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Effect of Streptozotocin Diabetes on Selected Enzymatic Activities in Rat Urine (39534)

MELVIN D. KUWAHARA, SHEILA A. LYONS, PAUL D. ROSENBLIT, AND ROBERT P. METZGER[1]

Departments of Physical Science and Chemistry, San Diego State University, San Diego, California 92182

Patients afflicted with certain pathological conditions, especially those of the kidneys, excrete in their urine significantly greater than normal levels of certain enzymatic activities, among them such acid hydrolases as *N*-acetyl-β-D-glucosaminidase (*N*-acetylglucosaminidase, EC 3.2.1.30), β-D-galactosidase (EC 3.2.1.23), and β-D-glucuronidase (EC 3.2.1.31) (1-4). Experimental animals given nephrotoxic agents also produce urines containing acid hydrolase activities at levels well above control values (5, 6). The loss in the urine of acid hydrolase activities, which are presumably of lysosomal origin, seems to be linked to the process of destruction of kidney tissue (2, 3). Recent reports state that diabetic patients excrete elevated levels of urinary lysozyme (EC 3.2.1.17), acid phosphatase (EC 3.1.3.2), *N*-acetylglucosaminidase, β-galactosidase, and α-D-glucosidase (EC 3.2.1.20) compared with nondiabetic subjects (2, 7, 8). Further studies are needed to determine the source of the excreted acid hydrolases from diabetic patients and to find out if there is a relationship between the levels of the activities of the urinary enzymes and the development of some of the vascular lesions of diabetes (8-10). In this paper, we suggest that streptozotocin-diabetic rats may provide a model for such studies. We report that these animals excrete significantly elevated levels of urinary *N*-acetylglucosaminidase, acid phosphatase, α-L-fucosidase (EC 3.2.1.51), β-D-galactosidase, and α-D-mannosidase (EC 3.2 .1.24) per 24 hr compared to controls. The diabetic rats also excrete significantly higher levels of the nonlysosomal enzymes alkaline phosphatase (EC 3.1.3.1) and glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1) per 24 hr than controls. Insulin treatment of the diabetic rats reduced the urinary enzymatic activities to control levels.

Experimental procedures. Chemicals. pNP (*P*-nitrophenyl)-α-D-mannoside, pNP-β-D-galactoside, pNP-α-L-fucoside, and pNP-β-2-acetylamino-2-deoxyglucoside were from Sigma Chemical Co. pNP-phosphate and GOT, LDH-L, and LDH-P Fast-Pack reagent kits were from Calbiochem. Protamine-zinc insulin was from Eli Lilly. Streptozotocin was from the Upjohn Co. Other reagents used were of the highest available purity.

Preparation of urine samples. Holtzman male Sprague-Dawley rats (100-300 g) were made diabetic by injection of 60 mg/kg of streptozotocin (in 0.1 *N* citrate, pH 4.5, administered via a tail vein). Three or more days after injection, samples of urine from diabetic and control rats were collected for 24 hr in bottles kept in ice. All animals had access to water. A group of diabetic rats and a control group were fed Purina rat chow during the collection period (fed group); a second group of diabetic rats and their controls were fasted during the urine collection period (fasted group). To induce nondiabetic polyuria, one group of fasted normal rats was given drinking water containing 30 g of glucose and 1.25 g of sodium saccharin/liter (11) and another group of fed normal rats was given drinking water containing 0.5% (w/v) NaCl.

The urine samples were centrifuged for 10 min at 480*g* and the pellet discarded. Ammonium sulfate (390 mg/ml of urine) was slowly added to the urine and the mixture was stirred for 20 min at 4°, then centrifuged for 30 min at 13,000*g*. The pellet was suspended in a minimal amount of 0.1 *M* acetate buffer, pH 5, then dialyzed for 4 hr against the same buffer and adjusted to 25 ml total volume with buffer prior to assay.

Assays. Blood sugar levels were estimated by the *o*-toluidine method (12). Data ob-

[1] Please address inquiries to this author at the Department of Physical Science.

tained from diabetic rats having blood sugar values less than 300 mg/100 ml of serum were not used. Protein levels were estimated using the biuret method (13).

Assays of acid hydrolase activities were conducted using 0.1 *M* acetate buffer adjusted to pH 5.0 for α-L-fucosidase, β-D-galactosidase, and *N*-acetylglucosaminidase, pH 4.6 for α-D-mannosidase, and pH 4.2 for acid phosphatase. One milliliter of buffer containing a 2 m*M* concentration of the appropriate substrate was mixed with 1.0 ml of urine preparation. This mixture and controls (1 ml of urine preparation plus 1 ml of buffer; 1 ml of buffer–substrate plus 1 ml of water) were incubated for 30 min at 37°. One milliliter of 10% trichloroacetic acid was added and the mixture was centrifuged. An equal volume of 0.5 *M* NaOH was added to the clear supernatant fraction and the absorbance at 410 nm was read. Alkaline phosphatase activity was assayed in 0.5 *M* glycine buffer, pH 10.5, containing 2 m*M* pNP-phosphate, incubated as above, then read directly at 410 nm. Calbiochem GOT, LDH-L, and LDH-P Fast-Pack kits were used as directed (14) to assay for GOT and lactate dehydrogenase activities in the urine preparations. In all cases, the activities measured were proportional to the amount of preparation assayed.

Results and discussion. The activities of acid phosphatase, alkaline phosphatase, *N*-acetylglucosaminidase, and β-D-galactosidase are sufficiently great to permit their detection in crude, dialyzed rat urine. However, the data from assays made using the enzyme activities precipitated from the urine by ammonium sulfate treatment were more precise. Concentration of the urinary protein by ammonium sulfate treatment was prerequisite to the measure of GOT and α-mannosidase activities.

We were unable to detect lactate dehydrogenase activity in the urinary protein fraction. All enzymatic activities were lost after tubes containing the urine preparations were placed in a boiling-water bath for 5 min. Sephadex G-200 column chromatography revealed that most of the urinary acid hydrolase activity was associated with the protein fraction having molecular weights between 100,000 and 220,000. Urines from rats treated with nephrotoxic agents possess glycosidases with similar molecular weights (15).

As seen in Table I, our fed diabetic rats produced almost 12 times more urine than fed controls per 24-hr period, and this urine contained activities of those enzymes of Ta-

TABLE I. ACTIVITIES OF SELECTED ENZYMES IN URINES FROM NORMAL AND FROM STREPTOZOTOCIN-DIABETIC RATS[a]

	Normal, fed	Diabetic, fed	Normal, fed, 0.5% saline[b]	Normal, fasted	Diabetic, fasted	Normal, fasted + saccharine[c]
Urine volume (ml/24 hr)	11 ± 2	129 ± 5	22 ± 4	9 ± 2	21 ± 3	190 ± 12
Protein (mg/24 hr)	39 ± 9	290 ± 98	53 ± 7	27 ± 2	34 ± 4	30 ± 4
N-acetylglucosaminidase	2.0 ± 0.2	13.2 ± 1.2	3.1 ± 0.6	1.5 ± 0.3	4.1 ± 0.5	1.6 ± 0.2
N-acetylglucosaminidase, untreated samples[d]	1.1 ± 0.4	8.9 ± 0.9	–	–	–	–
Acid phosphatase	12 ± 2	86 ± 9	23 ± 4	9 ± 2	27 ± 6	9 ± 2
Acid phosphatase, untreated sample[d]	26 ± 4	121 ± 23	–	20 ± 2	40 ± 3	–
Alkaline phosphatase	40 ± 10	160 ± 20	40 ± 10	30 ± 10	60 ± 10	22 ± 10
α-L-Fucosidase	1.2 ± 0.1	3.8 ± 0.3	–	0.8 ± 0.1	2.1 ± 0.2	1.2 ± 0.1
β-D-Galactosidase	4.1 ± 0.4	16.4 ± 2.5	5.4 ± 1.0	2.8 ± 0.4	5.3 ± 0.5	2.4 ± 0.2
β-D-Galactosidase, untreated samples[d]	1.5 ± 0.6	10.8 ± 3.4	–	–	–	–
α-D-Mannosidase	0.9 ± 0.1	4.2 ± 0.6	1.0 ± 0.3	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
Glutamate-oxaloacetate transaminase	0.11 ± 0.02	2.3 ± 0.9	0.17 ± 0.13	0.06 ± 0.02	0.23 ± 0.06	–

[a] Average values ± standard errors are presented for selected enzymatic activities in ammonium sulfate-precipitated urinary protein, prepared and assayed as described in the text. $N = 10$ in each case. Lactate dehydrogenase activity was not detected in our samples. Activities are reported as nanomoles of pNP/30 minute in 24-hr samples, except for GOT; activities of GOT are given as nanomoles of oxaloacetate/minute in 24-hr samples.

[b] The drinking water of this group contained 0.5% (w/v) sodium chloride.

[c] The drinking water of this group contained 30 g of D-glucose and 1.25 g of sodium saccharin/liter.

[d] Urines were dialyzed for 4 h against deionized water. For fasted groups, $N = 24$. For fed groups, $N = 15$–19. Urine was collected from pairs of normal control rats.

ble I that were elevated about sixfold over control values. Our fasted diabetic rats produced urinary enzymatic activity values and urinary volumes that were about two-fold greater than those yielded by fasted rats and by fed controls in 24 hr ($P_{av} < 0.005$ and 0.025, respectively). Urines from fasted diabetic rats and from fed diabetic rats both contained significantly less enzymatic activity per milliliter of urine than the control urines, as may be calculated from the data of Table I. The average specific activities of the enzymes listed in Table I in urine from fasted diabetic rats were twofold greater than that of controls; those of the fed diabetic rat urine were 0.66 that of control values. Values for the levels of enzymatic activities in urines collected from rats in the period from 3 to 30 days after injection with streptozotocin were similar. The 24-hr urinary volume and the levels of the urinary acid hydrolase activities (and the concentration of urinary glucose) returned to control values when fed diabetic rats were treated twice daily with 2 to 4 units of insulin per treatment. Upon cessation of insulin treatment, the 24-hr urinary volume and the levels of enzymatic activities contained therein gradually increased, reaching pretreatment levels about 3 days after the insulin injections were stopped. Results for *N*-acetylglucosaminidase and α-L-fucosidase, which are representative of the behavior of the acid hydrolases, are shown in Fig. 1.

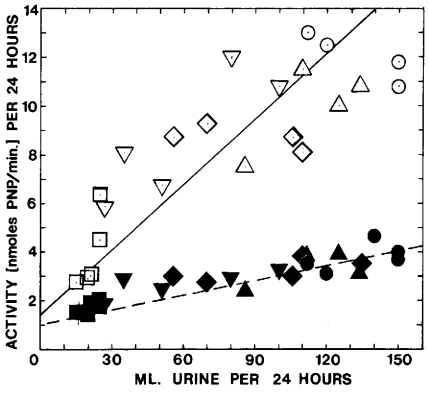

FIG. 1. The effect of insulin upon the excretion of daily total urinary acid hydrolase activity and upon urinary volume produced by fed, streptozotocin-diabetic rats. Ammonium sulfate preparations of urines from five diabetic animals collected at the appropriate times were studied. Mean enzyme activity excreted per 24 hr is plotted against the respective urine volume obtained from each animal for the enzymes *N*-acetylglucosaminidase (open symbols) and α-L-fucosidase (closed symbols). The lines derived from the least-squares equations obtained from analyses of all data (including that from fed and fasted normal rats, fed normal rats given 0.5% saline solution to drink, and fed and fasted diabetic rats) for *N*-acetylglucosaminidase (——) and α-L-fucosidase (— —) are also shown. The symbols represent values obtained from fed, streptozotocin-diabetic rat urines: prior to insulin treatment (○, ●); during insulin treatment (□, ■); 1 day after cessation of insulin treatment (▽, ▼); 2 days after insulin treatment (◇, ◆); and 3 days after insulin treatment (△, ▲). Similar results were obtained with acid phosphatase, β-D-galactosidase, and α-D-mannosidase.

The above data suggest a strong correlation between the volume of urine produced and the levels of urinary enzymatic activity excreted per 24 hr. Indeed, a linear relationship between the volume of urine produced by an individual rat and the level of enzymatic activity contained in that urine was readily apparent when these values for each individual rat were graphed. Data from a minimum of 40 animals were plotted for each enzyme studied. Least-squares analysis of the data for each enzyme yielded parameters for a linear equation which were essentially the same as those which may be calculated from the data of Table I. Correlation coefficients (r^2) ranged from 0.73 to 0.88. Results for two equations are shown in Fig. 1.

To determine if the increased levels of urinary enzymatic activities are related to diabetes or if large amounts of urinary enzymes are yielded by any animals which produce large volumes of urine, we attempted to induce normal rats to produce large quantities of urine by giving them either 0.5% NaCl (w/v) or a glucose and saccharine (11) mixture in their drinking water. Rats given the saline solution produced urinary volumes and enzymatic activities similar to those of fasted diabetic rats. However, rats given glucose and saccharin in their drinking water produced about 17 times more urine in 24 hr than controls, but their urine contained levels of enzymatic activities similar to control values, as seen in Table I. Therefore, it is diabetes itself that is responsible for certain enzymes existing in the 24-hr

urine of diabetic rats at levels well above those of normal control. The excretion rate of lysosomal and of nonlysosomal enzymes is affected by diabetes. The fact that lactate dehydrogenase, an enzyme found at concentrations above control values in urines from subjects having certain renal disorders (14, 16), is not elevated above control values in diabetic rat urine suggests that the effect of diabetes may be specific to certain cell types as the source of the urinary enzymes.

The source of the urinary enzymes under study is still unknown. This and the effect of the excessive loss of those enzymes upon the organ from which they came will require further study.

Summary. The 24-hr urine collected from streptozotocin-diabetic rats contained activities of acid phosphatase, alkaline phosphatase, *N*-acetyl-β-D-glucosaminidase, α-L-fucosidase, β-D-galactosidase, glutamate-oxaloacetate transaminase, and α-D-mannosidase that were significantly greater than control values. Lactate dehydrogenase activity was similar in urines from diabetic and from control animals. Results were similar with rats injected with streptozotocin from 3 to 30 days prior to use. The levels of urinary acid hydrolase activities present in diabetic rats returned to control values upon treatment with insulin and became elevated above control values once again upon cessation of insulin treatment. The levels of enzymatic activity measured in diabetic rat urine, except for LDH, were directly proportional to the volume of urine produced. However, normal animals with induced polyuria did not produce urines with enzymatic activities above control values.

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