ETHANOLAMINE PHOSPHOLIPID METABOLISM IN MYELINATING RAT BRAIN

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Abstract

^{3}H-labelled ethanolamine phospholipids were isolated from the brains of 13-day-old rats after intracerebral injection of [9,10-^{3}H]palmitic acid. Groups of rats of the same age were injected intracerebrally with preparations derived from the whole labelled fraction, the ethanolamine plasmalogen-depleted fraction, or [^{14}C]palmitic acid. A study of the distribution of radioactivity in phospholipids and sphingolipids, 2 hr after injection, indicates that the alkenyl chains of ethanolamine plasmalogen are effective precursors of the acyl groups of sphingomyelin and cerebroside. This role of plasmalogen is discussed in terms of the properties and distribution of plasmalogenase, aldehyde dehydrogenase, and fatty acid chain-lengthening enzymes in brain.

The poor incorporation of label from [^{3}H]phosphatidylethanolamine into phosphatidylcholine confirms previous reports that progressive methylation of the former is not significant in brain.

I. Introduction

The ethanolamine phospholipids of brain consist of ethanolamine plasmalogen, EP, (1-alkenyl-2-acyl ethanolamine glycerophosphatide), phosphatidylethanolamine, PE, (1,2-diacyl ethanolamine glycerophosphatide), and small amounts of 1-alkyl-2-acyl ethanolamine glycerophosphatide. Together with other phospholipids and sphingolipids they occur in plasma membranes and cellular organelles. Soon after birth, when brain myelination commences, EP and PE increase rapidly, these being major components of myelin (Wells and Dittmer 1967). However, the activity of brain plasmalogenase, which releases aldehydes specifically from EP alkenyl chains (Ansell and Spanner 1965; Ansell 1968), suggests that EP possesses more than a structural function. This is also indicated by the studies of Debuch (1966) in which [^{14}C]acetate was injected intracerebrally into 14-day-old rats. The radioactivity associated with the alkenyl chains of the glycerophosphatides was found to increase initially and then to decline steadily. A consideration of Debuch's data in terms of decreasing specific activities and the dilution of radioactivity by newly formed EP indicates that during myelination alkenyl chains are cleaved and are then further metabolized.

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This paper* reports on the metabolism of biosynthetically prepared [^{3}H]ethanolamine phospholipids injected intracerebrally into 13-day-old rats. Comparative studies were also made with [^{14}C]palmitic acid.

II. Materials and Methods

(*a*) *Animals*

Thirteen-day-old litter-mate male rats of the Wistar strain were used. Five to nine animals were used in each experiment.

(*b*) *Materials*

[9,10-^{3}H]palmitic acid (specific activity 500 mCi/mmole) and [U-^{14}C]palmitic acid (specific activity 495 mCi/mmole) were obtained from the Radiochemical Centre, Amersham. Tween 20 was a product of the Sigma Chemical Co., St. Louis, Mo., U.S.A. Lipids were emulsified in aqueous Tween 20 (36 mg/ml) for intracerebral injection. DEAE-cellulose (Whatman Chromedia) was grade DE23. Silica gel G (Merck) was used in all thin-layer chromatography procedures. The lipid standards were Sigma Chemical Co., and Koch-Light products.

(*c*) *Injection Procedure*

Animals were injected intracerebrally as described by Bickerstaffe and Mead (1967). The animals were lightly anaesthetized with ether and injected frontally at the level of the eyes using a Hamilton gas-tight syringe. Animals revived soon after return to their mothers.

(*d*) *Production of* [^{3}*H*]*Ethanolamine Phospholipid*

Each animal was injected with [9,10-^{3}H]palmitic acid (1 mCi) emulsified in aqueous Tween (50 μl). Incubation time was 2 days.

(*e*) *Lipid Extraction*

Animals were killed by decapitation, the whole brains quickly removed, pooled, chilled on ice, and weighed. Within 10 min of removal, the brains were extracted in a nitrogen atmosphere according to the method of Rouser *et al.* (1963). The brain tissue was thoroughly macerated in successive batches of chloroform–methanol (2 : 1 v/v) using 40 ml per gram brain tissue. Extracting solvent contained the anti-oxidant butyl hydroxytoluene (0·005%). Extracts were separated after each maceration by suction filtration and the solvent removed by vacuum distillation below 30°C.

(*f*) *Fractionation of Lipid Constituents*

The thoroughly dried total lipid was subjected to DEAE-cellulose and silicic acid column chromatography as described by O'Brien and Sampson (1965*a*). The front fraction eluted from the DEAE-cellulose by chloroform–methanol (9 : 1 v/v) contained cholesterol, ceramide, cerebroside, phosphatidylcholine, and sphingomyelin. Following this fraction, chloroform–methanol (3 : 2 v/v) eluted ethanolamine phospholipid. Front fraction constituents were separated by silicic acid column and thin-layer chromatography (TLC). For the latter process 20 by 20 cm plates of silica gel G (0·25 mm) were activated at 110°C for 30 min. For analysis and separation, chloroform–methanol–water (65 : 25 : 4 by vol) and chloroform–methanol–35% aqueous ammonia (14 : 6 : 1 by vol) were used and spots were visualized under ultraviolet light after spraying with rhodamine 6G (aqueous 0·05%). Purified lipids were recovered from TLC plates by collection into a glass aspirator under suction as described by Goldrick and Hirsch (1963) and eluted by sequential solvent extraction using (i) chloroform–methanol (2 : 1 v/v); (ii) chloroform–methanol (1 : 2 v/v); (iii) warm methanol.

* A brief account of this work was presented at a meeting of the Biochemical Society, Cambridge, 1970. [W. Segal and S. J. Wysocki, *Biochem. J.* **119**, 43P (1970).]

(*g*) *Gas–Liquid Chromatography* (*GLC*)

The aldehyde and fatty acid composition of [^{3}H]ethanolamine phospholipid was determined by GLC on 10% EGSS-X (ethylene glycol succinate polyester combined with a methyl silicone) on 100–120-mesh Aeropak 30 (Varian Aerograph) as described by Hansen, Tang, and Edkins (1969). Free aldehydes were derived from the alkenyl chains by mild acid hydrolysis (Anderson *et al.* 1969) and fatty acids converted to methyl esters by boron trifluoride treatment (Morrison and Smith 1964).

(*h*) *Assay of Radioactivity of Lipid Constituents*

Radioactivity was measured in a Nuclear-Chicago Corp. model 6860 (Mk. I) automatic liquid-scintillation spectrometer. Counting vials were monitored and a background contribution assigned to each to diminish errors in samples of low activity. Cerebroside was counted in diatol (Herberg 1960) containing 5 g PPO (2,5-diphenyloxazole) and 0·3 g dimethyl POPOP [1,4 bis-(4-methyl-5-phenyloxazol-2-yl)benzene] per 1000 ml. All other lipid samples were counted in toluene containing 5 g PPO and 0·3 g dimethyl POPOP per 1000 ml. Each sample was counted for sufficient time to accumulate 10,000 counts. Counting efficiencies were 60 and 30% for ^{14}C and ^{3}H respectively.

(*i*) *Distribution of Radioactivity in* [^{3}*H*]*Ethanolamine Phospholipids*

This was determined by measurement of specific radioactivity of a sample of [^{3}H]ethanolamine phospholipid and that of the [^{3}H]PE obtained after destroying [^{3}H]EP by acid hydrolysis. The ^{3}H-labelled aldehydes and [^{3}H]lyso-EP were separated from the [^{3}H]PE by TLC and radioactive assay of the hydrolysis products gave the ratio of alkenyl group activity to that of lyso-EP.

(*j*) *Plasmalogen Assay and Preparation of* [^{3}*H*]*Phosphatidylethanolamine*

The plasmalogen content of the [^{3}H]ethanolamine phospholipid was determined by the method of Rapport and Alonzo (1960). Treatment of the [^{3}H]ethanolamine phospholipid with 90% acetic acid at 38°C according to the method of Gray (1958) selectively hydrolysed the [^{3}H]EP and the [^{3}H]PE was separated by preparative TLC. The [^{3}H]PE was shown to be free of [^{3}H]EP by treatment with hydrogen chloride on a TLC plate as described by Horrocks (1968). Solvent development showed the absence of lyso-EP and aldehydes.

(*k*) *Metabolic Studies*

Three separate metabolic studies were made. Incubation times in each case was 2 hr:

(1) [^{3}H]ethanolamine phospholipid—i.e. [^{3}H]EP+[^{3}H]PE was emulsified in aqueous Tween 20. Each animal was injected intracerebrally with the phospholipid mixture (1 mg/50 μl; 194,000 counts/min/brain).

(2) [^{3}H]PE was emulsified in aqueous Tween 20. Each animal was injected intracerebrally (1 mg/50 μl; 212,500 counts/min/brain).

(3) [U-^{14}C]palmitic acid was emulsified in aqueous Tween 20. Each animal was injected intracerebrally (276,000 counts/min/brain).

(*l*) *Sphingolipid Degradation*

Sphingolipids (3–9 mg) were subjected to acid methanolysis by the method of Gaver and Sweeley (1965) and the products extracted by the procedure of Carter and Hirschberg (1968). Acyl groups of the sphingolipids were obtained as fatty acids and corresponding methyl esters free of sphingosine contamination, as shown by TLC and ninhydrin spray. Sphingosine bases contained traces of sphingosine methyl ether as found by Gaver and Sweeley (1965), but were devoid of acyl group derivatives.

III. Results

(*a*) *Biosynthetic* [^{3}H]*Ethanolamine Phospholipid*

This was prepared by DEAE-cellulose chromatography of the total brain lipid from six rats 2 days after intracerebral injection of [9,10-^{3}H]palmitic acid. The

Table 1

Properties of biosynthetic [^{3}H]ethanolamine phospholipid*

Average yield per brain (mg)	13·3
EP content (%)	39·2
Specific radioactivity (counts/min/mg)	194,000
Radioactivity distribution (%)	
PE	54·1
EP (by difference)	45·9
Alkenyl group: lysoplasmalogen† ratio	9 : 1

* The ethanolamine phospholipid fraction of 12-day-old rat brain contains a small amount (2·8%) of 1-alkyl-2-acylethanolamineglycerophosphatide (Wells and Dittmer 1967). This would be a minor constituent of the PE fraction.

† The product obtained by dealkenylation of EP.

product was chromatographically pure on TLC. Analytical data are shown in Tables 1 and 2. Because of difficulty in obtaining a clean separation of stearic and oleic

Table 2

Composition and distribution of radioactivity of fatty acids and aldehydes in brain [^{3}H]ethanolamine phospholipid after intracerebral injection of [9,10-^{3}H]palmitic acid into 13-day-old rats

Component*	Fatty acids		Fatty aldehydes	
	Composition (g/100 g)	Rel. sp. activity†	Composition (g/100 g)	Rel. sp. activity‡
$C_{16:0}$	11·8	1·0	38·7	1·0
$C_{16:1}$	1·8			
$C_{18:0}$	22·6	0·70	47·4	0·88
$C_{18:1}$	9·0		13·9	
$C_{18:2}$	0·6			
$C_{20:4}$	22·8			
$C_{22:5\omega6}$	7·4			
$C_{22:5\omega3}$	2·6			
$C_{22:6}$	21·4			

* Number of carbon atoms: number of double bonds.

† Relative to palmitic acid = 1·0.

‡ Relative to palmitaldehyde = 1·0.

acids (as methyl esters) and the corresponding aldehydes by preparative GLC, the $C_{18:0}$ and $C_{18:1}$ components were collected together for study of radioactivity distribution.

(*b*) *Labelling of Phospholipids and Sphingolipids after* [^3H]*Ethanolamine Phospholipid Injection* (*Expt. 1*)

Radioactivity distribution is shown in Table 3. Much of the recovered radioactivity remained in the ethanolamine phospholipid fraction. The incorporation of label into sphingomyelin, cerebroside, and to a minor degree into phosphatidylcholine indicates a rapid metabolism of ethanolamine phospholipid. The sphingolipids are labelled almost exclusively in the acyl groups, indicating that neither alkenyl nor acyl groups make a significant contribution to sphingosine biosynthesis. The poor labelling of phosphatidylcholine (also in expt. 2) shows that *N*-methylation of PE does not occur.

TABLE 3

DISTRIBUTION OF RADIOACTIVITY AFTER INTRACEREBRAL INJECTIONS

Each animal was intracerebrally injected with sufficient of each test material to give the brain approximately the same specific radioactivity. Incubation period 2 hr

Parameter	Expt. 1*	Expt. 2†	Expt. 3‡
No. of rats per group	8	5	9
Pooled brain weight (g)	8·4	5·2	9·7
Total lipid extracted (mg)	508	317	621
Distribution of radioactivity (counts/min/mg)			
Cerebroside	450	233	287
Sphingosine of cerebroside§	35	72	105
Acyl groups of cerebroside§	389	119	135
Sphingomyelin	371	77	449
Sphingosine of sphingomyelin‖	14	10	151
Acyl groups of sphingomyelin‖	266	57	201
Phosphatidylcholine	25	59	1264
Ethanolamine phospholipid	411	643	267

* [^{3}H](EP+PE) injected: 194,000 counts/min/brain.

† [^{3}H]PE injected: 212,500 counts/min/brain.

‡ [U-^{14}C]palmitic acid injected: 276,000 counts/min/brain.

§ Radioactivity expressed as counts/min/mg cerebroside hydrolysed.

‖ Radioactivity expressed as counts/min/mg sphingomyelin hydrolysed.

(*c*) *Labelling of Phospholipids and Sphingolipids after* [^3H]*Phosphatidylethanolamine Injection* (*Expt. 2*)

Table 3 shows that the isolated ethanolamine phospholipid is the most highly labelled lipid examined. The relatively lower activity of recovered ethanolamine phospholipid in experiment 1 compared with that in experiment 2 indicates that EP is metabolizing far more rapidly than PE. The relative labelling of sphingolipid acyl groups in experiments 1 and 2 indicates that the reactive alkenyl groups of plasmalogens are responsible for such labelling, as the β-acyl groups of plasmalogen correspond in nature and chain length to those of PE (Gray and Segal, unpublished data). Cerebroside acyl group labelling, although less than in experiment 1, is not insignificant especially when compared with corresponding labelling in experiment 3 in which the amount of injected radioactivity was somewhat greater.

(d) Labelling of Phospholipids and Sphingolipids after [U-^{14}C]Palmitic Acid Injection (Expt. 3)

Table 3 shows that in this case there is a general incorporation of label. Phosphatidylcholine labelling is considerably greater than that of other constituents. Although the EP content of the [^{3}H]ethanolamine phospholipid is only 40%, it is a better precursor of sphingolipid acyl groups than the injected palmitic acid. In experiment 3 the sphingosine and acyl-group labelling are comparable, quite unlike that found in experiment 1 when [^{3}H]ethanolamine phospholipid was injected.

These results indicate that plasmalogen alkenyl groups are significant precursors of sphingolipid acyl groups.

IV. Discussion

A labelled sample of rat brain ethanolamine phospholipid was prepared biosynthetically in order that this be identical in composition with the corresponding endogenous lipid of the recipient rat. The intracerebral injection of labelled phospholipid for metabolic studies has been reported by Bickerstaffe and Mead (1967) and more recently by Debuch, Friedemann, and Muller (1970). In the present study the incorporation of label from EP into acyl groups of a different lipid class indicates that intracerebral injection of phospholipids is a useful method for studying the metabolism of such compounds. This procedure possesses obvious advantages over *in vitro* methods which require highly demanding brain tissue preparations.

The nature and chain length of acyl and alkenyl groups of brain ethanolamine phospholipids are determined by the age and species (O'Brien and Sampson 1965*b*; Debuch 1966; and Bickerstaffe and Mead 1967). These workers have shown that the alkenyl groups of EP in brain at all ages are almost exclusively $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$. The acyl groups of the ethanolamine phospholipids are more variable with age. Acyl groups of the β-position derive mainly from long-chain polyunsaturated acids (Yabuuchi and O'Brien 1968) and are markedly different from those of sphingolipids. The foregoing data, together with the distribution of radioactivity in alkenyl chains and lysoplasmalogen of EP (Table 1), suggests that the alkenyl chains become involved in chain-lengthening processes prior to incorporation as sphingolipid acyl groups.

There are a number of reports in the literature which support such a hypothesis:

(1) Kishimoto and Radin (1966) have shown that chain elongation processes in brain utilize palmitoyl CoA derived from "complex lipids".

(2) Ansell (1968) has reported that brain plasmalogenase activity parallels the formation of ethanolamine plasmalogen.

(3) Erwin and Deitrich (1966) have shown that brain mitochondria and microsomes contain an aldehyde dehydrogenase of very broad specificity, the K_m for palmitaldehyde being $1 \cdot 3 \times 10^{-6}$M. Ansell and Spanner (1968) have found that plasmalogenase is associated with mitochondria and microsomes—the subcellular particles which Aeberhard and Menkes (1968) have designated as the loci for fatty acid chain elongation. It is significant also that chain elongation processes in brain involve $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$ fatty acids (Aeberhard and Menkes 1968; Dhopeshwarkar, Maier, and Mead 1969) corresponding to the $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$ alkenyl groups found in brain EP.

Experiment 2 indicates that PE may also supply intermediate chain-length acyl groups (C_{16} and C_{18}) for chain elongation, hydroxylation, and incorporation into cerebroside. The α-position acyl groups of PE consist almost entirely of C_{16} and C_{18} entities (Yabuuchi and O'Brien 1968) which correspond to those involved in chain elongation. Ansell (1968) has reported that rat brain plasmalogenase activity is is $2 \cdot 9$ μmoles/g fresh wt./hr while phospholipase activity is $0 \cdot 5$–$1 \cdot 9$ μmoles/g fresh wt./hr.

The injection of palmitic acid (expt. 3) leads to a general labelling of phospholipids and both sphingosine and acyl moieties of sphingolipids. The relative labelling of the acyl groups of sphingolipids is not very impressive compared with that derived from [^{3}H]EP. In this respect Kishimoto and Radin (1963) have reported that intracerebrally injected oleic acid is a poor precursor of nervonic acid, the latter being a product of chain elongation and is abundant in brain sphingolipids.

It is noteworthy that there are a number of correlations of defective myelination in mutant mice in which sphingolipids and EP are simultaneously depressed and lignoceric ($C_{24:0}$) and nervonic ($C_{24:1}$) acids, which are known to be derived by chain-lengthening mechanisms (Kishimoto and Radin 1966), were virtually absent (Nussbaum *et al.* 1968). The data of Galli and Galli (1968) and Baumann *et al.* (1968) for other myelin-deficient mutant mice also indicate that the depressed lipids were plasmalogens and sphingolipids while the concentrations of other lipids were either normal or somewhat elevated. The long-chain fatty acyl groups of sphingolipids, which are dependent on chain elongation processes rather than on diet, were considerably diminished.

The poor incorporation of label from [^{3}H]PE into phosphatidylcholine in experiment 2 (Table 3) indicates that a progressive methylation of PE does not occur in brain. This substantiates the data of Ansell and Dohmen (1957–58) and Dawson (1955) who drew the same conclusion on the basis of ^{32}P incorporation studies. In this connection it is significant that human brain ethanolamine phospholipid acyl groups are highly unsaturated, while those of human brain phosphatidylcholine are mainly saturated (O'Brien and Sampson 1965*b*).

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