those in dogs, plasma DHEA-SO_4 in the human might contribute significantly to steroid hormone production. Furthermore, Young and Hall (1968) observed the biosynthesis of cholesteryl sulfate by slices of rabbit testis. Based on these reports and other evidence steroid sulfates are present in gonadal tissue although their levels have not been documented.

Combinations of unconjugated steroids in low concentrations have not been tested for inhibitory effects on steroid sulfate cleavage but some kind of cumulative inhibition would be expected to occur. Furthermore, the concentrations and kinds of related steroid sulfates present will in part regulate the cleavage of any single steroid sulfate member. Clearly then, the contribution from steroid sulfates to steroid hormone production, although small, can be highly variable and well suited to be an auxiliary biosynthetic route.

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Synthesis of Cerebroside by Brain from Uridine Diphosphate Galactose and Ceramide Containing Hydroxy Fatty Acid*

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ABSTRACT: A crude microsomal fraction from the brains of young mice catalyzes the formation of galactosylceramide containing hydroxy fatty acids from 2-hydroxy fatty acid ceramide and uridine diphosphate galactose. The 2-hydroxy fatty acid ceramide is most effective as a substrate when it is spread over the large surface area offered by the diatomaceous earth, Celite, in the absence of detergent. The enzyme system exhibits high specificity: ceramide containing nonhydroxy fatty acids will not stimulate galactose incorporation and only uridine diphosphate galactose or a uridine diphosphate galactose generating system acts as a sugar donor. The product of the incubation mixture has been characterized as 2-hydroxy fatty acid galactosylceramide by a variety of chromatographic and chemical procedures. Some other properties of the system were investigated. The results are discussed and related to our present knowledge of sphingolipid metabolism.

An active psychosine[1]-synthesizing system has been demonstrated by Cleland and Kennedy (1960). Brady (1962) reported that psychosine was an acceptor for stearoyl-CoA in a rat brain microsomal system. On the basis of this work it has been generally accepted (Olson, 1966) that the biosynthetic route for cerebroside follows the route outlined in eq 1 and 2.

$$\text{sphingosine} + \text{UDPGal} \longrightarrow \text{pyschosine} + \text{UDP} \quad (1)$$

$$\text{psychosine} + \text{fatty acyl-CoA} \longrightarrow \text{cerebroside} + \text{CoA} \quad (2)$$

Since this scheme is at variance with a preliminary observation in this laboratory (Radin, 1959), that ceramide stimulates the incorporation of galactose into lipids, we have reinvestigated the biosynthesis of cerebroside.

Experimental Section

Materials.. UDPGal and dithiothreitol were purchased from Calbiochem (Los Angeles, Calif.). UDPGlc was a product of Sigma Chemical Co. (St. Louis, Mo.) and the nonionic detergent Tween 20 was obtained from Atlas Chemical Industries (Wilmington, Del.). Analytical grade Celite, a purified diatomaceous earth, was a Johns-Manville product. All other chemicals used were reagent grade and all solvents were redistilled before use. Radioactivity incorporation studies utilized uniformly labeled α-D-[^{14}C]glucose-1-P (10.3 mCi/mmole), uniformly labeled α-D-[^{14}C]galactose-1-P (5.0 mCi/mmole), and uniformly labeled [galactose-^{14}C]UDPGal (5.9 mCi/

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[1] Abbreviations not listed in the 1966 issue of *Biochemistry 5*, 1445, are: nfa and hfa, nonhydroxy fatty acid and 2-hydroxy fatty acid (chain length may be indicated in a prefix, *e.g.*, C_{18}-nfa); LCB, a mixture of long-chain bases (sphingosine and some dihydrosphingosine); ceramide, fatty acylamide of LCB (the fatty acid may be indicated by a prefix); psychosine, LCB galactoside; cerebroside, galactosylceramide; BSC, beef spinal cord sphingopids.

mmole), all obtained from Calbiochem and used without dilution. White mice (Swiss-Webster) were obtained from Spartan Laboratories, Haslett, Mich.

Analytical Methods. Thin-layer chromatography was done on 0.25- or 0.50-mm layers of silica gel G (Brinkmann Instruments, Westbury, N. Y.) using a streak applicator developed in part in this laboratory (Applied Science Laboratories, State College, Pa.). Separation of cerebrosides into four classes, C_{18} and long-chain hfa-cerebrosides and C_{18} and long-chain nfa cerebrosides, was accomplished by two developments with chloroform–methanol–water (144:25:2.8) (all solvent systems are given as volume ratios). To separate nfa ceramide from hfa ceramide, thin-layer chromatography with chloroform–methanol–acetic acid (90:2:8) was used (Bowen and Radin, 1968b). Sphingosine and dihydrosphingosine were identified by thin-layer chromatography in chloroform–methanol–water–NH_4OH (280:70:6:1) (Sambasivarao and McCluer, 1963). The same solvent system was utilized to separate cerebroside from glucosylceramide on borate-impregnated silica gel plates (Kean, 1966). Glucose and galactose were clearly separated on commercial silica gel plates (5763 of Brinkmann Instruments) by development in 1-butanol–pyridine–0.1 M HCl (5:3:2). Lipid spots were visualized with iodine vapors or with bromothymol blue and the sugars were located with aniline phthalate. When lipids were to be recovered from a thin-layer chromatography plate the powder was transferred to a small column and the lipid eluted with chloroform–methanol–water (7:7:1) (Davison and Graham-Wolfaard, 1963), then repurified in a chloroform–methanol–water partition system (Folch *et al.*, 1957). Radioautography of thin-layer chromatography plates utilized Kodak No-Screen X-Ray film, with exposures of 2–14 days.

Radioactivity was determined in a Packard Tri-Carb scintillation counter by evaporating the samples to dryness in vials and dissolving the residue in a toluene–absolute ethanol (95:5) mixture containing scintillators. When necessary, quench corrections were carried out using an internal standard.

Fatty acids were identified by programmed temperature gas–liquid partition chromatography using polyester and Apiezon columns (Radin, 1965). Nonhydroxy fatty acids from cerebroside and ceramide were chromatographed as the esters after methanolysis in methanolic HCl (Kishimoto and Radin, 1965). Hydroxy fatty acid methyl esters were acetylated with isopropenyl acetate before chromatography (Kishimoto and Radin, 1963). Infrared spectra were obtained from samples in KBr pellets using a Perkin-Elmer Infracord Model 237 spectrophotometer.

Preparation of Lipid Substrates and Standards. BSC and cerebrosides were prepared by the method of Radin and Brown (1960). Ceramides were prepared by the method of Carter *et al.* (1961) using cerebroside as starting material. The product was fractionated into nfa and hfa ceramides by column chromatography on Unisil silica gel (Clarkson Chemical Co., Williamsport, Pa.) using stepwise elution with chloroform, chloroform–methanol (99:1), chloroform–methanol (98:2), and chloroform–methanol (97:3). The fractions containing nfa and hfa ceramide were pooled separately. The preparations exhibited infrared spectra typical of ceramide. The fatty acids of nfa ceramide consisted primarily of stearate, lignocerate, and nervonate, while those from the hfa ceramide preparation consisted primarily of cerebronate and hydroxystearate. This distribution of fatty acids reflects that of the starting material (Kishimoto and Radin, 1959; Skipski *et al.*, 1959). Examination by thin-layer chromatography of the LCB remaining after methanolysis showed that both nfa ceramide and hfa ceramide contained mainly sphingosine, with some dihydrosphingosine.

The procedure of Kopaczyk and Radin (1965) was used to synthesize stearoyl LCB and stearoylpsychosine from LCB and psychosine, respectively. LCB was prepared from BSC by the procedure of Carter *et al.* (1951). [Galactose-6-^{3}H]stearoylpsychosine was prepared as described by Bowen and Radin (1968a). Glucosylceramide was isolated from a Gaucher spleen surgically removed from a patient.

Preparation of Crude Microsome Fraction. Mice (15–19-days old) were decapitated and the brains removed and homogenized with six volumes of 0.25 M sucrose containing 0.01 M nicotinamide. A Potter-Elvehjem homogenizer with a mechanically driven Teflon pestle was used. After the addition of three more volumes of homogenizing solution the homogenate was centrifuged at 11,000*g* for 15 min at 2°. The supernatant from this step was centrifuged for 45 min at 105,000*g* at 2°. The pellet was suspended in enough sucrose–nicotinamide to yield a final volume of 0.8 ml/g of brain.

Assay for Cerebroside Formation. To assay the ability of various lipids to stimulate the incorporation of [^{14}C]-galactose into cerebroside, we coated the substrate to be tested onto 50 mg of Celite by evaporation from chloroform–methanol (2:1) under nitrogen. This was followed by addition of 25 μmoles of Tris-HCl, 0.5 μmole of dithiothreitol, 1 μmole of ATP, 0.07 μmole of UDPGlc, 0.04 μmole of [^{14}C]galactose-1-P, and 0.25 ml of the crude microsomal fraction, all in a total volume of 0.5 ml. The ATP was neutralized with KOH before use and Tris-HCl was added as a 1 M solution at pH 7.4, measured at 25°. The incubation was carried out at 37° with violent agitation for 120 min.

The incubation was terminated by the addition of 0.5 mg of BSC in 10 ml of chloroform–methanol (2:1) and pressure filtration to remove protein. After a rinse of the incubation tube and filter with an additional 1 ml of chloroform–methanol (2:1), 2.3 ml of 2 M KCl was added to the filtrate, the resultant layers were separated by centrifugation, and the upper layer was discarded. The lower layer was washed twice more with 5 ml of water–methanol (1:1) containing 1 M KCl, and once more with water–methanol (1:1) containing 0.1 M citrate (an equimolar mixture of citric acid and trisodium citrate). The wash with an acidic citrate solution was designed to remove psychosine. When LCB was tested as a substrate for cerebroside formation, this wash was repeated several times. The remaining lower layer was then evaporated to dryness under nitrogen and subjected to alkaline methanolysis in a total volume of 3 ml as described by Kishimoto *et al.* (1965). The lipid-containing solvent layer

TABLE I: Requirements for Incorporation of [^{14}C]-Galactose into Cerebroside.[a]

Modification	[^{14}C]Galactose Incorp[b] (mμmole) I	II	III
None	2.0	2.9	3.0
− hfa ceramide	0.3	0.5	0.3
− Celite	0.2	0.6	0.9
− UDPGlc	0.6	0.8	0.4
− ATP	0.7	1.3	0.9
+ Mg (1.0 μmole)	2.0		3.0
+ Tween (added before microsomes)	0.7		1.3
+ Tween (added after microsomes)	1.4	2.1	2.5
+ Tween−hfa ceramide	0.1		

[a] Incubations, containing 0.5 mg of hfa ceramide coated on Celite, were conducted as described in the text. Where indicated Tween 20 (1.5 mg in aqueous solution) was added. [b] A millimicromole of [^{14}C]galactose represents 8800 cpm under our counting conditions.

was washed once with 1 ml of methanol–water (1:1) and the radioactivity in an aliquot was determined. When necessary, the rest of the sample was examined by thin-layer chromatography and radioautography was carried out.

Assay for Psychosine Formation. If psychosine was to be recovered, the incubation was treated as for cerebroside assay except that after initial addition of 2 M KCl, the mixture was adjusted to approximately pH 10 using phenolphthalein as an indicator. This procedure prevents psychosine from being removed although the unutilized [^{14}C]galactose is washed out (Cleland and Kennedy, 1960). Psychosine, present in the citrate washes, was recovered by adjusting the citrate solution to pH 10, adding a half-volume of 4 M KCl, and extracting the psychosine by two washes with a half-volume of chloroform.

Results

Requirements for Incorporation of [^{14}C]Galactose into Cerebroside. Table I describes three experiments demonstrating the effect of various variables on the formation of cerebrosides. Essential requirements include hfa ceramide as acceptor as well as a [galactose-^{14}C]UDPGal-generating system. ATP stimulates the reaction considerably. This requirement may arise from the need for regeneration of [^{14}C]galactose-1-P (Burton *et al.*, 1958) that might be degraded by phosphatase in our preparation. Metal cofactor requirements were not studied, except to demonstrate that the unwashed microsomal fraction did not require additional magnesium or manganese.

Presentation of Lipid Substrate to the Enzyme System. The problem of solubilizing lipid substrates for enzyme studies is usually met by varying the type and concentration of detergents which are used for emulsifying the substrate. The end result is a compromise between the emulsifying capabilities of the detergent for the substrate in question and unwanted side effects. Spreading the substrate over the large surface area offered by Celite (Radin, 1959) appears to make the ceramide available for biosynthetic reactions (compare lines 1 and 3 in Table I). Addition of relatively small amounts of nonionic detergent to the incubation mixture, prior to addition of the enzyme, has a pronounced inhibitory effect on galactose incorporation. This effect is greatly lessened if the order of addition is reversed (Table I).

TABLE II: Sugar Donor for Cerebroside Synthesis.[a]

Sugar Donor	[^{14}C]Sugar Incorp (mμmole) Acceptor	Control
[^{14}C]Galactose-1-P (40 mμmoles) + UDPGlc (70 mμmoles)	1.54	0.23
[^{14}C]Glucose-1-P (10 mμmoles) + UDPGal (70 mμmoles)	0.05	0.02
[Galactose-^{14}C]UDPGal (8.5 mμmoles)	0.77	0.10
[Galactose-^{14}C]UDPGal (17 mμmoles)	1.02	0.12

[a] Incubations were carried out as described in the text except that the only sugar and/or nucleotide sugar additions were as indicated above. The acceptor was 0.5 mg of hfa ceramide coated on Celite. Control incubations contained uncoated Celite.

Identification of Galactose Donor. Synthesis of UDPGal from galactose-1-P and UDPGlc by microsomes from the brains of young animals has been demonstrated by Cleland and Kennedy (1960). The involvement of this reaction in the biosynthesis of cerebroside was indicated by the work of Burton *et al.* (1958). The presence of galactose-1-P uridyl transferase activity was checked in our system in a series of incubations without lipid acceptor, where the formation of [galactose-^{14}C]-UDPGal was followed by isolating charcoal-adsorbable radioactivity (Ng *et al.*, 1964). The rate of this reaction is such that under our conditions (without acceptor) it supplies [^{14}C]UDPGal more rapidly than it is incorporated into cerebroside. The adsorbed radioactive material was eluted with ethanol–14 mM NH_4OH (1:1) and hydrolyzed by boiling in the presence of Amberlite IR-120-H^+ (Bourne *et al.*, 1965). The radioactive product of this hydrolysis was identified as galactose by thin-layer chromatography and radioautography. It was also demonstrated that [galactose-^{14}C]UDPGal could substitute for [^{14}C]galactose-1-P and UDPGlc (Table II). Incubation with a [glucose-^{14}C]UDPGlc-generating system ([^{14}C]glucose-1-P and UDPGal) did not bring about significant incorporation of radioactivity into the cerebroside-containing lipid fraction (Table II).

Specificity of the Enzyme System for Substrate. The experimental results tabulated in Table III indicated that

TABLE III: Acceptors for Incorporation of [^{14}C]Galactose into Cerebroside.[a]

Acceptor	[^{14}C]Galactose Incorp (mμmole)			
	I	II	III	IV
hfa ceramide	1.9	3.1	2.9	2.0
hfa ceramide (purified by thin-layer chromatography)	1.6	2.9		
nfa ceramide	0.4		0.7	
nfa ceramide (purified by thin-layer chromatography)	0.4	0.5		
Stearoyl LCB	0.5	0.5		0.4
LCB	0.6[b]			0.6[b]
nfa ceramide + hfa ceramide	2.2		3.2	
None	0.3	0.4	0.5	0.3

[a] Incubations, containing 0.5 mg of each of the indicated lipids coated on Celite, were conducted as described in the text. [b] When LCB was present as the acceptor approximately 1.2 mμmoles of [^{14}C]galactose was incorporated into psychosine (assayed as described in the text). No psychosine was formed in any of the other incubations.

only hfa ceramide was effective in stimulating synthesis of cerebroside. The nfa ceramide prepared from naturally occurring cerebroside and synthetic stearoyl LCB were both inactive as galactose acceptors, as was glucosylceramide (experiment not shown). The acceptor activity of hfa and nfa ceramide was not affected upon further purification by thin-layer chromatography. LCB was active as a galactose acceptor (Table III); however, the end product of this reaction was primarily psychosine. The slight stimulation by LCB of galactose incorporation into the lipid fraction containing cerebroside will be discussed below. No psychosine (less than 0.1 mμmole) was produced if ceramide was present as the galactose acceptor.

It has recently been shown that 3-ketodihydrosphingosine is an intermediate in the biosynthesis of sphingosine and dihydrosphingosine (Braun and Snell, 1968). To control for the possibility that the active acceptor in our incubation system was a ketoceramide contaminating the hfa ceramide preparation, a sample of substrate was treated with sodium borohydride as described by Gaver and Sweeley (1966). The hfa ceramide thus treated suffered no loss in activity as a galactose acceptor.

Identification of the Reaction Product. Figure 1 shows a typical thin-layer chromatography pattern of the cerebroside-containing lipid fraction, superposed on a radioautograph obtained from the same plate. Four cerebroside spots, A–D, are identifiable. In the standards lane (center) spots A, C, and D are from the BSC while spot B is synthetic stearoylpsychosine. In a separate experiment, preparative thin-layer chromatography was conducted on a sample of BSC and the lipids corresponding to spots A, C, and D were isolated. The three samples showed infrared spectra typical of cerebroside. The fatty acids from spot A were primarily the saturated and monounsaturated C_{24}-nfa, along with a variety of other long-chain nfa. The fatty acids isolated from spot C were predominantly the saturated C_{24}-hfa, along with small amounts of the other long-chain hfa. Spot D contained the C_{18}-hfa almost exclusively. The LCB remaining after hydrolysis was characterized by thin-layer chromatography. All the samples contained sphingosine as well as some dihydrosphingosine. On the basis of this work, as well as previous investigations into the separation of cerebrosides by thin-layer chromatography (Hooghwinkel *et al.*, 1964; Suomi and Agranoff, 1965), the lipids of spot A were identified as long-chain nfa cerebrosides, those of spot C as long-chain hfa cerebrosides, and the lipids of spot D as C_{18}-hfa cerebroside.

Figure 1 (right lane) demonstrates that if hfa ceramide is added as an acceptor, the primary reaction product cochromatographs with both long-chain and C_{18}-hfa cerebrosides. A total of about 15 radioautographs were run to identify the radioactive end product of various incubations similar to those described in Tables I and II. In all cases, whenever hfa ceramide or a mixture of ceramides was present, the end products were primarily the C_{18} and long-chain hfa cerebrosides. In some experiments a ceramide fraction enriched in long-chain hfa ceramide was used as a substrate. The primary product was long-chain hfa cerebroside, at the expense of C_{18}-hfa cerebroside. The enzyme system does not appear to have high specificity for chain length and it is of interest to note that C_{18}-hfa cerebroside is a very minor com-

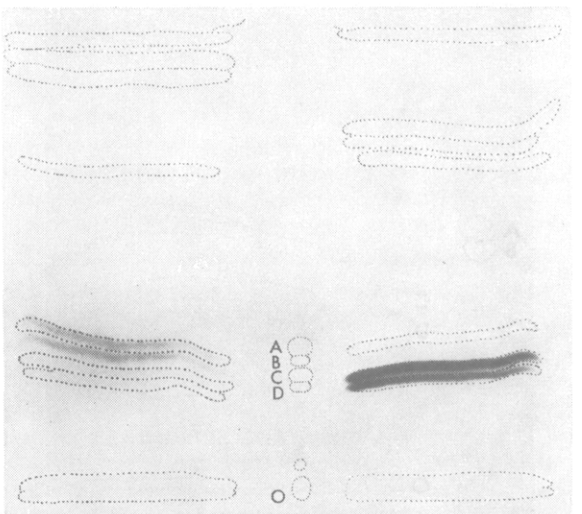

FIGURE 1: Radioautograph of cerebroside-containing fractions, chromatographed so as to differentiate several classes of cerebrosides. Lipids were applied to the origin (0) of silica gel thin-layer chromatography plates and developed twice in chloroform–methanol–water (144:25:2.8). The dotted lines indicate areas visualized by iodine vapors. The right lane contains lipids isolated from an incubation mixture containing 0.5 mg of hfa ceramide as galactose acceptor; the left lane contains lipids from an incubation tube containing 0.5 mg of nfa ceramide. The center lane shows BSC plus stearoylpsychosine. Spot A, long-chain nfa cerebroside; spot B, C_{18}-hfa cerebroside; spot C, long-chain hfa cerebroside; spot D, C_{18}-hfa cerebroside. For details, see text.

ponent of these mouse brains (P. Morell and N. S. Radin, unpublished observations).

The radioautograph obtained from the lipids of incubations without an acceptor or with added nfa ceramide (Figure 1, left lane) showed radioactivity in the long-chain and C_{18}-nfa cerebrosides. The presence of nfa ceramide did not appreciably affect the total incorporation or distribution of [^{14}C]galactose. If Tween 20 was present in control incubations, no nfa cerebroside synthesis at all was observable. Although LCB did cause some stimulation of galactose incorporation into the cerebroside-containing lipid fraction (Table II), radioautography demonstrated that this was accounted for by a slight increase in synthesis of some unidentified lipids migrating below cerebrosides on thin-layer chromatography.

To identify the product as being a galactosylceramide the cerebroside-containing fraction from a standard incubation mixture was chromatographed on a borate-containing thin-layer chromatography plate (Figure 2). Glucosyl- and galactosylceramides were clearly separated. Almost all of the radioactivity cochromatographed with the hfa cerebrosides from BSC; no radioactivity was observed in the glucosylceramide region.

To further identify the radioactive product as cerebroside, a sample was subjected to hydrolysis in a 1-butanol–water mixture containing 1 M KOH as described by Taketomi and Yamakawa (1963). The hydrolysis mixture was acidified with 0.1 M citric acid and extracted with C-M (2:1). Psychosine was isolated from the citrate layer as described in the Experimental Section and identified as such by means of thin-layer chromatography and radioautography. Only one radioactive spot corresponding to psychosine and clearly separated from cerebroside, could be seen. The yield of radioactive psychosine, based on the radioactivity in the cerebroside-containing lipid fraction, was 74%. A control hydrolysis with synthetic [galactose-^{3}H]stearoylpsychosine gave a 67% yield under the same conditions.

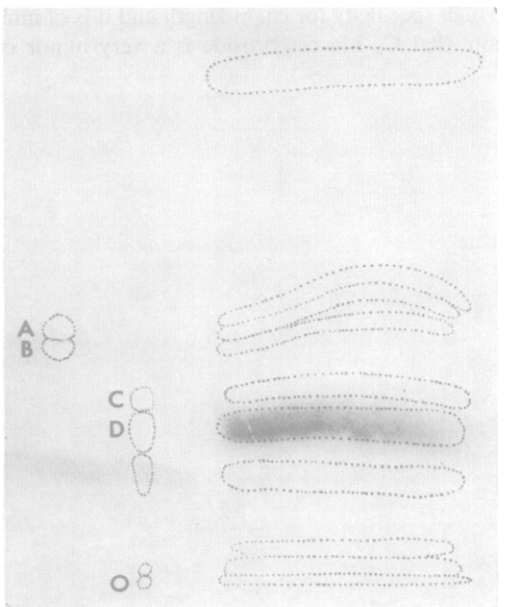

FIGURE 2: Radioautograph of a cerebroside-containing fraction, chromatographed so as to separate cerebroside from glucosylceramide. Lipids were applied to the origin (0) of a borate-impregnated silica gel plate and developed twice in chloroform–methanol–water–NH_4OH (280:70:6:1). The dotted lines indicate areas visualized by iodine vapors. The right lane contains lipids isolated from an incubation mixture containing hfa ceramide as a galactose acceptor. The center lane contains BSC and the left lane contains glucosylceramide. Spots A and B presumably represent the long-chain and short-chain glucosylceramides, respectively, while spots C and D probably represent the nfa and hfa cerebrosides, respectively.

Effect of Incubation Time and Amount of Substrate on Cerebroside Formation. Figure 3 indicates that, under our conditions, synthesis of hfa cerebroside is linear with time for at least 120 min. Substrate saturation experiments (Figure 4) indicate that the system approaches saturation at about 1.0 mg (about 1.4 μmoles) of hfa ceramide.

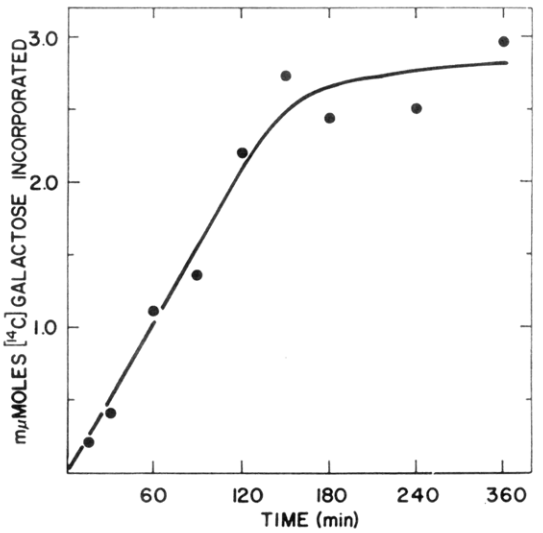

FIGURE 3: Time course of incorporation of [^{14}C]galactose into the cerebroside-containing lipid fraction. Incubation conditions are as described in the text (0.5 mg of hfa ceramide as acceptor) except that time of incubation was varied as indicated.

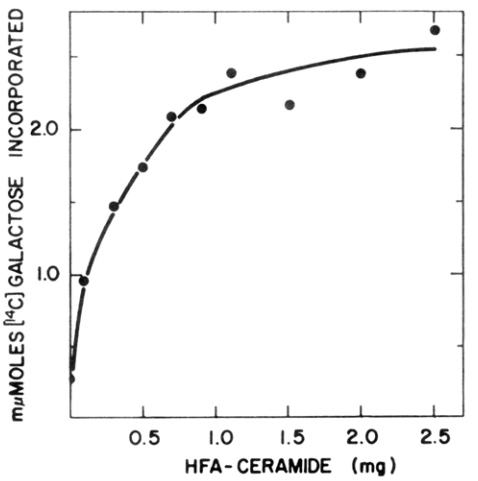

FIGURE 4: Effect of hfa ceramide concentration on incorporation of [^{14}C]galactose into the cerebroside-containing lipid fraction. Incubation conditions are as described in the text except that the amount of hfa ceramide (coated on 50 mg of Celite) was varied as indicated.

Discussion

There is evidence that sphingosine may be acylated to form nfa ceramide (Sribney, 1966):

$$\text{sphingosine} + \text{fatty acyl-CoA} \longrightarrow \text{nfa ceramide} + \text{CoA} \quad (3)$$

Another possible route for formation of nfa ceramide has been demonstrated (Gatt, 1966) (eq 4) although it is likely that the primary function of this enzyme is catabolic. The ability of nfa ceramide to serve as substrate

$$\text{sphingosine} + \text{fatty acid} \longrightarrow \text{nfa ceramide} + H_2O \quad (4)$$

for the formation of sphingomyelin (eq 5) has been well documented (Sribney and Kennedy, 1958; Kopaczyk and Radin, 1965; Fujino *et al.*, 1968) and a preliminary report (Basu, 1968) indicates that a nfa ceramide is precursor to glucosylceramide (eq 6), an intermediate in ganglioside biosynthesis.

$$\text{nfa ceramide} + \text{CDP-choline} \longrightarrow \text{sphingomyelin} + \text{CDP} \quad (5)$$

$$\text{nfa ceramide} + \text{UDPGlc} \longrightarrow \text{glucosylceramide} + \text{UDP} \quad (6)$$

The data presented in this paper offer evidence that the reaction shown in eq 7 is catalyzed by a crude microsomal fraction from the brains of young mice. The

$$\text{hfa ceramide} + \text{UDPGal} \longrightarrow \text{hfa cerebroside} + \text{UDP} \quad (7)$$

presence of this hfa galactosyl ceramide transferase activity has not previously been observed. The lipid substrate for this reaction, hfa ceramide, has been characterized by several chemical, physical, and chromatographic criteria. However, under our reaction conditions, the utilization of this substrate is less than 1% of the added hfa ceramide and we cannot completely discard the possibility that some unknown contaminant is the galactose acceptor. The linearity of the time course of the reaction (Figure 3) indicates that hfa ceramide is the immediate precursor and is not first converted, by a rate-limiting reaction, into another product which is the immediate precursor of hfa cerebroside. Up to 10% of the radioactive UDPGal was incorporated into hfa cerebroside under our incubation conditions. The radioactive lipid product was characterized by its stability to alkali at room temperature as well as its degradation by alkali at high temperature to yield psychosine. The strongest evidence was the excellent agreement shown between carrier hfa cerebroside bands and radioautograph bands in two different thin-layer chromatography systems. A complete characterization of the product, in particular the nature of the sphingosine base (sphingosine or dihydrosphingosine, *threo* or *erythro* configuration, etc.), awaits further experiments.

The presence of a small pool of hfa ceramide in brain has been demonstrated (Klenk and Huang, 1968). The hfa chain-length distribution in these ceramides was found to be similar to that of hfa cerebroside. These observations support the hypothesis that the reaction described by eq 7 takes place *in vivo*. A brain cerebroside galactosidase has been shown to be active on hfa cerebroside, forming hfa ceramide (Hajra *et al.*, 1966). Possibly hfa ceramide is an intermediate in *both* the synthesis and degradation of hfa cerebroside. A route for hfa ceramide synthesis is yet to be demonstrated.

Schneider and Kennedy (1968) injected tritiated dihydrosphingomyelin into tail veins of rats and observed that a significant portion of the radioactivity in peripheral organs could be recovered in a fraction identified as dihydrokerasin (nfa cerebrosides containing dihydrosphingosine). Radioactivity of this fraction was intermediate between that of dihydroceramide and dihydrosphingosine. They interpreted these results to support the hypothesis that nfa ceramide is a precursor of nfa galactosylceramide. However, these authors failed to identify the carbohydrate moiety of the dihydrokerasin fraction. In view of the results of Basu (eq 6) and the demonstration that glucosylceramide is a major component of the monohexosylceramides of peripheral organs (Makita and Yamakawa, 1962; Svennerholm and Svennerholm, 1963; Makita, 1964), it is possible that the observations of Schneider and Kennedy (1968) relate to glucosylceramide rather than to galactosylceramide formation.

Since control experiments in our incubation system demonstrated the formation of a significant amount of nfa cerebroside (Figure 1) we assume that the crude microsomal fraction contains the necessary enzyme system for such synthesis. However, as shown in the Results section, we were unable to stimulate galactose incorporation by adding nfa ceramide. In preliminary experiments where we attempted the fatty acid acylation of psychosine, under conditions similar to those described by Brady (1962), we were unable to stimulate the formation of nfa cerebroside significantly beyond control level. The possibility remains open that neither the direct galactosylation of nfa ceramide nor the acylation of psychosine represents the primary route of nfa cerebroside formation. This problem is currently under investigation in this laboratory.

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Incorporation of Glucosamine-^{14}C into Membrane Proteins of Reticulocytes*

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ABSTRACT: Reticulocytes from phenylhydrazine-treated rabbits were found to incorporate radioactive glucosamine into glycoproteins of the cell membrane. Hexosamines accounted for 90% of the bound activity, while less than 5% was found in sialic acid. Radioactive uridine diphosphate *N*-acetylglucosamine was identified in the acid-soluble fraction. The incorporation was inhibited by puromycin. Added glucose was also inhibitory. Labeled components were cleaved from the intact cell surface by trypsin. These components showed a number of radioactive peaks on DEAE-cellulose and were found to be associated with glycopeptides of high molecular weight. About 10% of the radioactivity was extractable from stroma by organic solvents.

The biosynthesis of proteins by reticulocytes has been studied by many investigators. It has been established that hemoglobin is the major protein synthesized (Dintzis *et al.*, 1958). However, reticulocytes may also have the capacity to make other proteins, among these glycoproteins, as suggested by the incorporation of glucosamine-^{14}C into these cells (Eylar and Matioli, 1965) and into related bone marrow cells (Dukes *et al.*, 1964; Dukes and Goldwasser, 1965). In connection with our studies of glycoproteins in erythrocyte membranes, it seemed plausible that the reticulocyte system may be used to investigate the biosynthesis of these complex substances. Toward this end we have studied the incorporation of radioactive glucosamine into reticulocytes hoping to gain some insight into a mechanism. A partial characterization of radioactive products has also been made.

Materials and Methods

Chemicals. Surfactant DN-65 was a gift from Rohm and Haas Corp., Chicago, Ill. Crystalline heparin (136 units/mg) was purchased from General Biochemicals Corp. UDP-*N*-acetylglucosamine was obtained from

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