Incorporation of Radioactivity of D-Glucosamine-1-C^{14}, D-Glucose-1-C^{14}, D-Galactose-1-C^{14}, and DL-Serine-3-C^{14} into Rat Brain Glycolipids*

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The incorporation *in vivo* of D-glucosamine-1-C^{14}, D-galactose-1-C^{14}, D-glucose-1-C^{14}, and DL-serine-3-C^{14} into gangliosides has been studied. Mild acid hydrolysis and selective degradation with neuraminidase of the labeled gangliosides indicates that the radioactivity of glucose and galactose is incorporated into all the carbohydrate moieties, while the glucosamine carbon-14 forms primarily *N*-acetylneuraminic acid and galactosamine, with no radioactivity appearing in the galactose or glucose portions. The serine radioactivity is found in *N*-acetylneuraminic acid and sphingosine moieties in addition to being incorporated into all of the carbohydrates present. The sequence in which the components are released during the degradative studies is consistent with the basic ganglioside structure that has been proposed, *i.e.* O^1-[(*N*-acetyl)-neuraminyl-(*N*-acetyl)galactosaminyl-((*N*-acetyl)neuraminyl))galactosyl]glucosyl-(*N*-acyl)-sphingosine.

In 1953 and 1954 the incorporation *in vivo* of C^{14}-acetate, C^{14}-formate, and C^{14}-ethanolamine into the sphingosine moiety of cerebral sphingolipids was reported (Zabin and Mead, 1953, 1954). The *in vitro* biosynthesis of sphingosine now has been shown to require palmitaldehyde and serine (Brady and Koval, 1957; Brady *et al.*, 1958). The incorporation *in vivo* of serine-3-C^{14} into myelin lipids of the rabbit was studied and the radioactivity was shown to persist in the sphingolipids for a considerable time (Davison *et al.*, 1959). Both the *in vivo* and *in vitro* incorporation of glucose and galactose into cerebrosides of the young rat brain have been investigated (Burton *et al.*, 1958, 1959). In addition, the incorporation *in vivo* of glucose and galactose into the cerebral glycolipids of young mice has been studied (Moser and Karnovsky, 1959). The last three investigations mentioned noted that young animals incorporated more radioactivity into glycolipids than older animals. In the rat, this age effect is particularly marked. The greatest incorporation of ratioactive sugars occurs between 8 and 14 days *post partum;* little radioactivity is found in the glycolipids with either younger or older animals.

In this paper the incorporation *in vivo* of D-glucosamine-1-C^{14}, D-glucose-1-C^{14}, D-galactose-1-C^{14}, and DL-serine-3-C^{14} into the cerebroside and ganglioside fractions of young rat brain tissue is reported. The radioactive gangliosides were selectively degraded and the radioactivity in most components determined or calculated. These isolation and degradation studies have yielded some insight into the structure of gangliosides.

Materials and Methods

Beef brain gangliosides for reference were prepared as described (*cf.* procedure II below and Burton, 1963).[1] Some preparations of gangliosides were dialyzed and some were passed over Sephadex to remove low-molecular-weight contaminants.

Ganglioside fractions from rat brain were obtained by one of two procedures after the rat was decapitated, the skull opened rapidly, and the brain (both cerebral and cerebellar portions) removed. *Procedure I:* After weighing, the whole brain was disrupted in 100 ml of chloroform-methanol[2] by a Waring Blendor and filtered through Whatman No. 1 filter paper. The blender and precipitate were washed with additional chloroform-methanol. The filtrate was taken to dryness and the residue transferred to thick-walled test tubes (1 × 10 cm) with three 1-ml portions of chloroform-methanol. Water (0.3 ml) was added and the tubes were thoroughly mixed mechanically ("buzzed") (Bessey *et al.*, 1946) or mixed with a Vortex Junior Mixer (Scientific Industries, Inc.). After centrifugation, the aqueous phase was removed and saved. A second extraction with 0.1 ml of water was performed. The two aqueous phases were combined and dialyzed against running tap water for 48 to 96 hours. The aqueous solution was lyophilized and then water was added to give a known volume. In some instances, the initial homogenization of the brain in chloroform-methanol was performed with a glass-Teflon homogenizer and 20 volumes of chloroform-methanol. *Procedure II:* Certain experiments were carried out with gangliosides prepared by homogenizing the brain tissue in 20 volumes of methanol, heating to 63°, filtering while hot, and collecting the glycolipids which precipitate at 4°. This precipitate, containing gangliosides, was then partitioned between chloroform-methanol and water as above. Both of these procedures were adapted from the directions of Folch-Pi (Folch-Pi and Lees, 1959). The gangliosides prepared by either of these

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[1] Anal.: calcd.: C, 55.8; H, 8.52; N, 3.33; found: C, 55.0; H, 8.30; N, 3.51; P, none detectable. *N*-Acetylneuraminic acid, 31.5% (34.7% calcd.). Chromatography indicated the presence of a major component, β, and a minor component, α. No δ or "fast" ganglioside or cerebroside were present (Fig. 1; *cf.* Fig. 1 in Burton, 1963). Some preparations contained a demonstrable trace glycolipid(s) (*cf.* footnote 4 and Fig. 6). Preliminary experiments on the separation of the ganglioside fraction by column chromatography on silica gel, with several solvents, and on cellulose consistently yielded only two major glycolipids corresponding to α and β in the approximate proportions of 1:2.

[2] Chloroform-methanol was used 2:1 (v/v) throughout.

methods appeared to be the same by chromatography, corresponding to beef brain α and β,[1] and they appeared to be degraded in the same manner by neuraminidase.

Cerebroside preparations were made from the organic phase and interface material obtained from procedure I above. The organic phase was taken to dryness, and the residue was dissolved in chloroform-methanol and passed over a mixed-bed column of Dowex-1, Dowex-50, and Florisil (Radin *et al.*, 1955; Burton *et al.*, 1958). Chromatography in several solvent systems, on both paper and thin-layer silica gel, failed to indicate any contaminations.

Paper chromatography was carried out with Schleicher and Schuell 589-red paper with either of two solvent systems (see legend to Fig. 1). The glycolipid areas were detected by a modified procedure employing cresyl violet (Bernheimer and van Heyningen, 1961). Carbohydrates were detected by the periodate-benzidine spray (Mowery, 1957) or aniline-diphenylamine (Sigma Chemical Co.), and *N*-acetylneuraminic acid was located by either spray above or by the periodate-thiobarbituric reagent (Warren, 1960).

Thin-layer chromatography was carried out with silica gel G (Merck, Germany) and one of three solvent systems, A, B (Fig. 1), or C (50% *n*-butanol, 33% pyridine, and 17% water by volume) (Klenk and Gielen, 1961). The chromatographed material was located by spraying with concentrated sulfuric acid and heating to 140° for 5 minutes, by Bials reagent (Klenk and Gielen, 1961), by the periodate-thiobarbituric reagent (Warren, 1960), or by Ehrlich's reagent (Block *et al.*, 1955). The chromatograms were photographed with a Polaroid Land camera.

D-Glucose-1-C^{14} was obtained from Volk Radiochemical Company and New England Nuclear Corporation, D-galactose-1-C^{14} from the National Bureau of Standards, and D-glucosamine-1-C^{14} hydrochloride and DL-serine-3-C^{14} from New England Nuclear Corporation. Neuraminidase was prepared from culture filtrates of *Clostridium perfringens* ATCC 10543 (Popenoe and Drew, 1957; Burton, 1963). The rats employed were from the Holtzman Farm, a variety of Sprague-Dawley rats. *N*-Acetylneuraminic acid was a gift from Dr. Luis Glaser. The cytolipin employed as a reference compound was the gift of Dr. Maurice Rapport. Synthetic sphingosine and ceramide were the gift of Dr. E. F. Jenny, Ciba, Switzerland.

Gangliosides were estimated as described (Warren, 1959; Burton, 1963); *i.e.*, one mole of ganglioside (α or β) is considered to contain one mole of enzyme or mild acid labile *N*-acetylneuraminic acid (see Table I and Discussion). Cerebrosides were determined by the Radin procedure (Radin *et al.*, 1955). Glucose and galactose were quantitated by the carbazole procedure (Dische, 1955). *N*-Acetyl-galactosamine was determined by the Morgan-Elson method (Reissig *et al.*, 1955) and galactosamine by the same procedure after acetylation with acetic anhydride.

Experimental

The ganglioside fraction of rat brain increases 2- to 3-fold from birth; adult levels are attained at about 25–30 days of age (ganglioside curve, Fig. 2). The ganglioside fractions from rats of various ages are similar in chromatographic pattern and appear identical to the beef brain ganglioside preparation (Fig. 1). Solvent B resolved the slow-moving gangliosides into two components labeled α and β (Fig. 1).[3] No fast-moving glycolipid, δ, or cerebroside were detected.

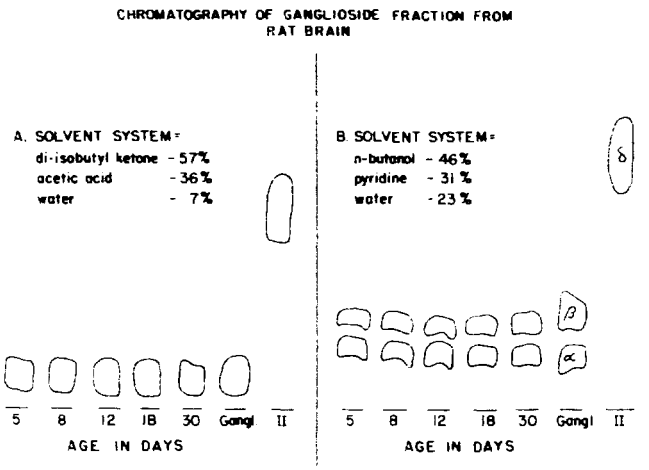

FIG. 1.—Chromatography of the ganglioside fraction from brains of young rats. The whole brain of young rats (2, 5, 8, 12, 18, and 30 days *post partum*) were quickly excised and homogenized in chloroform-methanol. The ganglioside fractions were prepared as described in Methods. Aliquots of 9.3 μl were chromatographed on Schleicher and Schuell 589-red paper with the solvent systems indicated by the ascending technique at 23° for 17 hours. The ganglioside areas were detected by dipping the paper in 0.02% cresyl violet in 1% acetic acid and washing immediately with cold 1% acetic acid (Bernheimer and Van Heyningen 1961). Reference compounds were ganglioside and ganglio-, side treated with neuraminidase, II (Burton, 1963).

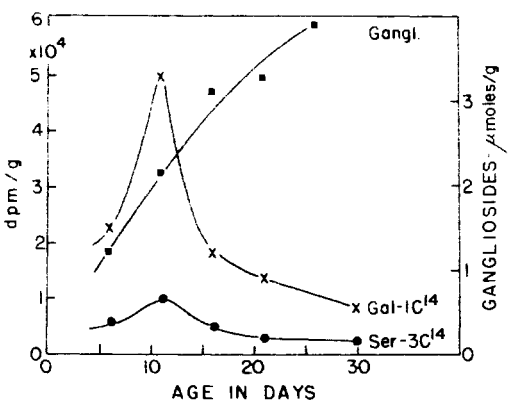

FIG. 2.—Incorporation of DL-serine-3-C^{14} and D-galactose-1-C^{14} radioactivity into rat brain gangliosides as a function of age. DL-Serine-3-C^{14} (3 mc/mmole) and D-galactose-1-C^{14} (0.9 mc/mmole) were administered intraperitoneally (1.8 μc/g body weight). After 24 hours, the rats were decapitated and the brains were excised and homogenized in chloroform-methanol. The ganglioside fraction was prepared as described in Methods. Radioactivity was determined and reported as dpm/g wet weight brain tissue. The ganglioside content was estimated as μmoles per g wet weight brain tissue (see Methods and Burton, 1963).

Incubation of the rat brain gangliosides with *C. perfringens* neuraminidase results in the conversion of the slow-migrating gangliosides to a faster-moving glycolipid, δ, as shown with both thin-layer and column

[3] The various gangliosides separated by paper chromatography were examined for a difference in net ionic charge by paper electrophoresis. The original ganglioside preparations and the two ganglioside fractions from the acidic solvent A migrated at the same rate toward the anode (*p*H 7). The several fractions, α, β, and δ, from the basic solvent system, B, failed to migrate. Subsequent experiments indicated that solvent system B esterified the gangliosides, rendering them incapable of migrating in an electric field. These esterified gangliosides still moved to the same R_F in both solvent systems as the original ganglioside preparations.

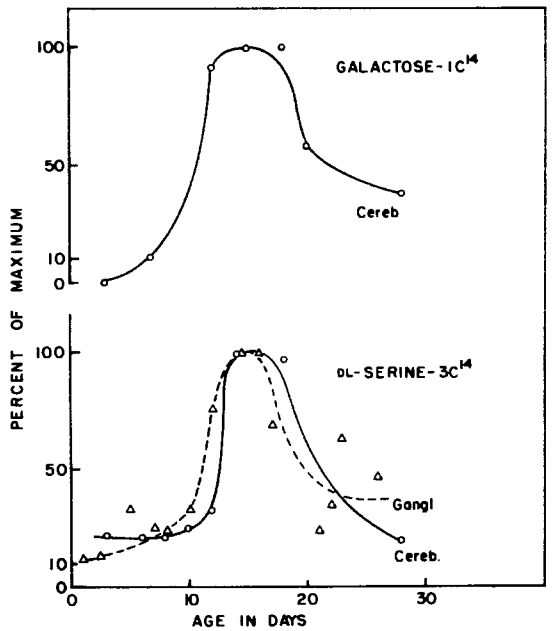

FIG. 3.—Incorporation of DL-serine-3-C^{14} and D-galactose-1-C^{14} radioactivity into rat brain cerebrosides as a function of age. The experimental details are described in the legend to Fig. 2. D-Galactose was given intraperitoneally (0.25 μc/g body weight) and DL-serine intracerebrally (0.5 μc/g). Cerebrosides were isolated as described in Methods.

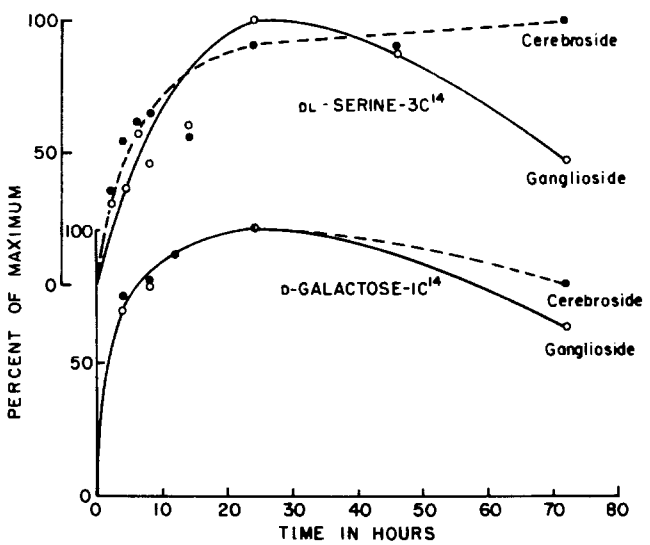

FIG. 4.—Incorporation of DL-serine-3-C^{14} and D-galactose-1-C^{14} radioactivity into cerebrosides and gangliosides of brains of young rats as a function of time after injection. The experimental details are given in the legends to Fig. 2 and 3. All rats were 14 days of age and of the same approximate body weight; they were taken from three litters. DL-Serine (3 mc/mmole) given intraperitoneally (0.8 μc/g body weight). Galactose (0.9 mc/mmole) given intraperitoneally (1.0 μc/g).

chromatography. Similar results were obtained with beef brain gangliosides (Burton, 1963).

Additional evidence which suggests that the qualitative composition of the ganglioside fraction may not vary appreciably with age in the young rat is shown by the fact that the following ratios remained relatively constant: (1) *N*-acetylneuraminic acid released by 1-hour acid hydrolysis *vs.* that released by 3-hour acid hydrolysis, and (2) *N*-acetylneuraminic acid released by exhaustive enzymatic hydrolysis *vs.* that released by 3-hour acid hydrolysis. If the composition of the ganglioside fraction had varied with age, this change might have been reflected in changes in these ratios (see Burton, 1963).[4]

Incorporation of D-*galactose-1-*C^{14} *and* DL-*serine-3-*C^{14} *into cerebral glycolipids.*

a. AGE STUDIES.– Radioactive serine and galactose were administered to rats of different ages and were incorporated into the rat brain gangliosides (Fig. 2). Maximum radioactivity was found in the ganglioside fraction in rats 10–12 days of age. Both older and younger animals showed less incorporation. Even though the same amount of radioactivity was administered, C^{14}-galactose labeled gangliosides more extensively than did serine. The incorporation of radioactivity from D-galactose-1-C^{14} and DL-serine-3-C^{14} into cerebrosides reached a maximum at 12–18 days *post partum* (Fig. 3).

b. TIME COURSE OF INCORPORATION.—A group of rats, 14 days *post partum*, were injected with radioactive galactose and serine. A few rats were killed after 4, 8, 12, 24, 48, and 72 hours. Both serine-3-C^{14} and galactose-1-C^{14} showed maximum incorporation 24 hours after the intraperitoneal injection (Fig. 4). Galactose was incorporated more rapidly than serine; after 4 hours, 70% of the maximum radioactivity from galactose had been incorporated, whereas only 35% of the maximum radioactivity derived from serine was in the ganglioside or cerebroside fractions.

Serine-C^{14}*-Labeled Ganglioside Peptide Fraction.*—A careful preparation of the ganglioside fraction by the chloroform-methanol procedure (omitting the air-drying step) leads to the isolation of gangliosides associated with a peptide fraction (Folch-Pi and Lees, 1959). When the rats were injected with DL-serine-3-C^{14}, this fraction was radioactive. Sealed-tube hydrolysis of this ganglioside-peptide fraction yielded a water-soluble substance with radioactivity. Chromatography showed that part of this radioactive water-soluble material was serine. Thus, in two experiments the isolated serine accounted for 19% and 33% of the radioactivity of the ganglioside-peptide fraction.

Incorporation of D-*Glucose-1-*C^{14} *and* D-*Glucosamine-1-*C^{14} *Radioactivity into Rat Brain Glycolipids.*—The greatest incorporation of radioactivity into gangliosides occurred with rats 6–13 days of age, as in the galactose and serine experiments. Maximum incorporation of radioactivity into gangliosides was found 12 hours after injection (Fig. 5).[5] The radioactivity incorporated into gangliosides from glucose and glucosamine persisted in the glycolipid at near maximal levels for 72 hours, the longest interval studied. Four times as

[4] In general this solvent system separates the ganglioside fraction into two major areas (Fig. 1). However, a third glycolipid area which migrates more rapidly than α and β is occasionally observed. As yet, we have not been able to demonstrate any difference in chemical composition between α and β. The nature of this third glycolipid is unknown.

[5] The blood serum from these rats injected with glucosamine-1-C^{14} was examined for radioactivity. Age did not affect the labeling of the blood serum. Over 90% of the radioactivity in the serum could be precipitated with 50% ethanol and probably represented labeled protein. The radioactivity of the blood serum protein from rats administered glucosamine-1-C^{14} rose to a maximum at 12 hours and then declined rapidly. About 17 to 19% of the serum protein radioactivity was observed to be *N*-acetylneuraminic acid which was released from the serum protein by neuraminidase. In contrast, glucose-1-C^{14} showed maximum serum radioactivity at the first time period examined instead of the peak at 12 hours observed for glucosamine-1-C^{14}. No radioactivity could be consistently detected in the *N*-acetylneuraminic acid of the serum protein.

TABLE I

PARTIAL DEGRADATION OF THE RADIOACTIVE GANGLIOSIDES

Aliquots of the gangliosides, labeled from the source noted, were chosen to contain approximately 6000 dpm. Experimental details are described in legends to Figures 2, 4 and 5 and in methods.

Fraction No.	*N*-Acetyl-neuraminic Acid (μM)	Galacto-samine (μM)	Carbohydrate as Galactose (μM)	Radioactivity Incorporated from DL-Serine-3-C^{14} (dpm)	D-Galactose-1-C^{14} (dpm)	D-Glucose-1-C^{14} (dpm)	D-Gluco-samine-1-C^{14} (dpm)
I	0.56	0	0	1052	642	765	2620
II	0.44	0.34	0	1472	1882	1412	3470
III	0	0.24	0.33[a]	942	1882	1353	765
R	0	0	0.61[b]	2680	1670	1835	0
Total	1.00	0.58	0.94	6146	6076	5365	6855

[a] Contains only galactose. [b] Contains both galactose and glucose.

much radioactivity was found in the gangliosides with glucosamine-C^{14} injection as with glucose-C^{14} injection. Cerebrosides, on the other hand, incorporated three to four times more radioactivity from glucose-1-C^{14} as from glucosamine-1-C^{14}.

Chromatography of the Radioactive Gangliosides Labeled from Glucose-1-C^{14} and Glucosamine-1-C^{14}.— Aliquots of the radioactive ganglioside fractions were chromatographed with solvent B, and the paper strips were scanned for radioactivity with the Forro strip counter or by counting sections of the paper in a liquid scintillation spectrometer. Three glycolipid areas were detected,[4] each containing radioactivity, from a ganglioside fraction labeled with radioactivity from glucosamine-1-C^{14} (Fig. 6). After this ganglioside fraction had been incubated with neuraminidase and then chromatographed, the glycolipid appeared in the position of the "fast" ganglioside and contained radioactivity. In addition, a radioactive area was found which corresponded to the *N*-acetylneuraminic acid reference spot. Similar data were obtained with a ganglioside fraction labeled with radioactivity from glucose-1-C^{14}.

Partial Degradation of the Radioactive Gangliosides.— Aliquots of the radioactive ganglioside fractions[6] from rats injected with serine-3-C^{14}, galactose-1-C^{14}, glucose-1-C^{14}, and glucosamine-1-C^{14} were mixed in solution with carrier beef brain ganglioside[1] (0.49 mg). These samples were then incubated in a final volume of 108 μl with an excess of neuraminidase for 3 hours at 37° and then dialyzed against 25 ml of distilled water for 21 hours at 20°. The dialysate (fraction I, Table I) was collected and shown to contain only *N*-acetylneuraminic acid. The dialysis tubing contents were removed, adjusted to a volume of 234 μl, and made 0.17 N with respect to HCl. Sample tubes were sealed and heated at 80° for 3.5 hours and the contents dialyzed against 25 ml distilled water for 43 hours. The dialysate (fraction II) contained *N*-acetylneur-

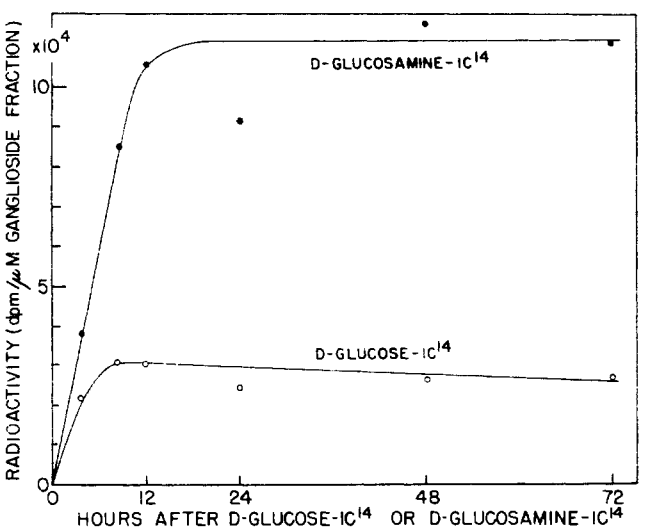

FIG. 5.—Incorporation of D-glucose-1-C^{14} and D-glucosamine-1-C^{14} radioactivity into gangliosides of brains of young rats. The experimental details are as described in the legends to Fig. 2 and 4. D-Glucosamine-1-C^{14} hydrochloride (0.56 mc/mmole) or D-glucose-1-C^{14} (2.0 mc/mmole) was administered intraperitoneally to rats 13 days of age at a dose of 0.7 μc/g of body weight. The ganglioside fraction was prepared as described in Methods.

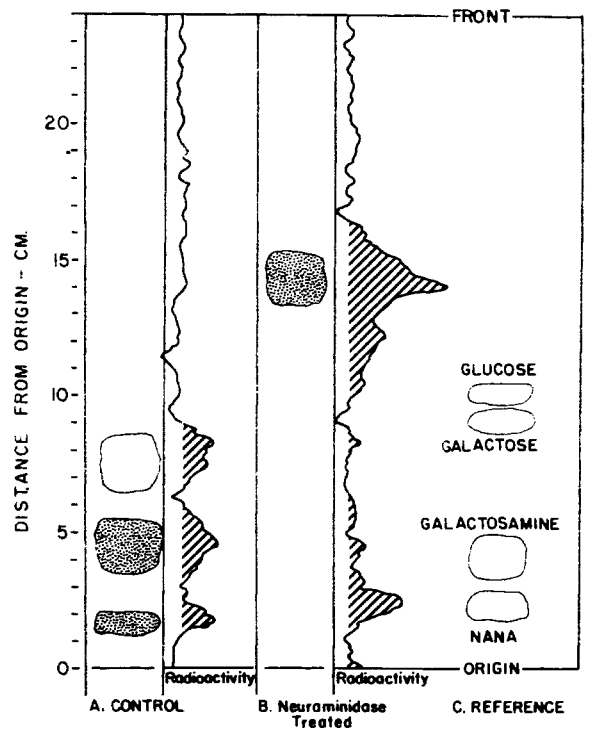

FIG. 6.—Chromatography of the ganglioside fraction of brains of young rats after D-glucosamine-1-C^{14} administration. The experimental details are described in the legend to Fig. 5. Aliquots of the ganglioside fraction were chromatographed as described in Fig. 1; solvent B was used. A second aliquot was incubated with neuraminidase prior to chromatography. Reference compounds as noted (NANA, *N*-acetylneuraminic acid). Radioactivity was determined with the Forro strip counter and is indicated by the shaded areas.

[6] All of these ganglioside fractions have been isolated free of the peptide residue and consist of α and β gangliosides (*cf.* footnote 1).

TABLE II

SPECIFIC ACTIVITY OF CARBOHYDRATES DERIVED FROM RADIOACTIVE GANGLIOSIDES

The experimental details are the same as those in Table I.

Radioactivity Administered as	Specific Activity (dpm/μmole)			
	N-Acetylneuraminic Acid	Galactosamine[a]	Galactose[a]	Residue R as Galactose
DL-Serine-3-C^{14}	1865	1935	1650	4380
D-Galactose-1-C^{14}	1135	4120	2770	2725
D-Glucose-1-C^{14}	1358	2420	2460	3000
D-Glucosamine-1-C^{14}	4660	4250	0	0

[a] Values calculated from the data of Table I and the *assumption* that the specific activity of each carbohydrate was the same throughout the ganglioside molecule, *i.e.*, *N*-acetylneuraminic acid in fraction I (serine-3-C^{14}) had an experimental specific activity of 1865 dpm/μmole, and it was therefore assumed that *N*-acetylneuraminic acid in fraction II had a specific activity of 1865.

aminic acid and galactosamine. The dialysis tubing contents were removed, brought to 0.17 N with respect to HCl, (final volume 281 μl), sealed in glass tubes, and heated at 80° for 48 hours. The samples were dialyzed against 25 ml of distilled water for 22 hours. The dialysate (fraction III) contained galactosamine and galactose as demonstrated by paper chromatography. The residue in the dialysis tubing (R) was removed and shown to contain carbohydrate by the carbazole reaction; both glucose and galactose could be shown to be present by paper chromatography. On thin-layer chromatography (solvent A) material could be detected at positions corresponding primarily to cerebrosides (R_F 0.37) and cytolipin (R_F 0.24). No ceramide (R_F 0.72) or sphingosine (R_F 0.32) could be detected. The quantitative results of this hydrolytic procedure are presented in Tables I and II. Sealed-tube hydrolysis of R fraction in 5.6 N HCl at 125° for 21 hours yielded a black residue and demonstrable sphingosine.

DISCUSSION

Central nervous system gangliosides are a family of complex sphingolipids containing sphingosine, fatty acid, glucose, galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid. The newborn rat has little ganglioside material, but by the 25th day of age adult levels of gangliosides are present, with a 10-fold increase in *total* gangliosides.[7] Our experiments, involving paper chromatography, thin-layer chromatography, and acid and enzyme hydrolysis, suggest a similarity of the ganglioside fractions from brain of the newborn and adult rat. The method of preparation, in either cold chloroform-methanol or boiling methanol, does not seem to affect the final quantitative or qualitative nature of the gangliosides from either rat or beef brain. Occasionally a third "rapid" ganglioside area can be detected migrating more than α or β. When gangliosides are carefully prepared, there is no detectable "fast" or δ-ganglioside present. However, solutions of gangliosides slowly form δ ganglioside from the presumably more native material even when frozen.

Gangliosides are rapidly hydrolyzed by *C. perfringens* neuraminidase, being converted from the chromatographically "slow"-moving to the δ form with a release of *N*-acetylneuraminic acid (see Burton, 1963). Thus, the "native" gangliosides are characterized by a high *N*-acetylneuraminic acid content. The data presented in Table I indicate a basic ratio of *N*-acetylneuraminic acid to galactosamine to hexose (calculated as galactose) of 2:1:2. About half of the *N*-acetylneuraminic acid is enzyme labile. Table I shows the order in which the various carbohydrates are liberated, with *N*-acetylneuraminic acid first, then galactosamine, followed by galactose. The residue, R, contains primarily cerebroside and cytolipin, as demonstrated by thin-layer chromatography. No sphingosine or ceramide was found. After hydrolysis of R with 5.6 N HCl, sphingosine was shown to be present by chromatography.

Coupled with the observations that cerebrosides derived from beef brain gangliosides contain glucose (Bogoch, 1957) and our observations that mild acid hydrolysis releases only galactose, the experiments reported here are consistent with the structure proposed by Klenk and Gielen (1960; structure I) for the basic ganglioside unit for "fast" ganglioside, O^1[(*N*-acetyl) neuraminyl-(*N*-acetyl) galactosaminyl-galactosyl]-glucosyl-(*N*-acyl)-sphingosine (see also Kuhn *et al.*, 1960). A major question remaining is whether the second *N*-acetylneuraminic acid attachment, which is neuraminidase labile, is a 3′ - (*N* - acetyl)neuraminyl galactosyl bond (Burton 1963).

The amount of radioactivity incorporated into gangliosides of young rat brain was greatest with the administration of glucosamine-1-C^{14} and less with galactose-1-C^{14}, glucose-1-C^{14}, and serine-3-C^{14}. When the data are normalized to 1 μc of radioactive substance injected per g of body weight, the values for radioactivity incorporated into the whole brain are: glucosamine-1-C^{14}, 80,600 dpm (65,600 dpm/g brain); galactose-1-C^{14}, 43,600 dpm (38,400 dpm/g); glucose-1-C^{14}, 21,100 dpm (18,350 dpm/g); and serine-3-C^{14} 6,140 dpm (5,760 dpm/g).[8]

If the extent of labeling is known then the per cent of saturation of precursor pools by the injected C^{14}-labeled compounds can be approximated. For example, the glucosamine-1-C^{14} shows maximum incorporation of radioactivity into ganglioside at 12 hours, with 65,600 dpm per g wet weight of brain and an average increase of 0.018 μmoles of ganglioside for this time interval. This corresponds to a specific activity of 3.6×10^6 dpm per μmole of *newly* synthesized ganglioside. The relative specific activities of the *N*-acetylneuraminic acid and galactosamine in the labeled ganglioside are 4660 and 4250 dpm/μmole (Table II). Since there are two moles of *N*-acetylneuraminic acid

[7] Examination of rat brains from birth to 56 days have shown a 10-fold increase in total gangliosides, a 2-fold increase on a wet-weight basis, and a 3-fold increase on a dry-weight basis. Typical average data as follows: Two days old, brain weight 0.35 g with about 10% solids; ganglioside fraction, 0.12 μmole total, 0.34 μmole/g wet weight and 1.2 μmoles/g dry weight brain tissue. Eight days old, brain weight 0.73 g with about 13% solids; ganglioside fraction, 0.30 μmole total, 0.41 μmole/g wet weight and 2.3 μmoles/g dry weight. Thirteen days old, brain weight 1.1 g with about 18% solids; ganglioside fraction, 0.48 μmole total, 0.44 μmole/g wet weight and 2.7 μmoles/g dry weight. Twenty-five days old, brain weight 1.5 g with about 27% solids; ganglioside fraction, 1.0 μmole total, 0.67 μmole/g wet weight and 3.8 μmoles/g dry weight. These data are in general agreement with the results reported by Greaney (1961).

[8] The incorporation of radioactivity from aspartic-4-C^{14} acid into gangliosides was studied as an example of nonspecific labeling. Radioactivity was found in all components of the glycolipid and in very low amounts, *e.g.*, total radioactivity was 769 dpm/g brain.

for each mole of galactosamine, the actual specific activity can be estimated to be 1.2×10^6 dpm/μmole for *N*-acetylneuraminic acid and 1.1×10^6 dpm/μmole for galactosamine. The specific activity of the injected glucosamine-1-C^{14} is 1.2×10^6 dpm/μmole. Therefore the precursor pools for *N*-acetylneuraminic acid and galactosamine incorporation were approximately saturated by the glucosamine-1-C^{14} injected. In the case of glucose-1-C^{14} similar calculations lead to the following approximations for the percentage saturation of precursor pools: *N*-acetylneuraminic acid 3%; galactosamine, 5%; galactose, 5%; and residual cerebroside glucose, 6%. Injected galactose-1-C^{14} saturates the precursor pools approximately as follows: *N*-acetylneuraminic acid, 10%, galactosamine, 36%; galactose, 25%; and residual cerebroside glucose, 24%. The figures for the injected glucosamine-1-C^{14} indicate that the precursor pools for *N*-acetylneuraminic acid and galactosamine synthesis must be very small. Thus during the incorporation of glucose-1-C^{14} and galactose-1-C^{14} into *N*-acetylneuraminic acid and galactosamine, the isotope must be diluted in pools prior to conversion of these precursors to amino sugars.

While glucose-1-C^{14} and galactose-1-C^{14} label other ganglioside components as well as the glucose and galactose, the specific activity of fraction III galactose compares well with the specific activity of the residue R, as galactose, being 2770 dpm/μmole in III compared with 2725 in R for galactose-1-C^{14} and 2460 to 3000 for glucose-1-C^{14}. However, with the C^{14} derived from serine-3-C^{14}, the residue R has a specific activity of 4380 compared to 1650 for galactose in fraction III. This indicates that the residue R was labeled with carbon-14 in components other than the carbohydrate. R was hydrolyzed to sphingosine and the sphingosine was shown to be radioactive.

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