The Association of the Galactosyl Diglycerides of Brain with Myelination

I. CHANGES IN THE CONCENTRATION OF MONOGALACTOSYL DIGLYCERIDE IN THE MICROSOMAL AND MYELIN FRACTIONS OF BRAIN OF RATS DURING DEVELOPMENT*

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SUMMARY

Through the use of a sensitive and specific gas chromatographic assay, the concentration of monogalactosyl diglyceride has been measured in whole brain and in fractions of brain from rats of varying age. The concentration of monogalactosyl diglyceride in whole brain was barely measurable before 10 days of age; increased sharply especially after 16 days up to about 20 days of age; and then decreased rather quickly to adult values. In adult brain, most of the monogalactosyl diglyceride was associated with myelin (64 to 68%); the next highest amount (15 to 18%) was recoverable from the microsomal fractions. In brains undergoing active myelination the greater part of the monogalactosyl diglyceride appeared in the microsomal fraction up to the age of 20 days. After 20 days the quantity of the galactosyl diglyceride decreased precipitously in the microsomal fraction, and increased sharply in the small myelin fraction. In the actively myelinating brain very little monogalactosyl diglyceride is found in the large myelin fraction; however in adult brain there are equal amounts in the small and large myelin fractions. Thus the galactosyl lipid is associated with an increasingly larger particle as the animal ages into adulthood. The enzyme activity responsible for the biosynthesis of the galactosyl diglyceride resided almost exclusively (88% of total) in the microsomal fraction.

These data indicate that a temporal relationship exists between the site of synthesis (microsomal) and site of deposition (myelin) of monogalactosyl diglyceride. This suggestion was confirmed by the finding that the specific radioactivity of monogalactosyl diglyceride in myelin from 23-day-old rats was the same as the specific radioactivity of monogalactosyl diglyceride derived from the microsomal fraction of brain from 20-day-old rats.

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Previous studies from our laboratory have shown that both monogalactosyl diglyceride (1,2-di-O-acyl-3-O-(β-D-galactopyranosyl)-sn-glycerol) and digalactosyl diglyceride (1,2-di-O-acyl-(3-O(α-D-galactosyl)-β-D-galactosyl)-sn-glycerol) are synthesized from two common precursors, UDP-galactose and 1,2-diglyceride in the presence of microsomal enzyme preparations from rat brain (1,2). These studies also demonstrated that the biosynthesis of monogalactosyl and digalactosyl diglyceride by the microsomal preparations of brain is almost nonexistent before 10 days of age, reaches a peak at 17 or 18 days of age, and then decreases to lower levels with increasing age. This age of maximum biosynthesis correlates well with the time of accumulation of galactosyl diglycerides as measured by Wells and Dittmer (3), and with the period of most active myelination, which in the rat or mouse occurs between about 10 to 20 days of life (4). This coincidence suggested that these biosynthetic enzymes and the galactosyl diglyceride products might be implicated in the process of myelination.

In this and the accompanying paper (5) we attempt to provide direct evidence for the participation of the galactosyl diglycerides in myelination. This first paper deals with the quantitative determination of monogalactosyl diglyceride by a sensitive gas-liquid chromatography technique in fractions of rat brain of varying age. A striking temporal relationship between the appearance of the galactosyl lipid in the microsomal fraction (site of biosynthetic enzyme) and in myelin (site of deposition) has been observed.

EXPERIMENTAL PROCEDURE

Preparation of Fractions of Rat Brain—Homogenates of brain from rats (bred and generously given by the Fels Research Institute, Temple University School of Medicine) of both sexes and of varying age groups were fractionated by slight modifications of the centrifugation procedures of Lapetina, Soto, and De Robertis (6) and Eichberg, Whittaker, and Dawson (7). Twenty percent (w/v) homogenates of whole brains of rats in 0.32 M sucrose were separated into four primary fractions: crude nuclear (particulate fraction from 900 x g centrifugation for 10 min, washed four times with 0.32 M cold sucrose), crude mitochondrial (particulate fraction from 11,000 x g centrifugation of 900 x g supernatants for 20 min, washed two times),
The sucrose acid dehydrogenase activity (nanomoles of formazan produced) and acetylcholine esterase activity (nanomoles of thioacetylcholine hydrolyzed) were measured using 40 to 80 µg of protein. The fractionation procedure was performed on brains from five rats each having a body weight of about 150 g (about 45 days old) as described under “Experimental Procedure.”

### Table I

| Fractions and subfractions | Total protein mg/fraction | Succinic dehydrogenase | | Acetylcholine esterase | |
|----------------------------|---------------------------|------------------------|---------|------------------------|---------|
|                            |                           | Total activity /mol/ fraction | % in fraction | Specific activity /mol/mg protein/min | Total activity /mol/fraction | % in fraction | Specific activity /mol/mg protein/min |
| Crude nuclear               |                           |                         |         |                        |                         |         |                        |
| Nuclei                      | 18.5                      | 300                     | 1.8     | 18.1                   | 1,868                  | 3.59  | 108                    |
| Large myelin                | 16.9                      | 115                     | 0.65    | 7.2                    | 615                    | 1.18  | 39                     |
| Cell debris                 | 94.0                      | 1,244                   | 7.14    | 20.8                   | 2,299                  | 4.42  | 38                     |
| Crude mitochondrial         |                           |                         |         |                        |                         |         |                        |
| M₂-A (small myelin)         | 21.1                      | 1,184                   | 6.79    | 63.8                   | 606                    | 1.17  | 30                     |
| M₂-B (synaptic membrane)    | 23.5                      | 4,036                   | 23.16   | 105.2                  | 1,908                  | 3.78  | 87                     |
| M₂-C (Mitochondrial)        | 28.0                      | 7,342                   | 42.13   | 298.6                  | 834                    | 1.60  | 31                     |
| M₃ (synaptic vesicles and membrane) | 10.5                   | 125                     | 0.72    | 7.0                    | 2,475                  | 4.70  | 150                    |
| M₃ (soluble)                | 22.7                      | Nil                     | Nil     | Nil                    | 431                    | 0.83  | 19                     |
| Microsomes                  | 286                       | 2,820                   | 16.18   | 10.6                   | 30,324                 | 58.31 | 114                    |
| Supernatums                 | 188                       | 232                     | 1.44    | 1.5                    | 10,584                 | 20.36 | 63                     |
| Total                       | 648                       | 17,425                  | 100     |                        | 52,004                 | 100   |                        |

The relative purity of the fractions was tested by measuring acetylcholine esterase activity (8) (marker enzyme for microsomal and soluble fractions) and succinate dehydrogenase activity (9) (marker enzyme for mitochondria), and by examining electron micrographs (for large and small myelin) prepared by Dr. Steven Phillips, Department of Anatomy, Temple University School of Medicine. The highest specific activity and the greater part of the succinate dehydrogenase activity was found in the mitochondrial fraction (M₂-C) and in the closely related (with respect to density) synaptic membrane (M₂-B) subfractions (Table I). The other fractions had relatively low succinic dehydrogenase activity indicating little contamination of mitochondria in these fractions. According to De Robertis et al. (10), the subfraction M₂-B contains some mitochondria which are in general slightly swollen. In agreement with the results of Lapetina, Soto, and De Robertis (6), the greatest amount of acetylcholine esterase activity was found in the microsomal fraction with a smaller but significant amount present in the 100,000 x g supernatant fraction. However the specific activity of this enzyme was greatest in the subfraction M₂ which consisted of synaptic vesicles and membranes. The association of acetylcholine esterase activity with synaptic membrane fraction was further revealed by the high specific activity of M₂-B which was a subfraction of M₂ consisting mainly synaptic membranes. Similar results were obtained by De Robertis et al. (10) and Trotter and Burton (11). The nuclear fraction also showed high specific activity. However, with the use of the acetylcholine esterase inhibitor, 284-C51, De Robertis et al. (10) were able to show that although most of the acetylcholine esterase activity of rat brain was the true type, the nuclear fraction had significant amounts of pseudochooline.
esterase due to the presence of blood capillaries. The endothelium of the latter contributes to pseudocholine esterase activity of this fraction (12). A very low amount of acetycholine esterase activity (total activity as well as specific activity) in both the small and large myelin fractions proved that there was relatively little contamination of microsomes in these fractions. Furthermore, electron microscopic examination of the small and large myelin revealed these fractions to be relatively pure. The small amount of contamination in the small myelin appears to include endoplasmic reticulum, fragments of nerve endings, and unidentified vesicles. Mitochondria and membranous fragments from cytoplasmic organelles made up the small amount of contamination in the large myelin.

**Extraction of Lipids from Tissues**—Whole brain, fractions of brain, or other tissues were extracted in a Waring Blender or a Potter-Elvehjem homogenizer with chloroform-methanol (2:1, v/v) in a ratio of 4 or 5 volumes of solvent per g wet wt. of tissue, but in no case was the volume less than 50 ml. The tissue-chloroform-methanol suspension was filtered through four layers of cheesecloth. The residue on the cheesecloth was washed twice with 2 M KCl (or 2 M NaCl) and twice with distilled water in a volume equal to about 1/4 the original volume of extractant. The residue was then extracted by the above procedure at least twice. The combined chloroform-methanol extracts were washed twice with 2 M KCl (or 2 M NaCl) and twice with distilled water in a volume equal to about 1/4 the original volume of extractant. The chloroform solution with any remaining aqueous phase and particulate matter was centrifuged at 5000 rpm for 10 min in a Sorvall RC-2B centrifuge. The volume of the chloroform phase was reduced to near dryness under a partial vacuum. The residue was dried overnight with high vacuum over P₂O₅.

**Alkaline Methanolysis of Lipids**—Lipids were decayed by treating the lipid in quantities no greater than 30 mg with a solution consisting of 1 mg of toluene, 1 ml of methanol, and 2 ml of 0.2 M methanolic KOH (freshly prepared) for 15 min at 37°. After adding 2 ml of water and 2 ml of chloroform, the mixture was centrifuged (10,000 × g for 10 min). The water phase was passed through a column of 1 g of Dowex 50, protonated form, layered on top of 1 g of Dowex 2, carbonate form. The column was washed with approximately 5 ml of water. The eluate, which had a pH of 5, was concentrated initially under reduced pressure and then dried in a stream of nitrogen.

**Gas-Liquid Chromatographic Assay for Monogalactosyl Diglyceride**—For analysis of monogalactosyl diglyceride, a portion of a chloroform-methanol (2:1, v/v) extract was decayed as described in the previous paragraph. The water-soluble products including monogalactosyl glycerol were converted to the O-trimethylsilyl ether derivatives according to the procedure of Swoolley, Wells, and Bentley (13). Aliquots of a hexane solution containing the O-trimethylsilyl ether derivatives were chromatographed on a Glowi (Willow Grove, Pennsylvania) model 310 gas chromatographed equipped with a hydrogen flame detector, a stream splitter, and a coiled glass column (6 foot × 1 inch diameter) containing 3% SE-30 on an acid-washed, silanized solid support (80 to 100 mesh). The compounds were injected onto the column at 200° of a temperature program of 4° per min beginning at 235° and held at 240°. The argon carrier gas flow rate was 65 cc per min. Radioactive compounds were collected and measured as previously described (1). Maltose was used as an internal standard. The areas under the standard maltose and monogalactosyl glycerol peaks were calculated by triangulation. The retention time (26 min) of monogalactosyl glycerol was frequently checked with standard monogalactosyl glycerol obtained from the decylation of monogalactosyl diglyceride either commercially (Supelco, Inc., Bellefonte, Pennsylvania) or isolated in our laboratory from spinach (1).

**Assays**—The biosynthesis of monogalactosyl diglyceride was determined by measuring the amount of [14C]galactose of UDP-galactose (New England Nuclear) incorporated into monogalactosyl diglyceride according to the procedure of Wenger, Petipas, and Pieringer (1). Digalactosyl diglyceride formation was measured in a similar way but with the modifications used by Wenger, Subba Rao, and Pieringer (2). The method of Ellman et al. (8) was used to assay acetylcholine esterase activity. For this assay 80 to 160 μg of protein of each fraction was used. Succinate dehydrogenase activity was measured using 40 to 80 μg of protein of each fraction by the method of Lee and Lardy (9). The molar extinction coefficient of 1.85 × 10⁴ M⁻¹ cm⁻² for the formazan derivative produced in the assay by Lee and Lardy (9) was used in our experiments. Protein was determined by the method of Lowry et al. (14).

**RESULTS**

**Effect of Age of Rat on Concentration of Monogalactosyl Diglyceride, Total Lipid in Brain, and Weight of Brain**—Our studies on the concentration of monogalactosyl diglyceride began with an attempt to repeat the earlier studies of Wells and Dittmer (3) by using a more specific assay (gas-liquid chromatography) for monogalactosyl diglyceride. In confirmation of the data of Wells and Dittmer (3), the concentration of monogalactosyl diglyceride in whole brain, which was found to be barely measurable before 10 days of age, increased sharply, especially after 16 days up to about 20 days of age (Fig. 1). In contrast to the earlier findings (3), however, the concentration of monogalactosyl diglyceride decreased to almost half the peak value by the 23rd day and then diminished at a considerably slower rate through adult life (Fig. 1). This decrease is the net concentration of monogalactosyl diglyceride after 20 days of age reveals the importance of the further metabolism of monogalactosyl diglyceride and in particular its degradation in controlling the concentration of monogalactosyl diglyceride in brain. The rise and fall of the monogalactosyl diglyceride concentration in brain correlated well with the rise and fall of the enzyme activity responsible for the synthesis of the galactosyl diglyceride (1, 2), except that the peak of the specific activity of the synthetic enzyme precedes the peak of the net accumulation of monogalactosyl diglyceride by about 3 days (that is, the peak of maximum enzyme activity occurs at 16 to 17 days of age). The maximum net accumulation of monogalactosyl diglyceride during the period of active myelination differs from the timing of the production of the total lipids of brain and the wet weight of the brain.

*The data of Wells and Dittmer (3), which showed galactosyl diglyceride to increase slightly rather than decrease with age after 20 days postpartum, is thus at greatest variance (same 13-fold difference) with our data in the older adult rat. We have no firm explanation of this discrepancy except to point out that we measured specifically monogalactosyl diglyceride and Wells and Dittmer measured total galactosyl diglyceride (their data as well as ours excludes other analogues). Unfortunately we have not been able to detect digalactosyl diglyceride or higher homologues in amounts that would account for this discrepancy in adult brain.*
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20 25 (100)(365)

AGE (DAYS)

Fig. 1. Change in concentration of brain weight, total lipids, and monogalactosyl diglyceride (MGD) in whole brains of rats of varying age. The extraction and gas chromatography techniques used are described under "Experimental Procedure." A group consisted of 8 rats for 6-, 9-, and 10-day-old rats, of 7 for 17-day-old rats, of 6 for 20-day-old rats, of 5 for 23-day-old rats, of 12 for 100-day-old rats, and of 4 for 365-day-old rats. The ages of 100 and 365 days are estimates made from body weights of the animals.

Table II

Concentration of monogalactosyl diglyceride in fractions of adult rat and mouse brain

Fraction of brain and gas chromatography techniques were done as described under "Experimental Procedure." In these experiments the brains from four adult rats and four mice were used.

| Fraction            | Rat               | Mouse              |
|---------------------|-------------------|--------------------|
|                     | nmoles MGD/g brain tissue | %      | nmoles MGD/g brain tissue | %      |
| Crude nuclear       |                   |                    |
| Nuclei              | 8.54              | 5.14               | 46.1 | 26.95 |
| Large myelin        | 55.6              | 33.4               |      |      |
| Cell debris         | 4.76              | 2.86               |      |      |
| Crude mitochondria  |                   |                    |
| Small myelin (M1-A) | 51.3              | 30.9               | 71.6 | 41.87 |
| Synaptic membrane (M1, B and M2) | 19.25 | 11.6 | 8.2 | 4.79 |
| Mitochondria (M1-C) | 1.24              | 0.75               | 15.1 | 8.85 |
| Microsomes          | 24.8              | 14.9               | 30.0 | 17.54 |
| Soluble             | 0.66              | 0.39               |      |      |
| Total of fractions  | 166.2             | 100                | 171.0 | 100  |

* Monogalactosyl diglyceride.

both of which increase more or less continually from birth through adult life (Fig. 1).

Predominance of Monogalactosyl Diglyceride in Myelin Fraction of Brains from Adult Rats and Mice—When each of the fractions obtained by the differential centrifugation technique described in "Experimental Procedure" was assayed for monogalactosyl diglyceride content by gas chromatography, 64% of the total monogalactosyl diglyceride was found in the combined myelin fractions of adult rat brain and 68% in the myelin of mouse brain (Table II; see also adult values, Fig. 2). These percentages, which should be minimum values if loss of myelin into other fractions is taken into account, demonstrate galactosyl lipid to be predominantly located in myelin of adult brain. These values agree closely with the data of Norton and Autilio (15) which showed that 72% of the minor galactolipids (a class in which monogalactosyl diglyceride would belong) of brain were in myelin.

Effect of Age of Rat on Concentration of Monogalactosyl Diglyceride in Fractions of Brain—In all experiments with adult rats the microsomal fraction was always observed to contain monogalactosyl diglyceride (Table II) in an amount which was higher than in any other fraction except myelin. This smaller quantity of monogalactosyl diglyceride in the microsomal fraction of adult brain might be attributed to myelin contamination. However, since this fraction contains almost all (88%) of the enzyme activity responsible for the biosynthesis of monogalactosyl and digalactosyl diglyceride (Table III), the microsomal fraction...
might be expected to retain some monogalactosyl diglyceride even in the adult rat brain. To test this possibility, brains of rats ranging in age from premelination (6 and 9 days old), through active myelination (16, 17, 20, and 23 days old), and postactive myelination (early and later adult) periods were fractionated and the concentration of monogalactosyl diglyceride was determined in each fraction (Fig. 2). The monogalactosyl diglyceride appears first in the 100,000 × g particulate or microsomal fraction (16-day value, Fig. 2A). The majority of the monogalactosyl diglyceride is associated with this fraction at the age of 20 days (Fig. 2A) when the highest amount of monogalactosyl diglyceride accumulates in brain (Fig. 1). However, within 3 days, at 23 days of age, the concentration of monogalactosyl diglyceride in the microsomal fractions falls to less than 1 of the 20-day value and most of the galactosyl diglyceride is made up of equal concentrations of small and large myelins (Fig. 2C). However, in the adult the total myelin, which contains 60 to 70% of the monogalactosyl diglyceride, is made up of equal concentrations of small and large myelin (Fig. 2B). The large myelin is a subfraction of a 900 × g particulate fraction. Thus the monogalactosyl diglyceride becomes associated with an increasingly larger and/or heavier particulate fraction of brain with increasing age. These data suggest but do not prove a temporal relationship between the site of enzymatic synthesis (microsomal) and the site of deposition (myelin) of monogalactosyl diglyceride.

Specific Radioactivity of Monogalactosyl Diglyceride in Microsomal and Small Myelin Fractions of Brain at 20 and 23 Days of Age—To investigate further a possible transfer of monogalactosyl diglyceride from a microsomal entity to myelin, an experiment was carried out in which the specific radioactivity of endogenous monogalactosyl diglyceride was measured in the microsomal and small myelin fractions at 20 and 23 days of age when the concentration of monogalactosyl diglyceride appeared to shift most dramatically from the microsomal to small myelin fraction (Fig. 2, A and B). Twenty-three rats, all of the same age, were given intraperitoneal injections of [U-14C]glucose (309 μCi per mmole). Each rat received a total of 1 μCi of [14C]glucose over 5 consecutive days according to the following schedule: at 15 days of age, 100 μCi; at 16 days of age, 100 μCi; at 17 days of age, 100 μCi; at 18 days of age, 400 μCi; and at 19 days of age, 250 μCi. Injections were stopped after the 19th day postpartum and 11 of the 23 rats with an average body weight of 30.3 g and average brain weight (wt) of 1.905 g were killed at 20 days of age. The brains were pooled and carefully fractionated, and the microsomal and small myelin fractions were extracted repeatedly with chloroform-methanol (2:1, v/v). A portion of the monogalactosyl diglyceride in the extracts was then prepared for gas-liquid chromatography by deacylating monogalactosyl diglyceride with mild alkali to monogalactosyl glycerol and then preparing the trimethylsilyl ether derivative of monogalactosyl glycerol (13). The radioactivity obtained from the trimethylsilyl ether derivative of the monogalactosyl glycerol peak was collected (13) and measured in a liquid scintillation spectrometer. At 23 days of age the remaining 12 rats with an average body weight of 44 g and average brain wet weight of 1.358 g were killed and the specific activity of the monogalactosyl glycerol derived from the monogalactosyl diglyceride in the microsomal and small myelin fractions was measured. The results were the following: at 20 days of age the specific activity of the monogalactosyl glycerol derived from monogalactosyl diglyceride in the small myelin was 930 dpm per pmole (55 dpm/59.2 nmole)

| Fractions and subfractions | MGD\(^a\) biosynthesis | DGD\(^b\) biosynthesis |
|----------------------------|------------------------|------------------------|
|                            | Specific activity      | Specific activity      |
|                            | mmole/mg protein/hr    | mmole/mg protein/hr    |
|                            | % in fraction          | % in fraction          |
| Total                      | 46.1                   | 433                    |

\(^a\) Monogalactosyl diglyceride.  
\(^b\) Digalactosyl diglyceride.
and in the microsomes was 910 dpm per μmole (189 dpm/298 nmoles); and at 23 days the specific activity of monogalactosyl glycerol derived from monogalactosyl diglyceride in small myelin was 920 dpm per μmole (212 dpm/230 nmoles) and in microsomes was 440 dpm per μmole (88 dpm/198 nmoles). Two points can be made about these data: first, the specific radioactivity of the monogalactosyl diglyceride in the small myelin fraction at 23 days of age is the same as the specific activity of the monogalactosyl diglyceride in the microsomes at 20 days of age. Second, the specific radioactivity of the monogalactosyl diglyceride in the myelin is unchanged at 20 days and 23 days of age even though the concentration of monogalactosyl diglyceride in small myelin increases significantly during this 3-day period (Fig. 2). When taken in conjunction with the finding of the enzyme for galactosyl diglyceride synthesis resides only in the microsomal fraction and not in myelin (Table III), these two observations support the idea of an age-dependent transfer of monogalactosyl diglyceride from a microsomal to a myelin entity. Unfortunately, the lack of large uptake of radioactivity into monogalactosyl diglyceride during the injection period and a diminution of the concentration of monogalactosyl diglyceride in the microsomes after the 20th day of age did not permit the experiment to be carried out on rats older than 23 days of age.

Presence of Monogalactosyl Diglyceride in Only Central and Peripheral Nerve Tissues—Using paper and thin layer chromatographic techniques, Steim (16) failed to detect the presence of monogalactosyl diglyceride in mammary glands, spleen, liver, intestine, and gray matter of brain; but did detect it in bovine cerebrum, cerebellum, spinal cord, and possibly bovine kidney. Using a gas-liquid chromatographic technique, which has both a qualitative and quantitative value, we repeated and extended the search for monogalactosyl diglyceride in animal tissues. In complete agreement with Steim (16), monogalactosyl diglyceride was found to be constituent of the central nervous system and, possibly, of kidney as well (Table IV). However, it is also found in sciatic nerve (Table IV) of the peripheral nervous system. It is absent from rat liver, spleen, serum, red blood cells, heart, lung, sternal bone, thigh muscle, stomach, intestine, skin, testis, epididymis, and epididymal fat pad (Table IV). The lower limit of the gas-liquid chromatography detection system was 4 to 5 nmoles per g of tissue, wet wt.

**DISCUSSION**

The pronounced rise and fall in the concentration of monogalactosyl diglyceride in rat brain between 10 and 23 days of age demonstrates the relatively active turnover of this galactolipid during the period of maximum myelination. These data further emphasize the probable importance of the balance between the synthetic and degradative enzymes regulating the concentration of the galactosyl diglycerides in brain. Both biosynthetic and catabolic enzyme systems which have been shown to exist in brain (1, 2, 17, 18) will have to be considered in any future studies on the control of the metabolism of the galactosyl diglycerides.

The temporal relationship between the microsomal and myelin fractions with respect to monogalactosyl diglyceride metabolism is of considerable interest. During the period of highest activity of the galactosyl diglyceride-synthesizing enzyme (1), which coincides with the period of most active myelination (4), the concentration of monogalactosyl diglyceride is highest in the 100,000 × g particulate or microsomal fraction. Since the microsomal fraction contains the enzyme for the biosynthesis of monogalactosyl diglyceride (Table III), the cell entity functioning as the site of synthesis must reside in this fraction. The likely candidate for this cell entity is the plasma membrane of oligodendroglia, which according to morphological evidence (19, 20) is responsible for the production of the myelin sheaths in the central nervous system and is sedimented in the microsomal fraction (21). Following this line of reasoning, one might logically expect to find the monogalactosyl diglyceride associated to the greatest extent initially with the microsomal fraction. However, after the burst of synthesis of monogalactosyl diglyceride at the onset of myelination has subsided (at about 20 days of age), the concentration of monogalactosyl diglyceride was observed to decrease rapidly in the high speed particulate microsomal fraction and to increase in the intermediate and low speed particulate myelin fractions. Myelin is practically devoid of galactosyl diglyceride biosynthetic activity even in the older animals in which the concentration of monogalactosyl diglyceride is highest in the myelin (Table III). Therefore, the most likely explanation of the above observation is that there is an age-dependent reaction causing the complex—of which the monogalactosyl diglyceride is an integral part—to change in physical dimensions (size and or weight) and to transfer from more slowly sedimenting, myelin fractions. This explanation is substantiated by the finding that the specific activity of the monogalactosyl diglyceride in the myelin of the 23-day-old rat is the same as the specific activity of the monogalactosyl diglyceride in the microsomes of brain of the 20-day-old rat.

The forerunner of the design of the experiments carried out in this paper was the experiment in which various lipids of brain were labeled in vivo by injection of a radioactive precursor (21–23). In each case, whether it was [14C]galactose incorporation

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### TABLE IV

**Concentrations of total lipids and monogalactosyl diglyceride in various tissues of rat**

| Tissue                          | Tissue wt | Total lipids | Monogalactosyl diglyceride |
|---------------------------------|-----------|--------------|-----------------------------|
|                                 | g/rat     | mg/rat       | μg/g tissue, wet wt         | nmoles/g tissue, wet wt |
| Brain                           | 1.68      | 122.2        | 72.8                        | 204                      |
| Spinal cord                     | 0.31      | 25.8         | 83.2                        | 600                      |
| Sciatic nerve                   | 0.06a     | 11.0         | 183.8                       | 464                      |
| Kidney                          | 2.65      | 198.8        | 75.0 (ca. 0.9)              | Nil                      |
| Liver                           | 9.91      | 433.0        | 42.7                        | Nil                      |
| Spleen                          | 1.03      | 94.8         | 94.1                        | Nil                      |
| Serum (ml)                      | 2.1       | 6.5          | 3.1                         | Nil                      |
| Red and white blood cells       | 2.92      | 11.0         | 3.8                         | Nil                      |
| Heart                           | 1.24      | 30.2         | 29.1                        | Nil                      |
| Lung                            | 1.65      | 51.2         | 31.0                        | Nil                      |
| Sternal bone                    | 1.10      | 40.8         | 37.1                        | Nil                      |
| Thigh muscle                    | 3.60a     | 85.8         | 23.8                        | Nil                      |
| Stomach and intestine           | 14.3      | 1272         | 89.9                        | Nil                      |
| Skin                            | 5.0*      | 400.0        | 81.2                        | Nil                      |
| Testis, epididymis, and epididy  | 6.74      | 1951.4       | 284.2                       | Nil                      |

*Portion of whole tissue. Four rats with a mean weight of 273 g were used in this study. The minimum amount of monogalactosyl diglyceride detected with accuracy by the gas-liquid chromatography technique would have been 5 nmoles per g, wet wt.
into neutral lipid, (22), [14C]glucose into cerebroside galactose (23), or [35S]sulfate into sulfatide (21), the accumulation of the radioactive precursor in brain was barely measurable before 10 days of age, greatest at about 20 days of age, and present in reduced amounts in adult animals. The close correlation of maximum uptake of radioactivity with the period of most active myelination made the relationship of the synthesis of these lipids in vivo to myelination rather obvious. Furthermore, the data of these earlier papers showed that the accumulation of [14C]galactose (22) and [35S]sulfate (21) was most active in microsomal fractions obtained from mice (22) and rats (22) at 15 to 20 days of age. Cuzner and Davison (24) have observed that the quantity of cerebroside in the microsomal fraction of brain of young rats (10, 16, and 21 days of age) is comparable with that of myelin, but later in development (30 days of age and older) relatively more cerebroside is found in myelin than in microsomes. Thus the temporal relationship between microsomal and myelin fractions demonstrated in this paper with monogalactosyl diglyceride may very well also exist in the biosynthesis of cerebrosides and sulfatides of brain.

From the observation that at least 88% of the galactosyl transferase activity can be recovered from the microsomal fraction (Table III), we should like to point out that the enzyme can be used as a "marker" for the microsomes of brain. There are apparently very few suitable enzymes in brain that can fulfill this function.

Cerebrosides have been used to identify myelin and to distinguish it from other cell constituents. The use of cerebrosides in this way is based on the fact that they make up a significant portion of the total lipid of myelin, they are formed during myelination, and they are found to a large extent in white matter. The work of Seminario, Hren, and Gomez (25) demonstrated that about 78% of cerebrosides present in brain is located in the crude nuclear (containing large myelin) and mitochondrial (containing small myelin) fractions of brain. About 61% of the cerebrosides of the crude mitochondrial fraction was recovered in further purification in myelin. The data of Table II show that about 85% of monogalactosyl diglyceride present in adult rat brain is recoverable in the crude nuclear and mitochondrial fraction. The remainder is associated with microsomes. Further fractionation revealed that 81% of monogalactosyl diglyceride associated with crude nuclei was recovered as large myelin and 72% of monogalactosyl diglyceride associated with crude mitochondria was isolated as small myelin (Table II). Using these studies as a basis for comparison, we believe that monogalactosyl diglyceride is at least as good as if not better than cerebroside as a "marker" for myelination. Although monogalactosyl diglyceride is present in adult brain in smaller amounts than cerebroside, this apparent deficiency is offset by the availability of a highly sensitive and specific gas-liquid chromatography assay for monogalactosyl diglyceride. In the actively myelinating brain there is much less difference in the concentration of two galactolipids (approximately 1 μmole of monogalactosyl diglyceride per g, wet wt and 2 to 4 μmole of cerebroside per g, wet wt, in brain (24, 26) from a 20-day-old rat) making monogalactosyl diglyceride of even more value at this age.

The availability of a sensitive gas-liquid chromatography assay and the fact that monogalactosyl diglyceride has as yet been detected in animals almost exclusively in myelinated nerve tissue (Table IV) (16), may make the analysis for monogalactosyl diglyceride a valuable diagnostic tool for a variety of neurological diseases.

Although digalactosyl diglyceride synthesis is readily demonstratable in vitro, we have not been able to detect digalactosyl diglyceride in brain by gas chromatography. Either the concentration of this galactolipid in brain is too low to be measured by gas chromatography or the method is not suitable for its detection. We are attempting to detect it by other means.

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REFERENCES

1. Wenger, D. A., Pettitpas, J. W., and Pieringer, R. A., Biochemistry, 7, 3700 (1968).
2. Wenger, D. A., Subrahmanya, K., and Pieringer, R. A., J. Biol. Chem., 245, 2513 (1970).
3. Wells, M. A., and Dittmer, J. C., Biochemistry, 6, 3109 (1967).
4. McIlwain, H., Biochemistry and the central nervous system, Ed. 3, London, J. and A. Churchill, Ltd., 1966.
5. Deshmukh, D. S., Inouye, T., and Pieringer, R. A., J. Biol. Chem., 245, 5935 (1971).
6. Lapetina, E. G., Soto, E. F., and De Robertis, E., Biochim. Biophys. Acta, 135, 33 (1967).
7. Eichberg, J. Jr., Whittaker, V. P., and Dawson, R. M. C., Biochem. J., 92, 91 (1964).
8. Elman, G. L., Courtnay, K. D., Andrews, V. Jr., and Featherstone, M., Biochem. Pharmacol., 7, 88 (1961).
9. Lee, Y. P., and Lardy, H. A., J. Biol. Chem., 240, 1427 (1965).
10. De Robertis, E., Pellegreno Delvaldi, A., Rodriguez De Lores Arnaiz, G. and Salganicoff, E., J. Neurochem., 9, 23 (1962).
11. Trotter, J. L., and Burton, R. M., J. Neurochem., 16, 805 (1969).
12. Brownman, M. W., and Alders, R. W., J. Neurochem., 4, 244 (1959).
13. Sweeney, C. C., Wells, W. W., and Bentley, R., Methods in enzymology, 8, 95 (1966).
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
15. Norton, W. T., and Autillo, L. A., J. Neurochem., 13, 213 (1966).
16. Steim, J. M., Biochim. Biophys. Acta, 144, 118 (1967).
17. Subra Rao, K., Wenger, D. A., and Pieringer, R. A., J. Biol. Chem., 245, 2200 (1970).
18. Subra Rao, K., and Pieringer, R. A., J. Neurochem., 17, 483 (1970).
19. Bunger, M. B., Bunger, R. P., and Ris, H., J. Biophys. Biochem. Cytol., 16, 67 (1961).
20. Hirano, A., and Dembitzer, H. M., J. Cell Biol., 34, 555 (1967).
21. Davison, A. N., and Gregson, N. A., Biochem. J., 98, 915 (1966).
22. Burton, R. M., Sodd, M. A., and Braddy, R. O., J. Biol. Chem., 233, 1053 (1958).
23. Moore, H. W., and Karmovsky, M. L., J. Biol. Chem., 224, 1990 (1956).
24. Cuzner, M. L., and Davison, A. N., Biochem. J., 106, 29 (1968).
25. Seminario, L. M., Hren, N., and Gomez, C. J., J. Neurochem., 11, 197 (1964).
26. Hauske, G., J. Neurochem., 15, 1237 (1968).
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