Metabolism of labeled dihydrosphingomyelin in vivo

PETER B. SCHNEIDER* and EUGENE P. KENNEDY

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Tritiated dihydrosphingomyelin was injected into rats, and after various time intervals the distribution of radioactivity in various organs and in the blood and urine was determined. The rate of catabolism of the administered dihydrosphingomyelin was relatively rapid, since about one-fifth of the tritium was recovered as body water after 6 hr. The liver appeared to be the principal site of metabolism of injected dihydrosphingomyelin, although other tissues were also labeled.

The radioactive lipids were extracted from each organ and fractionated by chromatography. The tritium label was found in diminishing amounts in the sequence dihydrosphingomyelin, dihydroceramide, dihydrosphingosine, which suggests that the catabolism of dihydrosphingomyelin proceeds in that sequence. An extensive incorporation of label into lipids other than sphingolipids was also observed.

KEY WORDS sphingolipids · metabolism · catabolism · dihydrosphingomyelin · ceramide · sphingosine · sphingosine phosphoryl choline · sphingomyelin

Sphingomyelin is an important constituent of mammalian lipids, but until recently little has been known about its catabolism in the mammal. Enzymes that catalyze the breakdown of sphingomyelin in vitro have been reported (1–3), but the pathways of catabolism in vivo, the organs in which breakdown principally occurs, and the dynamics of the process remain to be elucidated.

Enzymes of various rat (1, 2) and human (3) tissues that hydrolyze sphingomyelin by cleaving the bond between ceramide and phosphoryl choline have been described, as well as an enzyme in rat brain that releases fatty acid from ceramide to give sphingosine (4). From these studies it would appear that the in vivo catabolism of sphingomyelin probably proceeds first to ceramide and then to sphingosine. However, the formation of sphingosine phosphoryl choline first and then sphingosine is a theoretical possibility. Sphingosine phosphoryl choline does not seem to exist in measurable amounts in mammalian tissues (5) although a report of an enzyme system capable of converting it to sphingomyelin (6) keeps open the question of its physiological role.

Since we were unable to find data on the fate of sphingomyelin in vivo we undertook this study to provide basic information needed for an appreciation of sphingomyelin catabolism. This report describes the methods for separation and measurement of the sphingolipids and presents observations on the disposition in vivo in rats of intravenously injected tritiated dihydrosphingomyelin. Since dihydrosphingomyelin occurs naturally along with sphingomyelin, the metabolism of the two should be closely parallel.

MATERIALS AND METHODS

Bovine heart sphingomyelin obtained from Sylvana Chemical Company (Milburn, N.J.) was purified as previously described (3). From this we prepared dihydrosphingomyelin, tritiated dihydrosphingomyelin, ceramide, and dihydroceramide (3). Sphingosine and dihydrosphingosine were prepared from ceramide or the hydrogenated analogue by strong alkaline hydrolysis (7). Kerasin was the gift of Dr. Pierre Stoffyn. Sphingosine phosphoryl choline was prepared according to Kaller (8). Phosphorus was determined by Bartlett's method (9). Tritium was counted in a Packard liquid scintillation counter using a basic scintillation fluid of toluene with 0.4% diphenyloxazole (PPO) and 0.01% 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) to which additions were made as indicated below to effect solu-

Abbreviation: SPC, sphingosine phosphoryl choline.

* Postdoctoral Fellow of the American Cancer Society. Present Address: Department of Medicine, Beth Israel Hospital, Boston, Mass.

Downloaded from www.jlr.org by guest, on June 19, 2012

bility of the sample as required. Efficiency corrections were made with an automatic external standard.

Injection of Animals

Just before use, the ^{3}H-dihydrosphingomyelin was purified on a thin layer of Silica Gel H (E. Merck A.G., Darmstadt, Germany) with $CHCl_3$–CH_3OH–2M NH_4OH (70:30:4) as solvent. The area containing dihydrosphingomyelin, as determined by a concurrent marker, was scraped off and eluted with $CHCl_3$–CH_3OH 1:4. After addition of unlabeled dihydrosphingomyelin to adjust the specific activity (1–4 mc/μmole, 1.2–4.8 mc/mg) the eluate was dried under nitrogen and prepared for injection by dissolving it with gentle heating in propylene glycol. Enough 0.85% saline solution was added to make the propylene glycol concentration 30%. This produced a fine dispersion containing about 85% of radioactivity in particles that would pass through a 10 μ polyethylene Millipore filter and about 70% in particles passing through a 1.5 μ filter. Animals were injected either with the unfiltered dispersion or with that filtered through a 1.5 μ Millipore (see Table 1), so that observations could be made on the effect of particle size. The radioactivity injected was determined by counting a sample of the preparation at the time of injection.

The ^{3}H-dihydrosphingomyelin dispersion, containing 23–103 μg of lipid (see Table 1) in 1.0 ml, was injected into the tail veins of male rats of the Charles River strain, weighing 190–210 g (one animal per experiment). Urine was collected during the interval from injection to sacrifice and its radioactivity determined by counting in Buhler's solution (10). The animals were killed at various intervals after injection by exsanguination under light ether anesthesia. The heparinized blood was centrifuged, the hematocrit valve was determined, and the red cells were washed twice with saline. Measured volumes of plasma and packed red cells were treated as described below along with the other organs for lipid extraction. A portion of the plasma was distilled and the tritium content of the collected water was measured in Buhler's solution. Using a figure for total body water of 70% of body weight (11), we calculated the tritium content of that compartment.

Extraction of Lipids

Each organ was homogenized in a volume of methanol equivalent to five times its weight but at least 5 ml. The methanol was removed after centrifugation and replaced by an equal volume of $CHCl_3$–CH_3OH, 2:1, and the tissue was redispersed. After 1 hr the solvent was removed by centrifugation; the extraction was repeated for 1 hr and once again for 6–12 hr. The CH_3OH and $CHCl_3$–CH_3OH extracts were pooled, dried under a stream of air, and redissolved in $CHCl_3$–CH_3OH 2:1, and insoluble matter was removed by centrifugation. At this point less than 0.5% of the radioactivity could be extracted by an aqueous wash. A sample of the solution was added to a mixture of 1 ml of ethanol and 10 ml of the toluene-based scintillation fluid for determination of the total lipid radioactivity in each organ. A blood volume equal to 6% of body weight (12) was assumed for calculation of activity in plasma and red cells.

Fractionation of Lipids

Each lipid extract was dried in a stream of air, dissolved in 2 ml of 0.4 M methanolic KOH and incubated at 37°C for 1 hr after which 0.25 ml of 4 M HCl was added and incubation was continued for another hour. 4 ml of $CHCl_3$ was added, followed by 4 ml of water. After gentle mixing the phases were separated by centrifugation, the aqueous phase was removed (leaving any interfacial fluff with the lower organic phase), and the organic phase was dried and redissolved in 1 ml of $CHCl_3$–CH_3OH 2:1. A portion of each lipid extract was then applied in a series of spots at the origin of a thin layer of Silica Gel H (50 × 200 × 0.5 mm). The amount of lipid applied ranged from 1 to 15 μg and the radioactivity varied from 0.01 to 0.8 μc. A mixture of markers, consisting of 20 μg of dihydrosphingomyelin, 20 μg of dihydrosphingosine, 20 μg of kerasin, and 80 μg of ceramide, was applied both in a free side lane and in a central lane applied over the sample. After chromatography these marker compounds were removed along with the separated sample compounds and served then as carriers in subsequent radioactivity determinations. Ceramide was used as a marker rather than dihydroceramide since the two compounds have the same R_f in our system and the unsaturated compound is more easily seen with I_2 vapor staining.

The thin-layer chromatogram was developed in $CHCl_3$–CH_3OH–CH_3COOH 90:3.5:5 at 26°C in a tank lined with paper. The plate was dried under a stream of warm air and redeveloped at 26°C in a lined tank with $CHCl_3$–CH_3OH–2 M NH_4OH 70:30:4 until the front had advanced to a short distance below the level to which ceramide was moved by the first solvent. The plate was dried and exposed to I_2 vapor and on the basis of the positions of the marker compounds was divided into nine bands which included (Fig. 1) the origin, dihydrosphingomyelin, dihydrosphingosine (in this system not separated from sphingosine), kerasin, the second front, ceramide (dihydroceramide), neutral fats (including fatty acids and fatty acid esters derived from lecithin and the cephalins), and intervening bands. For the determination of radioactivity the two bands above the ceramide level were scraped into small columns and eluted with 5 ml of $CHCl_3$–CH_3OH 2:1; the eluate was dried,

Downloaded from www.jlr.org by guest, on June 19, 2012

JOURNAL OF LIPID RESEARCH ASBMB

ASBMB

JOURNAL OF LIPID RESEARCH

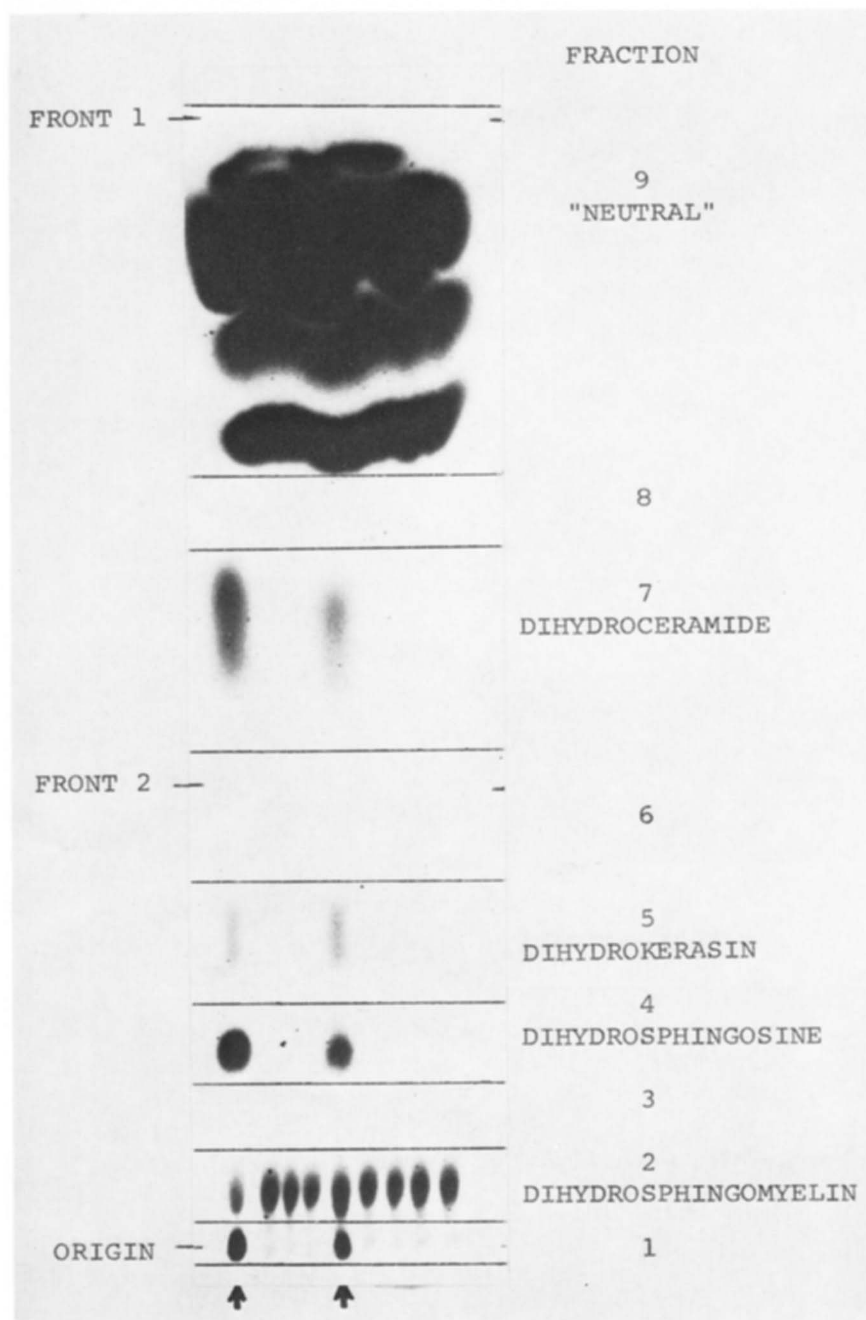

FIG. 1. Photograph of a 0.5 mm layer of Silica Gel H to demonstrate lipid separation and define the fractions which are discussed in the text. The plate was developed to the level of "Front 1" with $CHCl_3$–CH_3OH–CH_3COOH 90:3.5:5, and then, after drying, to the level of "Front 2" with $CHCl_3$–CH_3OH–2 M NH_4OH 70:30:4. For the purposes of this photograph the lipids were charred with H_2SO_4 and heating. Marker lipids were applied to the left and central lanes (arrows) to help define the nine bands. In this case sphingosine phosphoryl choline was added to the marker mixture and appears in band *1*.

and toluene scintillation fluid (10 ml) was added for counting. The bands at the level of ceramide and below were each scraped into a counting vial to which we added 2 ml of 0.1M ethanolic KOH, 0.16 ml of water, and 10 ml of the toluene scintillation fluid. The whole was shaken and counted without particular regard to phase heterogeneity. This empirically determined counting scheme, which had an efficiency for tritium of about 20%, gave quantitative recovery of counts applied to the plate and generally gave better recovery than could be obtained by any but the most exhaustive elution of the silica gel bands. The amount of water that must be added

Downloaded from www.jlr.org by guest, on June 19, 2012

is to some extent a function of the amount of silica gel but the 0.16 ml used in these experiments served for the range of gel areas encountered (2–10 cm^2).

Identification of Lipids

Since the above fractionation was based only on chromatography, further characterization of the sphingolipid bands was carried out. Because ^{3}H-dihydrosphingomyelin was the parent of all these compounds we assumed that the radioactivity we were dealing with was located in the dihydrosphingolipids although, rigorously, the identification procedures used below do not differentiate between the saturated and unsaturated lipids. It should be pointed out that in the initial tritiation of sphingomyelin, double bonds in the amide-linked fatty acid take up about 10% of the total label and, in addition, there may be recirculation of isotope in the fatty acids in vivo so that in the identification experiments part of the tritium label will be found in fatty acids.

The dihydroceramide band from the chromatography of a sample of liver lipids (approximately 100 μg of lipid and 10 μc of tritium) was scraped into a column and eluted with 5 ml of $CHCl_3$–CH_3OH 2:1. On rechromatography of a portion of the eluate about 95% of the radioactivity could again be recovered from the ceramide band. The bulk of the eluate was hydrolyzed with 100 μg of dihydroceramide carrier by refluxing with 1 M KOH in 90% CH_3OH for 8 hr (7). When the hydrolysate was chromatographed about 15% of the radioactivity was found in the ceramide band, and this presumably represented unhydrolyzed material. Of the remaining (hydrolyzed) activity 90% was found in either the dihydrosphingosine or fatty acid band (oleic acid used as a marker for the latter). The fatty acid band itself contained about 20% of the hydrolyzed activity. If hydrolysis was prolonged to 12 hr the unhydrolyzed portion fell to 1–3% but only 80% of the remaining activity was accounted for by the dihydrosphingosine and fatty acid bands. The rest of the activity was distributed along the chromatogram and probably represented side products of the hydrolysis. From these experiments it appears that at least 80% and probably considerably more of the activity of this band was indeed due to dihydroceramide.

The kerasin band from the chromatogram of liver lipids was eluted with 5 ml of $CHCl_3$–CH_3OH 2:1 and 5 ml of $CHCl_3$–CH_3OH 1:2 (1% H_2O). On rechromatography of a sample of the eluate with $CHCl_3$–CH_3OH–2 M NH_4OH 40:10:1 about 90% of the radioactivity traveled again in the kerasin band (R_f 0.56) and was clearly separated from phrenosin (R_f 0.48). A portion of the eluate with 1 mg of kerasin carrier was degraded by periodate oxidation, borohydride reduction, and mild acid hydrolysis (13) to remove the carbohydrate moiety, and the product was chromatographed according to the standard fractionation scheme. The ceramide band contained about 70% of the radioactivity, the remainder being distributed along the chromatogram. Another portion of the eluate, this time with hydrogenated phrenosin as carrier, was hydrolyzed by refluxing for 18 hr with 1 M KOH in dioxane–water 1:1 (14) to yield fatty acid and dihydropsychosine. On chromatography of the hydrolysis products about 50% of the activity appeared in the unhydrolyzed kerasin band and only 40% was accounted for by the sum of the fatty acid and dihydropsychosine bands. Of the hydrolyzed activity about 30% was in the fatty acid band, but since hydrolysis was incomplete this may be only an approximation of the tritium distribution in the kerasin. More drastic hydrolysis decreased the unhydrolyzed portion but increased the activity in unidentified side products without materially increasing that in the fatty acid and dihydropsychosine. Since the radioactivity of the cerebrosides in our experiments would be mainly in the hydrogenated analogue, the difficulty encountered in the alkaline hydrolysis may be due to the greater stability of the amide bond in the dihydrokerasin than in the unsaturated compound that was used in the original description of this hydrolysis scheme. Using primarily the data from the acid hydrolysis we find the content of identifiable cerebroside radioactivity in the kerasin band to be at least 70%. Again, it is probable that the content is really greater since the hydrolysis methods are not completely quantitative and some side products might be expected even if the starting material were pure cerebroside.

The dihydrosphingosine band was eluted as above and treated with fluorodinitrobenzene (15) to form the dinitrophenol derivative. On chromatography, about 75% of the radioactivity traveled in a band corresponding to the dinitrophenol derivative of authentic dihydrosphingosine with an R_f that of ceramide. On rechromatography of the eluate from the dihydrosphingosine band about 70% of the activity could be recovered from the same band.

Sphingosine Phosphoryl Choline Determination

The search for possible radioactivity in sphingosine phosphorylcholine (SPC) was carried out by adding a measured amount (about 0.8 μmole) of unlabeled SPC to the first methanol homogenate of liver, spleen, and plasma, continuing the lipid extraction as above, and then reisolating the SPC to determine its specific activity. Preliminary experiments with SPC and tritiated SPC showed that these behave identically in the fractionation and chromatographic procedures employed, so that SPC serves as a proper carrier for the saturated analogue. The SPC was isolated from the total lipid extracts by dissolving them in 2 ml of $CHCl_3$ and extracting the solution five times with 3 ml of CH_3OH–0.1 M HCl 1:2.

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 1 Distribution of Recovered Radioactivity in Rats after Injection of ^{3}H-Dihydrosphingomyelin

Experiment Number	1	2	3	4	5	6	7
Time after Administration		6 hr			12 hr		24 hr
Amount Administered (μg)	92	103	23*	43*	57	33*	62
				% of dose			
Lipids							
liver	42	45	56	45	26	36	12
spleen	2.5	3.1	3.2	5.5	1.3	3.4	3.3
lung	1.2	1.8	0.6	0.5	0.6	0.5	0.6
small intestine	0.8	0.8	0.8	1.3	1.4	1.2	0.1
kidney	0.3	0.2	0.3	0.4	0.4	0.4	0.2
heart	0.08	0.06	0.07	0.1	0.09	0.08	0.1
brain	0.03	0.02	0.02	0.03	0.03	0.03	0.02
plasma	1.4	1.1	1.0	2.0	1.0	0.7	0.6
red cells	0.2	0.1	0.3	0.3	0.4	0.3	0.4
Body water	21	17	22	25	49	38	—
Urine	0.6	0.5	0.7	0.8	1.5	1.4	2.6

* Animals 3, 4, and 6 were injected with ^{3}H-dihydrosphingomyelin dispersions which had been filtered through 1.5 μ Millipore filters.

Any interfacial fluff was left with the lower phase when the upper was removed. The upper (aqueous) phases were pooled and washed twice with 1 ml of $CHCl_3$ and then made alkaline with 0.5 ml of 4 M KOH. 5 ml of $CHCl_3$ and 2.5 ml of butanol were added and thoroughly mixed. After centrifugation, the lower phase containing the SPC was dried. This material was applied to a 0.25 mm thin layer of cellulose and run in ethanol–water–acetic acid 60:40:3. The chromatogram was stained with a spray of Rhodamine G and the spot corresponding to marker SPC (R_f 0.9) was removed and extracted three times with 0.5 ml of 80% ethanol. This extract was dried, dissolved in $CHCl_3$–CH_3OH 2:1, and applied as two separate but equal spots to a Silica Gel H thin layer for development in $CHCl_3$–CH_3OH–2 M NH_4OH 70:30:5. The chromatogram was exposed to I_2 vapor and the two sample spots corresponding to marker SPC were separately removed, one for phosphorus determination and the other for tritium counting. Blanks for these assays were taken from empty lanes at the same level.

The silica gel was removed from the phosphorus determination tubes by centrifugation just before the final color was read. Since SPC is extremely difficult to elute quantitatively from silica gel, radioactivity was determined by adding 1 ml of ethanol and 1 ml of 0.05 M KIO_4 to the silica gel, allowing this to stand overnight, and then adding 10 ml of the toluene scintillation fluid. This converts the SPC into long-chain aldehyde which is quantitatively counted in the toluene phase without the necessity for removing the aqueous phase. Using the specific activity of SPC and correcting to the amount of carrier initially added, we calculated the total activity that could be ascribed to the SPC in the organ.

The above scheme works very well if no lipids besides SPC are initially present but other lipids interfere with the selective partitioning of SPC into the first water phase and lead to low overall recoveries: 20–30% for spleen and plasma, 5–15% for liver.

RESULTS

The activity of SPC in the liver was found to be, in several experiments, 0.03–0.1% of the total radioactivity in the lipids, and in spleen or plasma, 0.01–0.05%. There was no significant difference between animals sacrificed 6 or 12 hr after the dihydrosphingomyelin injection. We cannot say whether the radioactivity measured represents SPC (dihydro-SPC) or is due to trace contaminants, but these measurements do set upper limits to the tritiated SPC.

In Table 1 are listed the amounts of radioactivity found in the total lipid extracts of the organs of rats killed at various times after the administration of ^{3}H-dihydrosphingomyelin. The activity found in the body water at the time of sacrifice and in the urine collected during the experiment is also listed, as well as the amounts of dihydrosphingomyelin administered. Because evaporation of urine during the collection period would lead to loss of activity in ^3H_2O, the figures for urine activity cannot be considered accurate. In one experiment, at 6 hr water contributed 25% of the urine activity while in another at 24 hr water accounted for 60%.

Table 2 indicates the results of lipid fractionation in terms of percentage of the total activity in the lipid extract of each organ. Brain and heart lipids were not fractionated because of insufficient activity. In the data for animal 7 the activities due to dihydrokerasin and dihydrosphingosine were not separated and the combined figure is listed.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 2 Distribution of Total Lipid Radioactivity among Lipid Fractions

	6 hr				12 hr		24 hr
Experiment Number	1	2	3	4	5	6	7
	% of total lipid radioactivity						
Liver							
neutral fraction	22	16	26	43	33	34	44
dihydroceramide	12	5	10	8	10	2	2
dihydrokerasin	4	3	3	4	7	5	7*
dihydrosphingosine	0.7	0.8	1	1	0.8	0.5	
dihydrosphingomyelin	58	73	56	40	43	57	40
Spleen							
neutral fraction	7	7	15	20	23	18	14
dihydroceramide	9	3	7	4	4	1	1
dihydrokerasin	5	3	3	3	7	4	5*
dihydrosphingosine	2	2	3	2	1	0.7	
dihydrosphingomyelin	73	82	66	65	62	72	74
Lung							
neutral fraction	8	6	28	52	50	53	33
dihydroceramide	3	2	10	6	4	2	2
dihydrokerasin	0.8	1	2	2	1	2	1.2*
dihydrosphingosine	0.4	0.8	1.6	1	1	0.7	
dihydrosphingomyelin	86	89	54	35	42	39	59
Small Intestine							
neutral fraction	63	73	74	81	71	78	70
dihydroceramide	3	2	3	2	2	2	3
dihydrokerasin	7	5	2	2	5	3	5*
dihydrosphingosine	1	0.6	0.7	1	1	0.5	
dihydrosphingomyelin	22	17	15	11	18	14	18
Kidney							
neutral fraction	47	64	67	77	77	76	69
dihydroceramide	5	3	5	3	2	1	2
dihydrokerasin	1	1	1	0.6	0.8	1	1.4*
dihydrosphingosine	0.7	0.5	0.7	0.4	0.7	0.6	
dihydrosphingomyelin	44	31	25	17	18	18	23
Plasma							
neutral fraction	19	37	50	47	36	46	35
dihydroceramide	1	2	2	1	2	1	1
dihydrokerasin	1	1	1	0.6	3	2	4*
dihydrosphingosine	0.4	0.1	0.3	0.2	0.2	0.3	
dihydrosphingomyelin	73	54	44	46	57	46	45
Red cells							
neutral fraction	15	24	23	35	23	34	22
dihydroceramide	2	4	3	5	2	2	2
dihydrokerasin	2	3	1	1	0.7	1	3*
dihydrosphingosine	0.7	0.9	0.7	0.8	0.3	0.6	
dihydrosphingomyelin	75	53	65	51	70	55	62

* These figures refer to combined dihydrokerasin and dihydrosphingosine.

DISCUSSION

The effect of the larger particles in the unfiltered dispersions (Table 1) was to increase the amount of radioactivity found in the lungs 6 hr after injection and to increase the proportion of lipid activity remaining as dihydrosphingomyelin (Table 2) at 6 hr in the lungs and (to a lesser extent) other organs. This is consistent with trapping of the larger particles by the capillaries and a slower degradation of these particles. All the particles of the filtered dispersions and most of those of the unfiltered dispersion were smaller than 1.5 μ and hence escaped removal from the plasma by simple mechanical filtration. Although the maximum size of these particles was about that of chylomicrons, it cannot be assumed that they duplicated the physiological state of the lipid since phospholipids are normally carried in plasma as lipoprotein complexes. However, some of the dihydrosphingomyelin did find its way into the physiological pool since, in our experiments, some of it was found in the red cells where it presumably appeared via an exchange between red cell and lipoprotein lipid (16).

Although our data may not reflect the normal time course of dihydrosphingomyelin clearance from the blood, they do suggest that the overall turnover of dihydrosphingomyelin (and presumably sphingomyelin) is relatively rapid. At 6 hr about 20%, and at 12 hr about 45% of the injected activity was already in the body water. This contrasts with a turnover time measured in months previously reported for brain sphingomyelin (17).

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

Having been removed from the blood and once inside the cell, the tracer ^{3}H-dihydrosphingomyelin should be exposed to the same catabolic steps as the endogenous lipid and hence our experiments should reflect this sequence. It is likely that because of the particulate nature of our lipid preparation most of it was removed by the reticuloendothelial system of the liver and, strictly speaking, our data may apply only to the Kupffer cells. However, it is not known to what extent the Kupffer or the polygonal cells of the liver catabolize endogenous sphingomyelin, and we assume the mechanisms are at least qualitatively similar in all cells where sphingomyelin is degraded.

The finding of the tritium label in diminishing amounts in the sequence dihydrosphingomyelin, dihydroceramide, dihydrosphingosine, is compatible with the view that dihydrosphingomyelin catabolism proceeds in that sequence. Dihydrosphingosine is presumably degraded further to fatty acid, represented in our experiments by the "neutral fat" pool of fatty acid esters. Because of its large size, this latter pool accumulates a considerable amount of tritium label before the label finally enters the body water.

Our data do not suggest any significant role for dihydro-SPC in the catabolic sequence since exceedingly little, if any, radioactivity was found in that compound. A minor role for dihydro-SPC cannot be ruled out but if this rather than dihydroceramide were the first degradation product of dihydrosphingomyelin it would require an exceedingly rapid turnover in the chemically undetectable pool or dihydro-SPC. It would also mean that the relatively large amount of activity in the dihydroceramide would have appeared there as a consequence of synthesis via dihydrosphingosine. Since dihydrosphingosine is synthesized from fatty acid and since the fatty acid pool, because of its large size, has a lower specific activity of the ^{3}H label, the tritium in the dihydrosphingosine is being continuously diluted. This would tend to decrease the specific activity of any dihydroceramide formed from the dihydrosphingosine and make it unlikely that the large amount of label observed in the dihydroceramide came from dihydrosphingomyelin via dihydro-SPC rather than directly.

An interesting finding is the relatively large amount of activity in the dihydrokerasin fraction. The amount of this activity was generally between those of the dihydroceramide and dihydrosphingosine fractions, although in some cases, particularly in spleen and small intestine, it exceeded that of the dihydroceramide. Experiments in vitro (18) and in vivo (19) with brain tissue suggest that cerebroside is formed from sphingosine via psychosin rather than via ceramide. The distribution of radioactivity in the present experiments with more in the dihydrokerasin than in the dihydrosphingosine suggests that cerebroside may be formed directly from ceramide. Definitive conclusions about this series of steps would require information on the actual pool sizes of the various compounds to permit application of the Zilversmit product–precursor relationships (20).

This work was supported by grants from the U.S. Public Health Service, National Institute of Neurological Diseases and Blindness, NB-02946, National Institute of General Medical Sciences, GM-13952, and the Life Insurance Medical Research Foundation.

Manuscript received 2 June 1967; accepted 14 September 1968.

References

1. Heller, M., and B. Shapiro. 1966. *Biochem. J.* **98:** 763.
2. Kanfer, J. N., O. M. Young, D. Shapiro, and R. O. Brady. 1966. *J. Biol. Chem* **241:** 1081.
3. Schneider, P. B., and E. P. Kennedy. 1967. *J. Lipid Res.* **8:** 202.
4. Gatt, S. 1963. *J. Biol. Chem.* **238:** PC 3131.
5. Dawson, R. M. C. 1958. *Biochem. J.* **68:** 357.
6. Brady, R. O., R. M. Bradley, O. M. Young, and H. Kaller. 1965. *J. Biol. Chem.* **240:** PC 3693.
7. Groom, V., and M. Sribney. 1965. *J. Lipid Res.* **6:** 220.
8. Kaller, H. 1961. *Biochem. Zeit.* **334:** 451.
9. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
10. Buhler, D. R. 1962. *Anal. Biochem.* **4:** 413.
11. Dittmer, D. S. Blood and Other Body Fluids. 1961. Federation of American Societies for Experimental Biology, Washington, D. C. 353.
12. Dittmer, D. S. Blood and Other Body Fluids. 1961. Federation of American Societies for Experimental Biology, Washington, D. C. 5.
13. Carter, H. E., J. A. Rothfus, and R. Gigg. 1961. *J. Lipid Res.* **2:** 228.
14. Carter, H. E., and Y. Fujino. 1956. *J. Biol. Chem.* **221:** 879.
15. Wheeldon, L. W., and F. D. Collins. 1957. *Biochem. J.* **66:** 435.
16. Sakagami, T., O. Minari, and T. Orii. 1965. *Biochim. Biophys. Acta.* **98:** 356.
17. Davison, A. N., R. S. Morgan, M. Wajda, and G. Payling Wright. 1959. *J. Neurochem.* **4:** 360.
18. Cleland, W. W., and E. P. Kennedy. 1960. *J. Biol. Chem.* **235:** 45.
19. Kopaczyk, K. C., and N. S. Radin. 1965. *J. Lipid Res.* **6:** 140.
20. Zilversmit, D. B., C. Entenman, and M. C. Fishler. 1943. *J. Gen. Physiol.* **26:** 325.

Downloaded from www.jlr.org by guest, on June 19, 2012