Smuckler, E. A. (1966), *Lab. Invest. 15*, 157.
Soo-Hoo, T. S., and Brown, A. D. (1967), *Biochim. Biophys. Acta 135*, 164.
van Deenen, L. L. M., and de Gier, J. (1964), *in* The Red Blood Cell, Bishop, C., and Surgenor, D. M., Ed., Academic, N. Y., p 278.
Warburg, O., Christian, W., and Velick, S. F. (1930), *Biochem. Z. 303*, 40.
Ways, P., and Hanahan, D. J. (1964), *J. Lipid Res. 5*, 318.
Weed, R. I., Reed, C. F., and Berg, G. (1963), *J. Clin. Invest. 42*, 581.
Zlatkis, A., Zak, B., and Boyle, A. J. (1953), *J. Lab. Clin. Med. 41*, 486.

The Metabolism of Glyceride Glycolipids. II. Biosynthesis of Monogalactosyl Diglyceride from Uridine Diphosphate Galactose and Diglyceride in Brain*

David A. Wenger,† Joan W. Petitpas, and Ronald A. Pieringer‡

ABSTRACT: Microsomal enzyme preparations of brain from rats 13 to 20 days of age catalyze the synthesis of 1,2-di-*O*-acyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol from uridine diphosphate galactose and 1,2-diglyceride. The enzyme requires the 1,2 isomer of the diglyceride substrate and prefers diglycerides with long-chain-saturated fatty acid constituents. Consistent and relatively uniform stimulation of the biosynthesis by exogenous diglyceride was best achieved by adsorbing the diglyceride substrate directly to a lyophilized enzyme preparation. The activity of the enzyme is highest in preparations made from brains of rats 14–18 days old. The age of highest enzymatic activity corresponds to the age during which the concentration of monogalactosyl diglyceride in brain increases most rapidly (Wells, M. A., and Dittmer, J. C. (1967), *Biochemistry 6*, 3169), and to the age during which myelination occurs at a maximal rate (McIlwain, H. (1966), Biochemistry and the Central Nervous System, 3rd ed, London, Churchill). This coincidence suggests that the enzyme responsible for the biosynthesis of monogalactosyl diglyceride in brain may function significantly in myelination.

The presence of monogalactosyl diglyceride in brain tissue was first reported by Steim and Benson (1963), Norton and Brotz (1963), and Rouser *et al.* (1967). The initial findings of these workers have been confirmed and extended by studies appearing in more recent publications (Rumsby and Gray, 1965; Pelick *et al.*, 1965; Rumsby, 1967; Steim, 1967). The monogalactosyl diglyceride of animal origin, which has been convincingly demonstrated thus far to occur only in tissues of the central nervous system, has been characterized as 1,2-di-*O*-acyl-3-*O*-(β-galactopyranosyl)-*sn*-glycerol.

The interest of this laboratory in monogalactosyl diglyceride of brain has been in the enzymatic processes responsible for the synthesis of this glycolipid. The results to be reported in this paper demonstrate that microsomal preparations of brains of rats of age greater than 13 days catalyzed the formation of 1,2-di-*O*-acyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol from UDP-galactose[1] and 1,2-diglyceride in the presence of magnesium ion. The enzymatic synthesis of monogalactosyl diglyceride is greatest during the period of most active myelination. A portion of this work has been presented in a preliminary report (Wenger *et al.*, 1967).

Experimental Procedure

Enzyme Source and Preparation. Fractionation of whole homogenates of brain from 13- to 18-day-old rats (bred and generously given by The Fels Research Institute, Temple University School of Medicine) was carried out according to the method of DeRobertis and coworkers (1962). All fractions were suspended in 0.32 M sucrose and were lyophilized overnight in small round-bottom flasks. The residues obtained after lyophilization were then extracted four times with 15 ml of acetone at 0° for 10 min with stirring. Acetone was completely removed from the residues by evaporation at reduced pressure. Microsomal fractions of brain were

* From the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania. *Received June 14, 1968.* Aided by a grant from the United Cerebral Palsy Research and Education Foundation.

† Predoctoral fellow of the U. S. Public Health Service. The material in this paper has been included in a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Organic Chemistry Department, Weizmann Institute of Science, Rehovoth, Israel.

‡ The author to whom inquiries are to be made.

[1] See *Biochemistry 5*, 1445 (1966), for a list of abbreviations.

employed as the source of enzyme in most experiments. Protein was determined by the method of Lowry *et al.* (1951).

Chromatography Systems. Gas–liquid partition chromatography of the *O*-trimethylsilyl ether derivatives of galactose, α-D-galactosyl-(1→3)glycerol, and β-D-galactosyl-(1→3)glucose was carried out on a Glowall (Willow Grove, Pa.) Model 310 chromatograph equipped with a hydrogen flame detector, a 20:1 stream splitter, and a 6 ft × 1/8 in. diameter coiled-glass column containing 3% SE-30 on an acid-washed, silanized solid support (80–100 mesh). The compounds were injected onto the column at 140° of a temperature program of 3°/min begun at 125° and held at either 185 or 215°. The argon carrier gas flow rate was 58 cc/min. Radioactive compounds were collected as described by Sweeley *et al.* (1966) by inserting a slightly modified Pasteur pipet into the effluent port for the duration of each peak registered on the recorder. The radioactive compounds collected in the pipet were transferred to scintillation spectrometer vials by washing the Pasteur pipet with 15 ml of scintillation fluid consisting of 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 l. of toluene.

The following paper chromatography systems were employed. Intact lipids were chromatographed on silicic acid impregnated paper (Whatman SG81) developed with the following solvent systems: (A) diisobutyl ketone–acetic acid–water (40:25:5, v/v) (Marinetti *et al.*, 1957), (B) chloroform–methanol–water (65:25:4, v/v) (Pelick *et al.*, 1965), (C) chloroform–methanol–water (45:10:1, v/v) (Pelick *et al.*, 1965). Water-soluble acid and mild alkaline hydrolysis products of the lipids were chromatographed on Whatman No. 1 paper developed with the following solvent systems: (E) pyridine–ethyl acetate–water (40:100:100, v/v, upper phase, descending) (Jermyn and Isherwood, 1949), (F) 1-butanol–pyridine–0.1 N HCl (50:30:20, v/v) (Lennarz, 1964), and (G) pyridine–1-butanol–water (20:36:32, v/v).

Thin-layer chromatography used to identify the carbohydrate moiety of the glycolipid was carried out on Eastman Chromagrams (Distillation Products Industries, Rochester, N. Y.) developed with 1-butanol–acetone–water (20:25:5, v/v) (system H).

Assay for Synthesis of Galactolipids. The incorporation of radioactivity from either UDP-galactose ([U-^{14}C]galactose) or diglyceride ([1,3-^{14}C]glycerol) into galactolipid was measured as follows. The enzymatic reaction was stopped by the addition of 1 ml of methanol. The tube containing the methanolic solution was placed in a boiling-water bath for 1 min and then cooled. Chloroform (2 ml) and distilled water (2 ml) were added to the tube and the contents were mixed. After centrifugation, most of the upper aqueous layer was removed by aspiration through a Pasteur pipet and discarded. An aliquot of the chloroform layer was taken either for quantitation of total galactolipid synthesis (when UDP-galactose was the radioactive substrate) or for chromatography on a column of silicic acid or on silicic acid impregnated paper. The amount of synthesis of the galactolipid was determined by counting an aliquot of the fractions from the column in a gas-flow Geiger counter (Nuclear-Chicago, Des Plaines, Ill.), or by cutting out the radioactive spot that cochromatographs with monogalactosyl diglyceride on the paper chromatogram and counting the paper directly on planchets of the Geiger counter or in vials of a scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.)

The radioactivity on the chromatograms was detected by a strip scanner, Model 363 (Atomic Accessories, Inc., Valley Stream, N. Y.), equipped with an integrator directly linked to the detector and independent of recorder, peak pen.

Sources of Diglyceride. 1,2(2,3)-Dipalmitin was prepared from 1(3)-tetrahydropyranyl glyceryl ether and palmityl chloride according to the procedure of Krabisch and Borgstrom (1965). The dipalmitin product had a melting point slightly lower (60°) than the reported value (62°). It showed only one major spot on thin-layer plates of silica gel developed with hexane–ethyl ether–acetic acid (80:20:1, v/v). [^{14}C]Diglycerides (dicaprin, dilaurin, dimyristin, dipalmitin, distearin, diolein, and dilinolein) labeled in the 1 and 3 positions of the glycerol moiety were synthesized from [1,3-^{14}C]-glycerol and the correct fatty acyl chlorides by a previously described technique (Pieringer *et al.*, 1967). The [^{14}C]diglycerides cochromatographed with authentic standards in the above thin-layer system and were used without further purification. 1,2-Dipalmitin and 2,3-dipalmitin, synthesized by a stereospecific chemical reaction (Lands *et al.*, 1966), were the generous gifts of Dr. William E. M. Lands, Department of Biological Chemistry, University of Michigan.

Preparation of Standard Monogalactosyl Diglyceride. Monogalactosyl diglyceride of spinach, which has been characterized as 1,2-di-*O*-acyl-3-*O*-(β-D-galactosyl)-*sn*-glycerol (Zill and Harmon, 1962), was employed as a standard in cochromatography experiments. The galactolipid was extracted from spinach with chloroform and partially separated from other lipids on a column of silicic acid according to the technique of Vorbeck and Marinetti (1965). The major portion of the monogalactosyl diglyceride was found in the chloroform–acetone (1:1, v/v) fraction. Because of pigmented impurities in this fraction, further purification was carried out by chromatography on silicic acid impregnated paper developed with solvent system A. Only one spot was detectable with the relatively specific periodate–starch stain (white spot on blue-purple background) (Metzenberg and Mitchell, 1954) at R_F 0.58. This material also fluoresced yellow under ultraviolet light after staining with Rhodamine G. The galactolipid was eluted from the paper with 10 ml of chloroform–methanol (2:1, v/v) and analyzed for fatty acid ester, galactose, and glycerol content. After the fatty acid ester quantity had been determined by the method of Stern and Shapiro (1953), the lipid was hydrolyzed with dilute alkali under conditions described by Tarlov and Kennedy (1965). The water-soluble product was analyzed by the periodate–chromotropic acid technique (Frisell *et al.*, 1954), which specifically measures the release of formaldehyde. For every 2 moles of lipid ester hydrolyzed with alkali, 0.97 mole of formaldehyde was released from the water-soluble alkaline hydrolysis product after

treatment with periodate. Descending paper chromatography of the alkaline hydrolysis product in system E produced one spot as detected by periodate–starch stain. Treatment of the material at this spot with 3 N HCl for 1 hr at 100° and rechromatography in solvent system E produced two spots which cochromatographed with glycerol and galactose ($R_{glycerol}$ 0.33). The glycerol and galactose produced in the acid hydrolysis were eluted quantitatively from the paper with water. Analysis of the galactose by a reducing sugar technique (Nelson, 1944) and glycerol by periodate oxidation (Frisell *et al.*, 1954) revealed a galactose to glycerol ratio of 1:0.93. Acid hydrolysis (3 N HCl at 100° for 3 hr) of the intact lipid produced diglyceride as revealed by chromatography on silicic acid paper developed in solvent system A, and galactose as shown by chromatography in solvent system E. These data demonstrate that the lipid extracted from spinach and employed as a chromatography standard is monogalactosyl diglyceride.

UDP-[U-^{14}C]galactose was purchased from either New England Nuclear Corp., Boston, Mass., or International Chemical and Nuclear Corp., City of Industry, Calif.

Source of Monogalactosylglycerol. α-D-Galactosyl-(1→3)-glycerol and β-D-galactosyl-(1→3)-glycerol were generous gifts of Dr. Pierre Stoffyn, McClean Hospital, Harvard Medical School. Trimethylsilyl ether derivatives of the α isomer and β isomer had retention times of 27 and 29 min, respectively, in the gas–liquid partition chromatography system previously described. Each isomer gave a single peak. The β-D-galactosyl-(1→3)-glycerol cochromatographed with the mild alkaline hydrolysis product of 1,2-di-*O*-acyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol obtained from spinach. The α-D-galactosyl-(1→3)-glycerol cochromatographed with the product of any enzymatic reaction formed in the presence of purified α-galactosidase (green coffee bean) and relatively high concentrations of glycerol and D-galactose.

Galactosidase Preparations. Partially purified α-galactosidase was prepared from green coffee beans (Brazilian variety purchased from Campbell Coffee Co., Philadelphia, Pa.) according to the procedure of Courtois and Petek (1966). β-Galactosidase was purchased from Calbiochem, Los Angeles, Calif. The activities of both enzyme preparations were checked against *p*-nitrophenyl α-D-galactoside and *o*-nitrophenyl β-D-galactoside. The α-galactosidase preparation was not contaminated with detectable amounts of β-galactosidase activity under conditions of maximum α-galactosidase activity. The β-galactosidase preparation was contaminated with approximately a 10% α-galactosidase activity.

Results

Identification of the Radioactive Product Biosynthesized from UDP-galactose and Diglyceride. Incubation of UDP-galactose (uniformly labeled with ^{14}C in the galactose moiety), dipalmitin, and magnesium ion, in the presence of the microsomal fraction from the brains of young rats, produced a radioactive compound that was readily extractable into chloroform. Chromatography of an aliquot of the chloroform phase on silicic acid impregnated paper resulted in a single radioactive spot in four different solvent systems. The radioactive lipid had R_F values of 0.58 in system A, 0.87 in system B, 0.85 in system C, and 0.83 in system D. In each system the radioactive lipid cochromatographed with authentic monogalactosyl diglyceride that had been isolated from spinach. When [^{14}C]dipalmitin (labeled in the 1 and 3 positions of the glycerol moiety) was the radioactive substrate in the incubation, a radioactive compound was produced which also cochromatographed with standard monogalactosyl diglycerides in the above systems.

When the radioactive lipid (labeled with [^{14}C]galactose) was subjected to mild alkaline hydrolysis according to the procedure of Tarlov and Kennedy (1965), 90% of the radioactivity was found in the water-soluble fraction. Almost all of the counts that remained as lipid after alkaline hydrolysis were found to cochromatograph with standard monogalactosyl diglyceride. Within the limits of the radioactive detection system, none of the radioactive lipid remaining after alkaline hydrolysis was found to cochromatograph with the monoalkyl ether of glycerol galactoside (R_F 0.58 in system D) (kindly supplied by Dr. W. T. Norton). Tarlov and Kennedy (1965) have indicated that the mild alkaline hydrolysis does not produce 100% hydrolysis. These findings also would appear to rule out any major incorporation of the [^{14}C]galactose into monoalkyl monoacyl glyceride galactoside or galactosphingolipid, since both of these types of compounds would retain chloroform solubility after mild alkaline hydrolysis. The resulting water-soluble radioactive material was then chromatographed in system E (Figure 1). The radioactive water-soluble compound was found to cochromatograph with characterized monogalactosylglycerol prepared from spinach monogalactosyl diglyceride. Cochromatography with carrier monogalactosylglycerol was also obtained in two other paper chromatography systems: system F (R_F 0.15) and system G (R_F 0.30). Identical cochromatography results were obtained with the alkaline hydrolysis product of the monogalactosyl [^{14}C]diglyceride derived from dipalmitin ([1,3-^{14}C]glycerol).

Treatment of the radioactive alkaline hydrolysis product (labeled in the carbohydrate moiety) with 3 N HCl for 1 hr at 100° produced only one radioactive compound. This compound cochromatographed with carrier galactose in system E (Figure 1) and in the thin-layer chromatography system H (R_F 0.37). Both of these chromatographic systems separate glucose (R_F 0.45 in H) and galactose. Thus, any conversion of galactose into glucose during the biosynthetic process can be ruled out. Acid hydrolysis of the radioactive alkaline hydrolysis product (labeled in the glycerol moiety) with 3 N HCl at 100° for 2 hr produced glycerol as the only radioactive product as determined by paper chromatography in sytem E (38.5 cm from origin when run overnight). When the galactolipid ([1,3-^{14}C]glycerol) having the R_F of the carrier monogalactosyl diglyceride was subjected to acid hydrolysis (3 N HCl for 1 hr at 100°) the resulting radioactive product cochromatographed with

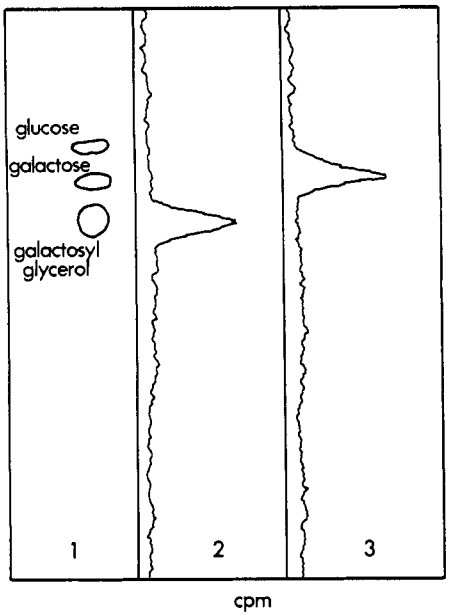

FIGURE 1: Cochromatography of carrier monogalactosylglycerol with the radioactive water-soluble alkaline hydrolysis product of the [^{14}C]glycolipid from UDP-[U^{14}C]galactose, and cochromatography of galactose with the radioactive product resulting from the acid hydrolysis of the alkaline hydrolysis product. Chromatography was carried out on Whatman No. 1 paper and developed in a descending manner with pyridine–ethyl acetate–water (40:100:100, v/v, upper phase). Lane 1 contains the standards: monogalactosylglycerol from the alkaline hydrolysis of spinach monogalactosyl diglyceride, galactose and glucose from commercial sources. Lane 2 is the ^{14}C product produced on alkaline hydrolysis of the radioactive lipid, and lane 3 is the product of the acid hydrolysis of the radioactive mild alkaline hydrolysis product from lane 2. Radioactive spots were detected on a radiochromatogram scanner, and the standards were visualized with periodate–starch spray.

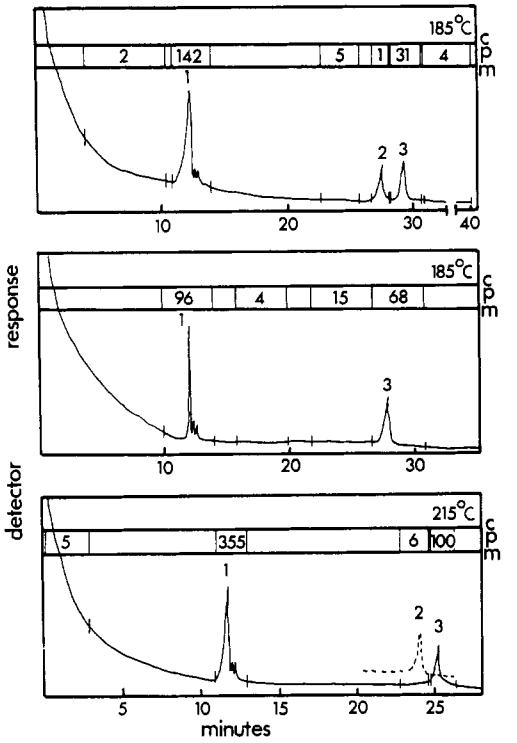

FIGURE 2: Gas–liquid partition chromatography of the radioactive water-soluble alkaline hydrolysis product from the [^{14}C]galactolipid. Conditions of chromatography were as described in the experimental portion of the text. Standard peaks: 1, galactose isomers, 2, α-galactosylglycerol, and 3, β-galactosylglycerol. The maximum temperature of each run is shown in the upper right corner of each figure. The dotted line in the 215° program shows the retention time of α-monogalactosylglycerol from a different experiment run under identical conditions.

standard dipalmitin in a thin-layer system, petroleum ether (bp 30–60°) (Skelly B)–ethyl ether–acetic acid (40:10:0.5, v/v) (R_F 0.8) and silicic acid paper system A (R_F 0.92). A very small amount of [^{14}C]monopalmitin was detected by this technique. The monopalmitin impurity probably can be attributed to a slight hydrolysis of the fatty acid ester bonds of the product. These data demonstrate that the radioactive lipid synthesized from UDP-galactose and dipalmitin in the presence of magnesium ion and an enzyme from young rat brain is monogalactosyl diglyceride.

Characterization of the Galactosidic Linkage of Monogalactosyl Diglyceride. The chloroform-soluble counts from ten incubation tubes were pooled together and subjected to mild alkaline hydrolysis by a procedure modified from Neufeld and Hall (1964). After passing the aqueous phase through a column containing 1 g of Dowex 50 (H^+) layered over 1 g of Dowex 2 (CO_3^{2-}), the solution was evaporated in a conical tube and converted into the trimethylsilyl ether derivative by the addition of 0.2 ml of tetramethylsilane reagent (Sweeley *et al.*, 1966). The retention time of this material was compared with the retention time for the trimethylsilyl derivatives of α-monogalactosylglycerol and β-monogalactosylglycerol (Figure 2). Figure 2 shows that the alkaline hydrolysis product of the [^{14}C]galactolipid had the same retention time as the β-monogalactosylglycerol. Relatively few counts were recovered at the time corresponding to α-monogalactosylglycerol. The radioactivity observed at the time corresponding to carrier galactose indicates that some hydrolysis of the monogalactosylglycerol had occurred during the work-up procedure. These results provide good evidence that the galactosidic linkage of the enzymatically prepared [^{14}C]monogalactosyl diglyceride is of the β configuration.

Further evidence for a β-galactosidic linkage was obtained using specific galactosidases. Radioactive monogalactosyl diglyceride was isolated after typical incubations with UDP-[U-^{14}C]galactose, magnesium ion, dipalmitin, and enzyme from rat brains. The chloroform-soluble material was subjected to paper chromatography in system A, and the radioactive material corresponding to authentic monogalactosyl diglyceride was eluted from the paper with chloroform and counted. This enzymatically synthesized monogalactosyl diglyceride was then subjected to the action of either α- or β-galactosidase (Table I). In all the experiments performed, the β-galactosidase catalyzed the hydrolysis of galactose from the [^{14}C]monogalactosyl diglyceride to a much greater extent than did the α-galactosidase. The α-galactosidase failed to produce water-soluble counts

TABLE I: Action of α- and β-Galactosidases on Monogalactosyl Diglyceride (Labeled with [^{14}C]Galactose).[a]

Incubation	Cpm in Aq Phase after Incubn
Experiment 1	
β-Galactosidase	69
α-Galactosidase buffer	39
(no enzyme)	36
Experiment 2	
β-Galactosidase	120
α-Galactosidase buffer	60
(no enzyme)	60
Experiment 3	
β-Galactosidase	45
β-Galactosidase (zero time control)	13
β-Galactosidase (boiled-enzyme control)	23
α-Galactosidase buffer	28
(no enzyme)	10

[a] Experiment I: To each of three tubes was added 180 cpm of [^{14}C]monogalactosyl diglyceride followed by 0.1 ml of 10% cutscum. The mixture was stirred until the residue of lipid appeared to be well suspended, and then 0.4 ml of water was added. To tube 1 was added 2.7 mg of β-galactosidase, to tube 2 was added 1.2 mg of dialyzed α-galactosidase, and to all three tubes was added 1 ml of buffer (0.2 M disodium phosphate–0.1 M citric acid, pH 6.0). After incubation at 37° for 22 hr, the tubes were placed in a 100° water bath for 3 min and cooled on ice. Chloroform (2 ml) was added and the mixture was centrifuged to separate the layers. A 0.5-ml aliquot of the aqueous phase from the three tubes was counted. Experiment 2: Each of three tubes contained 400 cpm of galactose-labeled monogalactosyl diglyceride. The incubation was carried out for 40 hr using the same concentrations and conditions given above. Experiment 3: Each of five tubes received 500 cpm of [^{14}C]monogalactosyl diglyceride. The concentrations of ingredients were the same as those given above. Tube 2 of expt 3 did not receive the β-galactosidase until the time of extraction, and tube 3 was given enzyme that had been placed in a 100° water bath for 10 min prior to starting the incubation. After incubation for 21 hr at 37 (except in the zero time control), the tubes were extracted by adding 1 ml of methanol and heating the mixture in a 100° water bath for 2 min followed by the addition of 2 ml of chloroform. After centrifuging to separate the layers, an aliquot of the upper aqueous phase was counted.

TABLE II: Biosynthesis of Monogalactosyl Diglyceride (UDP-[U-^{14}C]galactose as the Radioactive Substrate).[a]

Conditions	mμmoles of Monogalactosyl Diglyceride/10 mg of Protein per hr
Experiment 1	
Complete system	5.18
Minus dipalmitin	1.56
Experiment 2	
Complete system	3.40
Minus dipalmitin	2.65
Minus magnesium ion	1.66
Zero-time enzyme control	0
Boiled enzyme control	0

[a] The complete incubation mixture contained 0.41 mM $MgCl_2$, 0.54 mM UDP-[U-^{14}C]galactose, 81.6 mM sodium phosphate buffer (pH 7.89), and 3.16 mg of protein from the 30,000*g* particulate fraction of young rat brain. After incubation for 1 hr at 37°, the lipid was isolated as described in the text and an aliquot was counted.

in amounts significantly higher than the no-enzyme controls. Aliquots of the aqueous phases were taken for chromatography on Whatman No. 1 paper in a descending manner in system F. In this solvent system a radioactive peak was detected at the R_F of galactose only in the tube treated with β-galactosidase. Incubation of monogalactosyl diglyceride (labeled in positions 1 and 3 of the glycerol moiety with ^{14}C) with β-galactosidase produced a radioactive product that cochromatographed with dipalmitin in system A. Under identical incubation conditions α-galactosidase failed to produce [^{14}C]diglyceride.

These results demonstrate that an enzyme in a particulate fraction from the brains of young rats catalyzes the incorporation of galactose from UDP-[U-^{14}C]galactose into a lipid identified as β-D-galactopyranosyl diglyceride.

Conditions Required for the Biosynthesis of Monogalactosyl Diglyceride. Optimum biosynthesis of monogalactosyl diglyceride was attained when UDP-[U-^{14}C]-galactose was incubated with dipalmitin, magnesium ion, and a 30,000*g* particulate fraction of brain from young rats (Table II). Although suspensions of 30,000*g* or 100,000*g* particulate fractions of rat brain can be used directly in the incubation, consistently better stimulation of the synthesis of monogalactosyl diglyceride by exogenous diglyceride was attained if the particulate preparation of brain was lyophilized and washed with acetone prior to being employed. Optimum interaction between enzyme and exogenous diglyceride was obtained by using a slight variation of a technique developed for the biosynthesis of monoglucosyl and diglucosyl diglyceride of *Streptococcus faecalis* (Pieringer, 1968). In this modified method the dry enzyme preparation of known protein content is saturated with an aliquot of a benzene solution of diglyceride of known concentration at room temperature. The benzene is removed by thorough evap-

TABLE III: Requirement for 1,2 Isomer of Diglyceride Substrate.[a]

Acceptor	Δmμmoles of [14C]Monogalactosyl Diglyceride/10 mg of Protein per hr
Benzene only	
2,3-Dipalmitin	0.06
1,2-Dipalmitin	0.80

[a] To each tube containing 1.67 mg of protein in an acetone powder from the microsomal fraction of brains from 17-day-old rats was added 0.05 ml of benzene containing either no diplamitin, 200 mμmoles of 2,3-dipalmitin, or 200 mμmoles of 1,2-dipalmitin. After overnight storage in the freezer, the benzene was evaporated, and the reaction was carried out with reagents given in Table II. After incubation for 1 hr and extraction, an aliquot of the chloroform phase was counted on a planchet. The value obtained for benzene alone was subtracted from the tubes incubated in the presence of 2,3- and 1,2-dipalmitin.

TABLE IV: Biosynthesis of Monogalactosyl Diglyceride (Dipalmitin ([14C]1,3-Glycerol) as Radioactive Substrate).[a]

Conditions	Monogalactosyl Diglyceride Formed (mμmoles)/Protein (10 mg) per hr
Complete system	3.13
UDP-galactose omitted	2.07

[a] The incubation was performed by adding 0.05 ml of benzene solution containing 83 mμmoles of dipalmitin ([14C]1,3-glycerol) to 1.77 mg of protein of an acetone powder of a microsomal fraction of brains from young rats. The benzene was completely evaporated at room temperature by a stream of nitrogen. Sodium phosphate buffer (1 ml of 1 M) (pH 8.0), $MgSO_4$ (0.01 ml of 1.0 M), and UDP-galactose (0.125 ml of 0.02 M) were added in that order at 4°. After incubating for 30 min at 37° and extracting 1.7 ml of 2.0 ml of chloroform was chromatographed. The R_F's of the radioactive galactolipids were detected with a strip scanner and the spots containing the radioactivity in each lane were cut out and counted in a gas-flow geiger counter. The 14C on both sides of the paper was counted and summed. The specific activity of the [14C]dipalmitin was 806 cpm/mμmole under these conditions.

oration either immediately or preferably for this particular reaction after having been in contact with the enzyme in the freezer overnight. Aqueous solutions of buffer, UDP-galactose, and magnesium ion are then added to the residue. Using this method it was possible to demonstrate that the enzyme was stereospecific for 1,2-diglyceride (Table III).

With dipalmitin as the radioactive substrate, the incorporation of [14C]dipalmitin (labeled at the 1 and 3 carbons of the glycerol moiety) into monogalactosyl diglyceride is stimulated by added UDP-galactose (Table IV). The incorporation of radioactivity from either UDP-galactose or diglyceride into monogalactosyl diglyceride and the stimulation of the incorporation of this radioactivity by the presence of the other compound demonstrate that UDP-galactose and diglyceride are the direct precursors of monogalactosyl diglyceride of brain.

Cellular Location of Biosynthetic Enzyme. Whole homogenates of rat brains were separated into nuclear, crude mitochondrial, microsomal, and soluble fractions by a slight modification of the procedure of DeRobertis *et al.* (1962). The specific activities of each of the fractions expressed as millimicromoles per milligram of protein per hour were nuclear, 0.1; crude mitochondrial, 0.09; microsomal, 0.18; and soluble, 0.016. Because the microsomal fraction appeared to have the highest specific activity, it was usually employed as the source of enzyme.

Comparison of Different Diglycerides as Substrates. Of seven diglycerides (labeled with 14C at the 1 and 3 carbon atoms of the glycerol moiety) dipalmitin and distearin appeared to function best as substrates in the synthesis of monogalactosyl diglyceride (Table V). It is interesting to note that the best substrates are the least water soluble of the diglycerides. This finding indicates an apparent true enzyme specificity for the diglyceride substrate having more saturated and longer chain fatty acids.

TABLE V: Comparison of Different Diglycerides as Substrates.[a]

[14C]Diglyceride Substrate	Monogalactosyl Diglyceride Formed (mμmoles)/Protein (10 mg) per hr
Dicaprin	0.86
Dilaurin	0.75
Dimyristin	0.80
Dipalmitin	2.33
Distearin	2.15
Diolein	1.53
Dilinolein	1.14

[a] The incubation containing 120 mμmoles of each diglyceride ([14C]1,3-glycerol) (2016 cpm/mμmole) was carried out as described in Table IV. The radioactivity of each lipid was quantitated by counting spots cut out of the chromatogram in a scintillation spectrometer. The above values are averages of two experiments.

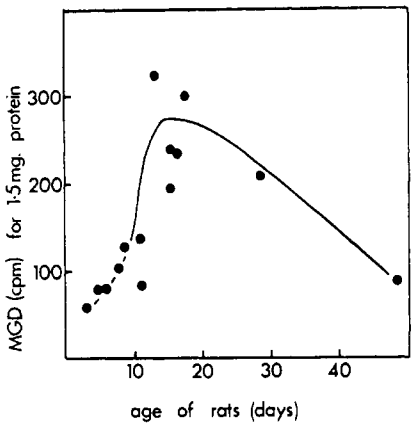

FIGURE 3: Effect of age on the synthesis of [^{14}C]monogalactosyl diglyceride *in vitro* using enzyme prepared from the brains of rats. Enzyme concentration curves were made using the 100,000*g* particulate fraction of brains of rats of varying age. Four to six brains of rats of each age were combined for each enzyme preparation. All of the experiments were carried out in exactly the same manner. Each tube contained weighed amounts of acetone powder (ranging from 0.165 to 7.5 mg of protein) from the age of the rat to be tested. To each tube was added 100 mμmoles of 1,2(2,3)-dipalmitin, 10 mμmoles of $MgSO_4$, 356 mμmoles of UDP-[U-^{14}C]-galactose (391 cpm/mμmole), and 0.1 ml of sodium phosphate buffer (pH 7.9), all in a total volume of 0.15 ml. After incubation at 37° for 1 hr, the lipid was extracted as usual and an aliquot of the chloroform was counted. At 1.5 mg of protein, the slopes of all the curves resulting from a plot of milligrams of protein against counts per minute in lipid phase were linear. The monogalactosyl diglyceride (counts per minute) synthesized with 1. 5mg of protein is shown plotted against the age of the rat from which the enzyme from brain was prepared.

Other Properties of the Biosynthetic Reaction. The incorporation of galactose from UDP-galactose into monogalactosyl diglyceride proceeded at a linear rate up to at least 2 hr of incubation time. Maximum synthesis was achieved when the final concentration of UDP-galactose was 2.25 mM in the presence of saturating concentrations (0.1 μmole) of dipalmitin. The concentration of diglyceride actually in aqueous solution could not be determined with accuracy. For the latter reason a concentration-curve type of experiment for diglyceride was not attempted. The presence of exogenous magnesium ion, while not being absolutely essential, did stimulate the enzymatic reaction. The optimum concentration of added magnesium sulfate was 0.091 M. Concentrations of 0.12 and 0.06 M magnesium sulfate produced approximately one-half as much product as did the optimum concentration.

Effect of Age of Rat on Activity of Brain Enzyme. Microsomal preparations of the enzyme were made from brains of rats varying between 3 and 49 days of age. Incubations were carried out to test the ability of the microsomal preparations to catalyze the incorporation of galactose from UDP-[U-^{14}C]galactose into monogalactosyl diglyceride using the typical incubation system. Enzyme concentration curves were prepared by plotting milligrams of protein *vs.* amount of monogalactosyl diglyceride synthesized for the enzyme preparations from different ages. All of the curves were linear up to at least 1.5 mg of protein. The counts per minute at 1.5 mg of protein were plotted against the age of the rat from which the enzyme was prepared (Figure 3). The enzyme activity was relatively low in very young rats and in rats over about 20-days old. Maximum synthesis of monogalactosyl diglyceride was obtained with the microsomal fraction of brain from rats between 14- and 18-days *post partum*. This age of maximum enzyme activity occurs within the period of maximum rate of myelin formation in the rat (10–20 days) (McIlwain, 1966), and the period in which the concentration of monogalactosyl diglyceride in brain is increasing most rapidly (Wells and Dittmer, 1967).

Discussion

The data presented in this paper demonstrate that a particulate enzyme from rat brain catalyzes the synthesis of monogalactosyl diglyceride according to the following reaction: UDP-galactose + 1,2-diglyceride → 1,2-di-*O*-acyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol.

The structure of the galactolipid biosynthesized *in vitro* is identical with the structure of the monogalactosyl diglyceride isolated from bovine spinal cord by Steim (1967). The enzyme responsible for the formation of this galactolipid displayed some specificity for the diglyceride substrate. The enzyme required the 1,2 isomer and preferred diglycerides containing longer chain and saturated fatty acid constituents.

Under the conditions of incubation employed in this study at least 90% of the radioactive galactose of UDP-galactose is incorporated into monogalactosyl diglyceride. No detectable amounts of synthesis of a sphingogalactolipid or of a monoalkyl ether derivative of monogalactosyl diglyceride were found. The conversion of practically all of the radioactivity of the glycolipid into a water-soluble compound (identified as monogalactosylglycerol) upon treatment with dilute alkali precludes the possibility of the presence of these two types of nonsaponifiable lipid.

The activity of the biosynthetic enzyme was dependent upon the age of the rat from which the brain enzyme preparation was made. The activity of the enzyme was greatest between 14- and 18-days *post partum*. These data compliment the findings of Wells and Dittmer (1967) who reported that the concentration of monogalactosyl diglyceride in brain increased very slowly up to 10 days of age, showed a dramatic rise between day 12 and day 24, and rose rather slowly after the 24th day. The age (14–18 days) during which the biosynthetic enzyme is quite active corresponds to the age generally agreed upon to be the period of maximum myelination in the rat (McIlwain, 1966). These results indicate that monogalactosyl diglyceride and the enzyme responsible for its synthesis may have an important role in the process of myelination.

Acknowledgment

The authors are indebted to Mrs. Margot Gruenstein of the Fels Research Institute who voluntarily agreed

to breed and grow the rats used in this study. Without her cooperation this study would have been considerably more difficult to carry out.

References

Courtois, J. E., and Petek, F. I. (1966), *Methods Enzymol. 8*, 565.

DeRobertis, E., Pellegrino deIraldi, A., Rodriguez De Lores Arnais, A., and Salganicoff, L. (1962), *J. Neurochem. 9*, 23.

Frisell, W. R., Meech, L. A., and MacKenzie, C. G. (1954), *J. Biol. Chem. 207*, 709.

Jermyn, M. A., and Isherwood, F. A. (1949), *Biochem. J. 44*, 402.

Krabisch, L., and Borgstrom, B. (1965), *J. Lipid Res. 6*, 156.

Lands, W. E. M., Pieringer, R. A., Slakey, P. M., and Zschocke, A. (1966), *Lipids 1*, 444.

Lennarz, W. J. (1964), *J. Biol. Chem. 239*, PC3110.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Marinetti, G. V., Erbland, J., and Kochen, J. (1957), *Federation Proc. 16*, 837.

McIlwain, H. (1966), Biochemistry and The Central Nervous System, 3rd ed, London, Churchill.

Metzenberg, R. L., and Mitchell, H. K. (1954), *J. Am. Chem. Soc. 76*, 4187.

Nelson, N. (1944), *J. Biol. Chem. 153*, 375.

Neufeld, E. F., and Hall, C. W. (1964), *Biochem. Biophys. Res. Commun. 14*, 503.

Norton, W. T. and Brotz, M. (1963), *Biochem. Biophys. Res. Commun. 12*, 198.

Pelick, N., Wilson, T. L., Miller, M. E., Angeloni, F. M., and Steim, J. M. (1965), *J. Am. Oil Chemists' Soc. 42*, 393.

Pieringer, R. A. (1968), *J. Biol. Chem.* (in press).

Pieringer, R. A., Bonner, H., Jr., and Kunnes, R. S. (1967), *J. Biol. Chem. 242*, 2719.

Rouser, G., Kritchevsky, G., Simon, G., and Nelson, G. J. (1967), *Lipids 2*, 37.

Rumsby, M. G. (1967), *J. Neurochem. 14*, 733.

Rumsby, M. G., and Gray, I. K. (1965), *J. Neurochem. 12*, 1005.

Steim, J. M. (1967), *Biochim. Biophys. Acta 114*, 118.

Steim, J. M., and Benson, A. A. (1963), *Federation Proc. 22*, 299.

Stern, I., and Shapiro, B. (1953), *J. Clin. Pathol. 6*, 158.

Sweeley, C. C., Wells, W. W., and Bentley, R. (1966), *Methods Enzymol. 8*, 95.

Tarlov, A. R., and Kennedy, E. P. (1965), *J. Biol. Chem. 240*, 49.

Vorbeck, M. L., and Marinetti, G. V. (1965), *J. Lipid Res. 6*, 3.

Wells, M. A., and Dittmer, J. C. (1967), *Biochemistry 6*, 3169.

Wenger, D. A., Petitpas, J. W., and Pieringer, R. A. (1967), *Federation Proc. 26*, 675.

Zill, L. P., and Harmon, E. A. (1962), *Biochim. Biophys. Acta 57*, 573.