

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

GIHAN I. TENNEKON, 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 make up 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  $\text{Na}^+/\text{K}^+$  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 evi-

dence 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  $\text{Na}^+/\text{K}^+$  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

[<sup>35</sup>S]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 Supelco, Inc. (Bellefonte, Pa.); and Triton X-100 was obtained from Schwarz/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,<sup>1</sup> and only a brief description is given here. The enzyme protein from 100–200 rat kidneys (obtained from Pel-Freez 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 galactosylceramide and lactosylceramide, but did not transfer sulfate to other tested glycolipids, glycoproteins, or mucopolysaccharides.

## Preparation of Antiserum

A 500- $\mu$ g 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  $\mu$ g of enzyme protein emulsified with incomplete Freund's adjuvant as well as 100  $\mu$ g 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., Travenol 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 antisera 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 MgCl<sub>2</sub>, 4 mM dithiothreitol, 2.5 mM ATP,  $\sim 6 \times 10^5$  dpm of [<sup>35</sup>S]PAPS (1.0–2.8 mCi/mmol), 40  $\mu$ l of enzyme preparation (containing 100–150  $\mu$ g of protein), and 8  $\mu$ l of a cerebroside suspension (80  $\mu$ M) in a final volume of 100  $\mu$ l. The mixtures were incubated in siliconized test tubes, 70  $\times$  7 mm, at 37°C for 60 min. The reactions were stopped by adding 10 vol of chloroform:methanol, 1:1 (vol/vol), and the mixtures were allowed to stand overnight at 4°C. The lipid extract was treated as described by Folch et al. (9), and the radioactivity of samples was determined as previously described (44) by standard techniques.

## Protein Determinations

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

<sup>1</sup> G. Tennekoon and S. Aitchison. Submitted for publication.

TABLE I  
Purification of CST from Rat Kidney

Steps	Kidney CST	
	Total protein mg	Specific activity dpm/min/mg
Homogenate	11,700	90
Triton extraction of microsomes	171	4,600
Ammonium sulfate fractionation	57	13,300
DEAE-cellulose	11.4	51,570
Sucrose gradient centrifugation	1.2	154,700
Sephacrose 4B	0.096	189,000

The purified protein is extremely stable at  $-70^{\circ}\text{C}$ . Galactocerebroside and lactosylceramide are equally good substrates for this enzyme.

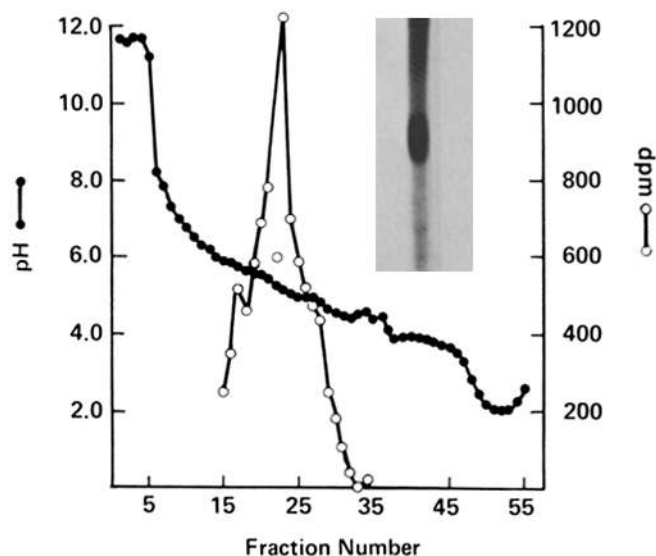


FIGURE 1 Isoelectric focusing of the purified kidney CST. The inset is an SDS polyacrylamide gel (10%) which was overloaded with protein to demonstrate the homogeneity of the protein.

## Immunocytochemical Studies

At 2, 5, 10, and 40 d postnatal, female Sprague-Dawley rats were anesthetized with chloral hydrate and perfused via the ascending aorta with cold 1% glutaraldehyde and 0.1% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.3). After a 15-min fixation *in situ*, the kidneys were removed, bisected, and immersed in fixative (4°C) for 1 h. The tissues were washed three times in PBS over a 2-h interval at 4°C, and stored in PBS with 20% sucrose overnight (4°C) for cryostat sectioning and in PBS without sucrose for electron microscopy studies. Immunocytochemical studies were carried out by the method of Sternberger et al. (41).

For light microscopy, frozen sections (6–8  $\mu$ m) of kidney were cut on a cryostat, placed on albuminized slides, allowed to dry momentarily to ensure adhesion, and immersed in cold PBS until all sectioning was completed. The sections were then washed in 0.5 M Tris buffer (pH 7.6) at 4°C for 30 min and then transferred to a moisture chamber. Additional sections were cut on either a Sorvall tissue chopper (100  $\mu$ m) (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) or a Vibratome (Oxford Laboratory-Searles Co., Foster City, Calif.) (10–40  $\mu$ m); these sections were not placed on slides but were incubated in the free state in small petri dishes at 25°C with agitation. The sections (both free and fixed to slides) were incubated in 0.25% Triton X-100 in 0.5 M Tris buffer for 30 min, briefly washed in buffer alone, and then incubated in 3% normal goat serum in 0.5 M Tris buffer for 30 min. After a brief wash in 0.5 M Tris buffer and 1% normal goat serum, the sections were incubated for 2 h in rabbit anti-sulfotransferase antibody, 1:100 dilution, with 0.5 M Tris buffer and 1% normal goat serum, followed by a wash in buffer and goat serum alone. Preimmune rabbit serum was used as a control. To localize the antigen, two techniques were used. For the two-layer technique, the sections were incubated for 2 h with goat anti-rabbit antibody conjugated to peroxidase (Miles Labora-

tories, Miles Research Products, Elkhart, Ind.), 1:100 dilution, in Tris buffer containing 0.5% Triton X-100. In the three-layer technique (29, 41), sections were exposed to goat anti-rabbit IgG, 1:50 dilution, in 0.5 M Tris buffer and 1% normal goat serum for 2 h; after washing, tissues were incubated for 2 h in rabbit peroxidase antiperoxidase (N. L. Cappel Laboratories Inc., Cochranville, Pa.), 1:100 dilution, in 0.5 M Tris buffer and 1% normal goat serum. The free sections, but not those attached to slides, required additional postfixation in 1% glutaraldehyde in 0.1 M PBS for 30 min at 4°C. Both slides and sections were then incubated in a fresh solution of diaminobenzidine (0.5 mg/ml) and 0.01% hydrogen peroxide in 0.05 M Tris buffer (pH 7.6) for 10 min. Tissue sections obtained with the Sorvall chopper or Vibratome were osmicated (1% osmium tetroxide buffered for 1 h), dehydrated, and embedded in Araldite. Cryostat sections were observed without any counterstaining, whereas the 1  $\mu$  plastic sections were stained lightly with toluidine blue. Tissue sections were examined in a Zeiss photomicroscope; the plastic-embedded regions showing immunocytochemical staining were then thin sectioned and collected on copper grids. Unstained and lightly stained (uranyl acetate and lead citrate) sections were photographed with an AEI 801 electron microscope.

## 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 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 Meth-

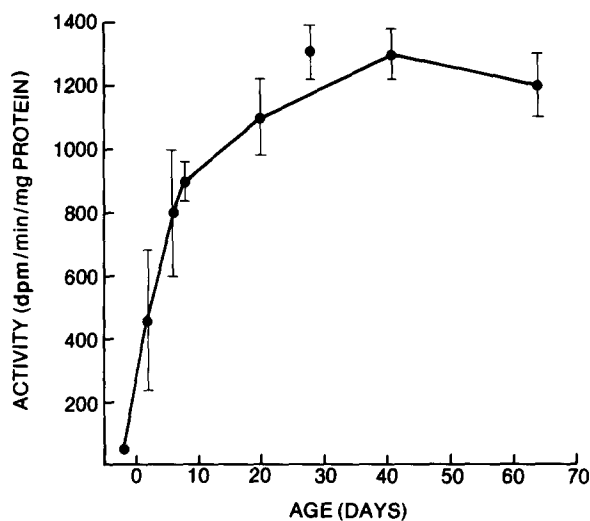


FIGURE 2 Specific activity of CST in the developing rat kidney. Each assay was performed in duplicate and the profile was repeated four times. The values are the averages from the four profiles.

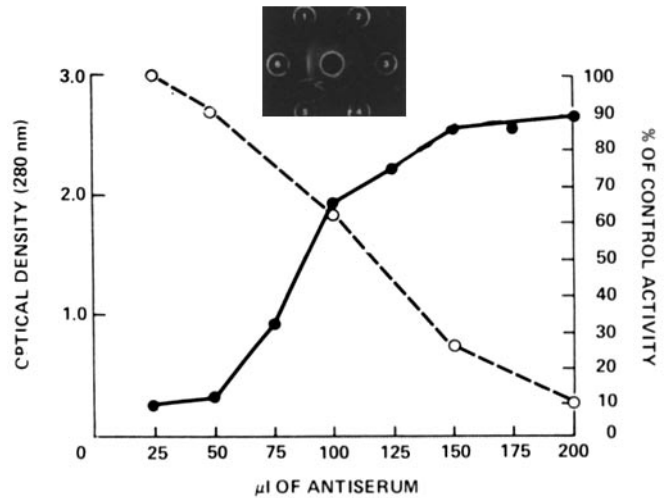


FIGURE 3 Inhibition of kidney CST activity by antibody raised against the protein. The precipitate was quantified by resuspending the precipitate in 0.25 M acetic acid and then reading the optical density at 280 nm. The inset shows a double diffusion plate. The center well contains purified rat kidney CST. (a) preimmune serum; (b) anti-CST absorbed with purified enzyme; (c) anti-CST (after the third immunization); (d) anti-CST absorbed against galactosylceramide; (e) anti-CST absorbed against purified myelin; and (f) anti-CST (IgG fraction after the sixth immunization).

ods. Since Triton X-100 is essential for the preparation of the enzyme, we first ensured that this detergent was not antigenic per se and that it did not interfere with the formation of immune complexes in this system. As shown in Fig. 3, with the use of the double immunodiffusion technique, both crude serum and the IgG fraction from treated rabbits gave visible precipitin lines against both crude and purified kidney enzyme protein. However, when the antiserum was tested against Triton X-100 extracts of CST from rat brain, only a weak cross-reaction was observed on immunodiffusion plates (data not shown).

Antiserum to kidney CST was tested after absorption with a series of lipids, fractions of myelin, and a homogenate of liver. None of the lipids tested, including galactocerebroside, sulfatide, digalactosyl ceramide, phosphatidylcholine (which is thought to be associated with CST in the native state), and GM<sub>1</sub> ganglioside showed any absorption, as evidenced by the presence of precipitin lines when the absorbed antisera were tested against the purified enzyme protein on immunodiffusion plates (Fig. 3). Since myelin contains a variety of glycolipids, we tested the antiserum against purified myelin and "heavy" and "light" fractions. Myelin was purified according to the standard procedure (30). None of these adsorbed the antiserum. Moreover, none of the myelin fractions contained CST activity. Liver homogenates, which do not contain CST, did not absorb, but, as expected, kidney homogenates effectively absorbed the antiserum, and no precipitin lines could be detected on the immunodiffusion plates.

The antiserum obtained against kidney CST was tested for its ability to inactivate CST in Triton X-100 extracts of adult rat kidneys or Triton X-100 extracts of calcium-precipitated microsomes from kidney. Since such crude enzyme extracts were unstable even at 4°C and lost up to 20% of their activity in 24 h, we incubated these extracts with preimmune serum obtained from rabbits or with antiserum to BSA and used these values as controls. Thus, any loss in activity that occurred in

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. 4A–D

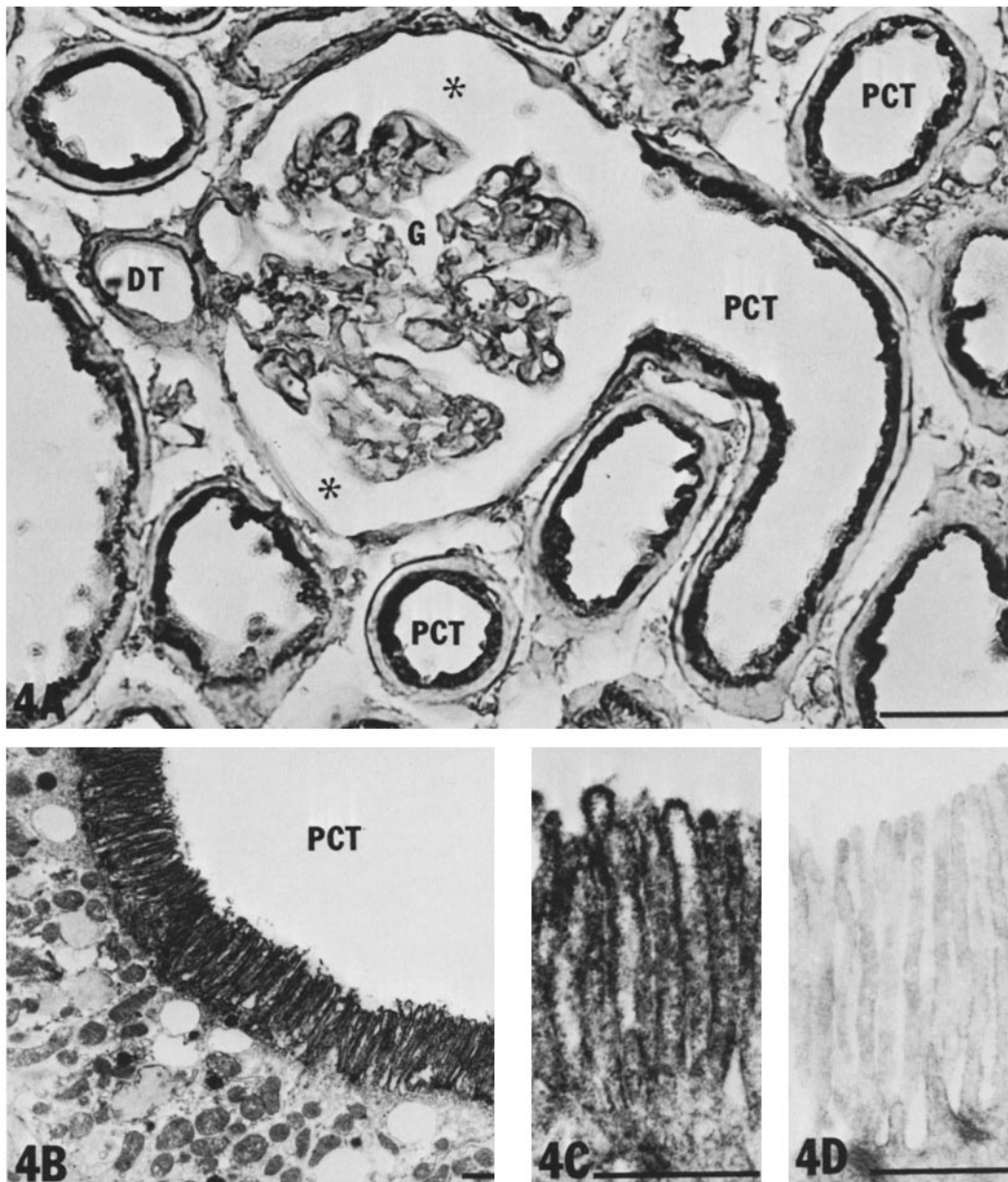


FIGURE 4 Immunocytochemical studies of CST in adult, 40-d-old rat kidney by the three-layer technique. A, a glomerulus (G) occupies the center of the field and is surrounded by Bowman's space (\*). The proximal convoluted tubule (PCT) arises at the urinary pole of Bowman's space. Note the dark staining of the brush border of the epithelial cells lining the PCTs which are cut in longitudinal and cross sections. The cells of the glomerulus and distal tubule (DT) are not stained by immunoperoxidase.  $\times 720$ . B, electron micrograph showing portion of cells lining the PCT. Note the dark reaction product staining microvilli of the brush border. The epithelium of the distal tubule did not show reaction product, making it unlikely that the pattern of staining was due to entrapment of horseradish peroxidase. (Lightly stained with uranyl acetate and lead citrate.)  $\times 4,600$ . C, electron micrograph of microvilli of the brush border showing reaction product indicating the location of CST. (Unstained.)  $\times 23,000$ . D, electron micrograph of microvilli as shown in Fig. 4 C, but prepared with preimmune goat sera instead of anti-CST antibody. (Unstained.)  $\times 20,000$ . Bars, 1  $\mu\text{m}$ .

and 5 G). 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

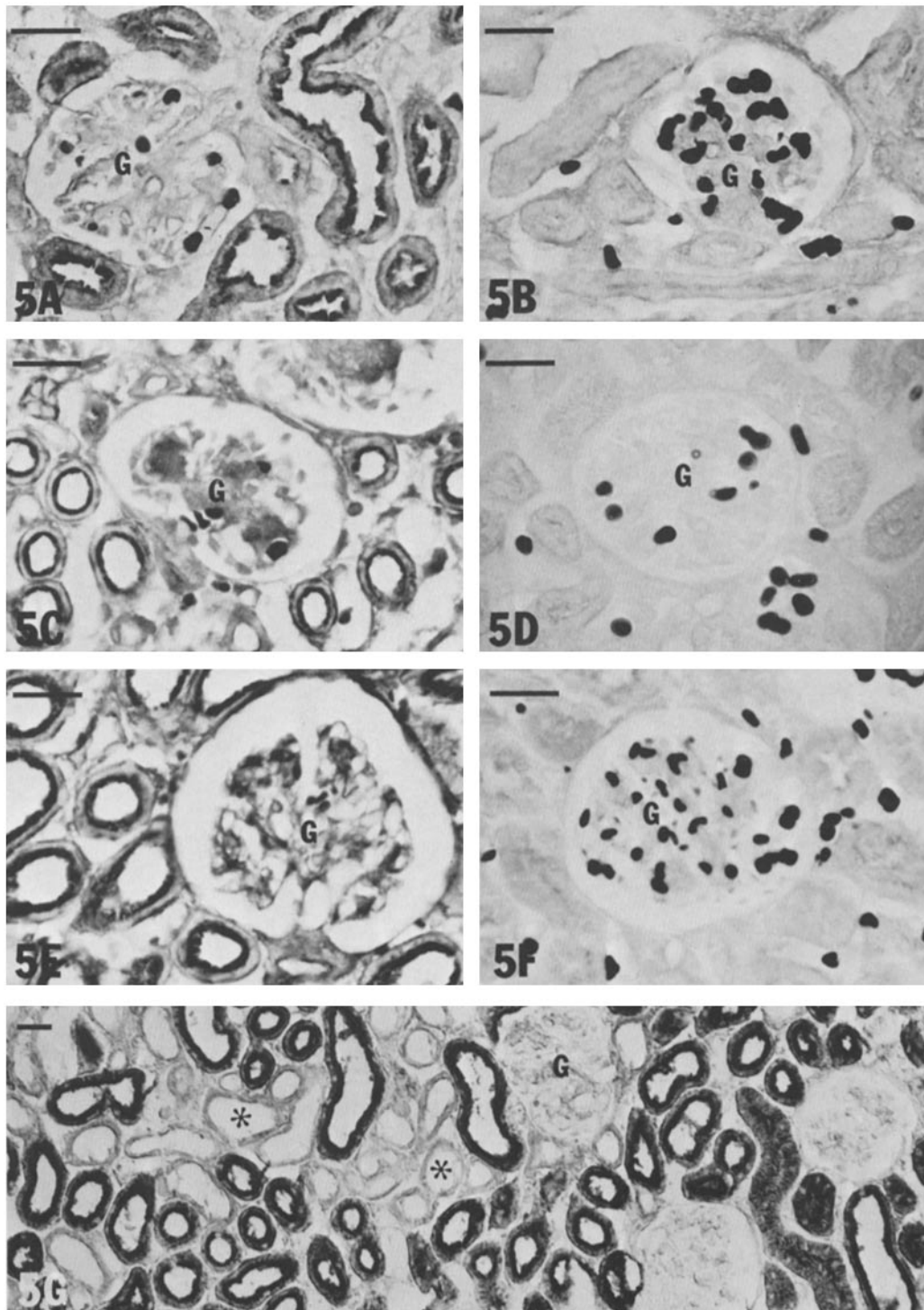


FIGURE 5 Immunocytochemical studies of the distribution of CST in developing rat kidney by the two-layer technique. *A* and *B*, 5 d postnatal,  $\times 400$ . *C* and *D*, 8 d postnatal,  $\times 400$ . *E* and *F*, 20 d postnatal,  $\times 400$ . *G*, 40 d postnatal,  $\times 200$ . Note the darkly 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  $\mu\text{m}$ .

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  $\mu$ m in length), and the microvilli were elongated and thinner. These structures were the only elements stained by the immuno-

peroxidase 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

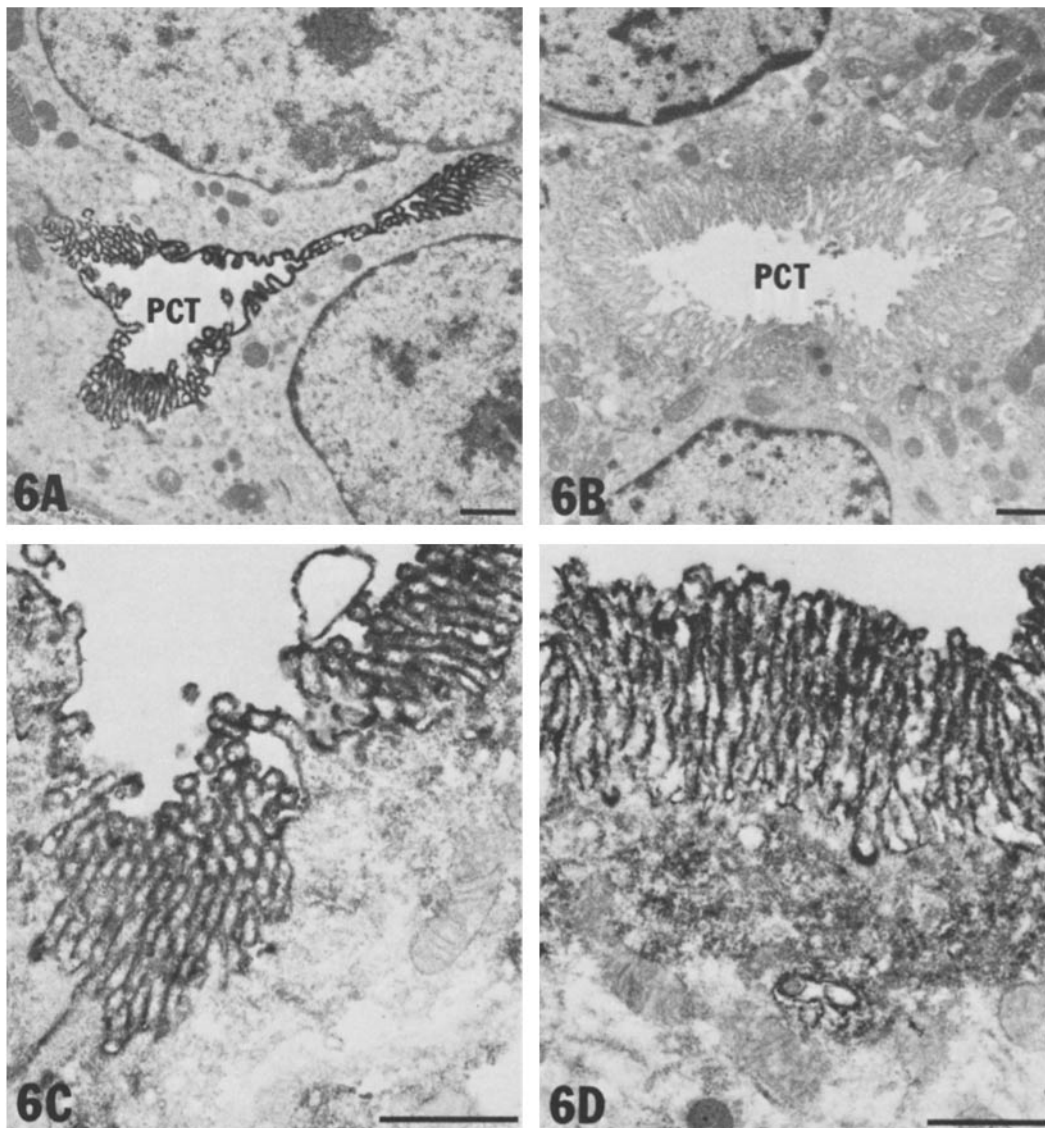


FIGURE 6 Ultrastructural immunocytochemical studies of CST in the epithelial cells of the proximal convoluted tubule of the kidney by the three-layer technique. Cell preparations were lightly stained with uranyl acetate and lead citrate. A, 2 d postnatal. Reaction product is confined to the small irregular microvilli lining the PCT.  $\times 7,100$ . B, 2 d postnatal. This section was prepared with preimmune sera. The microvilli show no reaction product.  $\times 7,100$ . C, 5 d postnatal. The microvilli show reaction product on their surface. Mitochondria are not stained.  $\times 18,400$ . 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.  $\times 16,300$ . Bar, 1  $\mu$ m.



subunit of Na<sup>+</sup>/K<sup>+</sup> (20). These conclusions were reached from correlating compositional studies with the activity of Na<sup>+</sup>/K<sup>+</sup> ATPase. For example, in brush border cells, where Na<sup>+</sup>/K<sup>+</sup> 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<sup>+</sup>/K<sup>+</sup> ATPase was purified, attempts to reactivate the enzyme with sulfatide produced only partial activation. Thus, the role of sulfatide in the functioning of Na<sup>+</sup>/K<sup>+</sup> ATPase is at best uncertain. Therefore, we have directed our attention to CST, which catalyzes the last step in the synthesis of sulfatide. Our immunocytochemical 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 believe that the enzyme appears with the differentiation and maturation of the brush border cells and that the high level of activity in mature cells is associated with active turnover of sulfatides in this tissue. Thus in the kidney, the localization of CST in the luminal surface of proximal tubules would argue against a role for sulfatide in Na<sup>+</sup>/K<sup>+</sup> ATPase, as the latter enzyme is located on the antiluminal surface of the cells. However, the localization to the luminal border does suggest that sulfatide has a specific role, such as in transport of ions or other molecules or possibly in maintaining the conformation and integrity of the membrane or the microvilli as has been suggested for the maintenance of myelin in the CNS.

The authors thank Dr. G. M. McKhann for the very useful discussions during the course of this study. We also thank Dr. Pamela Talalay for assistance in preparing the manuscript and Ms. J. Safchuck for her expert help in typing the manuscript.

This work was supported by the National Institutes of Health grants NS 14760, NS 10920, NS 15721, and NS 10580.

Received for publication 5 January 1981, and in revised form 2 June 1981.

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