Turnover of the fatty acids of rat brain gangliosides, glycerophosphatides, cerebrosides, and sulfatides as a function of age

YASUO KISHIMOTO, W. EWART DAVIES, and NORMAN S. RADIN

Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan

SUMMARY Rats of three different ages (7, 13, and 22 days) were given a single injection of acetate-1-^{14}C, then sacrificed at intervals (4 hr, 2 days, 10 days, and 30 days). The fatty acids of brain gangliosides, glycerophosphatides, cerebrosides, and sulfatides were isolated and counted. Some were decarboxylated and the resultant carbon dioxide was counted.

The data indicate that the palmitate of gangliosides and glycerophosphatides is made de novo from acetate, but that the stearate of these lipids is made by elongation of palmitate. The palmitate used for elongation is not the freshly synthesized acid but rather the acid recently liberated by breakdown of the complex lipids. Lignocerate also appears to be made by elongation, but it is possible that the stearate of cerebroside is made de novo. No qualitative difference was seen in the turnover curves or modes of biosynthesis in the three rat groups. The turnover rates for ganglioside fatty acids were similar to those for the phosphoglycerides. Turnover data for sphingosine in cerebrosides and sulfatides are presented.

KEY WORDS brain · fatty acid · biosynthesis · turnover · elongation · gangliosides · glycerophosphatides · cerebrosides · cerebroside sulfate · rat · cerebroside/sulfatide separation · age · sphingosine turnover

STUDIES IN THIS LABORATORY (1–3) and elsewhere (4) have shown that the fatty acids of brain cerebrosides are synthesized by chain elongation of shorter fatty acids by successive addition of acetate residues. The 16:0 of whole brain lipids was shown to be made de novo from acetate, while whole brain 18:0 was shown to be made by chain elongation. The fatty acids of the cerebrosides were found to undergo turnover, one portion breaking down quite rapidly and the other portion very slowly. Whole brain 16:0 and 18:0 were metabolized at intermediate rates.

This paper reports an extension of the earlier studies to individual fatty acids of gangliosides, glycerophosphatides, and sulfatides. Rats of different ages were examined to see if there is a qualitative difference in the routes of fatty acid synthesis at different points in the sequence of myelin formation. Data are furnished for the turnover of sphingosine in cerebrosides and sulfatides.

Abbreviations: Fatty acids are identified by the carbon number and number of double bonds (18:0 = stearate); C, chloroform; M, methanol; W, water; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

METHODS

Some of the materials and equipment have been described previously (1–3). The rats were of the Sprague-Dawley strain, from Camm Research Institute (Wayne, N.J.). The sodium acetate-1-^{14}C had a specific activity of 2 mc/mmole. A solution in isotonic saline (0.1 mc/ml) was injected intraperitoneally into three groups of male rats, aged 7, 13, and 22 days (groups A, B, and C). The volumes injected were 0.15, 0.25, and 0.40 ml respectively. The younger rats were kept with their mothers until they were 21 days old. At intervals of 4 hr, 2 days, 10 days, and 30 days after injection, a rat from each of 5 litters was sacrificed and the brains were pooled and extracted with C–M 2:1. Aliquots corresponding to two rat brains were processed to isolate the various lipids.

Isolation of the Ganglioside Acids

The procedure is adapted from a new analytical method for determining gangliosides (5). It involves isolation of gangliosides by solvent partitioning, treatment with 1 N

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KOH at 37° for 24 hr to saponify ester-type lipids, then acidification with HCl and extraction with hexane to remove the interfering fatty acids. The ganglioside solution is evaporated to dryness with a nitrogen stream, a known amount of methyl 22:0 (Applied Science Laboratories, State College, Pa.) is added as internal standard, and the mixture is methanolyzed. The HCl–methanol is removed with nitrogen, water is added, and the esters are extracted with hexane. The trace of oleate present is removed with a silver nitrate–silica gel column (6) and the individual saturated esters are isolated by GLC.

A thermal conductivity gas chromatograph (Model 720, F & M Scientific Corporation, Avondale, Pa.) was used with an 84 × 1/4 inch o.d. column packed with 9% SE-30 on Chromosorb W. Quantitative collection was made with a Millipore filter collector (7).

The isolated 18:0 ester was saponified; one portion of the acid was counted and the other portion was decarboxylated. The isolated 16:0 and 20:0 were counted in toto.

Isolation of the Glycerophosphatide Acids

The lower phase remaining after the gangliosides had been extracted was evaporated to a small volume with benzene and lyophilized. The residue was stirred for 1 hr with chloroform–0.2 N methanolic NaOH 2:1 (100 ml/g lipids) to methanolyze the fatty acids bound in ester linkage, and the pH was adjusted to about 7 with 0.2 volume of aqueous acetic acid. After the lower layer had been centrifuged and evaporated to dryness, the lipids were passed through a Florisil column in hexane–benzene 9:1 (40 mg of adsorbent per mg of lipid). The methyl esters come out with the same solvent, 25 ml/g. More details of this procedure have been published already (8).

The methyl esters were further fractionated on silver nitrate–silica gel; hexane yielded the saturated esters, and hexane–benzene 9:1 the monoenoic esters. The 16:0 and 18:0 were isolated by GLC, counted, and decarboxylated.

Isolation of the Cerebroside and Sulfatide Acids

The Florisil column used to isolate the glyceride fatty esters was eluted further with hexane–ether 8:2 (to yield cholesterol) and C–M 3:1 (35 ml/g). The latter solvent eluted cerebrosides plus sulfatides, which were then separated with a silica gel column. When necessary, the radioactive lipids were augmented with carrier glycolipid to bring the weight of each component to 5–10 mg. The sample was applied to 1 g of Unisil (Clarkson Chemical Co., Williamsport, Pa.) in C–M 98:2 and eluted with 20 ml of the same solvent. Further elution with 40 ml of C–M 94:6 yielded the cerebrosides; 40 ml of C–M 85:15 yielded the sulfatides. A small multibore column was used to improve the separation (9).

The glycolipids were then methanolyzed and the HCl–methanol was evaporated off under nitrogen. The residue was suspended in chloroform and separated into esters and sphingosine on a 1-g Unisil column, using 20 ml of chloroform and 20 ml of C–M 1:4 (10). Only the saturated nonhydroxy esters were isolated from the ester fraction, by means of a silver nitrate–silica gel column. GLC was used to isolate the 18:0 and 24:0, which were then counted and decarboxylated.

Thin-Layer Chromatographic Methods

Silica Gel G was used either with C–M–W 24:7:1 (for polar lipids) or with hexane–ether 85:15 or 70:30 (for esters). Sphingosine was examined with C–M–W–concd. ammonia 280:70:6:1 (11). The spray was bromothymol blue in 0.01 N NaOH.

RESULTS

Validation of the Methodology

The method of isolating the ganglioside fatty acids is discussed in a separate paper (5).

The alkaline methanolysis procedure is said to attack the fatty acid residue of plasmalogen only slowly (12), but we found no detectable plasmalogen on checking with TLC. When HCl was used to neutralize the alkali, we found some aldehydes in the methyl esters. However, when acetic acid was used for the neutralization, no aldehyde could be detected, either by GLC or TLC. On TLC a very faint spot accompanied the esters at R_F 0.78 (esters appear at 0.50); possibly this is a brain hydrocarbon. The ester spot was elongated and the color of the lower part was distinctly yellower after standing for a few days. By testing with standards we found that the polyunsaturated methyl esters move more slowly than 18:0 and 18:1, and give the same characteristic color.

The procedure for separating cerebrosides from sulfatides is more convenient than are previously published methods, such as ion exchange. It yields a mixture of cerebrosides which shows no other spots on TLC, and a mixture of sulfatides showing less than 1% cerebroside (estimated by comparison with known spots). Ceramides are discarded in the initial elution of the silica gel column.

The acidic methanolysis procedure was checked in two ways. Stearoyl-1-^{14}C cerebroside was processed through the HCl–methanol treatment and the nonhydroxy esters were recovered from a column of Florisil (8) with H–B 9:1 (25 ml/g Florisil). All the radioactivity was recovered. In a second test, a cerebroside preparation was methanolyzed on a large scale and the yields of all four groups of esters (saturated nonhydroxy and hydroxy, unsaturated

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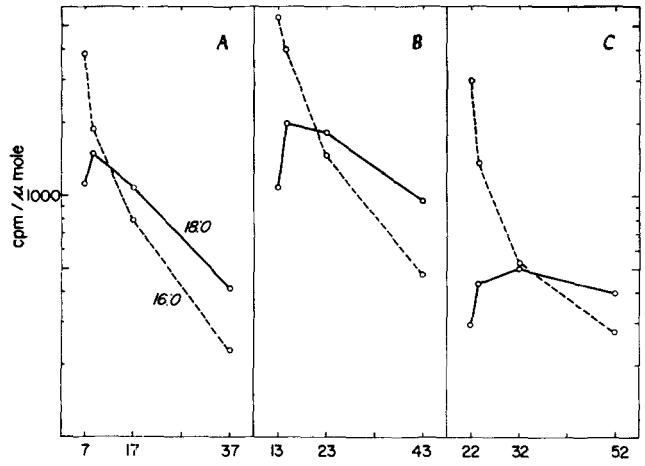

FIG. 1. Specific activity in the ganglioside 18:0 (solid line) and 16:0. Logarithmic ordinates are used for improved clarity in this and the other graphs. The abscissa values refer to the ages of the animals (in days) in each of the three groups. Thus, rats of group A were injected with acetate-1-^{14}C when 7 days old; of group B at 13 and of group C at 22 days.

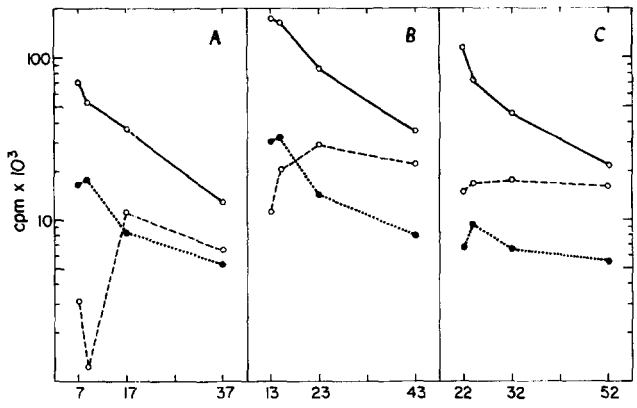

FIG. 2. Radioactivity in the glycerophosphatide fatty acids. The solid line represents the saturated acids; the dashed line, the monoenes; the dotted line, the polyenes. Units are cpm/rat brain.

nonhydroxy and hydroxy) were found to agree gravimetrically with the yields obtained by a previously used method (13).

The sphingosine isolation was followed by TLC, which revealed no cross-contamination with the methyl esters. The latter showed a very faint spot slower than the hydroxy esters and another, faster than the nonhydroxy esters. The sphingosine fraction showed four spots, giving the white and blue reaction characteristic of bases, similar to our sphingosine preparation. Recovery of radioactivity from the brain cerebrosides ranged between 88.4 and 98.5% for esters plus sphingosine; considering that the galactose must contain some ^{14}C, this seems reasonable.

Radioactivity in the Ganglioside Fatty Acids

The specific activities of the 18:0 and 16:0 of gangliosides are shown in Fig. 1. The data reveal several distinct features. (*a*) Both acids, in all three age groups, show a decline in activity following a maximal value; that is, they exhibit turnover. The *total* activity in these ganglioside fatty acids declines even while net accumulation of gangliosides occurs, as can be calculated from the specific activities reported here and the data in the accompanying paper (14). Thus there is actual destruction of gangliosides during this accumulation. (*b*) In each age group, the 16:0 reaches its maximal value first, at about 4 hr as compared to 2–10 days for 18:0. (*c*) The highest specific activity is seen in the group of 13-day old rats, an age at which myelin deposition appears to be most rapid. (*d*) Even at an age at which ganglioside deposition has slowed down considerably, namely 22 days, there was still much incorporation of ^{14}C. (*e*) Judging by the lower downward slope in the 18:0 curve for the C group, the rate of breakdown of stearate in ganglioside is somewhat smaller in later life.

The specific activities of ganglioside 20:0 were similar to those of the 18:0. The time courses were similar also, but the 20:0 of the last group of rats showed no sign of leveling off (compared with group B) in total activity.

TABLE 1 RELATIVE CARBOXYL ACTIVITY* IN GANGLIOSIDE STEARATE

Time of Sacrifice	Age of Rats at Time of Injection		
	7 days (A)	13 days (B)	22 days (C)
4 hr	17.7	18.4	21.6
2 days	10.6	11.6	17.3
10 days	6.6	7.3	11.0
30 days	5.5	6.2	8.7

* (Carboxyl activity/total activity) × 100%. Value expected for de novo synthesis, 11.1%.

Relative Carboxyl Activity in Ganglioside Stearate

The results of the decarboxylation study are shown in Table 1. The observed values are to be compared with that expected if 18:0 were made by de novo synthesis. In such an event, the radioactive carbon would be evenly distributed in each odd-numbered position and $^1/_9$ or 11.1% of the activity would be in the carboxyl group. It may be seen that in each group of rats the initial or first two values are above this value, and the remaining values are below. Moreover, there seems to be a distinct trend toward higher relative carboxyl activities with increasing age at time of injection.

Radioactivity in Glycerophosphatide Fatty Acids

Figure 2 shows the amount of activity in each fraction of the ester-linked acids: saturated, monoene, and polyene, obtained with the silver nitrate–silica gel column. The

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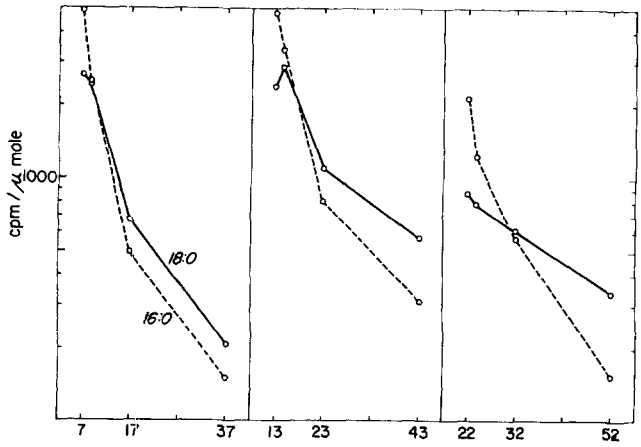

FIG. 3. Specific activities of the glycerophosphatide 18:0 (solid line) and 16:0.

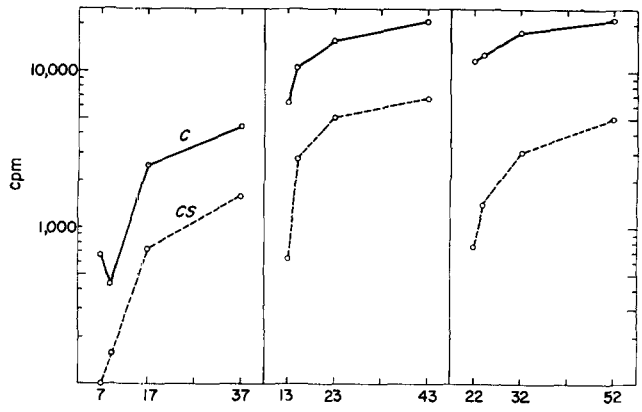

FIG. 4. Radioactivity in the whole cerebrosides (solid line) and sulfatides.

polyenes were not isolated; their activities were calculated by difference.

It may be seen that here, as with the gangliosides, the most active uptake of labeled acetate appears in the B group. The highest rate of decline is also in the B group. The time interval required to reach the maximal activity is less than 4 hr for the saturated acids, 2 days for the polyenoic acids, and 10 days for the monoenoic acids (primarily 18:1).

Figure 3 shows the specific activities of the 16:0 and 18:0 of the phosphoglycerides. It may be seen that 16:0 initially has a much higher specific activity than 18:0 but is exceeded later. As with the gangliosides, the crossover points seem to come later with increasing age of rats at the time of injection. The curves for the C group agree well with the previously published curves for similar rats (1).

The specific activities of the glycerophosphatide acids and ganglioside acids are unexpectedly similar, and the descending arms of the turnover curves have rather similar slopes.

TABLE 2 RELATIVE CARBOXYL ACTIVITY (%) IN STEARATE AND PALMITATE OF RAT BRAIN PHOSPHOGLYCERIDES

		Age of Rats at Time of Injection		
	Time of Sacrifice	7 days (A)	13 days (B)	22 days (C)
Palmitate:	4 hr	12.2	12.1	12.1
	2 days	12.7	12.2	12.2
	10 days	12.9	13.1	12.2
	30 days	13.6	13.5	—
Stearate:	4 hr	15.3	15.8	18.8
	2 days	8.0	11.0	17.1
	10 days	4.8	5.9	9.2
	30 days	4.3	5.0	7.2

Values expected for de novo synthesis: palmitate, 12.5%; stearate, 11.1%.

Relative Carboxyl Activity in Ester-Linked Acids

The results of decarboxylating 16:0 and 18:0 are shown in Table 2. As pointed out for the gangliosides, the values expected for de novo synthesis are 11.1% for 18:0 and 12.5% for 16:0. It can be seen that the relative activity in the carboxyl group of 16:0 in all three rat groups is quite close to the de novo value. The 18:0 carboxyl activity, in contrast, is much like that found in ganglioside 18:0. The first one or two values are well above the de novo ratio and the later values are below. Again, the older rats show higher relative carboxyl activities at corresponding time points.

Radioactivity in Cerebrosides and Sulfatides

Figure 4 shows the activities found in the total cerebroside and sulfatide fractions of the rat brains. Unlike the lipids described above, these lipids show no sign of a drop in activity following a maximum (except perhaps for an early point in the A group). Moreover, in the case of the cerebrosides, the highest amount of ^{14}C incorporation is not in the B group but in the C group. It may be seen that the sulfatide activity is initially much lower than the cerebroside activity but tends to catch up with time.

The activities in the total fatty esters and sphingosine of the two glycolipids were determined. Figure 5 shows the data for cerebrosides. The curves for both moieties of cerebroside are similar in shape, that for the sphingosine being lower. However, the *specific* activities (per atom of carbon) are similar for the two moieties. The curves for cerebroside sulfate (not shown) are similar, except that group C showed a marked continuous rise in activity up to the last time point.

The activities found in the 18:0 and 24:0 of the two glycolipids are shown in Figs. 6 and 7. Unlike the fatty acids of gangliosides and glycerophosphatides, 24:0 shows a continuously rising total activity. (The curve for cerebroside 24:0 of the B group does seem to flatten off.)

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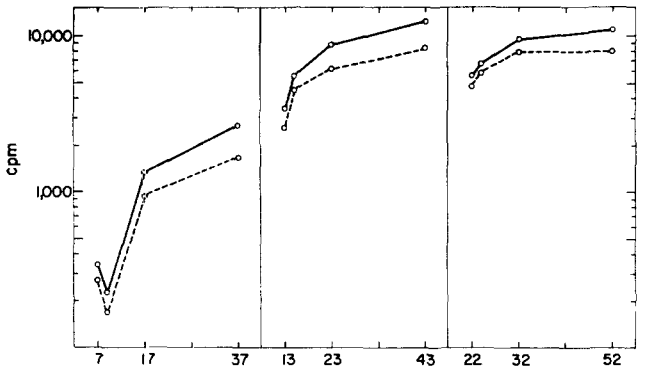

FIG. 5. Radioactivity in the cerebroside fatty acids (solid line) and sphingosine.

There is a striking difference in the case of cerebroside stearate, which shows a period of decline in activity (except in group A). The stearate of sulfatide, however, follows a time course like 24:0.

The relative carboxyl activity in the 24:0 of the cerebrosides is shown in Table 3. Only the data from groups B and C are reported as there was too little activity in group A for accurate measurement. The ratio expected for de novo synthesis of 24:0 is 2/24, or 8.3%. As with the 18:0 samples from gangliosides and glycerophosphatides, the values are higher than this in the early samples and (in the case of the B group only) go below the de novo value. Also similar to the data in Tables 1 and 2 is the finding that the older rat group has higher relative carboxyl activities at each time point.

DISCUSSION

Biosynthetic Mechanisms for Palmitate and Stearate

The evidence from in vitro studies with brain and other tissues points to de novo synthesis of 16:0 and chain elongation by successive acetate (or malonate) condensations to yield 18:0, 20:0, etc. (15). Studies with whole animals in this laboratory confirm that this is the mechanism actually operating in the brains of weanling rats (1).

TABLE 3 RELATIVE CARBOXYL ACTIVITY (%) IN CEREBROSIDE LIGNOCERATE

Time of Sacrifice	Age of Rats at Time of Injection	
	13 days (B)	22 days (C)
4 hr	13.4	18.2
2 days	9.4	13.2
10 days	6.5	12.1
30 days	5.5	11.2

Value expected for de novo synthesis, 8.3%.

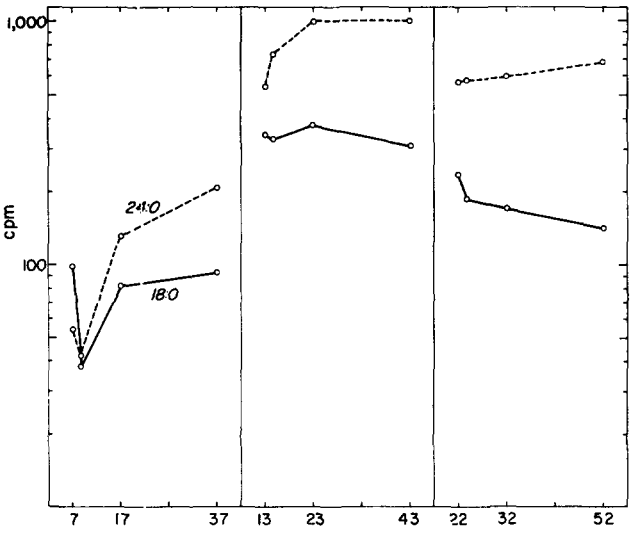

FIG. 6. Radioactivity in the cerebroside 18:0 (solid line) and 24:0.

The experiments reported in this paper provide additional evidence for the two routes of biosynthesis, particularly for the fatty acids of gangliosides and phosphoglycerides. The decarboxylation data for the 16:0 of the latter lipids are quite consistent with a mechanism of de novo biosynthesis, for the expected relative radioactivity is found in the carboxyl group over a wide range of times and activities. The curves for 16:0 of gangliosides and glycerophosphatides (Figs. 1 and 3) have the shape one would expect. The specific activity of brain acetate must reach its peak very shortly after injection, then decline rapidly. The 16:0 activity, derived only from labeled acetate, must likewise reach its peak very shortly after injection and then decline, but less rapidly. This type of curve was found also with *total* brain 16:0 in 22-day rats (1).

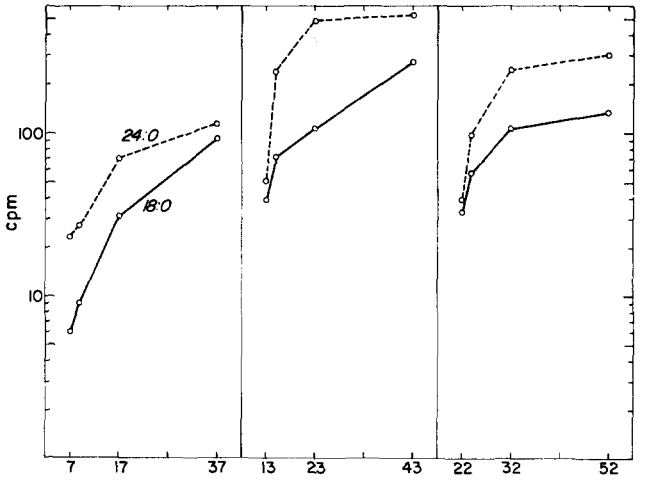

FIG. 7. Radioactivity in the sulfatide 18:0 (solid line) and 24:0.

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Mead and Fulco have proposed that the 18:0 of brain is made by elongation of 16:0, the latter being elongated immediately after its synthesis and without dilution by previously synthesized 16:0 molecules (16). Such a biosynthetic route would yield a relative carboxyl activity of 11.1%, independently of the time of sacrifice. Our data for ganglioside and phosphoglyceride show that this route cannot be of appreciable significance. They are consistent with the hypothesis that freshly synthesized 16:0 is immediately incorporated into complex lipids, that the complex lipids then break down with the liberation of 16:0, and that this 16:0 enters a pool of free fatty acids some of which are elongated. This sequence of events explains the initial high relative carboxyl activities in 18:0, then the low ones found later. It is consistent with the finding in vitro that much of the fatty acid synthesized from labeled acetyl CoA by liver microsomes and mitochondria is directly incorporated into phospholipids (17, 18). It is consistent also with the demonstration that a pool of free fatty acids exists in brain[1] (19).

Additional evidence for the conversion of 16:0 to 18:0 in rat brain may be seen in the shapes of their specific activity curves (Figs. 1 and 3). The curves show that much of the 16:0 and 18:0 of ganglioside and phosphoglyceride is indeed liberated from the complex lipids, presumably making them available for elongation. The peaks for the 18:0 curves are reached later than the 16:0 peaks, in accordance with the prediction of kinetic analysis of precursor–product relations (20).

Mead and Fulco drew their conclusion about the direct elongation of freshly synthesized 16:0 from the finding that brain glycerophosphatide 18:0 had a relative carboxyl activity in their experiment of 11.7% (16). As indicated above, no conclusion can be drawn from a single time point, since the values may change with time.

In our previous study of cerebroside fatty acids (1), decarboxylation of 18:0 yielded values (except for the first one) that were close to the de novo value, 11.1%. In the present study, only the B group yielded enough activity in the cerebroside 18:0 to provide moderately reliable relative carboxyl activities: 11.7, 9.8, 10.8, and 9.4% at the four time points. Thus there is still some reason to believe that the 18:0 of cerebrosides is made in a novel way. This is in agreement with the strikingly different time course for the 18:0 in this lipid (Fig. 6) as compared with the 18:0 of sulfatides or the 24:0 of the glycolipids.

Biosynthetic Mechanisms for Lignocerate

In a previous study of the biosynthesis of the cerebroside acids with labeled acetate (1), we found the relative carboxyl activity of cerebroside-bound 24:0 to range from 15.7% to 7.8%. It is evident that these rats made their 24:0 by chain elongation of fatty acids recently released from the complex lipids. This conclusion was strengthened by injecting labeled 16:0 and 18:0 directly into rat brains and later isolating 24:0 which had a very low relative carboxyl activity (2).

However, there is the possibility that *some* 24:0 is made directly from acetate, either by a one-step de novo process or by immediate elongation of freshly synthesized 16:0 or 18:0. Fulco and Mead, as part of the above-mentioned experiment with 18:0, also isolated the cerebroside 24:0 and found a relative carboxyl activity of 10.3%, from which they drew the conclusion that the latter mechanism operates (21). While such a conclusion cannot be drawn from a single time point, it must be noted that they used 13-day old rats and that in our earlier study (1) we used only 22-day old rats. Perhaps the younger rats utilize the direct elongation mechanism in preference to the indirect mechanism. However, the data in Table 3 show that 13-day old rats, like the older ones, utilize the indirect mechanism. The last two values for the younger rats are well below the de novo value. We have also shown that 13-day old rats have free 16:0 in their brain free fatty acids.[1]

The Unsaturated Fatty Acids

The shapes of the curves in Fig. 2 can be explained in terms of the presently held beliefs on biosynthesis. The monoenes (mainly oleate) are made by dehydrogenating the corresponding saturated acids (mainly 18:0) so that the early time points should show low radioactivities. As the 18:0 rises in specific activity, the 18:1 rises also. An important constituent of the monoenes is *cis*-vaccenic acid, which is formed from 16:0 by desaturation and elongation (22, 23). This relationship also explains the observed shapes of the curves in Fig. 2. It is interesting to note that the monoenes in the glycerophosphatides show turnover, but the rate of breakdown seems to be much lower in the oldest group of rats.

The polyenoic acids, mainly 20:4 and 22:6, are made from the essential C_{18} polyenes by condensation with radioactive acetate. Since the labeled acetate reaches its peak activity very soon after injection and drops rapidly, the polyenes reach their maximum very early also. Their rate of drop is somewhat faster than that of the monoenes, but this may reflect the difference in breakdown rates in acetate and stearate pools rather than differences in metabolic turnovers.

Relation Between Age and Lipid Metabolism

A number of other workers have found that various precursors of lipids — sulfate, glucose, galactose, and serine — are incorporated into rat brain lipids most efficiently

[1] W. E. Davies, Y. Kishimoto, and N. S. Radin, unpublished work with rat brain.

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during the early stages of myelination, at about 13 days of age. Our own data with acetate in the three groups of rats are similar [except in the case of cerebrosides (Fig. 4)]. This type of observation has generally been interpreted to mean that maximal utilization reflects maximal synthetic rates. However, this explanation is too simple. Brain gangliosides accumulate at a steady pace through the first three weeks of life (14, 24), yet their fatty acids show the usual high activities in the B group. The same appears to be true of the phosphatides and cholesterol (14). Cerebrosides show a more complex course of accumulation, which may explain why the activity in the B group is atypical.

A number of other explanations for the greater incorporation of labeled precursors in 13-day rats can be offered: (*a*) The brain lipids may be turning over more rapidly at this age, so that the synthetic rate really is higher even though the accumulation rate is unchanged; (*b*) the body pool of acetate, serine, etc., may be smaller at this age so that the specific activity of the precursor available to the brain is higher; and (*c*) the small molecules may penetrate the blood–brain barrier more readily at this age, thus allowing the brain to get a higher share of the building blocks.

Comparison of the Different Glycolipid Residues

The turnover curves for the fatty acids shown here indicate only that these acids are removed from brain in the course of their metabolism. The possibility exists that the other residues that make up the lipids remain in the cells and only the fatty acid is removed and replaced. However, our previous study with labeled galactose in weanling rats (25) showed that the galactose portion of ganglioside and cerebroside also turns over, and an unpublished study[2] with tritium-labeled acetate showed that the sphingosine residue also undergoes turnover. [The latter study also confirmed the existence of a cerebroside fraction having a high turnover rate (1).] The galactose experiment showed that the gangliosides undergo destruction faster than the cerebrosides, in agreement with the conclusions from the fatty acid data of this paper. Evidently the glycolipids undergo complete breakdown when one part is split off.

The above findings are also consistent with the idea that part of the cerebrosides are sulfated in toto to form sulfatides (25). Direct sulfation of cerebroside by brain in vitro has recently been reported (26).

[2] Y. Kishimoto, and N. S. Radin, unpublished work with whole rats.

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