Metabolism of Palmitaldehyde-1-^{14}C in the Rat Brain*

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ABSTRACT: Following injection of palmitaldehyde-1-^{14}C into the brains of young rats, the incorporation of label into the various brain lipids was studied at 2, 6, 48, and 72 hr. Evidence is presented that the label appearing in the phosphatidalethanolamines arose not by direct incorporation of palmitaldehyde but by oxidation of the palmitaldehyde to palmitic acid and incorporation of the latter into phosphatidylethanolamine, followed by reduction of the acyl group to the alkenyl ether.

The origin of the alkenyl ether[1] link in the plasmalogens is under active investigation in a number of laboratories but is still obscure. Several biosynthetic routes appear possible and have been proposed. The reaction of long-chain aldehydes with the α-hydroxyl group of glycerol to form a hemiacetal which, on dehydration, would give the alkenyl ether is logical and has been proposed by Burton (1959) and by Craig and Horning (1960) and, indeed, it has been shown by Vignais and Zabin (1958) that brain tissue is capable of reducing palmitate to palmitaldehyde. However, Carr *et al.* (1963), using a rat brain homogenate, were unable to demonstrate incorporation of palmitaldehyde-1-^{14}C into the unsaturated ether moiety of plasmalogens either in the presence or absence of CoA[2] and ATP. Baumann *et al.* (1965) concluded from experiments with *Clostridium butyricum* that although palmitaldehyde-1-^{14}C was incorporated into both alkenyl and acyl chains of glycerophospholipids, it was not incorporated specifically into the α position as aldehyde.

A second route to the alkenyl group is the reduction of the acyl group in the α position of the diacylphospholipids. However, the stage at which such a reduction might take place is completely unclear. Kiyasu and Kennedy (1960) provided evidence that a particulate fraction from rat liver could convert α-alkenyl-β-acyldiglyceride ("plasmalogenic diglyceride") to choline and ethanolamine plasmalogens in the presence of CDP choline or CDP ethanolamine. McMurray (1964) has also observed that "plasmalogen diglyceride" stimulates the incorporation, by brain homogenates, of CDP choline and CDP ethanolamine into the corresponding plasmalogens. However, there is no information presently available on the biosynthesis of such diglycerides.

A third possible route is the dehydrogenation of glyceryl ether phospholipids. Preliminary results with bovine bone marrow suggested that plasmalogens might be the precursors of glyceryl ether phospholipids (Thompson and Hanahan, 1963). However, recent experiments with the slug *Arion ater* provide very strong evidence that the ether-linked side chain of glyceryl ether phospholipids is the direct precursor of the α,β-unsaturated side chain of plasmalogens (Thompson, 1966).

The incorporation of long-chain fatty aldehydes into plasmalogens under *in vivo* conditions has not been investigated in animal tissues. This paper reports the results obtained following injection of palmitaldehyde-1-^{14}C into 18-day-old rat brains and allowing several periods of time for the aldehyde to be metabolized.

Experimental Section

Materials. The solvents used were ACS reagent grade and were redistilled prior to use. Ethanol (1%) was added to chloroform as a preservative. The nitrogen used contained less than 5 ppm of oxygen (high-purity nitrogen, Linde Co.). All solvent ratios given in the text are on a volume basis.

Palmitaldehyde-1-^{14}C. The palmitaldehyde-1-^{14}C was prepared by the method of Weygand *et al.* (1953). From 50.6 mg of palmitic-1-^{14}C acid containing 330 μc was obtained, after purification *via* thin layer chromatography on silicic acid using ether–pentane (2:98) as developing solvent, 13.5 mg of palmitaldehyde containing 100 μc.

Experimental Animals. Four groups of 18-day-old rats (male albino) of the Sprague–Dawley strain

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[1] The term alkenyl ether denotes those 1-*O*-ethers of glycerol in which there is unsaturation between the α- and β-carbon atoms of the hydrocarbon chain.

[2] Abbreviations used: CDP, cytidine diphosphate; CoA, coenzyme A; ATP, adenosine triphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.

were obtained, together with lactating mothers, from the Berkeley Pacific Laboratories and were fed on Wayne Lab-Blox (for mice and rats).

Injection of Palmitaldehyde. Approximately 1 μc (0.13 mg) of prepared palmitaldehyde-1-^{14}C suspended in 25 μl of an aqueous Tween 20 solution (36 mg/ml) was injected into each animal. The weanling rats were anesthetized lightly with ether, and the needle of a 50-μl Hamilton gas-tight syringe was inserted through the sutura sagittalis, on a level with the connecting line between the eyes at about a 45° angle with the frontal plane to a depth of about 5 mm. The rats recovered quickly and always appeared normal. They were kept with their mothers throughout the experiment. Groups A, B, C, and D were left to metabolize the injected palmitaldehyde for 2 hr, 6 hr, 2 days, and 3 days, respectively.

Extraction of Lipids. The animals from the various groups were sacrificed by CO_2 asphyxiation. The brains were removed immediately and frozen on Dry Ice. The pooled brains of each group were extracted with chloroform–methanol (2:1) under nitrogen as described by Rouser *et al.* (1961). During the extraction procedure and all subsequent steps, the lipid fractions were kept in a nitrogen atmosphere to prevent oxidation of unsaturated fatty acids. Evaporation of the chloroform–methanol extract to dryness on a rotary flash evaporator at 30° followed by drying *in vacuo* over KOH gave the total lipid.

Column Chromatography. Phosphatidylethanolamine (PE), which contains the largest proportion of brain plasmalogens, was isolated by fractionation of the lipid extract on a DEAE-cellulose column essentially as described by Rouser *et al.* (1961, 1963). The eluting solvents and lipids in each eluate were as follows: chloroform–methanol (9:1): cholesterol, ceramide, cerebroside, phosphatidylcholine (PC), and sphinomyelin; chloroform–methanol (7:3): PE, methanol, and water-soluble nonlipid material; glacial acetic acid: free fatty acids, phosphatidylserine (PS), gangliosides, and methanol to wash out acetic acid; and chloroform–methanol–28% ammonium hydroxide (4:1:0.2): phosphatidylinositol and other acidic lipids.

Thin Layer Chromatography. The eluates from the column chromatography were monitored continuously by thin layer chromatography. The solvent system chloroform–methanol–28% ammonium hydroxide (150:49:1) was used for monitoring PC and PE. PC was detected by spraying with bromophenol blue and PE by ninhydrin. Identification and purity of PE and PC were confirmed by comparison with the pure substances obtained from Applied Science Laboratories, Inc.

Gas–Liquid Partition Chromatography (GLPC) of Fatty Acids and Fatty Aldehydes. Isolated PE was subjected to methanolysis according to the procedure of Morrison and Smith (1964) except that diethyl ether instead of pentane was used as the extracting solvent. The resulting mixture of methyl esters and dimethyl acetals was analyzed directly by glpc or the dimethyl acetals were isolated by saponification of the methanolysate with 5% methanolic KOH at 60° for a 1-hr period. The dimethyl acetals were extracted into petroleum ether (bp 30–60°) and weighed before analysis. The aqueous phase was acidified and the free fatty acids were extracted with petroleum ether. The acids were reesterified in ether with freshly prepared diazomethane, weighed, and analyzed.

Fatty acid methyl esters and dimethyl acetals were analyzed by gas–liquid partition chromatography on a Barber Colman instrument (Model 10) with a hydrogen flame detector. Two 6-mm i.d. columns were used. The first, a 41-in. column packed with 14% (w/w) ethylene glycol succinate on siliconized Chromosorb P (60–80 mesh), operated at 186°, was used since it gave short retention times and allowed the complete fatty acid spectrum to be determined. The second, a 10-ft column packed with 16.7% (w/w) ethylene glycol succinate on siliconized Chromosorb (60–100 mesh) with a 2.5-cm foresection of 10% Apiezon L on siliconized West Coast firebrick (60–100 mesh), operated at 196°, was used because it completely decomposed the dimethyl acetals to the corresponding vinyl ethers (Stein and Slawson, 1966). At the start of the experiment, the fatty acid methyl esters and dimethyl acetals were run separately, but when it was found that the vinyl ethers obtained from the dimethyl acetals could be completely separated from the methyl esters on the 10-ft column, the fatty acid esters and dimethyl acetals were chromatographed together. In addition, as the retention times were quite long on the 10-ft column, and a stream splitter was present between the column and the detector, it was possible to determine the relative specific activity of the radioactive substances. The gas stream emerging from the column was split in the ratio 4:1 with four parts proceeding to the outlet and one part to the detector. The gas eluate was collected in a U tube with its first few millimeters heated to the temperature of the exit tube (200°) by a heating element and its U portion immersed in Dry Ice. In this way, samples were collected in a U tube and then washed out with 15 ml of scintillation solution into a vial for counting in a scintillation counter. The relative specific activity of a substance was taken as the ratio of the counts per minute in the collected fraction to the peak area of the substance obtained during collection. The gas split and collection is assumed to be constant; from the application of known radioactive substances, the over-all error is not more than $\pm 5\%$.

Purified fatty acid esters and synthetic dimethyl acetals were used as reference standards for identification. Fatty acid esters for which standards were not available were tentatively identified by their carbon number (Woodford and van Gent, 1960). Peak areas were estimated by triangulation. Detector linearity and relative detector response for different molecular weights were checked on prepared quantitative mixtures from Applied Science Laboratories or on standards supplied by the Hormel Instruments, Austin, Minn.

Glyceryl ether phospholipids were analyzed by first

TABLE I: Radioactivity Recovered in Total Lipid Extract after Intracerebral Injection of Palmitaldehyde-1-^{14}C into 18-Day-Old Rat Brains.

Group of Rats	A	B	C	D
No. of rats/group	10	11	11	11
Activity of palmitaldehyde-1-^{14}C injected/rat $\times 10^6$ cpm	0.725	1.04	0.727	0.725
Time of exposure to palmitaldehyde	2 hr	6 hr	2 days	3 days
Pooled weight of brains (g)	14.38	15.46	15.4	16.72
Extracted lipid (% of wet brain wt)	6.1	6.7	5.6	6.5
Activity in total lipid (cpm $\times 10^6$)	1.45	1.95	1.11	0.705
Per cent activity of injected palmitaldehyde in total lipid	19.94	17.1	13.8	8.84

obtaining the glyceryl ethers by acetolysis as described by Thompson (1965). The isopropylidene derivatives of the glyceryl ethers were prepared by the method of Hanahan *et al.* (1963) and were analyzed by gas–liquid partition chromatography on the previously described ethylene glycol succinate 41-in. column.

Neutral lipids were analyzed for saturated ether and α,β-unsaturated components by two procedures. The first involved isolation of the glyceryl ethers by refluxing the neutral lipids with 2 N KOH in 95% tehanol (Thompson, 1965). The isopropylidene derivates were formed and analyzed by gas–liquid partition chromatography. Methanolysis of the neutral lipids with BF_3–MeOH, as previously described for phospholipids, was used to liberate dimethyl acetals from any α,β-unsaturated links. The second procedure was that recently described by Thompson (1966), who used it principally for phospholipids. The method consisted of treating the lipid with $LiAlH_4$. The products, which were glyceryl ethers and glyceryl alkenyl ethers, were separated by thin layer chromatography using the system diethyl ether–petroleum ether–glacial acetic acid (70:30:1). In the present work the glyceryl ethers were analyzed as their isopropylidene derivatives while the glyceryl alkenyl ethers were methylated and the dimethyl acetals analyzed by gas–liquid partition chromatography.

Results

Four groups of 18-day-old rats (A–D) were injected intracerebrally with approximately 1 μc of palmitaldehyde-1-^{14}C/animal. The weights and radioactivities of their brain lipids are shown in Table I. These results show that radioactivity from palmitaldehyde is incorporated into the brain lipids of 18-day-old rats and that it decreases with time after injection. To investigate the nature of the active products formed in the brains, the total lipid extracts were chromatographed on DEAE-cellulose columns (Table II).

It is apparent that the radioactivity of the palmitaldehyde injected into the brains is distributed throughout the lipid fractions recovered from the DEAE-cellulose column. There seems to be no significant difference in the weight distribution of the lipids obtained from the 18- or 21-day-old rats. There is, however, a difference in the distribution of radioactivity. This varies according to the fraction and length of time after injection. For all cases studied, the front fraction, which contains cholesterol, ceramide, cerebroside, lecithin, sphingomyelin, and lysolecithin, had an average of 71% of the counts found in the lipids. PE had the next highest level of activity except in the case of the 2-hr period in which its activity is lower than that of PS.

TABLE II: Concentration and Radioactivity of Lipids in Rat Brains after Injection of Palmitaldehyde-1-^{14}C.[a]

	Group A (2 hr)		Group B (6 hr)		Group C (2 days)		Group D (3 days)	
Total Lipid Chromatographed	791 mg	1.14 $\times$ 10^6 cpm	1004 mg	1.69 $\times$ 10^6 cpm	743 mg	0.87 $\times$ 10^6 cpm	1030 mg	0.56 $\times$ 10^6 cpm
Front fraction	48.18	71.43	46.35	69.32	52.27	74.78	48.36	69.75
Phosphatidylethanolamine fraction	24.93	12.31	23.62	15.97	22.26	11.91	17.49	14.75
Phosphatidylserine fraction	9.53	13.04	11.56	10.40	12.05	6.84	10.69	8.86
Inositide fraction	9.28	1.43	8.17	2.22	3.92	2.64	4.81	4.59
% recovery	108	84.7	106	88	99	92.9	108	78.3

[a] All values are expressed as a per cent of the total weight or radioactivity obtained from the column. Results at various time intervals after substrate injection.

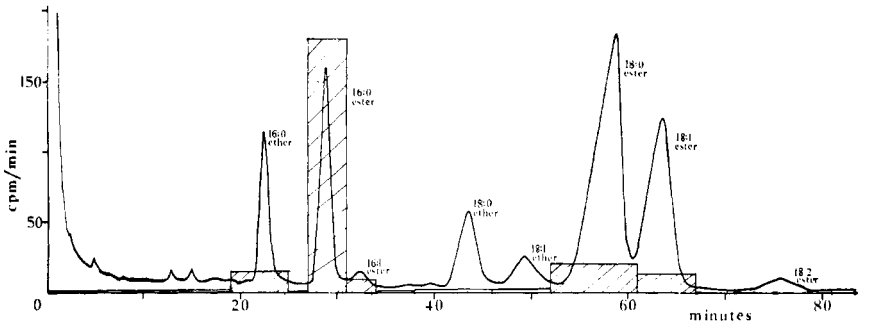

FIGURE 1: Bar graph of collected radioactivity with superimposed gas–liquid partition chromatogram of the methyl esters and vinyl ethers obtained from the methylation of phosphatidylethanolamine isolated from rat brains 6 hr after injection of palmitaldehyde-1-^{14}C. Chromatography was on a 10 ft × 0.25 in. i.d. column packed with 16.7% (w/w) ethylene glycol succinate on siliconized Chromosorb (60–100 mesh) with a 2.5-cm foresection of 10% Apiezon L on siliconized West Coast firebrick (60–100 mesh). Operating conditions: column temperature 192°, flash heater 178°, detector 255°, and collector 220°, and inlet pressure 12 psi.

These results indicate that PE actively incorporates radioactivity from injected palmitaldehyde within 2–12 hr after administration. To determine whether the radioactivity in PE was a result of direct incorporation of the palmitaldehyde into ethanolamine plasmalogens, the PE was subjected to transmethylation, and the methyl esters and dimethyl acetals were isolated. The aldehydes are assumed to be liberated from the α position of any phosphatidalethanolamine in the PE fraction.

The fatty aldehyde content of ethanolamine plasmalogens is shown in Table III and the complete fatty acid spectrum in Table IV. A typical gas–liquid partition spectrum and the radioactivity peaks collected during the chromatography are shown in Figure 1. Separation between 16:0/16:1 and 18:0/18:1 methyl esters and vinyl ethers was complete. The relative specific activities of each component up to 18:1 methyl ester were calculated, and are represented in histogram form in Figure 2. The activity in 16:1 methyl ester was so small in comparison to that in 16:0 that even the slightest tailing of the 16:0 peak would give spurious results for 16:1. Thus, 16:1 methyl ester, as it contained little if any radioactivity, was not considered further.

The data in Figure 2 indicate that both the methyl esters and vinyl ethers contain radioactivity, the amount of which is related to the time after injection

TABLE III: Fatty Aldehyde Composition of Ethanolamine Glycerophosphatide.[a]

Fatty Aldehyde	Source of Glycerophosphatide: Group A Rats (18-day old)	Group B Rats (18-day old)	Group C Rats (20-day old)	Group D Rats (21-day old)
14:0	*b*	*b*	*b*	*b*
16:0	43.3	41.9	43.7	40.8
18:0	40.5	39.6	43.2	42.8
18:1	16.2	18.6	13.1	16.4

[a] Each fatty aldehyde is expressed as per cent of total fatty aldehyde composition. Each value is the average of duplicate determination on two isolated samples. [b] Trace amounts detected.

TABLE IV: Fatty Acid Composition of Ethanolamine Glycerophosphatide.[a]

Fatty Acid	Source of Glycerophosphatide: Group A rats (18-day old)	Group B rats (18-day old)	Group C rats (20-day old)	Group D rats (20-day old)
14:0	*b*	*b*	*b*	*b*
16:0	8.2	11.1	9.7	6.9
16:1	0.7	1.2	0.3	0.5
18:0	23.6	24.4	20.7	22.2
18:1	14.0	13.6	9.6	14.0
18:2	0.5	0.9	0.7	0.6
18:3	0.5	0.4	0.7	0.4
20:1	1.9	1.5	1.0	1.6
20:2	*b*	*b*	*b*	0.5
20:3	0.6	0.4	0.7	0.6
20:4	18.2	18.4	21.7	19.5
22:4	8.4	6.7	8.2	8.4
24:1	3.4	3.9	2.9	3.9
22:6	19.4	17.7	25.9	22.3

[a] Each fatty acid is expressed as per cent of total fatty acid composition. Each value is the average of duplicate determinations on two isolated samples. [b] Trace amounts detected.

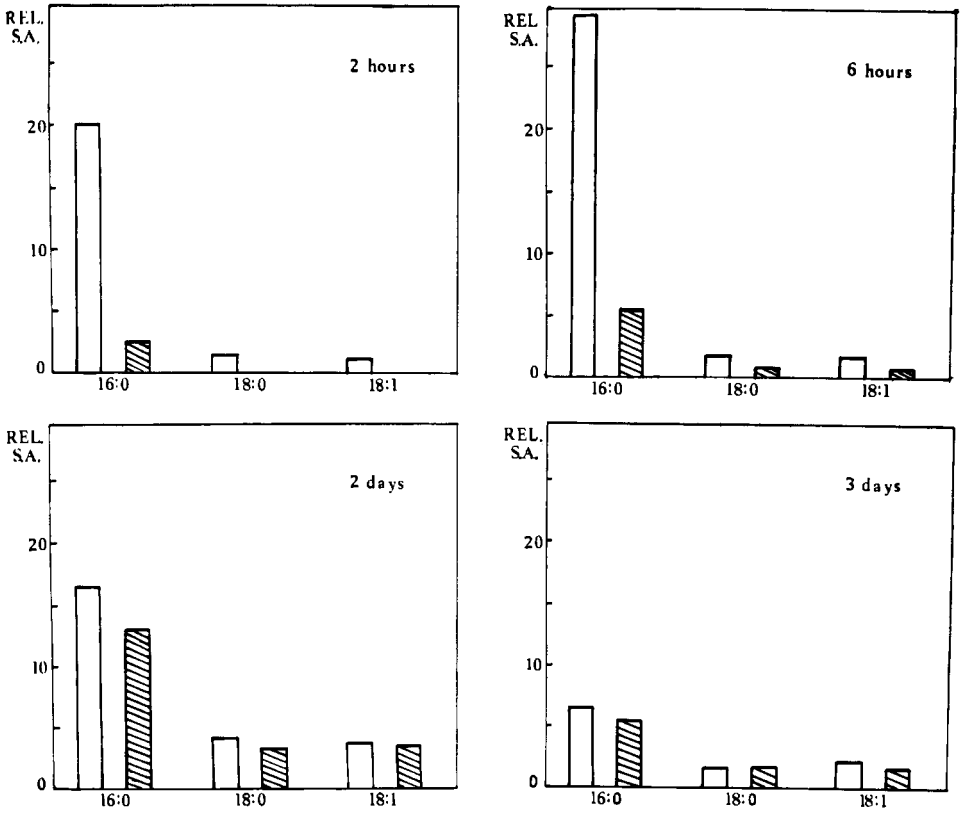

FIGURE 2: Changes in the distribution of radioactivity in ethanolamine glycerophosphatide at various hours after injection of palmitaldehyde-1-^{14}C into 18-day-old rat brains. (open bars) methyl esters; (cross-hatched bars) vinyl ethers. Abbreviation: Rel. S.A., relative specific activity.

of palmitaldehyde. The PE from rat groups A, B, and D was also analyzed for glyceryl ether phospholipids. The estimated specific activities of the 16:0, 18:0, and 18:1 isopropylidene derivatives of these fractions are shown in Table V. Although the above results were concerned only with the phospholipid fraction, it was also considered that the actual synthesis of the vinyl ether or saturated ether links does not take place at the phospholipid stage but rather at the diglyceride stage as postulated by Thompson (1966).

For this reason, the active front fractions isolated on the DEAE-cellulose column were analyzed for saturated ether or alkenyl ether compounds. The fractions were first separated on silicic acid–silicate–water columns according to the procedure of Rouser *et al.* (1961, 1963). The weight and radioactivity in each fraction obtained from the chromatography of the extracts from rats of groups B and C are presented in Table VI.

In both cases the majority of radioactivity is located in the lecithin and sterol fractions. The lecithin frac-

TABLE V: Estimated Relative Specific Activity Figures for the Methyl Esters, Dimethyl Acetals, and Isopropylidene Derivatives from Rat Brains at Various Times after Injection of Palmitaldehyde.

	16:0			18:0			18:1		
Time	Methyl Ester	Acetal	Isopropylidene	Methyl Ester	Acetal	Isopropylidene	Methyl Ester	Acetal	Isopropylidene
2 hr	20.0	2.5	6.1	1.5	0.0	0.0	1.2	0.0	0.0
6 hr	29.0	5.5	7.5	1.8	0.7	1.0	1.6	0.6	*b*
2 days	16.5	13.0	NA[a]	4.2	3.4	NA	3.6	3.4	NA
3 days	6.3	5.2	3.1	1.5	1.4	1.1	2.0	1.3	*b*

[a] NA, not analyzed. [b] Trace amounts detected.

TABLE VI: Concentration and Radioactivity of Lipids in the Front Fractions Obtained from DEAE-Cellulose Columns.[a]

	Group B (6 hr)		Group C (2 days)	
	465.8 mg	1.03 × 10^6 cpm	268 mg	418.3 × 10^3 cpm
Sterol fraction	37.8	42.0	35.2	35.3
Ceramide	*b*	0.0	2.8	3.0
Cerebroside	11.7	5.4	8.8	10.6
Lecithin	37.9	45.7	47.8	38.7
Sphingomyelin–lecithin	3.8	0.8	4.7	2.9
Sphingomyelin	8.8	6.1	2.7	1.2

[a] All values are expressed as a per cent of the total weight or radioactivity obtained from the column. [b] Trace amounts detected.

tion was analyzed by thin layer chromatography and was found to consist entirely of phosphatidylcholine. The sterol fraction, which would be expected to contain any traces of glyceryl ether derivatives or unreacted palmitaldehyde, was subjected to thin layer chromatography using the system benzene–petroleum ether (20:80) (Morrison and Smith, 1964). No material was detected in the position of the fatty aldehydes (R_F 0.35). Furthermore, on the addition of cold palmitaldehyde followed by gas–liquid partition chromatography of the fraction, no radioactivity was collected from the gas–liquid partition chromatograph corresponding to palmitaldehyde. A small amount of activity present was due entirely to trace amounts of fatty acid methyl esters. Second, an attempt was made to determine by the techniques already outlined whether saturated ether or alkenyl ether compounds were present in the fraction. In neither case could these compounds be detected by the methods used.

Discussion

Currently, three major biosynthetic pathways are favored for the synthesis of plasmalogens: reduction of the corresponding diacyl phospholipids, dehydrogenation of the glyceryl ether phospholipids, or *via* plasmalogenic diglyceride. The origin of the alkenyl ether link in plasmalogenic diglyceride is unknown but it could arise by reduction of the α-acyl group of the corresponding diglyceride or by condensation of a long-chain aldehyde with a glycerol derivative. Carr *et al.* (1963) attempted to investigate the latter pathway by incubating a rat brain homogenate with radioactive palmitaldehyde, but unfortunately such a small amount of material was incorporated into plasmalogens that it was considered negligible. The present experiment tests the same point *in vivo*. The 18-day period was chosen as the period of most active myelination since evidence has accumulated indicating that plasmalogens are concentrated in myelin (Berry *et al.*, 1965; Webster, 1960; McMurray, 1964) and are synthesized actively during myelination (McMurray, 1964; Beith *et al.*, 1962; Korey and Orchen, 1959). In fact, evidence has been presented that the ethanolamine phosphatides of myelin are largely of the alkenyl ether acyl form (O'Brien and Sampson, 1965). Myelin, once formed, is widely believed to have a low metabolic turnover (Davison and Dobbing, 1960; Cuzner *et al.*, 1965), and, hence, during the nonmyelination period, enzyme activity should be low, although recent evidence suggests the contrary (Vandenheuval, 1965; Smith and Eng, 1965; Nicholls and Rossiter, 1964).

If injected palmitaldehyde is directly incorporated into the plasmalogens, the activity should be located in the α-alkenyl chain. Analysis of PE fraction after a 2-hr period revealed only a small amount of activity in the α-alkenyl chain, as indicated by the low relative specific activity of palmitaldehyde dimethyl acetal. Most of the activity was located in the fatty acid residue of the plasmalogens and diacylphospholipids as indicated by the high relative specific activity of the palmitic acid. These results suggest that the palmitaldehyde is first converted to palmitic acid by oxidative enzymes in the brain. This suggestion is strengthened by the detection of radioactive 18:0 and 18:1 acids which are products of the metabolism of the radioactive palmitic acid. No activity was found in 18:0 or 18:1 dimethyl acetal, indicating that the highly labeled injected palmitaldehyde was not converted to 18:0 or 18:1 aldehydes by the brain, and that the aldehydes were not directly incorporated into the plasmalogens.

After 6 hr, the actual incorporation of radioactivity into the ethanolamine fraction was greater than that for the 2-hr period, the major increase occurring in the palmitic acid of plasmalogens and diacylphosphatides. There was also an increase in activity in the palmitic dimethyl acetal, but this did not prove direct synthesis from the administered palmitaldehyde since the relative specific activity was still low when compared to palmitic acid. Small increases in the specific activity of 18:0 and 18:1 acids also occurred and a small amount of radioactive 18:0 and 18:1 dimethyl acetals was also detected. These results suggest that the major portion of administered palmitaldehyde is oxidized to palmitic acid and some of its elongation products, which are then incorporated into the acyl side chains of phosphatidal- and phosphatidylethanolamines.

After 2 days, a reduction in the radioactivity of the ethanolamine fraction was found. The relative specific activity of palmitic acid dropped in comparison to the 6-hr period, but the specific activity of the dimethyl acetal rose to a level nearly equal to that of the acid. These results indicate that the metabolic turnover of the diacylphosphatide resulted in the lowering of the specific activity of palmitic acid. In contrast, the

specific activities of certain metabolic products, the 18:0 and 18:1 acids and the 18:0 and 18:1 dimethyl acetals, rose.

Three days after administration of the palmitaldehyde, the specific activities of 16:0, 18:0, and 18:1 acids and 16:0, 18:0, and 18:1 dimethyl acetals had decreased below those of the 2-day period, indicating the continuing metabolism of both phosphatidyl- and phosphatidalethanolamines. The over-all picture that emerges for the metabolism of palmitaldehyde injected into 18-day-old rat brains is as follows. First, a substantial part of the palmitaldehyde is converted to palmitic acid within the first 6 hr. This is indicated by the disappearance of free radioactive palmitaldehyde and the appearance of radioactive palmitic acid and its metabolic products, such as 18:0 and 18:1 acids, in the ethanolamine phosphatide fraction. During this time there was a considerable drop in radioactivity of the PS fraction, while the activity of the ethanolamine fraction rose. These changes may find an explanation in the rapid incorporation of the palmitic acid thus formed into the PS followed by the decarboxylation of PS and the resulting increase in the radioactivity of PE (Borkenhagen *et al.*, 1961; Ansell and Spanner, 1962). The presence of a radioactive fatty acid pool is also indicated by the appearance of activity in the front fraction and inositide fraction obtained from the DEAE-cellulose column since both fractions contain fatty acid components. Second, the radioactive palmitic acid and metabolites are incorporated into the acyl side chains of phoshatidyl- and phosphatidalethanolamines. This incorporation is maximal around the 6-hr period for palmitic acid and thereafter decreases as the acyl chains of the ethanolamine fraction are metabolized. Third, at the time when incorporation of radioactivity into the alkenyl ether moiety of the plasmalogens reached a maximum, no radioactive palmitaldehyde could be detected, indicating that this was not the source of radioactivity in the plasmalogens.

The most logical interpretation of the experimental facts is that the plasmalogens arise as a result of the reduction of the diacylphosphatides. If this is true the radioactivity would be expected to appear in the diacyl phosphatides before the plasmalogens. As reported, the activity of palmitic acid liberated from phosphatidylethanolamine was much higher than the corresponding dimethyl acetal liberated from phosphatidalethanolamine up to 6 hr. After 2 days, the specific activity of the 16:0 acetal rose and nearly attained the same specific activity as 16:0 acid. This evidence suggests that radioactive diacylphosphatide is formed within 6 hours and that it is then reduced to plasmalogen. This conversion pathway is supported by the results of the 2-hr experiment, as activity was present in the 18:0 and 18:1 acids, but not in the corresponding acetals. With longer metabolic periods, activity was found in both the acetals and acids, confirming the idea that the diacylphospholipid was formed before the plasmalogen. Debuch (1966) reported similar results after intracerebral injection of acetate-1-^{14}C into rat brains. She found that the fatty acids of the isolated lipids were labeled before the corresponding aldehydes of the plasmalogens.

Recent evidence by Thompson (1966) suggests that in the slug *A. ater*, the acyl group in the α position of glycerides is converted by a reductive mechanism to the saturated ether moiety, which is then dehydrogenated to vinyl ether. Thompson favors the initial formation of a diacyl glyceryl ether, which gives rise to a glyceryl ether phospholipid before dehydrogenation to plasmalogens occurs. In the present experiments, no neutral glyceryl ethers were found and certainly no radioactivity after injection of palmitaldehyde-1-^{14}C was located in the α position of such substances. However, analysis did yield glyceryl ether phospholipids and that with a C_{16} side chain had a higher relative specific activity than did the corresponding plasmalogen up to the 6-hr period. After this time, the radioactivity of the alkyl chain fell below that of the alkenyl chain. It is therefore conceivable that the glyceryl ether phospholipids are the precursors of the plasmalogens, but since the pool size of the glyceryl ether phospholipids is small and their relative specific activity is not markedly greater than that of the plasmalogens, it is difficult to draw such a conclusion from this series of experiments. An equally acceptable explanation is that the glyceryl ether phospholipids and plasmalogens are on separate pathways in which the diacylphospholipids serve as a common precursor. It is obvious, however, that the plasmalogens are not the precursors of the glyceryl ether phospholipids. This observation is supported by the recent work of Horrocks and Ansell (1965), who injected intercerebrally ethanolamine-^{14}C into rat brains, and that of Thompson (1966), who fed various radioactive precursors to slugs.

Thus, although these experiments deny the direct incorporation of palmitaldehyde into the alkenyl ether moieties of the plasmalogens and suggest that the latter are formed by reduction of the corresponding acyl groups, they do not furnish firm evidence as to the stage at which such a reduction may take place. The indication, however, is that it occurs after formation of the phospholipid.

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