Cerebroside Sulfotransferase: Preparation of Antibody and Localization of Antigen in Kidney

GIHAN I. TENNEKOON, JAMES FRANGIA, SUE AITCHISON, and DONALD L. PRICE
Departments of Neurology and Pathology, and the Neuropathology Laboratory, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT This immunohistochemical study describes the localization of the enzyme cerebroside sulfotransferase (phosphoadenosine phosphosulfate: galactosylceramide sulfotransferase, EC 2.8.2.11) in rat kidney. The enzyme was purified from kidney and the preparation was used to raise antibodies for immunocytochemical investigations. In the kidney, the antigen was present only on the brush border of the epithelial cells of the proximal tubules, suggesting that sulfation of glycolipids occurs in the cytoplasm and plasma membranes of these specific cells. Moreover, biochemical and immunocytochemical studies of cerebroside sulfotransferase during development indicate that catalytic activity is correlated with the appearance of enzyme protein.

Glycolipids makeup a large proportion of all cell membranes. Recent studies have shown that these lipids are localized to the outer half of the lipid bilayer, where they apparently form a mechanical barrier to selected solutes (1). The lipids are synthesized in stages in different areas of the cell. Thus, the ceramide backbone is formed in the endoplasmic reticulum and carbohydrate residues are added predominantly in the Golgi complex (36). Sulfation of glycolipids, glycoproteins, and glycosaminoglycans occurs both in the Golgi complex and on the cell surface (8, 48). In the latter location, glycolipids play a role in cell-cell interactions (19, 26, 36), in differentiation during development (43), and in the functional properties of differentiated cells (14, 16, 21, 37, 39, 40, 42, 47), such as receptors for toxins (3, 6, 18, 33, 34, 37). Abnormalities in the composition of glycolipids have been noted in situations where cell-cell interactions are disturbed, such as in transformed cells (2, 7, 13, 15, 26, 28, 32, 35).

Sulfatide (sulfogalactosylceramide) is a sulfated glycolipid found primarily in membrane components of the gastrointestinal and genitourinary systems and the central nervous system (CNS) (4, 5, 20, 31, 38). In the former two systems, it has been hypothesized that sulfatide may play a role in the transport of potassium ions (17, 20) in the reaction mediated by Na+/K+ ATPase located in the cells of the intestine and kidney. In the CNS, where sulfatide is a constituent of myelin, it is thought to interact specifically with the basic protein of myelin to protect this protein from the degradation by proteases (46). The evidence for these functions is largely circumstantial, however, and the definitive role of sulfatide remains uncertain.

The last reaction in the synthesis of sulfatide, the sulfation of galactocerebroside, is catalyzed by the enzyme galactocerebroside sulfotransferase (phosphoadenosine phosphosulfate: galactosylceramide sulfotransferase, EC 2.8.2.11). Since the level of sulfatide in tissues depends, in part, on the activity of this enzyme, the ability to localize the sulfotransferase in specific tissues, particularly during development, would permit investigation of the regulation of the enzyme in differentiating cells, and thus provide potential information on the role of sulfatide itself. A survey of tissues in the rat showed highest activity in the kidney. The fact that sulfatide has been implicated as having a role in the transport of potassium ions and Na+/K+ ATPase in the kidney makes it particularly interesting to study the enzyme in this tissue.

This study describes the preparation of an antibody to purified cerebroside sulfotransferase (CST) from rat kidney. By using this antibody, we showed that the CST was localized to the brush border of the proximal tubule of the kidney. A preliminary report of some of these studies has been presented (45).

MATERIALS AND METHODS
Sprague-Dawley rats (Charles River Farms, Boston, Mass.) were obtained between 7 and 60 d postnatal. For younger ages, mothers at 13-15 d of gestation...
were obtained, and, on the day of delivery, each litter was reduced to ten pups. The adult animals were fed ad libitum on standard laboratory rodent diet.

Materials

[35S]Phosphoadenosine phosphosulfate (PAPS) was obtained from New England Nuclear (Boston, Mass.); imidazole was obtained from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.); galactocerebroside was obtained from Spulco, Inc. (Belleville, N.J.); and Triton X-100 was obtained from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). All solvents were reagent grade.

Purification of Enzyme Proteins

Detailed descriptions of the purification of the enzyme protein from rat kidney are described elsewhere, and only a brief description is given here. The enzyme protein from 100-200 rat kidneys (obtained from Pel-Freeze Biologicals Inc., Rogers, Ark.) was solubilized by treatment with Triton X-100 followed by ammonium sulfate fractionation and column chromatography (Table I). Homogeneity of the protein was confirmed by PAGE and isoelectric focusing (Fig. 1). The purified protein catalyzed the transfer of sulfate from phosphoadenosine phosphosulfate to both galacsoylceramide and lactosylceramide, but did not transfer sulfate to other tested glycolipids, glycoproteins, or mucopolysaccharides.

Preparation of Antiserum

A 500-μg sample of the purified protein from kidney was emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and subcutaneous injections were given to New Zealand white rabbits at multiple sites. Preimmune serum was obtained from each animal before immunization. 3 wk after the first injections, the animals were boosted with 100 μg of enzyme protein emulsified with incomplete Freund's adjuvant as well as 100 μg intravenously. Similar boosting was repeated at 3-wk intervals. 10 d after each immunization, the rabbits were bled and sera were obtained for characterization.

Characterization of the Antiserum

The serum obtained was characterized by double immunodiffusion on Ouchterlony plates (Hyland Diagnostics Div., Traveler Laboratories, Inc., Costa Mesa, Calif.). Since the enzyme protein had been solubilized in Triton X-100, we first established that Triton X-100 itself was not antigenic and that it did not interfere with antigen-antibody interactions. This was done by administering Triton X-100 in Freund's adjuvant to rabbits; no antibodies to Triton X-100 were detected by the double-diffusion method. The interaction of bovine serum albumin (BSA) with antibodies against BSA was also studied in the presence of Triton X-100. At a number of different concentrations of Triton X-100, there was no interference with antigen-antibody interactions.

The antisera were further characterized by studying their ability to inhibit the activity of CST. The Triton X-100-solubilized enzyme protein was incubated with a series of increasing amounts of antiserum in 0.9% NaCl such that the concentration of Triton X-100 in the mixture was kept constant (Triton X-100 to protein ratio, 5:1). The enzyme protein and the antiserum were incubated for 24 h at 4°C, after which the mixtures were centrifuged and the supernatant fraction was assayed for CST activity. The precipitate was also extracted with Triton X-100 and assayed for enzyme activity. The precipitate was washed twice with buffer containing no detergent and then resuspended in 1.0 ml of 0.25 M acetic acid followed by reading the optical density at 280 nm.

Enzyme Assay

Incubation mixtures contained 100 mM 2-[N-morpholino]ethane sulfonic acid buffer (pH 6.2), 20 mM MgCl₂, 4 mM diithiothreitol, 2.5 mM ATP, ~6 × 10⁴ dpm of [35S]PAPS (1.0-2.8 mCi/mmol), 40 μl of enzyme preparation (containing 100–150 μg of protein), and 8 μl of a cerebroside suspension (80 μg) in a final volume of 100 μl. The mixtures were incubated in a waterbath at 37°C for 60 min. The reactions were stopped by adding 10 vol of 10% trichloroacetic acid followed by reading the optical density at 280 nm.

Protein Determinations

Protein was assayed by the method of Lowry et al. (27), with BSA as standard.

G. Tennenagoon and S. Aitchison. Submitted for publication.
RESULTS

Activity of Galactocerebroside Sulfotransferase in Rat Kidney during Development

The activity of CST was examined in rat kidneys obtained from 18-d-old embryos up to 65 d after birth. For embryos and animals up to 4 d postnatal, kidney tissue from about 10 animals was required, while sufficient tissue could be obtained from kidneys of two animals older than 4 d. At each age, the pooled tissue was homogenized in the presence of Triton X-100, centrifuged at 12,000 g for 20 min, and the supernatant fraction was assayed for CST as described in Materials and Methods. The profile in Fig. 2 shows that low activity was obtained from kidneys of two animals older than 4 d. At each age, the pooled tissue was homogenized in the presence of Triton X-100, centrifuged at 12,000 g for 20 min, and the supernatant fraction was assayed for CST as described in Materials and Methods. The profile in Fig. 2 shows that low activity was detected even in 18-d embryos and that activity rapidly increased until ~20 d after birth. After this period, there was a gradual increase in specific activity until 40 d, when activity remained constant. Total activity continued its rise until 65 days after birth.

Preparation and Characterization of Antiserum

CST (~64-000 mol wt) was purified to homogeneity from rat kidney, as described in Materials and Methods. Antibody to the purified sulfotransferase was obtained from rabbits by conventional techniques as described in Materials and Methods.

Preparation and Characterization of Antiserum

CST (~64-000 mol wt) was purified to homogeneity from rat kidney, as described in Materials and Methods. Antibody to the purified sulfotransferase was obtained from rabbits by conventional techniques as described in Materials and Methods.
the presence of antiserum to CST could be attributed to inhibition by the antibody rather than loss of activity upon storage. Antisera from rabbits, obtained at different times after CST antibody production had been initiated, were tested for their ability to inhibit CST activity. No inhibition was observed by antisera obtained in early stages after the initiation of antibody production (one or two injections of CST), although weak precipitin lines were seen at this time. However, as soon as strong precipitin lines were detected on immunodiffusion plates, inhibition (60–95%) of CST activity was found (Fig. 3).

**Immunocytochemical Localization of CST in the Kidney**

In the kidney, CST was localized by incubating kidney sections from 40-d-old (adult) rats with antikidney CST antibody and visualizing the antigen by the immunoperoxidase technique. Light microscopic examination of these sections showed dark reaction product predominantly over the brush border and also some staining over the cytoplasm of the epithelial cells of the proximal convoluted tubules (Figs. 4 A–D).
The elements of the glomerulus, the juxtaglomerular apparatus, the loop of Henle, and the distal tubule did not stain. More detailed studies by high resolution immunocytochemistry showed reaction product staining the microvilli of the brush border and, to a lesser degree, small apical vesicles of the epithelial cells. The nucleus, cytoplasm, mitochondria, endoplasmic reticulum, and dense bodies of these cells did not stain. In the microvilli the staining intensity was greater over the apical portions than in the crypts of these villi.

Our second approach was to analyze the localization of CST.

Figure 5 Imnunocytochemical studies of the distribution of CST in developing rat kidney by the two-layer technique. A and B, 5 d postnatal, X 400. C and D, 8 d postnatal, X 400. E and F, 20 d postnatal, X 400. G, 40 d postnatal, X 200. Note the darkly stained brush border of epithelial cells of the proximal convoluted tubules and the unstained glomeruli (G) and distal convoluted tubules (*). A, C, E, and G show reaction product over the brush border of PCT. B, D, and F were prepared with normal goat sera and show no reaction product in kidney tissue, but peroxidase activity is present in red blood cells. In G, reaction product is abundant on the brush border of cells lining the PCT, but no reaction product over the glomeruli (G) or cells lining the distal convoluted tubules (*). Bar, 1 μm.
in the developing animal and to correlate the appearance of the antigen with biochemical studies of enzymatic activity. At 2 d (Fig. 6A and B), the brush border was not well formed, corresponding to Stage III (25), and there was considerable variation in the number, length, and distribution of microvilli. Reaction product was associated with these microvilli, particularly over membranes of the apical surface of these structures. At 5 d (Figs. 5A-B and 6C) the brush border was taller and showed immunocytochemical staining. The stain was confined to the microvilli and apical vesicles of the epithelial cells of the proximal convoluted tubule. There was no staining of cisternae of the Golgi apparatus or other organelles in proximal tubule cells. By 8-20 d (Figs. 5C-F and 6D), the cells of the proximal tubule showed a more uniform brush border (~1.6 mm in length), and the microvilli were elongated and thinner. These structures were the only elements stained by the immunoperoxidase method. By 40 d, the cells lining the lumen of the tubules were flattened slightly so that both the diameter of the lumen and of the whole tubule were increased. The brush border remained about the same size, accounting for 25% of the height of the cell. The pattern of immunocytochemical staining remained the same, although the amount of stained material increased during the period between 8 and 40 d.

DISCUSSION

Sulfated glycolipids are enriched in certain membranes. In mammalian systems their role remains unresolved (4, 5), although in plants and halophilic bacteria sulfolipids are related to cation transport (20). It has been hypothesized that, in mammals, sulfatide binds potassium ions from the extracellular space and transfers them to the smaller molecular glycoprotein

---

FIGURE 6  Ultrastructural immunocytochemical studies of CST in the epithelial cells of the proximal convoluted tubule of the kidney by the three-layer technique. Cell preparations were lightly stained with uranyl acetate and lead citrate. A, 2 d postnatal. Reaction product is confined to the small irregular microvilli lining the PCT. X 7,100. B, 2 d postnatal. This section was prepared with preimmune sera. The microvilli show no reaction product. X 7,100. C, 5 d postnatal. The microvilli show reaction product on their surface. Mitochondria are not stained. X 18,400. D, 10 d postnatal. The microvilli are taller, of more consistent height, and are uniformly stained. Note plane of section passes through the base of several microvilli; this accounts for staining in these regions. X 16,300. Bar, 1 μm.
subunit of Na+/K+ (20). These conclusions were reached from correlating compositional studies with the activity of Na+/K+ ATPase. For example, in brush border cells, where Na+/K+ ATPase is active, 46% of the dry weight is lipid (38), and of the total lipids, 50% is glycolipid (10-12). Analysis showed that the major glycolipids in the microvilli were cerebroside, ceramide di- and trihexosides, sulfatide, and several gangliosides. However, when Na+/K+ ATPase was purified, attempts to reactivate the enzyme with sulfatide produced only partial activation. Thus, the role of sulfatide in the functioning of Na+/K+ ATPase is at best uncertain. Therefore, we have directed our attention to CST, which catalyzes the last step in the synthesis of sulfatide. Our immunochemical studies showed that CST is confined to a specific cell type in the kidney, the proximal convoluted tubule. In the tubule, CST has a distinct subcellular distribution in the microvilli of the brush border. Since CST is both enriched in kidney and is membrane-bound, it is perhaps not surprising that the reaction product is associated with the membranes of microvilli. The immunocytochemical staining appeared to be highly localized to microvilli of the proximal convoluted tubule, and reaction product was not seen in association with the bulbous microvilli of the cells comprising the distal tubule. Moreover, the increase in staining intensity in the proximal tubule during development correlates with the increase in CST activity. We were unable to correlate the initial appearance of enzyme activity with the synthesis of the enzyme protein in the kidney, as even 18-d-old embryos had catalytic activity. Preliminary studies on the brain show that the appearance of immunocytochemical reaction product correlated with detection of catalytic activity.

The epithelial cells of the proximal convoluted tubule in the kidney are concerned with unidirectional transport of solutes and fluids from the tubule lumen to the bloodstream (24, 38). The single layer of epithelial cells lining the proximal convoluted tubules have a brush border made up of large numbers of microvilli on their apical surface, and it is at this site that transport processes are initiated. There are ultrastructural studies on the development of the proximal tubule (22-25), but no information regarding their sulfolipid composition during maturation. We are now studying the development of the proximal tubule in the rat kidney. J. Ultrastruct. Res. 51:119-139.

Larsson, E., E. 1975. Ultrastructure and permeability of intracellular contacts of developing proximal tubules in the rat kidney. J. Ultrastruct. Res. 45:100-113.

Larsson, E., and M. Horster. 1976. Ultrastructure and net fluid transport in isolated perfused developing proximal tubules. J. Ultrastruct. Res. 54:276-285.

Larsson, E., and A. B. Maunsbach. 1976. Differentiation of the vascular apparatus in cells of the developing proximal tubule in the rat kidney. J. Ultrastruct. Res. 53:234-270.

Lingwood, C. A., and S.-I. Hakomori. 1977. Selective inhibition of cell growth and associated changes in glycolipid metabolism induced by monovalent antibodies to glycolipids. Exp. Cell Res. 108:385-391.

Lowry, O., H. J. Rosebro, A. B. Farr, and R. L. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Morris, C., J. M., and D. Dept. 1961. Thiocholine and other anticholinesterase agents. J. Pharmacol. Exp. Ther. 127:177-197.

Naoum, K., and T. Kawamura. 1975. Peroxidase labeled antibody: a new method of immunocytochemistry. J. Histochem. Cytochem. 23:471-480.

Norcon, W. T. 1975. Isolation of myelin from nerve tissue. In Methods in Enzymology, edited by A. Fleischer and L. Packer. Academic Press, Inc., New York. 435-444.

Norton, W. T. 1976. Formation, structure and biochemistry of myelin. In Basic Neurochemistry. G. J. Seigel, S. R. Albers, R. Katzman, and B. W. Agranoff, editors. Little, Brown and Co., New York. 74-99.

Podlasky, D., E. K., and K. J. Iselbacher. 1978. Inhibition of growth of transformed cells and tumors by an endogenous acceptor of galactosyltransferase. Proc. Natl. Acad. Sci. U S A. 75:4426-4430.

Potter, E. N. R., and E. J. Fuller. 1979. Cell surface receptors for lymphokines. 1. The possible role of glycolipids as receptors for macrophage migration inhibitory factor (MIF) and macrophage activation factor (MAF). Cell Immunol. 44:71-88.

Radke, R. A., and B. D. Anderson. 1977. Specific interaction of Concanavalin A with glycolipid monosaccharides. Biochim. Biophys. Acta. 470:325-330.

Ramsdell, F. J. 1978. Antimicrobial activity of mycobacterial glycolipids. Infect. Immun. 29:914-917.

Schachter, H., and S. Rosenman. 1980. Mammalian glycosyltransferases: their role in the synthesis and function of complex carbohydrates and glycolipids. In Biochemistry of Glycolipids and Proteoglycans. W. J. Lennard, editor. Plenum Press, Corp. New York. 85-160.

Schnaitman, D. 1978. Role of calcium and glycoconjugates in opiate-membrane interactions. Proc. Clin. Biol. Res. 20:201-207.

Schnaitman, D. 1977. Brush border of the renal proximal tubule and the intestinal mucosa. In Mammalian Cell Membranes, Vol 4, Chap. 10: Membranes and Cellular Functions, edited by G. A. Janmey and D. M. Robinson, editors. Brittenmark, London. 221-254.

Schnaitman, A. R., T. T. Astrup, and J. H. Schwartz. 1979. Membrane glycoprotein regional synthesis and axonal transport in a single identified neuron of Aplysia californica. Science (Wash. D. C). 203:78-80.

Serra, P. F. 1979. Capping of exoglycosaminidase on cells. J. Biol. Chem. 254:829-833.

Sternberger, L. A., A. A. Hardy, Jr., and J. J. Cuculis. 1970. The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-
antibody complex and its use in identification of spirochetes. J. Histochem. Cytochem. 18: 315-333.

42. Svennerholm, L. 1980. Gangliosides and synaptic transmission. In Structure and Function of Gangliosides. L. Svennerholm, P. Mandel, H. Dreyfus, and P. F. Urban, editors. Plenum Publishing Corp., New York. 533-544.

43. Svennerholm, L. 1974. Sphingolipid changes during development. Mod. Probl. Paediatr. 13:104-115.

44. Tennekoon, G., and G. M. McKhann. 1978. Galactocerebroside sulfotransferase: further characterization of the enzyme from rat brain. J. Neurochem. 31:329-340.

45. Tennekoon, G., J. Frangia, and G. Aitchison. 1980. Immunocytochemical localization of cerebroside sulfotransferase. Transactions of American Society of Neurochemistry. 11:75.

46. Van Deenen, L. L. M. 1975. Lipid-protein interaction in membrane and model systems. In Functional Linkage in Biomolecular Systems. F. O. Schmitt, D. M. Schneider, and D. M. Crothers, editors. Raven Press, New York. 106-112.

47. Waag, T. J., W. W. Freimuth, H. C. Miller, and W. J. Esselman. 1978. Thy-1 antigenicity is associated with glycolipids of brain and thymocytes. J. Immunol. 121:1361-1365.

48. Young, R. W. 1973. The role of the Golgi complex in sulfate metabolism. J. Cell Biol. 57: 175-189.