Effect of Diabetes on In Vivo Metabolism of [^{35}S]-labeled Glomerular Basement Membrane

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SUMMARY

Glomerular basement membrane (GBM) was labeled in vivo by the injection of tracer amounts of [^{35}S]-sulfate into normal and streptozotocin-diabetic rats. The biosynthesis and turnover of sulfated glycosaminoglycans in the GBM was determined from the specific activity of [^{35}S] after pronase digestion of basement membranes purified from glomeruli isolated 1–7 days after injection. Peak radiolabeling of both normal and diabetic GBM occurred 24 h after injection and, when corrected for differences in serum sulfate specific activities, was less in diabetic than in normal samples. The specific activity of GBM sulfate, expressed as cpm/μg uronic acid, progressively diminished over the ensuing period of study in both normal and diabetic samples. The rate of decrease in specific activity of [^{35}S]-labeled GBM was not significantly different in diabetic preparations compared with that in normal controls. The findings are compatible with diminished sulfation and/or production but normal turnover of glycosaminoglycans in the renal GBM in experimental diabetes. DIABETES *33*:8–12, January 1984.

The identification of glycosaminoglycans in glomerular basement membrane (GBM)[1–5] has raised several questions concerning the role of these substances in normal glomerular function and in certain diseases that characteristically affect the renal GBM. These questions are particularly relevant with respect to the renal disease associated with diabetes mellitus. The nephropathic lesions in diabetes encompass both morphologic and functional changes, manifested, respectively, as an accumulation of basement membrane and an increased permeability of the filtration barrier. Since glycosaminoglycans possess a high net negative charge, which may influence the electrochemical properties and charge-selective nature of the capillary filtration barrier,[6,7] it has been suggested that changes in basement membrane glycosaminoglycans may contribute to the defective function of the glomerular filtration barrier in diabetes and other proteinuric states.[1,8] The recent demonstration that removal of heparan sulfate by an in situ enzymatic digestion leads to a dramatic increase in the permeability of GBM to ferritin lends support to this hypothesis.[8]

We recently reported that glomeruli isolated from streptozotocin-diabetic rats and incubated in vitro with [^{35}S]-sulfate incorporate significantly less radiolabel into basement membrane glycosaminoglycans than do glomeruli from nondiabetic animals.[9] Heparan sulfate was identified as the major [^{35}S]-labeled glycosaminoglycan species in rat GBM,[4,9] and the findings were compatible with decreased sulfation of basement membrane by diabetic glomeruli in vitro. In the present experiments, we examined the in vivo synthesis and turnover of [^{35}S]-labeled GBM in normal and diabetic rats. The results indicate that the incorporation of [^{35}S]-sulfate into basement membrane in vivo is diminished in experimental diabetes, whereas the rate of turnover of [^{35}S]-labeled glycosaminoglycans in GBM is not significantly different in normal versus diabetic animals.

MATERIALS AND METHODS

Experimental animals and tissue preparation. Male white rats maintained on Purina chow and water ad libitum were used in all experiments. The animals were injected in groups of two with a single intraperitoneal dose of [^{35}S]-sulfate (New England Nuclear, Boston, Massachusetts, 880 mCi/mmol; 60 μCi/100 g body wt). Radioisotope was injected within 1 wk after receipt from the manufacturer. In four separate experiments, animal groups were killed 1, 2, 5, and 7 days after injection. At the designated times, the animals were killed by placement in a carbon dioxide chamber; kidneys

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were quickly removed from each rat, placed in cold 0.85% NaCl, and immediately processed for glomerular isolation.

Diabetic animals were injected with radioisotope 17 days after the induction of diabetes via intraperitoneal injection of streptozotocin, 65 mg/kg body wt. The diabetic rats, which were age-matched with littermate controls, weighed approximately 120 g when injected with streptozotocin and were markedly hyperglycemic at the time of death.

After gross dissection to separate the renal cortices, glomeruli were isolated by sieving through a series of stainless-steel meshes.[10] The basement membranes were purified with osmotic lysis in the presence of protease inhibitors, followed by sequential treatment with detergents as previously reported.[11,12] In each experiment, pooled GBM was isolated from groups of two control or two diabetic rats at the specified intervals after injection and was lyophilized before further analysis.

Specific activity of serum sulfate. Normal and diabetic rats were bled from tail veins at 30, 60, 120 min, and 4 and 24 h after injection of radioisotope. Sera collected from these rats in each experimental group were individually processed and were deproteinized by precipitation with 5% trichloroacetic acid (TCA). After centrifugation, trichloroacetic acid was removed from the aqueous phase by extraction with ether. Aliquots of the protein-free sera were taken for measurement of radioactivity in a liquid scintillation counter. Sulfate concentrations were measured by the benzidine method.[13]

Sample analysis. The basement membranes were digested with pronase (1 mg/ml with an enzyme : protein ratio of ≈1 : 5) in 0.12 M Tris HCl, pH 7.2, containing 0.01 M $CaCl_2$ for 24 h at 37°C.[14] Digested proteins were removed by precipitation with 5% TCA and excess TCA was extracted from the supernatant, which was devoid of protein, with ether. One-half of the aqueous samples was taken for determination of radioactivity in a liquid scintillation counter and the other half was used for measurement of uronic acid by the carbazole method.[15] Aliquots contained about 10 μg of uronic acid to ensure accurate determination and yielded the characteristic pink color on reaction with carbazole. The basement membrane preparations are devoid of DNA, and overestimation of the amount of uronic acid by the carbazole method was therefore not a problem.

Since [^{35}S] may be incorporated into noncollagen proteins as well as glycosaminoglycans of basement membrane,[5] removal of the former with TCA precipitation was corroborated by subjecting resultant supernatants to cellulose acetate electrophoresis, with and without prior digestion (hyaluronidase or chondroitininase ABC) or nitrous acid oxidation. About 35% of the incorporated [^{35}S] liberated with pronase digestion was precipitated with TCA. All of the radioactivity in the supernatant was nondialyzable, using dialysis bags of both 2000- and 12,000-mol wt exclusion limits. On cellulose acetate electrophoresis, glycosaminoglycans in the supernatant migrated as a single band that was resistant to enzymatic digestion but sensitive to oxidation with nitrous acid.

RESULTS

The streptozotocin-diabetic rats were markedly hyperglycemic and manifested typical untreated insulin-deficient diabetes. Mean blood glucose concentrations in diabetic animals killed 1, 2, 5, and 7 days after radioisotope injection were 18.6, 18.8, 21.2, and 21.8 mM/L; blood glucose concentrations were all less than 8 mM/L in control animals. Body weights in diabetic rats were significantly less than those of control animals, but were about 11% greater at the termination of the study period than at the time of radioisotope injection. Body weights of control animals were about 16% greater at the end of the study than at the time of radiosulfate injection. Renal cortical weights were greater in diabetic animals and were about 12% higher at day 7 than at day 1 after injection; renal cortical weights in normal animals had increased about 17% by the end of the study. Corrections for possible dilution of radioisotope due to animal growth, calculated from the weights at time of death relative to the initial body weights, were therefore made for the GBM specific activity data obtained on days 5 and 7 in both normal and diabetic rats. Correction for renal growth, as discussed below, was estimated by dividing the GBM specific activity data by the renal cortical weights at the time of death.

Circulating radioactivity, measured in deproteinized samples of peripheral blood, rapidly declined within the first 4 h after injection in both normal and diabetic rats (Figure 1). Circulating radioactivity was very low at 24 h and undetectable thereafter, indicating that significant reutilization of extracellular label did not occur in either experimental group. Values for the specific activity of serum sulfate were lower in diabetic than in control rats at each time interval, but the rate of decrease in the serum sulfate specific activities was similar in diabetic and normal rats (Figure 1). Serum sulfate concentrations were not significantly different in normal versus diabetic animals, suggesting that the lower serum [^{35}S]-specific activity in diabetic rats resulted from accelerated excretion of the radioisotope due to osmotic diuresis (measured for the first 24 h after injection in two normal and two diabetic rats). These differences in the specific activities of serum sulfate influence relative tissue availability of the labeled precursor after radioisotopic injection into normal and diabetic rats. The ratio of the area under the curves of serum [^{35}S]-specific activities in normal versus diabetic samples

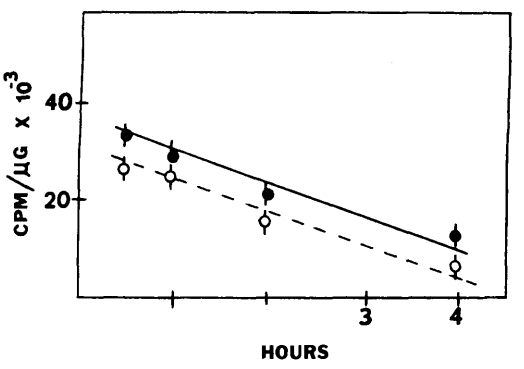

FIGURE 1. Specific activity of sulfate in the sera of normal (●——●) and diabetic (○----○) rats at various times after the injection of [^{35}S]-sulfate (60 μCi/100 g body wt). Each point represents the mean ± SEM of three determinations obtained from three individual rats in each group. Results expressed as cpm/μg sulfate. Serum sulfate concentrations (mean ± SEM) in normal and diabetic animals were 3.27 ± 0.51 and 2.75 ± 0.30 mg/dl, respectively.

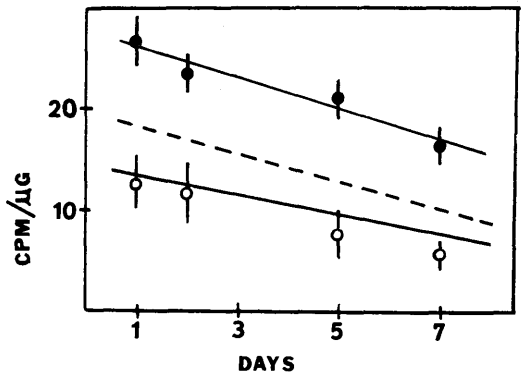

FIGURE 2. Specific activity of [35S]-sulfate in glomerular basement membrane (GBM) of normal (●——●) and diabetic (○——○) rats. Values expressed as cpm/μg uronic acid 1–7 days after injection of radiotracer. Results represent mean ± SEM of four separate experiments in each of which renal cortices from two normal and two diabetic animals were pooled, respectively, at each time period for preparation of pronase digests from the isolated GBM. Results were corrected for change in body weight (see text) and, as indicated by the dashed line, for differences in the precursor pool size in diabetic animals as estimated from the specific activity curves of serum sulfate. The respective slopes for normal, diabetic, and corrected diabetic curves are −1.49, −1.25, and −1.86.

was 1.49. This ratio approximated that obtained by comparing the specific activities of the glomerular tissue fluid sulfate, measured in the water supernatant after osmotic lysis of isolated glomeruli in normal and diabetic samples 24 h after injection; the ratio of normal to diabetic tissue fluid sulfate specific activities was 1.70. The correction factor of 1.49, obtained from the ratio of serum sulfate specific activities, was applied to the GBM specific activity data in diabetic preparations to take into account the differences in precursor pool sizes.

Peak appearance of labeled sulfate in GBM occurred within 24 h after radioisotope injection in both normal and diabetic samples (Figure 2). Incorporation of [35S]-sulfate was sufficient for reproducible results, with about 800 cpm recovered at 24 h and about 300 cpm recovered 7 days after injection in normal samples (background < 20 cpm). Maximum [35S]-sulfate incorporation into diabetic GBM was significantly less than that in nondiabetic animals. This finding pertained even when differences in pool sizes, estimated from the differences in serum sulfate specific activities, were taken into account (Figure 2, dashed line).

After maximum incorporation, the specific activity of sulfate (cpm/μg uronic acid) in glycosaminoglycans of GBM progressively declined during the remaining period of study. The relationship between the decrease in radioactivity and time was linear in both normal and diabetic samples (Figure 2). Diminution with time in the actual cpm indicated that the decline in [35S]-specific activity arose from disappearance of label in the membrane and was not only due to dilution by new glycosaminoglycan synthesis. The amount of uronic acid recovered was not significantly different in samples at each time period, suggesting that turnover of [35S]-radiolabel was in concert with turnover of the glycosaminoglycan polysaccharide. With application of the correction factor for differences in precursor pool sizes (Figure 2, dashed line), the rate of decrease in [35S]-specific activity of diabetic GBM was similar to that of normal (diabetic membrane = −1.86; normal membrane = −1.49). Thus the slightly faster disappearance of diabetic compared with normal [35S]-labeled GBM calculated from these data (6.5 days versus 8.4 days, respectively, for one-half the specific activity observed at 24 h) primarily derives from the lower initial specific activity in diabetic samples rather than a slower rate of turnover.

Additional evidence that [35S]-incorporation into diabetic GBM is diminished whereas [35S]-turnover in normal and diabetic basement membranes is similar derives from calculation of the specific activity of sulfate per gram of renal cortex at each time interval (Figure 3). The data in these curves take into account the growth of the kidneys during the course of the experiments, and hence correct for apparent diminution in specific activities due to tissue growth. The interval for one-half the initial specific activity estimated from the data in these curves is 6 days and 5.4 days, respectively, for normal and diabetic samples.

Renal cortical mass and GBM glycosaminoglycans were preserved in diabetic rats despite loss of peripheral subcutaneous tissue and its extracellular matrix. Results of a previous study indicated that the uronic acid content of diabetic basement membrane, calculated as μg uronic acid/glomerulus, did not differ significantly from that of controls,[9] and these findings were confirmed in the present study.

DISCUSSION

The experiments presented herein describe the effect of diabetes on the in vivo incorporation and turnover of [35S]-sulfate in GBM, and extend previous in vitro findings that indicated that [35S] incorporation into basement membrane glycosaminoglycans is diminished in glomeruli isolated from rats with experimental diabetes. There is little doubt that [35S]-sulfate labels integral components of GBM, since the techniques employed for purification of this extracellular matrix yield preparations that are devoid of cellular material and cell membranes and have appropriate compositional features. The predominant [35S]-labeled glycosaminoglycan, which is liberated after pronase digestion and separated from glycopeptides after TCA precipitation, migrates similar to heparan sulfate standard on cellulose acetate electro-

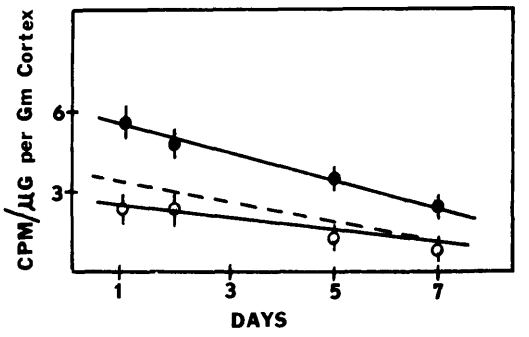

FIGURE 3. Specific activity of [35S]-sulfate in glomerular basement membrane of normal (●——●) and diabetic (○——○) rats expressed as cpm/μg uronic acid per gram of renal cortex. Results represent mean ± SEM of four separate experiments calculated from the data given in Figure 2. The corrected diabetic values, obtained by multiplication of observed values by 1.49 (see text), are indicated by the dashed line. The respective slopes for normal, diabetic, and corrected diabetic curves are −0.53, −0.30, and −0.45.

phoresis,[4,9] is resistant to enzymatic digestion within chondroitinase ABC and hyaluronidase, and is sensitive to nitrous acid.[4] These findings are compatible with the presence of heparan sulfate. Although small amounts of chondroitin sulfate and dermatan sulfate have been identified in detergent-isolated GBM,[16] heparan sulfate is clearly the principal species[17] and accounts for ≈80% of the [^{35}S] incorporated into basement membrane glycosaminoglycans. Basement membranes also contain sulfated glycoproteins and [^{35}S] may be incorporated into such substances. For example, immunofluorescent techniques have demonstrated the presence of entactin, a sulfated glycoprotein purified from the extracellular basement membrane-like matrix elaborated by an embryonal carcinoma endodermal cell line, in areas adjacent to the basement membrane in mouse and rat kidney glomeruli.[18] Lemkin and Farquhar reported that about 32% of the [^{35}S] incorporated into rat GBM is recovered in glycopeptides, whereas 68% is recovered in sulfated glycosaminoglycan.[5] Our results agree with these findings.

Information on the turnover of GBM components in normal or diabetic kidneys is incomplete. Price and Spiro found that the diminution in the amount of radioactivity incorporated into rat GBM after intraperitoneal injection of tritiated amino acids was very slow, without appreciable change in the specific activities of glycine, proline, and hydroxyproline over study periods of ≥ 200 h.[19] In an extension of this work, Brownlee and Spiro compared the in vivo metabolism of normal and diabetic basement membrane protein, reporting that while the specific activities of proline and hydroxyproline were increased in GBM prepared from diabetic rats, there was no difference in the rate of basement membrane collagen turnover in diabetic rats during the 42–50 h after injection of radioisotope followed in that study.[20] The authors, on the other hand, reported that the turnover of rat GBM collagen, labeled in vivo by radioactive glycine or proline, was similar to that of newly synthesized fibrillar collagen in normal animals[12] and was prolonged in streptozotocin diabetes.[21] Other investigators have also found that the rate of turnover of GBM collagen is diminished in experimental diabetes.[22] Only one study examined the in vivo turnover of glycosaminoglycans in renal tissue.[23] In that work, the decrease in specific activity, calculated as cpm/μg uronic acid of [^{35}S]-labeled glycosaminoglycan in crude renal cortex, was analyzed after intraperitoneal injection of labeled sulfate into normal rats. The rate of decrease in specific activity of [^{35}S]-glycosaminoglycans was nonlinear with time, and the turnover was considered broadly biphasic with half-lives estimated for the first and second phases at just over 2 days and at 5–6 days, respectively. In the present work, there was a linear relationship between time and the rate of decrease in [^{35}S]-specific activity in purified GBM during the 1–7 days after radioisotope injection examined. The estimated half-life of [^{35}S]-labeled GBM was similar to that of the second phase in whole renal cortex reported by Barry and Bowness,[23] as well as to the half-lives reported for sulfated glycosaminoglycans in other tissues.[24–27] The rates of turnover of [^{35}S]-labeled normal and diabetic GBM were not significantly different, a finding that was surprising in view of reports that renal glycosidase[28–30] and aryl sulfatase[31] activities are decreased in diabetic animals.

The results reported herein demonstrate that the in vivo incorporation of [^{35}S] into GBM is diminished in experimental diabetes. Since the isolation procedure employed in this investigation preserves mesangial basement membrane-like material as well as peripheral GBM, it is not possible to distinguish whether diabetes affects [^{35}S]-incorporation into mesangial versus peripheral extracellular matrix. The absence of significant changes in the uronic acid content of basement membranes from diabetic animals may reflect an interval after induction of diabetes that was too short for development of detectable quantitative changes. Alternatively, the differences in sulfate incorporation may reflect undersulfation of glycosaminoglycan chains rather than a decrease in glycosaminoglycan production in diabetes. The net result of decreased glycosaminoglycan production and/or sulfation coupled with increased collagen synthesis and prolonged turnover in diabetic GBM would be a matrix relatively rich in collagen components and relatively poor in sulfated glycosaminoglycan. The quantitative or qualitative changes in GBM glycosaminoglycans, particularly heparan sulfate, which is the predominant species, may contribute to the proteinuria associated with diabetic nephropathy.

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REFERENCES

[1] Kanwar, Y. S., and Farquhar, M. G.: Presence of heparan sulfate in the glomerular basement membrane. Proc. Natl. Acad. Sci. USA 1979; 76:1303–1307.

[2] Kanwar, Y. S., and Farquhar, M. G.: Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. Proc. Natl. Acad. Sci. USA 1979; 76:4493–97.

[3] Cohen, M. P.: Glycosaminoglycans are integral constituents of renal glomerular basement membrane. Biochem. Biophys. Res. Commun. 1980; 92:343–48.

[4] Cohen, M. P., Wu, V. Y., and Surma, M. L.: Non-collagen protein and proteoglycan in renal glomerular basement membrane. Biochem. Biophys. Acta 1982; 678:322–28.

[5] Lemkin, M. C., and Farquhar, M. G.: Sulfated and nonsulfated glycosaminoglycans and glycopeptides are synthesized by kidney in vivo and incorporated into glomerular basement membranes. Proc. Natl. Acad. Sci. USA 1981; 78:1726–30.

[6] Comper, W. D., and Laurent, T. C.: Physiologic function of connective tissue polysaccharides. Physiol. Rev. 1978; 58:255–315.

[7] Brenner, B. M., Hotstetter, T. H., and Humes, H. D.: Molecular basis of proteinuria of glomerular origin. N. Engl. J. Med. 1978; 298:826–33.

[8] Kanwar, Y. S., Linker, A., and Farquhar, M. G.: Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. J. Cell Biol. 1980; 86:688–93.

[9] Cohen, M. P., and Surma, M. L.: [^{35}S]-sulfate incorporation into glomerular basement membrane glycosaminoglycans is decreased in experimental diabetes. J. Lab. Clin. Med. 1981; 98:715–22.

[10] Cohen, M. P., and Klein, C. V.: Glomerulopathy in rats with streptozotocin diabetes. Accumulation of the glomerular basement membrane analogous to human diabetic nephropathy. J. Exp. Med. 1979; 149:623–31.

[11] Carlson, E. C., Brendel, K., Hjelle, J. T., and Meezan, E.: Ultrastructural and biochemical analyses of isolated basement membrane from kidney glomeruli and brain and retinal microvessels. J. Ultrastruct. Res. 1978; 62:26–53.

[12] Cohen, M. P., and Surma, M. L.: Renal glomerular basement membrane. In vivo biosynthesis and turnover in normal rats. J. Biol. Chem. 1980; 255:1767–70.

[13] Kent, P. W., and Whitehouse, M. W.: Micro-determination of ester sulphate and free sulphate ions. Analyst (London) 1955; 80:630.

[14] Linker, A., and Hovingh, P.: The heparitin sulfates (heparan sulfates). Carbohydr. Res. 1973; 29:41–62.

[15] Bitter, T., and Muir, H.: A modified uronic acid reaction. Anal. Biochem. 1962; 4:330–34.

[16] Brown, D. M., Michael, A. F., and Oegema, T. R.: Glycosaminoglycan synthesis by glomeruli in vivo and in vitro. Biochim. Biophys. Acta 1981; 674:96–104.

[17] Linker, A., Hovingh, P., Kanwar, Y. S., and Farquhar M. G.: Characterization of the heparan sulfate isolated from dog GBM. Lab. Invest. 1981; 44:560–65.

[18] Carlin, B., Jaffe, R., Bender, G., and Chung, A. E.: Entactin, a novel basal lamina-associated sulfated glycoprotein. J. Biol. Chem. 1981; 256:5209–14.

[19] Price, R. G., and Spiro, R. G.: Studies on the metabolism of the renal glomerular basement membrane. Turnover measurements in the rat with the use of radiolabeled amino acids. J. Biol. Chem. 1977; 252:8597–8602.

[20] Brownlee, M., and Spiro, R. G.: Glomerular basement membrane metabolism in the diabetic rat. In vivo studies. Diabetes 1979; 28:121–25.

[21] Cohen, M. P., Surma, M. L., and Wu, V. Y.: In vivo biosynthesis and turnover of glomerular basement membrane in diabetic rats. Am. J. Physiol. 1982; 242:F385–89.

[22] Romen, W., Lange, H. W., Hempel, K., and Hecke, T.: Studies on collagen metabolism in rats. II. Turnover and amino acid composition of the collagen of glomerular basement membrane in diabetes mellitus. Virchows. Archiv. (Cell Pathol.) 1981; 36:313–20.

[23] Barry, D. N., and Bowness, J. M.: Identification and turnover of glycosaminoglycans in rat kidneys. Can. J. Biochem. 1975; 53:713–20.

[24] Davidson, E., and Small, W.: Metabolism in vivo of connective-tissue mucopolysaccharides. II. Chondroitin sulfate B and hyaluronic acid of skin. Biochim. Biophys. Acta 1962; 69:453–58.

[25] Handley, C. J., and Phelps, C. F.: Biosynthesis in vitro of chondroitin sulfate in neonatal rat epiphysial cartilage. Biochem. J. 1972; 126:417–32.

[26] Kraemer, P. M., and Tobey, R. A.: Cell-cycle dependent desquamation of heparan sulfate from the cell surface. J. Cell Biol. 1972; 55:713–17.

[27] Schiller, S., Mathews, M. B., Cifonelli, J. A., and Dorfman, A.: Metabolism of mucopolysaccharides in animals: further studies on skin utilizing [14C]-glucose, [14C]-acetate, [35S]-sodium sulfate. J. Biol. Chem. 1956; 218:139–45.

[28] Fushimi, H., and Tarui, S.: Kidney and serum β-N-acetylglucosaminidase activities in streptozotocin-diabetic rats and their responses to insulin and glucagon. J. Biochem. 1974; 76:225–27.

[29] Fushimi, H., and Tarui, S.: β-glycosidases and diabetic microangiopathy. I. Decreases of β-glycosidase activities in diabetic rat kidney. J. Biochem. 1976; 79:265–70.

[30] Fushimi, H., Shibata, M., and Tarui, S.: Glycosidase activities in the liver and kidney of hereditary diabetic mice. J. Biochem. 1980; 87:941–49.

[31] Ou, S. L., Shah, S. V., Velosa, J. A., Abboud, H. E., and Dousa, T. P.: Lysosomal hydrolases in glomeruli of rats with experimental diabetes. Abstract. Clin. Res. 1979; 27:668A.