labelling slightly. The incorporation into phosphatidylinositol was strongly stimulated by CMP and CTP and slightly by CDP-choline. The small amount of radioactivity found in the neutral glycerides was not particularly affected by the cytidine nucleotides. With longer incubation periods, the incorporation into the neutral lipids reflects the labelling of phosphatidic acid (Possmayer & Mudd, 1971).

Subcellular distribution of the enzymes responsible for the acylation of sn-[^{14}C]glycerol 3-phosphate

The distribution of the enzyme markers of the various subcellular organelles of rat brain and of the phospholipid synthesis *de novo* among the fractions obtained as outlined in the Methods section is listed

Table 3. *Cytidine nucleotide effect on the incorporation of sn-[^{14}C]glycerol 3-phosphate into the individual glycerolipids of the various different regions of the rat central nervous system*

Samples incubated as described in Table 2 were analysed as described in the Methods section.

Region	Lipid / Addition ...	Incorporation of *sn*-[^{14}C]glycerol 3-phosphate (nmol/min per mg of protein) None (control)	CMP	CTP	CDP-choline
Cerebral hemispheres	Phosphatidic acid	0.191	0.060	0.025	0.206
	Phosphatidylinositol	0.020	0.067	0.064	0.028
	Diglyceride	0.009	0.007	0.007	0.007
	Triglyceride	0.009	0.005	0.005	0.007
Midbrain	Phosphatidic acid	0.132	0.072	0.035	0.145
	Phosphatidylinositol	0.018	0.056	0.067	0.032
	Diglyceride	0.009	0.011	0.011	0.007
	Triglyceride	0.008	0.007	0.006	0.008
Cerebellum	Phosphatidic acid	0.129	0.027	0.024	0.154
	Phosphatidylinositol	0.021	0.057	0.064	0.028
	Diglyceride	0.011	0.010	0.009	0.010
	Triglyceride	0.009	0.007	0.006	0.011
Medulla oblongata	Phosphatidic acid	0.141	0.055	0.031	0.144
	Phosphatidylinositol	0.019	0.063	0.052	0.021
	Diglyceride	0.010	0.011	0.007	0.009
	Triglyceride	0.010	0.008	0.005	0.009

in Table 4. The relative specific activity was calculated as follows:

$$\text{Relative specific activity} = \frac{\%\text{ of activity or amount of component in the subcellular fraction}}{\%\text{ of total protein in the subcellular fraction}}$$

The distributions of 5′-nucleotidase and $Na^+ + K^+$-stimulated ATPase activities are quite similar. In liver these enzymes are primarily associated with the plasma membrane (Neville, 1960; Novikoff *et al.*, 1961; Bodansky & Schwartz, 1968; Berman *et al.*, 1969). The enzymic activity corresponding to these enzymes was most highly concentrated in the myelin (P_2) and microsomal (P_5) fractions. The nuclear and the nerve-ending fractions also possessed considerable 5′-nucleotidase and $Na^+ + K^+$-stimulated ATPase activity reflecting the plasma-membrane content of these fractions. The high 5′-nucleotidase activity observed in the supernatant may be due to plasma serum 5′-nucleotidase (Bodansky & Schwartz, 1968). Succinate dehydrogenase activity appears to be uniquely associated with mitochondria (Whittaker, 1965; Roodyn, 1967). The electron micrographs revealed mitochondria within the pinched-off nerve endings (see also Gray & Whittaker, 1962; de Robertis *et al.*, 1963) and in the cellular debris of the crude nuclear fraction. Lactate dehydrogenase activity, which has a distribution similar to that of K^+ (Johnson & Whittaker, 1963; Whittaker, 1965), has been used to assess the contamination of other fractions by supernatant. It is not known whether the lactate dehydrogenase activity observed in the other fractions is due to a natural or artificial inclusion by these membranes (nuclei and synaptosomes may have glycolytic activity) or to an adsorption of the enzyme to the surface of these particles. Since it was observed that good recovery of the enzymic activity required the presence of Triton X-100 or freezing and thawing (F. Possmayer & J. B. Mudd, unpublished work; see also Whittaker, 1965; Marchbanks, 1967), it appears that the lactate dehydrogenase was occluded within these structures. Although the pattern of the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into the total lipids of these fractions corresponded most closely to that observed for NADPH-cytochrome *c* reductase, the P_1 fraction exhibited a somewhat greater incorporation of *sn*-[^{14}C]glycerol 3-phosphate than would be expected from the NADPH-cytochrome *c* reductase activity observed. Since NADPH-cytochrome *c* reductase activity appears to be specifically associated with the cytoplasmic membranes of the endoplasmic reticulum (Sottocasa *et*

al., 1967; Dallner *et al.*, 1968; Berman *et al.*, 1969), it was not surprising to find that the microsomal fraction (P_5) had the highest relative specific activity. A considerable amount of NADPH-cytochrome *c* reductase activity was observed in the fractions prepared from the crude mitochondrial preparation. Eichberg *et al.* (1964) have pointed out that, although the centrifugation of fraction S_1 at 17300*g* for 60min results in considerable contamination of the crude mitochondrial pellet with endoplasmic-reticulum elements, this treatment is necessary to eliminate contamination of the microsomal fraction by small synaptosomes. The distribution of the subcellular markers shown in Table 4 is similar to previously published results with brain (Kai *et al.*, 1966; Keough & Thompson, 1970; Lapetina & Hawthorne, 1971). Since these workers did not include an assay for an enzymic marker for the endoplasmic reticulum, it is difficult to assess the cross-contamination by cytoplasmic membranes in these studies.

The purities of the different fractions can be more conveniently assessed in Fig. 1. The close fit observed between the profiles of NADPH-cytochrome *c* reductase activity and the incorporation of *sn*-[^{14}C]glycerol 3-phosphate indicated that the phospholipid synthesis *de novo* in the other fractions could be largely explained by contamination of these fractions with cytoplasmic membranes. The similarity between the distribution of RNA, a ribosomal marker, and these activities corroborates this suggestion. DNA, on the other hand, was almost exclusively limited to the nuclear fraction. Further inspection of the relative-specific-activity plots from the individual experiments revealed that the NADPH-cytochrome *c* reductase activity and the *sn*-[^{14}C]glycerol 3-phosphate incorporation profiles paralleled one another in the individual experiments. This was particularly notable in the preliminary experiments, in which the profile of these activities was markedly different from the more typical results recorded here. These preliminary experiments, conducted before it was realized that the dispersing conditions are quite critical (Whittaker, 1965; J. Harwood, personal communication), employed a longer and more vigorous homogenization.

Previous studies in liver (Smith & Hübscher, 1966; Stoffel & Schiefer, 1968; Zborowski & Wojtczak, 1969; Sarzala *et al.*, 1970; Daae & Bremer, 1970) have strongly indicated that hepatic mitochondria possess enzyme systems capable of acylating *sn*-[^{14}C]glycerol 3-phosphate. Therefore it was somewhat disconcerting to note that a comparison of the relative-specific-activity profiles for *sn*-[^{14}C]glycerol 3-phosphate incorporation, NADPH-cytochrome *c* reductase activity and RNA revealed little evidence for mitochondrial phosphatidate production under the conditions used here. Therefore a more extensive

Table 4. *Subcellular distribution of enzyme markers from rat forebrain*

The results of the assays conducted as described in the text are listed as the mean ± S.D. of the recovered activity and the relative specific activity. The results are based on five fractionations carried out as described in the Methods section.

Fraction	Protein (% of total)	5'-Nucleotidase (% of total)	5'-Nucleotidase Relative specific activity	$Na^+ + K^+$-stimulated ATPase (% of total)	$Na^+ + K^+$-stimulated ATPase Relative specific activity	Succinate dehydrogenase (% of total)	Succinate dehydrogenase Relative specific activity	Lactate dehydrogenase (% of total)	Lactate dehydrogenase Relative specific activity	NADPH-cytochrome *c* reductase (% of total)	NADPH-cytochrome *c* reductase Relative specific activity	Incorporation of *sn*-[^{14}C]glycerol 3-phosphate (% of total)	Incorporation of *sn*-[^{14}C]glycerol 3-phosphate Relative specific activity
Crude nuclear fraction (P_1)	21.6±3.6	33.6±3.9	1.09±0.06	24.9±1.3	1.16±0.04	16.4±9.2	0.72±0.23	7.0±1.4	0.35±0.09	17.3±3.1	0.72±0.26	24.2±7.1	1.56±0.36
Myelin fraction (P_2)	13.4±2.5	20.6±4.9	1.47±0.18	23.5±3.8	1.72±0.37	2.0±1.1	0.15±0.06	8.2±1.8	0.63±0.15	18.4±7.0	1.19±0.29	11.6±4.3	0.79±0.30
Nerve-ending fraction (P_3)	17.1±4.5	16.1±3.3	1.02±0.49	19.0±1.6	1.05±0.23	26.2±10.1	1.47±0.34	16.0±3.6	0.87±0.20	16.0±6.5	0.85±0.22	11.6±3.1	0.68±0.26
Mitochondrial fraction (P_4)	10.5±0.6	5.2±3.7	0.48±0.32	8.4±3.2	0.81±0.36	51.5±3.3	4.90±0.41	3.8±0.9	0.37±0.09	8.7±2.4	1.00±0.30	10.8±1.0	1.09±0.14
Microsomal fraction (P_5)	12.3±1.1	20.9±1.4	1.71±0.14	19.7±3.2	1.62±0.14	1.7±1.3	0.12±0.09	10.5±1.2	0.85±0.12	39.4±11.2	3.44±0.97	41.5±10.2	3.71±1.15
Supernatant (S)	24.4±4.7	13.0±4.5	0.59±0.11	4.0±2.0	0.24±0.08	2.3±0.8	0.10±0.04	54.5±6.3	2.37±0.38	2.3±1.3	0.09±0.02	0.01±0.00	0.0
Recovery (% of homogenate)	89.6±12.8	76.8±8.1		67.2±1.5		81.0±22.8		89.3±10.7		98.6±9.4		58.4±9.8	
Absolute amounts and activities													
Homogenate (per g of brain)	122.7± 10.6mg	2970±657nmol/min		30630±8000nmol/min		4381±892nmol/min		68.8±15.7nmol/min		73.0±8.5nmol/min		25.3±4.6nmol/min	
(per mg of protein)	—	24.2±5.0nmol/min		249.6±65nmol/min		38.1±11.3nmol/min		0.603±0.094nmol/min		0.434±0.043nmol/min		0.214±0.040nmol/min	

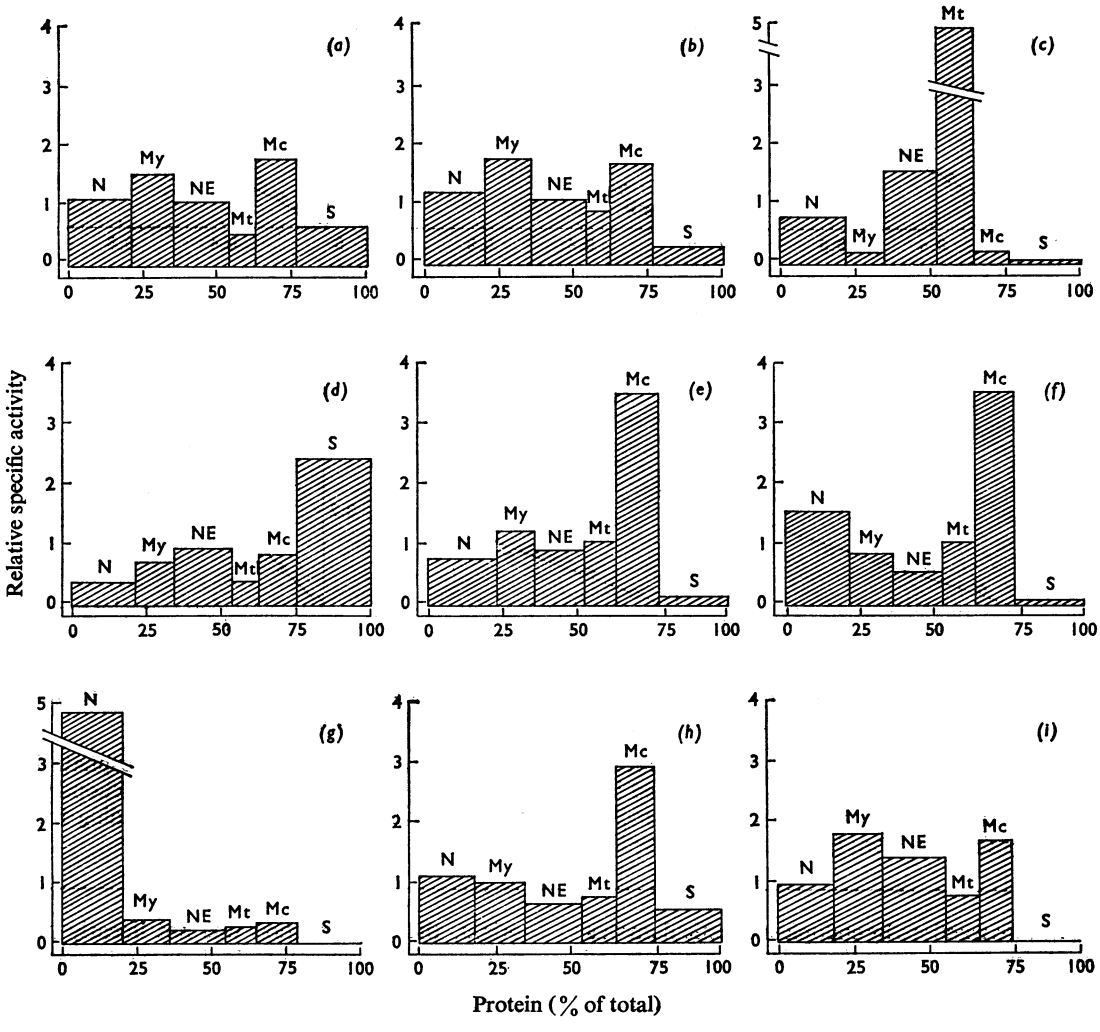

Fig. 1. *Subcellular distribution of the incorporation of sn-[^{14}C]glycerol 3-phosphate and marker enzymes in rat brain*

The relative specific activity is defined as the ratio of the percentage of the component in the subcellular fraction over the percentage of the total protein in that fraction. The abbreviations for the subcellular fractions are: N, crude nuclear fraction; My, myelin fraction; NE, nerve-ending fraction; Mt, mitochondrial fraction; Mc, microsomal fraction; S, supernatant. (The results are based on five fractionations, carried out as described in the Methods section.) (*a*) 5′-Nucleotidase; (*b*) $Na^+ + K^+$-stimulated ATPase; (*c*) succinate dehydrogenase; (*d*) lactate dehydrogenase; (*e*) NADPH-cytochrome *c* reductase; (*f*) *sn*-[^{14}C]glycerol 3-phosphate incorporation; (*g*) DNA; (*h*) RNA; (*i*) phospholipid phosphorus.

study of the ability of these subcellular fractions to incorporate *sn*-[^{14}C]glycerol 3-phosphate was conducted to determine the true nature of this incorporation.

The first part of this experiment is depicted in Fig. 2, which shows that the incorporation of *sn*-[^{14}C]-glycerol 3-phosphate is proportional to the amount of protein added for each fraction. The second part of the experiment (Fig. 3) made use of an experimental approach devised by McMurray & Dawson (1969). The incorporation of *sn*-[^{14}C]glycerol 3-phosphate was measured with various amounts of microsomal fraction added to a fixed amount of the fraction being studied. The degree of contamination

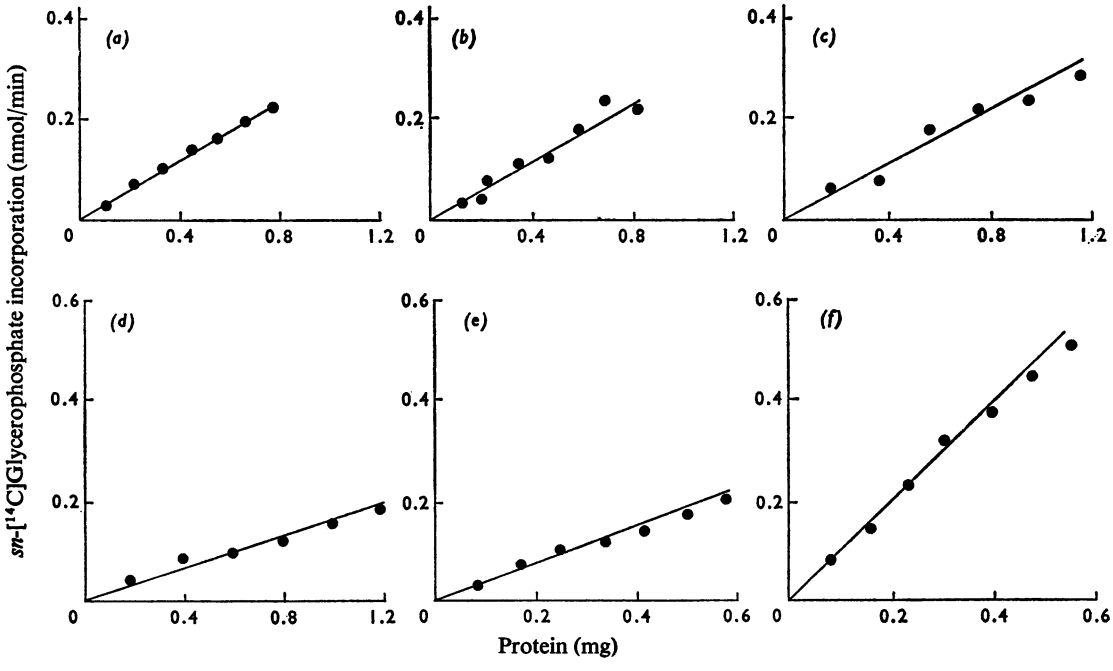

Fig. 2. *Effect of various amounts of protein on the incorporation of sn-[^{14}C]glycerol 3-phosphate into the lipids of rat brain homogenate and subcellular fractions*

The whole homogenate and subcellular fractions were incubated with *sn*-[^{14}C]glycerol 3-phosphate in the presence of ATP, $MgCl_2$ and CoA as described in the text. Note the different scale for the mitochondrial and microsomal fractions. (*a*) Homogenate; (*b*) nuclear fraction; (*c*) myelin fraction; (*d*) nerve-ending fraction; (*e*) mitochondrial fraction; (*f*) microsomal fraction.

of the various fractions by endoplasmic reticulum was assessed by comparing the relative amounts of NADPH-cytochrome *c* reductase activity in the microsomal and the other fractions. The incorporation of *sn*-[^{14}C]glycerol 3-phosphate was then plotted against the amount of microsomal fraction present, and by extrapolation to zero microsomal-fraction contamination, an estimate was obtained of the ability of the fractions to incorporate *sn*-[^{14}C]glycerol 3-phosphate. In this way it was discovered that the incorporation catalysed by the myelin (P_2), synaptosomal (P_3) and mitochondrial (P_4) preparations plus added microsomal fraction was proportional to the amount of cytoplasmic membranes present and within experimental error the plots could be extrapolated through the origin, indicating that incorporation in these fractions was entirely caused by the presence of endoplasmic reticulum. Further, the rate of incorporation observed was essentially the same as that for the microsomal fraction alone.

However, although the radioactive lipid formation observed in the presence of a fixed amount of the nuclear fraction could be extrapolated through the origin, the incorporation was not linearly proportional to the amount of endoplasmic reticulum. This latter effect, which was observed in several but not all experiments, may be due to the presence of relatively intact whole cells in the crude nuclear fraction.

Subcellular distribution of the cytidine nucleotide effects on the incorporation of sn-[^{14}C]glycerol 3-phosphate into rat brain lipids

Table 5 lists the results of the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into the total and the individual lipids of rat brain subcellular fractions. CMP and CTP produced a definite inhibition of the labelling of the total lipids of the whole homogenate. Quantitatively similar effects were noted in all of the subcellular fractions except for the mitochondrial preparation, in which there was no decrease compared with the control. CDP-choline produced a slight stimulation in the homogenate and the microsomal fraction, but this increase was not statistically significant.

The main effects of CMP and CTP are to decrease

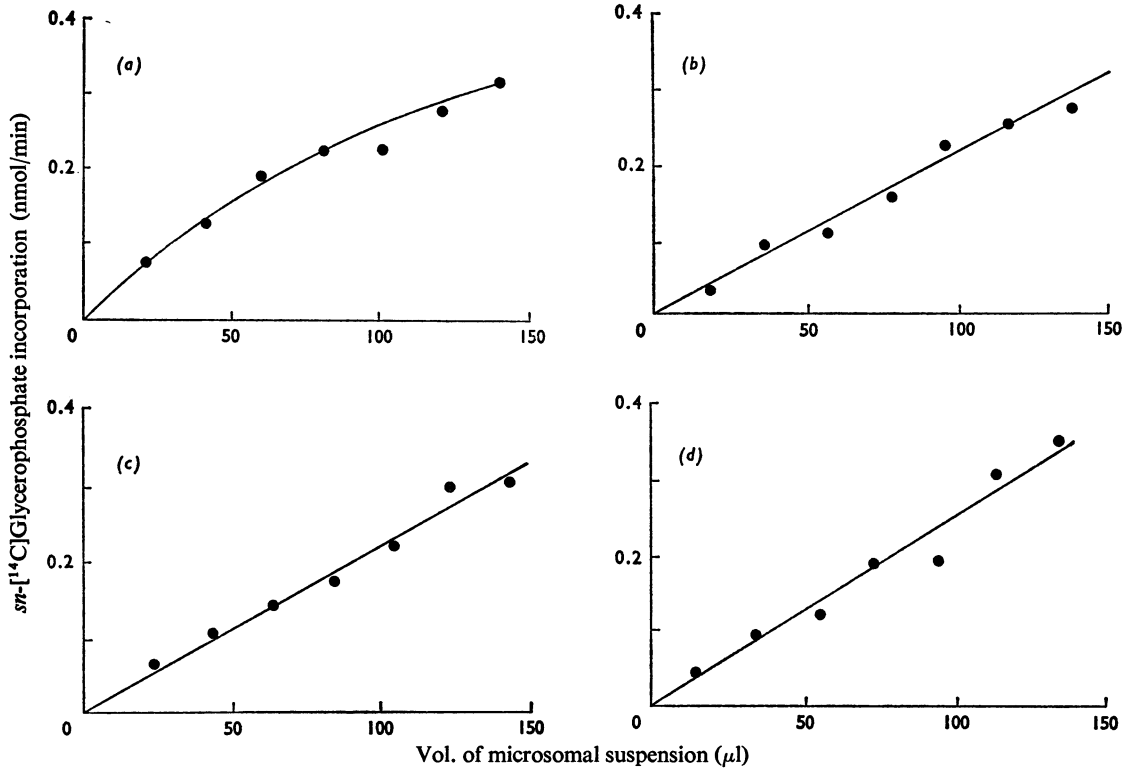

Fig. 3. *Effect of adding increasing amounts of the microsomal fraction to a fixed amount of each of the other subcellular fractions on the incorporation of sn-[^{14}C]glycerol 3-phosphate into rat brain lipids*

The subcellular fractions were incubated with *sn*-[^{14}C]glycerol 3-phosphate in the presence of ATP, $MgCl_2$, and CoA as described in the Methods section. The first point on each curve contains no added microsomal preparation. The microsomal-fraction content of the other fractions was calculated from the amount of NADPH-cytochrome *c* reductase activity present relative to that of the microsomal fraction. (*a*) Nuclear fraction; (*b*) myelin fraction; (*c*) nerve-ending fraction; (*d*) mitochondrial fraction.

markedly the incorporation into phosphatidic acid. The results in the subcellular fractions are very similar to those in the whole homogenate, except for the mitochondrial fraction. The incorporation into phosphatidylinositol is stimulated by all of the cytidine nucleotides in the whole homogenate. Whereas CMP and CTP produce identical stimulations in the whole homogenate, CMP has a lesser or even a negligible effect on the formation of phosphatidylinositol in the isolated fractions. The CTP effect on the labelling of phosphatidylinositol was observed in all fractions, including the mitochondria. CDP-choline produced a definite stimulation in the whole homogenate and in the crude nuclear and the microsomal fractions. Previous results have demonstrated that a marked stimulation of the labelling of phosphatidylinositol occurs with incubation times longer than the 10min used in the experiments reported here (Possmayer & Strickland, 1967*a*,*b*; Possmayer & Mudd, 1971).

Only a small amount of radioactivity was incorporated into the glycerides in the presence of the isolated fractions. These values are included to present the complete incorporation pattern. However, the variation in these small quantities defied statistical analysis.

It is evident from the results in Table 5 that most of the effects of the cytidine nucleotides observed in the whole homogenate can be explained in terms of the incorporation supported by the cytoplasmic membranes of the microsomal fraction or contamination of the other fractions by the cytoplasmic membranes. The only discrepancies appear to be the lack of a cytidine nucleotide effect on the incorporation

Table 5. *Effect of cytidine nucleotides on the incorporation of sn-[^{14}C]glycerol 3-phosphate into the individual glycerolipids of the different subcellular fractions of rat brain*

The assay procedure is described in the text. Cytidine nucleotide concentration was 0.2mM. The results are expressed as nmol incorporated/min per mg of protein±S.D. for four separate assays except for the whole homogenate where five assays were conducted.

		Total lipid		Phosphatidic acid		Phosphatidylinositol		Glycerides	
Preparation	Addition	Incorporation	(% of control)	Incorporation	(% of control)	Incorporation	(% of control)	Incorporation	(% of control)
Homogenate	—	0.214±0.040	—	0.160±0.043	—	0.023±0.015	—	0.010±0.002	—
	CMP	0.160±0.013	75.1±7.9	0.062±0.016	39.1±6.3	0.067±0.017	409.0±241.7	0.007±0.002	71.6±14.9
	CTP	0.134±0.033	62.1±5.5	0.042±0.008	26.8±4.0	0.071±0.017	431.5±265.5	0.004±0.001	45.7±20.7
	CDP-choline	0.227±0.039	106.2±4.3	0.167±0.047	103.2±3.7	0.031±0.018	150.5±45.8	0.006±0.002	57.7±15.6
Nuclear fraction (P_1)	—	0.193±0.065	—	0.119±0.065	—	0.008±0.003	—	0.003±0.002	—
	CMP	0.146±0.044	76.9±6.9	0.087±0.044	76.7±15.6	0.015±0.007	170.4±45.5	0.004±0.003	—
	CTP	0.115±0.031	61.3±4.8	0.040±0.015	38.3±14.1	0.045±0.026	507.1±170.6	0.003±0.002	—
	CDP-choline	0.198±0.068	102.1±3.0	0.125±0.060	109.8±19.9	0.014±0.007	164.9±50.9	0.004±0.003	—
Myelin fraction (P_2)	—	0.096±0.019	—	0.062±0.014	—	0.006±0.002	—	0.003±0.004	—
	CMP	0.066±0.008	70.0±7.3	0.046±0.007	76.8±18.0	0.006±0.005	100.3±68.7	0.003±0.002	—
	CTP	0.052±0.015	49.6±6.8	0.015±0.005	26.8±13.0	0.018±0.049	295.6±96.9	0.002±0.001	—
	CDP-choline	0.102±0.017	100.5±6.9	0.077±0.024	124.4±24.9	0.007±0.004	107.6±45.4	0.002±0.000	—
Nerve-ending fraction (P_3)	—	0.096±0.021	—	0.080±0.015	—	0.006±0.001	—	0.003±0.002	—
	CMP	0.074±0.017	80.3±25.1	0.058±0.021	72.5±21.3	0.007±0.002	124.1±51.4	0.004±0.002	—
	CTP	0.062±0.009	70.0±15.6	0.022±0.005	29.2±9.3	0.024±0.009	407.7±168.4	0.003±0.001	—
	CDP-choline	0.092±0.018	95.9±10.2	0.079±0.020	98.6±15.6	0.010±0.006	177.1±122.4	0.003±0.002	—
Mitochondrial fraction (P_4)	—	0.139±0.025	—	0.111±0.023	—	0.010±0.002	—	0.005±0.005	—
	CMP	0.135±0.042	96.6±16.4	0.107±0.031	96.1±14.6	0.010±0.003	103.2±31.5	0.004±0.002	—
	CTP	0.134±0.039	97.4±16.3	0.087±0.024	78.3±14.5	0.032±0.003	348.1±76.9	0.003±0.002	—
	CDP-choline	0.151±0.043	108.8±17.0	0.117±0.025	106.1±15.5	0.010±0.002	110.6±13.5	0.004±0.003	—
Microsomal fraction (P_5)	—	0.480±0.143	—	0.370±0.097	—	0.031±0.022	—	0.007±0.002	—
	CMP	0.370±0.153	76 7±13.3	0.275±0.074	74.6±6.4	0.028±0.020	91.6±26.2	0.011±0.006	—
	CTP	0.308±0.140	64.2±15.9	0.123±0.082	32.6±14.8	0.139±0.031	673.1±462.7	0.004±0.001	—
	CDP-choline	0.496±0.150	103.8±1.6	0.380±0.104	102.4±9.5	0.037±0.023	134.1±36.2	0.006±0.002	—

of *sn*-[^{14}C]glycerol 3-phosphate into the mitochondrial preparation and the inability of CMP to stimulate the labelling of phosphatidylinositol.

Discussion

It has been known for some time that most of the enzymes involved in the formation of the complex lipids are primarily localized in the endoplasmic reticulum (Wilgram & Kennedy, 1963; Eibl *et al.*, 1969; McMurray & Dawson, 1969; Sarzala *et al.*, 1970; Porcellati *et al.*, 1970; Williams & Bygrave, 1970). However, the marked ability of mitochondrial preparations to incorporate a variety of radioactive precursors into phosphatidic acid led to the conclusion that this lipid could be synthesized by two independent systems, one in the mitochondria and the other in the endoplasmic reticulum. Incorporation of $^{32}P_i$ into phosphatidic acid is dependent on the formation of [^{32}P]ATP and the mitochondria undoubtedly contribute to this process. Since the subsequent incorporation of $^{32}P_i$ from [^{32}P]ATP into phosphatidic acid (McMurray & Dawson, 1969; Jungalwala & Dawson, 1971; Jungalwala *et al.*, 1971; Miller & Dawson, 1972*a*) could conceivably be limited by glycerol kinase (EC 2.7.1.30) or diglyceride kinase (EC 2.7.1.–) activity (Lapetina & Hawthorne, 1971; Lapetina & Michell, 1972), this evidence will not be discussed here. Nevertheless, the incorporation of *sn*-[^{14}C]glycerol 3-phosphate or the glycerol 3-phosphate-dependent incorporation of radioactive fatty acids observed by many workers with mitochondrial preparations from rat liver (Smith & Hübscher, 1966; Smith *et al.*, 1967; Stoffel & Schiefer, 1968; Zborowski & Wojtczak, 1969; Shephard & Hübscher, 1969; Sarzala *et al.*, 1970; Daae & Bremer, 1970), adipose tissue (Roncari & Hollenberger, 1967) and brain (Mårtensson & Kanfer, 1968), appears to be greater than can easily be explained by contamination. Initially, it was believed that the results reported here represented a specific difference between brain mitochondria and those of other tissues. However, during the preparation of this manuscript it was learned that similar results have been obtained by Davidson & Stanacev (1972) with mitochondrial preparations from guinea-pig liver. Eibl *et al.* (1969) have also reported that acyltransferase activity was mainly associated with the microsomal fraction. Despite a careful re-examination of the previous publications, it has not been possible to explain the discrepancy between the results reported by different workers. Such comparisons are complicated by differences in the mitochondrial preparations used (i.e. various amounts of contaminating membranes and in some cases the addition of supernatant), the incubation conditions (i.e. pH, $MgCl_2$ concentration), the nature and source of the activated acyl group (i.e. added acyl-CoA or endogenously generated acyl-CoA from ATP or palmitoylcarnitine plus CoA), the extraction procedures and the radioactive products (i.e. lysophosphatidic acid, phosphatidic acid and neutral glycerides). It appears likely that some of these studies were not conducted with optimum incubation conditions. Therefore, it is only possible to conclude that the results of Davidson & Stanacev (1972), Eibl *et al.* (1969) and those reported here clearly demonstrate that the primary reaction in the biosynthesis of all glycerolipids is still not completely understood. Since the experimental technique devised by McMurray & Dawson (1969) in which various amounts of the microsomal fraction are added to a fixed amount of mitochondria permits a more accurate evaluation of the source of the enzymes involved in phosphatidate synthesis, it is considered that a thorough re-evaluation of the role of the mitochondrion in the production of phosphatidic acid will be required.

At least part of the difficulty of interpretation may arise from apparent differences in the microsomal fractions from different tissues and species. In addition to endoplasmic reticulum, this fraction contains variable amounts of plasma membrane, Golgi-apparatus components, and other membrane fragments. Further, neither the smooth nor the rough endoplasmic reticulum appears to be homogeneous (Dallner *et al.*, 1969). Consequently the specific activity of an enzyme, localized in the endoplasmic reticulum or a portion of it, may be significantly higher that can be appreciated by measurements on the entire microsomal fraction. Therefore, it is extremely important to use a marker enzyme having a distribution in the endoplasmic reticulum approximating to that of the particular enzyme under investigation. Although NADPH-cytochrome *c* reductase activity appears to be a particularly useful marker for measuring the contamination by endoplasmic reticulum corresponding to phospholipid synthesis in rat liver (McMurray & Dawson, 1969) and guinea-pig brain (Miller & Dawson, 1972*a*,*b*), this may not be the case for all tissues. For example, Omura *et al.* (1966) have observed that adrenal-cortex mitochondria contain an iron–sulphur (non-haem Fe^{2+}) protein that can function in the reduction of cytochrome *c* by NADPH. This mitochondrial system appears to be uniquely associated with steroid hydroxylations in adrenal cortex and reproductive organs and has not been observed in nervous tissue (R. H. Wickramasinghe, personal communication). However, it should be emphasized that the validity of the conclusions reported here is dependent on the ability of NADPH-cytochrome *c* reductase activity to act as a true marker for endoplasmic reticulum.

Consequently, the observation that the incorporation of *sn*-[^{14}C]glycerol 3-phosphate in the presence of synaptosomes paralleled the NADPH-cytochrome *c* reductase activity in this fraction (P_3) must be

cautiously interpreted. Miller & Dawson (1972*a*) have reported that synaptosomes contain occluded NADPH-cytochrome *c* reductase activity. They suggest that this activity may represent smooth endoplasmic reticulum present in synaptosomes *in vivo*. If this conclusion is valid, synaptosomes may be capable of independently producing the phospholipids necessary for the more distant portions of brain neurones. Part of the phospholipid requirement of nerve endings could also be met by the transfer of phospholipid from supporting cells or from other regions. In this context, it is noteworthy that Ansell & Spanner (1971) have suggested that the major source of brain choline is phosphatidylcholine or lysophosphatidylcholine transported from liver.

The question of phosphatidic acid synthesis by brain mitochondria is further complicated by the possibility that this lipid could arise via the acylation of triose phosphates (Hajra, 1968; Hajra & Agranoff, 1968) through reactions previously demonstrated with liver preparations. Although the triose phosphate pathway does not appear to function in the incorporation of free glycerol into phosphatidic acid in liver (Okuyama & Lands, 1970), this route may contribute to the formation of glycerides via fructose metabolism (Rao *et al.*, 1971). However, attempts to demonstrate these reactions in brain have not been successful (T. B. Ong, personal communication).

It should be noted in these studies that the incorporation of *sn*-[^{14}C]glycerol 3-phosphate was dependent on endogenously generated rather than added acyl-CoA. The acyl-CoA generating system was chosen because previous reports (Sánchez de Jiménez & Cleland, 1969; Zahler & Cleland, 1969; Kuwahara, 1972) indicated that it was impossible to establish optimum conditions promoting an incorporation of glycerol 3-phosphate directly proportional to time and protein concentration with exogenously added acyl-CoA. Further, it was considered that true autonomy of a particular organelle with respect to phospholipid production would require the independent activation of fatty acids. The conditions cited here are optimum for the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into rat brain homogenates (Possmayer & Mudd, 1971). Preliminary experiments demonstrated that the addition of fatty acid did not further stimulate this incorporation. Apparently the free fatty acid content of rat brain homogenates is sufficient to promote maximal acylation. However, it is possible that the free fatty acid concentrations in the individual fractions (Lunt & Rowe, 1968) may be low enough to limit the acylation. However, since the incorporation of *sn*-[^{14}C]glycerol 3-phosphate supported by the individual fractions is directly proportional to the amount of protein present (Fig. 2), this objection may not be too serious. Nor would the slight difference in fatty acid concentration observed be expected to compromise the conclusions based on the experiment in Fig. 3, in which the increasing amounts of the microsomal fraction were added to the other fractions.

The evidence presented in Table 4 and Figs. 1 and 3 indicate that the enzymes responsible for the acylation of *sn*-[^{14}C]glycerol 3-phosphate are primarily limited to the endoplasmic reticulum. The similarity of the cytidine nucleotide effects on the total incorporation in the whole homogenate and the various subfractions is consistent with this view. However, it will be noted that the total incorporation of *sn*-[^{14}C]glycerol 3-phosphate supported by the mitochondrial fraction was not particularly responsive to the presence of cytidine nucleotides. In contrast to the other fractions where the relative effects were quite consistent from experiment to experiment, there was a considerable variation in the cytidine nucleotide effect on the total incorporation into the mitochondria. For example, in some experiments all cytidine nucleotides produced a stimulation of the total incorporation. The reason for this variation is not appreciated at the present time. Presumably there must be some interaction between the mitochondria and the cytidine nucleotides or the phosphatidic acid.

The effect of the cytidine nucleotides on the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into phosphatidylinositol is similar in each of the isolated fractions including the mitochondria. Owing to the t.l.c. procedure used in these studies (Possmayer & Mudd, 1971), a small portion of the radioactivity associated with phosphatidylinositol in the mitochondrial fraction may be due to phosphatidylglycerol and phosphatidylglycerophosphate (Possmayer *et al.*, 1968). However, no radioactivity was found associated with lysophosphatidic acid.

It is noteworthy that although CMP has no effect on the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into phosphatidylinositol in the isolated fractions, the effect of CMP on the incorporation into phosphatidic acid is still evident. This indicates that the inhibitory effect of the cytidine nucleotides on the incorporation of *sn*-[^{14}C]glycerol 3-phosphate is not secondary to the production of CDP-diglyceride and phosphatidylinositol. The ability of CMP to inhibit the acylation of *sn*-[^{14}C]glycerol 3-phosphate has also been reported in rat liver microsomal (Fallon & Lamb, 1968) and bacterial preparations (Kito & Pizer, 1969). On the other hand, Zborowski & Wojtczak (1969) have observed that CMP stimulated the glycerol 3-phosphate-dependent incorporation of [^{14}C]palmitic acid into the lipids of rat liver mitochondrial preparations. Marinetti and his colleagues have also observed that the presence of cytidine nucleotides can influence the rate and the pattern of the incorporation of [^{14}C]glycerol into the neutral and complex glycerophosphatides of rat liver

(Marinetti *et al.*, 1964) and bone calvaria (Dirksen *et al.*, 1970*a*,*b*) preparations. However, despite numerous attempts, it has not been possible to demonstrate consistent effects of cytidine nucleotides on the acylation of *sn*-[^{14}C]glycerol 3-phosphate by rat liver microsomal preparations in this laboratory (F. Possmayer & J. B. Mudd, unpublished work).

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