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Cerebroside Sulfate (Sulfatide A) in Some Organs of the Rat and in a Mast Cell Tumor*

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A noteworthy characteristic of cerebroside sulfate (2) (sulfatide A (3)) is its continual accumulation in the brain of rats (4), human beings (5), and mice (6). Recent experiments (7) with radioactive galactose and sulfate showed that cerebroside sulfate of rat brain, unlike other galactolipids, turns over slowly, if at all.

Sulfolipids have been described in organs other than brain, including skeletal muscle (8), submaxillary gland (8), testis (8), liver (8, 9), lung (10), kidney (11), and, inferentially (12), in spleen, blood, and adrenal gland. The identity of the sulfolipids extracted from these organs as cerebroside sulfate has not been established. In this paper evidence is presented that cerebroside sulfate is present in kidney, liver, spleen, and possibly, heart of the rat, and also is present in neoplastic murine mast cells. The turnover rate of cerebroside sulfate in these organs of the rat and in the mast cells was compared with that in the brain. The intracellular distribution of this compound in brain, kidney, and liver was also determined.

EXPERIMENTAL

Extraction and Fractionation Procedures—Rats weighing 150 g were injected intraperitoneally with 1 mc of S^{35}-sulfate, obtained from the Oak Ridge National Laboratories. At stated time intervals, the animals were decapitated, the blood was collected in 8% sodium citrate, and the organs were removed and weighed. For turnover studies, the organs from two rats were pooled. The citrated blood was centrifuged, and the cells were lysed in water. Brain, kidney, and liver were fractionated in 0.25 M sucrose (13).

Mast cells were obtained from the P-815-X-1 mastocytoma (14) grown in (AKR X DBA/2) F_1 hybrid mice. For turnover studies, cells in the ascitic form were used. Each mouse was given 1 mc of (0.004 μg) sodium S^{35}-sulfate intraperitoneally and, at appropriate intervals, the animals were killed by cervical separation. The mast cells were removed, and, after three washings in 0.9% NaCl, were counted and sonically fragmented. One million cells weighed approximately 1 mg.

Cerebroside sulfate was extracted by the method of Lees *et al.* (3).

Materials—ψ-Heparin (*i.e.* heparin bereft of amide-sulfate) was prepared by hydrolyzing commercial heparin in 0.04 N HCl (15). The sulfate esters of ethanolamine and choline were synthesized (16).

Analytical Methods—Radioactivity of extracts was measured in a liquid scintillation counter. Galactose was measured by the anthrone method (17). To detect galactose chromatographically, extracts were first hydrolyzed in 6 N HCl in a sealed tube for 1 hour in a boiling water bath.

To measure sulfate, the sample of cerebroside sulfate was placed in a sealed tube containing 1 ml of glacial acetic acid and 1 ml of 6 N HCl and kept in a boiling water bath for 48 hours. Acid was carefully removed from the hydrolysate by repeated heating; the neutral hydrolysate was taken to dryness and the residue taken up in 0.5 ml of H_2O. To the aqueous extract was added 0.5 ml of 0.1 M acetate buffer (pH 4.0), 1 ml of a mixture of chloroform-methanol (2:1), 10 mg of barium chloranilate (18) and 1 ml of ethanol. The mixture was shaken for 15 minutes, centrifuged and the supernatant solution read in a Beckman DU spectrophotometer at a wave length of 530 mμ. This method reliably detects 40 μg of inorganic sulfate. Optical density at 530 mμ is linearly related to the concentration of inorganic sulfate up to at least 200 μg.

Reversed phase paper chromatography was carried out in diisobutyl ketone-acetic acid-water (40:25:5), a solvent system useful in the resolution of phosphatides (19); the Whatman No. 3 paper used was impregnated with either silicic acid (19) or silicone (20). R_F values were the same in papers coated with either substance. Heparin was chromatographed in isopropyl alcohol-0.04 M ammonium formate (14). Other solvent systems for paper chromatography were *n*-propyl alcohol-ethyl acetate-water (70:10:20) (21), butanol-acetic acid-water (2:1:1), and aqueous 2,4-lutidine (22); the latter was prepared by diluting 67 ml of 2,4-lutidine to 100 ml with water and allowing the mixture to stand for several minutes until a single phase formed.[1] Radioactivity on the paper chromatograms was measured either directly with the aid of an end window Geiger-Müller counter or, after elution with methanol-petroleum ether (1:1), in a liquid scintillation apparatus. Some papers were stained with Azure A (14), ninhydrin (21), or alkaline silver nitrate (21).

RESULTS

Presence of Cerebroside Sulfate in Tissues—Kidney, liver, and spleen of the rat and the mastocytoma from the mouse were extracted for cerebroside sulfate. The final extract was reprecipitated three times (3). Hydrolysates of these extracts were chromatographed on *n*-propyl alcohol-ethyl acetate-water (70:10:20) along with authentic glucose and galactose. Papers were stained with alkaline silver nitrate. Only galactose was de-

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[1] J. Awapara, personal communication.

TABLE I

Galactose-sulfate molar ratios of cerebroside sulfate extracts

Tissue	Galactose:Sulfate
Brain	1.2
Kidney	0.94
Liver	0.90
Spleen	1.1
Mastocytoma	1.3

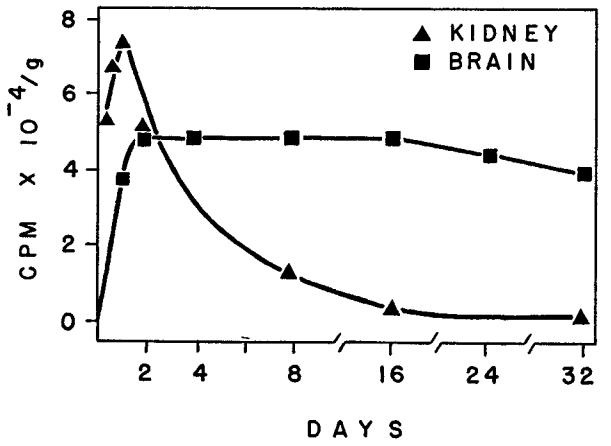

FIG. 1. S^{35}-content of cerebroside sulfate as a function of time

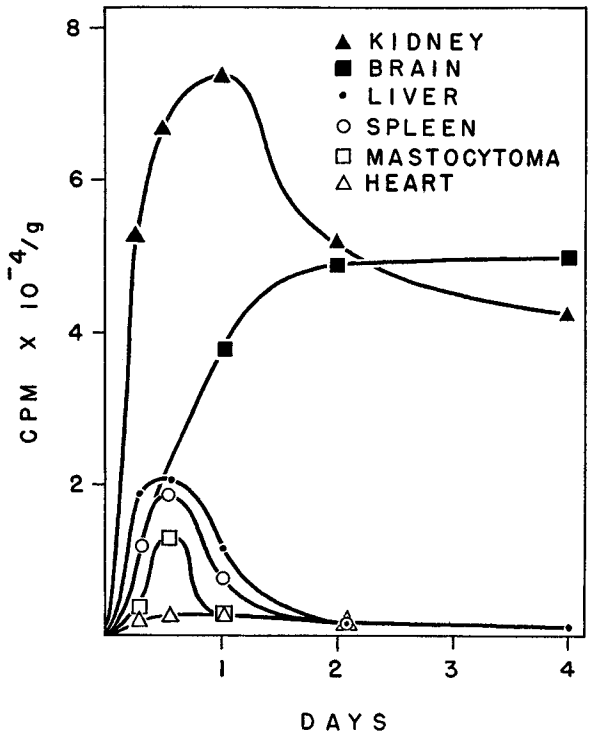

FIG. 2. S^{35}-content of cerebroside sulfate as a function of time

tected. Table I shows the molar ratios of galactose and sulfate obtained on these extracts to be identical within experimental error to that of known cerebroside sulfate isolated from brain. Insufficient material precluded similar experiments on rat heart.

Reversed-phase paper chromatography of purified brain cerebroside sulfate prepared from rats given S^{35}-sulfate showed the presence of a single radioactive spot at R_F 0.50. This area reacted with Azure A to yield a dark blue spot. Identically prepared extracts from the kidney, liver, spleen and heart of the rat and from the mouse mastocytoma, chromatographed in the same manner, also showed a single spot with R_F 0.50, identical to that of brain cerebroside sulfate. No sulfatide could be extracted from blood cells or plasma.

Chloroform-methanol extract—Experiments were carried out to determine at which step in the extraction procedure the extract was free of S^{35}-labeled compounds other than cerebroside sulfate. The chloroform-methanol extracts of brain, kidney, liver, spleen, and heart of the rat and of the mastocytoma were washed with water (Step 1 of the procedure for extracting cerebroside sulfate (3)). With all tissues, reversed phase paper chromatography of the organic phase showed a single radioactive spot at R_F 0.50, corresponding to known cerebroside sulfate. Furthermore, in neither the butanol nor the lutidine system was a radioactive spot other than cerebroside sulfate revealed.

The aqueous phase was also radioactive and this activity was chromatographed in butanol-acetic acid-water, a solvent system in which cerebroside sulfate travels with the solvent front. A radioactive, ninhydrin-positive spot was detected at R_F 0.18 and 0.32, corresponding to cysteic acid and taurine. In the aqueous lutidine system, both radioactivity and ninhydrin reactivity were found at R_F 0.39 and 0.44, corresponding to the known values for taurine and cysteic acid, respectively. Chromatography of the aqueous phase in admixture with taurine and cysteic acid indicated the identity of the S^{35}-labeled compounds in the aqueous phase as taurine and cysteic acid. No choline sulfate (23) or ethanolamine sulfate could be detected.

Turnover of Cerebroside Sulfate—Since cerebroside sulfate was the only S^{35}-labeled compound present in the washed chloroform-methanol extracts of tissues after the administration of S^{35}-sulfate, an aliquot of this extract was used to measure cerebroside sulfate turnover. The turnover of cerebroside sulfate in brain, kidney, liver, spleen, and heart of the rat and in the mastocytoma of the mouse is shown in Figs. 1 and 2. In brain, the maximal incorporation of the isotope occurred 2 days after injection of S^{35}-sulfate, after which time the activity remained constant through the 16th day, but slowly fell thereafter; thus, on the 32nd day, the level was still $\frac{3}{4}$ of that found on the 2nd day.

In comparison, in liver, spleen, and heart the concentration of labeled cerebroside sulfate was highest at 12 hours, with only a small fraction present at 48 hours. After 4 days, the radioactivity had virtually disappeared. In kidney, however, incorporation of S^{35}-sulfate was highest 24 hours after injection of S^{35}-sulfate and measurable radioactivity remained on the 32nd day. The mast cells showed maximal incorporation 12 hours after administration of S^{35}-sulfate and the activity fell rapidly.

TABLE II

Intracellular distribution of cerebroside sulfate in brain, kidney, and liver of the rat

Fraction	Brain	Kidney	Liver
	%	%	%
Debris and nuclei	33	38	29
Mitochondria	50	13	10
Microsomes	10	38	22
Supernatant fraction	7	11	39

Distribution of Cerebroside Sulfate—Table II shows that cerebroside sulfate was distributed differently in the three organs studied. In brain the mitochondrial fraction was richest, whereas in the liver it was the supernatant, particle-free fraction; in the kidney the microsomal fraction had the same amount as the nuclei and debris, whereas the content of the mitochrondria and microsomes was quite low. To test the possibility that the distribution of cerebroside sulfate within cells of different organs is a function of time, cerebroside sulfate was extracted from all three organs at intervals of 12, 24, and 48 hours, respectively, after the injection of S^{35}-sulfate. The distribution was the same at all three periods. Calculations of the intracellular distribution of cerebroside sulfate, based on nitrogen content or mass of the various fractions (24), did not significantly alter the relationship.

Failure to Show Degradation of Cerebroside Sulfate—An aqueous suspension of cerebroside sulfate-S^{35} was incubated for 3 hours with homogenates of either 5 g of the solid mastocytoma or 2 g of rat brain. In neither case was there any detectable degradation of cerebroside sulfate. The presence of cerebroside sulfate in mast cells, which are rich in heparin (14), suggested that these cells might have the capacity to transfer S^{35}-sulfate from cerebroside sulfate to ψ-heparin, but incubation of homogenates of the mast cell tumor with labeled cerebroside sulfate and ψ-heparin resulted in no labeling of the heparin.

DISCUSSION

The preparations of cerebroside sulfate from the various organs, like brain cerebroside sulfate, yielded galactose on hydrolysis, had the same galactose-sulfate ratios as brain cerebroside sulfate, and had R_F values on paper chromatography identical to brain cerebroside sulfate. These observations strongly imply that the compound in the various organs is cerebroside sulfate. Turnover studies revealed several salient differences between brain and other organs. Brain required 48 hours for maximal deposition of S^{35}-sulfate into cerebroside sulfate, whereas kidney required 24 hours, and the other organs and the mast cells only 12 hours. An analogous difference between brain and other organs in the incorporation of radioactive phosphate into phospholipids (25) is partly attributable to slow passage of the isotope into brain (26–28), but primarily due to a slow rate of incorporation of the isotope into the lipid (28). Similarly, the relatively slow incorporation of sulfate into the cerebroside by brain may be, in part, a reflection of the slow penetration of sulfate into brain (29).

A more remarkable difference between brain and the other organs was the exceedingly slow turnover rate found in brain. In this organ, as has been shown by others (7), cerebroside sulfate does not turn over for at least 16 days. However, at 24 days there was a measurable loss of radioactivity and this was more marked at 32 days. By contrast, cerebroside sulfate in other organs and in the mast cells had a half-life of 10 to 96 hours. The exceedingly slow turnover of cerebroside sulfate in brain is not a characteristic of other galactolipids (7). However, the brain phospholipid fraction resembles brain cerebroside sulfate in that it turns over very slowly in both adult (25, 28) and young brain (25, 28, 30), as contrasted with a relatively rapid turnover in other organs (25, 28, 31).

An interesting finding was the distribution of the major portion of cerebroside sulfate in different cellular fractions of the three organs examined: in brain, it sediments with mitochondria; in kidney, with microsomes; and in liver it remained in the supernatant fraction. These observations are consonant with the observation (9) that in brain it is the particulate fraction that incorporates S^{35}-sulfate into a sulfolipid, whereas in liver it is the supernatant fraction.

The finding of S^{35}-labeled taurine and cysteic acid after the administration of S^{35}-sulfate warrants comment. The reaction has been noted before (32, 33) and attributed to the bacterial flora (32), but it appeared possible that in mammalian tissue a pathway may exist for forming S^{35}-cysteic acid from S^{35}-sulfate by sulfonation and subsequent amination of pyruvate. However, homogenates of either brain or mast cells in a fortified system were unable to convert either S^{35}-sulfate or pyruvate-2-C^{14} to cysteic acid or taurine; moreover, taurine and cysteic acid extracted from mast cells grown in culture with S^{35}-sulfate in the medium were not labeled.[2]

SUMMARY

The turnover of cerebroside sulfate (sulfatide A) in brain, kidney, liver, spleen and heart of the rat and in a mast cell tumor of the mouse has been measured. The compound turned over rapidly in the mast cells and in all organs of the rat except the brain. Fractionation of brain, kidney, and liver demonstrated that the distribution of cerebroside sulfate differs in these organs: in brain, the substance sedimented with mitochondria; in kidney, with microsomes; whereas in liver it remained in the soluble fraction. No cerebroside sulfate could be detected in rat blood cells or plasma.

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