PHARMACOLOGICAL STUDIES ON EXPERIMENTAL NEPHRITIC RATS (9). CHANGES IN ACTIVITIES OF URINARY ENZYMES IN THE MODIFIED TYPE OF MASUGI'S NEPHRITIS AND THEIR SOURCES

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Abstract—Using a modified model of Masugi's nephritis of rats, various enzymatic activities in urine, serum and renal tissue (glomeruli or cortex) were determined at appropriate intervals after the administration of anti-kidney serum and compared with the urinary protein content and the kidney weight. In the urine, alkaline phosphatase (Al-Phosase), acid phosphatase (Ac-Phosase) and N-acetyl-β-glucosaminidase (NA-β-Gase) activities remarkably increased after the induction of nephritis, reached their peaks on the 10th day and reverted to almost the normal levels on the 30th day. The patterns of time course of these enzymatic activities were similar to patterns seen in the urinary protein content and the kidney weight. In the serum, the Al-Phosase activity decreased slightly, while NA-β-Gase activity increased slightly. The Ac-Phosase activity in serum remained at normal levels during the experimental periods. In the glomeruli, the bound activities of these three enzymes decreased with nephritis, showing a negative correlation with results in the urine. On the other hand, fibrinolytic activities in the urine (plasmin-like enzyme) and renal cortex (plasminogen activator) also paralleled the urinary protein content and the kidney weight in the course of the disease. These results suggest that the Al-Phosase, Ac-Phosase and NA-β-Gase excreted into urine in cases of nephritis may be mostly derived from damaged renal cells and one part of Al-Phosase may also come from the plasma. Moreover, the increase of plasmin-like enzyme in urine is considered to be due to the increase of plasminogen activator in the renal cortex. Thus, the determination of these enzymatic activities in the urine should be useful for evaluating effects of drugs for the treatment of nephritis.

With the development of enzymatic chemistry, these activities in the serum or urine have been frequently determined as a supplementary means of diagnosis of various clinical diseases. It has been demonstrated in humans (1, 2) and experimental animals (3–5) that enzymatic excretions into the urine are increased in proportion to the degree of renal damage. We also found that the excretions of alkaline phosphatase (Al-Phosase) and lactic dehydrogenase into urine and glucose-6-phosphate dehydrogenase activity in kidney were increased in cases of Masugi's nephritis of rats (6).

The present work was an attempt to detect the enzymes which are useful for estimating the effect of drugs for treatment of nephritis and in addition to Al-Phosase activity, we determined acid phosphatase (Ac-Phosase), N-acetyl-β-glucosaminidase (NA-β-Gase),
acid protease (Ac-Proase), neutral protease (N-Proase) and fibrinolytic (FL) activities in urine at various intervals after the i.v. administration of anti-kidney serum in the modified type of Masugi’s nephritis of rats. We then compared our findings with urinary protein content and kidney weight. These enzymatic activities were then studied in renal tissue (glomeruli or cortex) and serum to determine whether sources of enzymes excreted into urine are mainly from damaged renal cells or circulating blood.

MATERIALS AND METHODS

Induction of modified type of Masugi’s nephritis

The anti-rat kidney rabbit serum (anti-kidney serum) was prepared as we previously reported (7) and nephritis was induced in male Wistar rats weighing approx. 140 g by a single i.v. administration of 0.5 ml/100 g of body weight of anti-kidney serum.

Preparation of enzymatic materials

Urine, serum and renal cortex: Groups of 10 rats were sacrificed on the 1st, 5th, 10th, 15th, 20th and 30th days after the administration of anti-kidney serum, blood was taken from the abdominal aorta and the serum separated as the enzymatic material. Animals were kept in individual metabolic cages for 24 hr before sacrifice for blood and urine collection. The urine was then centrifuged at 3,000 rpm for 15 min and the supernatant used as the enzymatic material. Immediately after the urine and blood collections, both kidneys were removed and weighed. The kidney was divided into halves and the medulla was excised. The cortex thus obtained was used for the determination of FL activity. All enzymatic materials were kept at 0-4°C and enzymatic activities were determined within 24 hr.

Glomeruli: Groups of 16 rats were sacrificed at the same intervals as in the case of determination of enzymes in urine, serum and renal cortex after the anti-kidney serum administration. The kidneys from each rat were perfused in situ with physiological saline solution until blanched. The perfused kidneys from 2 animals were mixed and glomeruli were isolated in accordance with the method of Chow and Drummond (8). The glomeruli obtained were suspended in 0.2% Triton X-100 dissolved in physiological saline solution and then allowed to stand for 60 min with occasional saking. The suspension was centrifuged at 12,000 rpm for 20 min at 0°C and the supernatant was used as the enzymatic material.

Determination of urinary protein content and kidney weight

The protein content in the urine used for determination of enzymatic activities was determined by the method of Kingsbury et al. (9) and expressed as mg per 24 hr urine. On the other hand, the wet weight of both kidneys was determined and expressed as g per kg of body weight.

Determination of Al-Phosase, Ac-Phosase, Na-β-Gase, Ac-Proase and N-Proase

Al-Phosase and Ac-Phosase activities were determined at pH 10.5 and 4.5, respectively, by the method of Bessey et al. (10) by using disodium ρ-nitrophenyl phosphate (Daiichi Kagaku Co. Ltd.) as substrate. Na-β-Gase activity was determined at pH 4.5 by the method of Hasebe (11) using ρ-nitrophenyl-N-acetyl-β-D-glucosaminide (BDH Chemicals
Co. Ltd., Poole, England) as substrate. Ac-Proase and N-Proase activities were determined at pH 3.1 and 7.0, respectively, by the method of Bertelli et al. (12) using 2% denatured hemoglobin (Sigma) solution as substrate. The activity of each enzyme was expressed as units, as shown in the Tables.

**Determination of FL activity**

FL activity in the renal cortex and urine was determined by the fibrin plate method described by Astrup and Müllertz (13). For the assay of FL activity, two kinds of plates were used: standard fibrin plate, containing plasminogen and heated fibrin plate, prepared by treating the standard fibrin plate at 85°C for 30 min to destroy fibrin-associated plasminogen. In the case of renal cortex, the central part of the tissue was punched out with a metabolic cylinder of 5 mm in diameter and the tissue thus obtained was placed on a 37°C standard fibrin plate. On the other hand, 0.03 ml of urine was put on either the standard or fibrin plate at 37°C. The FL activity was expressed as the multiplied product (mm²) of longitudinal and abscissal lengths (mm) of lysis area after 30 hr.

**RESULTS**

*Changes in protein content in urine and wet weight of both kidneys after i.v. administration of anti-kidney serum (Fig. 1)*

The urinary protein content (mg/24 hr urine) in the nephritic group produced an increase of 372% on the day after the administration of anti-kidney serum and reached a peak (1944% higher than in the normal group) on the 10th day. Thereafter, the rate of increase was reduced gradually but was still over 100% higher than the normal level on the 30th day.

On the other hand, the wet weight of both kidneys per kg of body weight in the nephritic group was significantly higher than that in the normal group from the 5th day and reached a peak (58% higher than in the normal group) on the 15th day. Later, the weight decreased gradually and returned to nearly normal levels by the 30th day.

![Fig. 1. Changes in protein content in urine and wet weight of both kidneys after intravenous administration of anti-kidney serum to rats. Each plot denotes mean value with S.E. obtained from 10 rats. Asterisk indicates a significant difference from normal (***: P<0.001, **: P<0.01).](image)
Changes in various enzymatic activities in urine and serum after the administration of anti-kidney serum

Al-Phosase activity (Fig. 2): The Al-Phosase activity in urine (μM ρ-nitrophenol/24 hr urine/hr) in the nephritic group brought about a marked increase from the 5th day, reached a maximum (746% higher than the normal level) on the 10th day and reverted to practically the normal level on the 30th day. The patterns of time course of changes in the enzymatic activity in the urine were similar to those in the urinary protein excretion and the kidney weight.

On the other hand, the enzymatic activity in serum (mM ρ-nitrophenol/1000 ml/hr) resulted in a slight but significant reduction from the 10th day and was lower by 45%, as compared with that of the normal group on the 15th day.

Ac-Phosase activity (Fig. 2): The Ac-Phosase activity in urine (μM ρ-nitrophenol/24 hr urine/hr) of the nephritic group induced pronounced increase of over 200% the 5th to the 15th days.

In the serum, however, the enzymatic activity (mM ρ-nitrophenol/1000 ml/hr) showed little change throughout the experimental periods up to the 30th day.

NA-β-Gase activity (Fig. 2): The NA-β-Gase activity in the urine (μg ρ-nitrophenol/24 hr urine/hr) in the nephritic group increased gradually with time, attained to a peak (140% higher than the normal level) by the 10th day and returned to the normal level by the 30th day. Thus, in the urine, this activity changed in much the same manner as did the urinary protein excretion and the kidney weight.

In the serum, the enzymatic activity (μg ρ-nitrophenol/ml/hr) showed a slight but significant increase of approx. 20% from the 5th to the 20th days.

Ac-Proase activity (Fig. 3): The Ac-Proase activity [O.D. (280 nm) of TCA soluble peptide/24 hr urine and ml, respectively, for urine and serum/hr] in the nephritic group showed a significant increase from the 5th to the 30th day.

Fig. 2. Changes in alkaline phosphatase, acid phosphatase and N-acetyl-β-glucosaminidase activities in urine and serum after intravenous administration of anti-kidney serum to rats. Each plot denotes mean value with S.E. obtained 10 rats. Asterisk indicates a significant difference from normal (***: P<0.001, **: P<0.01, *: P<0.05).
tended to increase from the 5th to the 20th days in the urine and from the 10th to the 20th days in the serum.

**N-Proase activity (Fig. 3):** The N-Proase activity in the urine (O.D. (280 nm) of TCA soluble peptide/24 hr urine/hr] in the nephritic group usually tended to be higher as compared with that of the normal group during the 30 day observation period, in particular, on the 5th day, it was insignificantly higher by 128%.

Enzymatic activity in the serum was too low for detection.

**Changes in Al-Phosase, Ac-Phosase and NA-β-Gase activities in glomeruli after the administration of anti-kidney serum (Fig. 4)**

Al-Phosase, Ac-Phosase and NA-β-Gase activities in glomeruli (mM or µg p-nitrophenol/g renal cortex) were significantly reduced, compared with those of the normal groups from the 5th to the 15th days. In particular there were maximal reductions on the 15th day, viz., Al-Phosase, Ac-Phosase and NA-β-Gase activities were lower by 78, 69 and 81%, respectively, than the normal levels. Changes in these enzymatic activities in the glomeruli during the process of nephritis showed a negative correlation with findings in the urine.

**Changes in FL activity in urine and renal cortex after the administration of anti-kidney serum (Fig. 5)**

The FL activity in urine [lysis area (mm²)/30 hr] of the nephritic group showed no significant increase on the standard fibrin plate, compared with findings in the normal group. In contrast, with the heated fibrin plate method, the FL activity in the urine of the nephritic group induced a marked increase, with over 800% increase on the 10th and 15th days.

On the other hand, the FL activity in the renal cortex [lysis area (mm²)/30 hr] was detectable with the standard fibrin plate method, but not with the heated fibrin plate method. In the nephritic group, the FL activity on the standard fibrin plate significantly increased.
from the 5th day, reached a maximum (305% higher than the normal level) on the 15th day and returned to almost normal levels on the 30th day. Thus, FL activities in the urine with the heated fibrin plate method and in the renal cortex with the standard fibrin plate method showed much the same patterns of change as seen in urinary protein excretion and the kidney weight, during the course of nephritis.

DISCUSSION

These experiments have shown that Al-Phosase, Ac-Phosase and NA-β-Gase activities markedly increase in the urine of rats with the modified type of Masugi's nephritis. In attempts to detect the sources of these three enzymes, the activities were also determined in serum and glomeruli. In the serum, the Al-Phosase activity was reduced slightly but significantly after the induction of nephritis. Thus, the Al-Phosase excreted into the urine may partly originate in the circulating blood as the result of an enhanced filtration in glomerular basement membrane (GBM). In the case of glomeruli, we determined bound activities of these enzymes which correspond to those of the enzymes linked to the lysosome particles in the glomerular cells. In contrast with the results in urine, the bound activities of these enzymes in the glomeruli resulted in striking reductions in the nephritic rats. These results
indicate that the main source of these enzymes excreted into urine is renal damaged cells. Concerning enzymatic distribution within the kidney, Bonting et al. (14) showed histo-
chemically that proximal tubular cells of rabbit kidneys were rich in Al-Phosase, thereby indicating injury of not only glomerular cells, but also the proximal tubular cells. On the other hand, the Ac-Phosase activity showed neither increase nor decrease in the serum of the nephritic rats. Therefore, it would seem unlikely that increase in this enzymatic activity in the urine results from injured organs other than the kidneys. As glomeruli in the healthy human kidney have been shown (15) to be rich in Ac-Phosase, the enzymatic activity in urine may be a more sensitive index to determine the extent of injury of the glomerular cells. As to the NA-β-Gase, Price et al. (1) separated isozymes of the enzyme to A and B forms and found that the latter was not detectable in normal urine and serum, although present in the kidney, and that in addition to the A form, the B form was remarkably excreted into urine of patients with renal diseases. As the NA-β-Gase activity in our experiment revealed a significant increase in the serum of the nephritic rats, the increase of this enzymatic activity in the urine is assumedly due to the injury of the renal cells rather than to acceleration of the filtration of this enzyme through the GBM. Shibata et al. (16) reported that a number of polymorphonuclear leukocytes (PMN) appeared in the glomeruli 2 hr after the injection of nephrotoxic serum. It has been known that phagocytosis by the PMN induces an extracellular release of PMN lysosomal enzymes. Accordingly, the possibility remains that these enzymes excreted into the urine in nephritic rats may originate in the PMN which have infiltrated to the glomeruli. However, in the present experiment, the activities of Ac-Proase and N-Proase showed no increase in the urine of the nephritic rats. Thus, the enzymes released from the PMN may have little effect on increases in these enzymes in the urine.

The role of coagulation and fibrinolysis in glomerulonephritis has been recently discussed in relation to progression of the disease. In our work, we investigated only the fibrinolysis system and found that the urinary samples obtained from normal rats possessed the ability to lyse the standard fibrin plate (plasminogen-rich fibrin plate) but little or no ability to lyse the heated fibrin plate (plasminogen-free fibrin plate). It has been reported that ε-amino-caproic acid (EACA) inhibits both plasmin (P) and plasminogen activator (PA) but not nonspecific proteases such as trypsin (17, 18). In the preliminary experiment, the lytic activity of normal urine was markedly inhibited by EACA (1 × 10⁻³ M or 7.63 × 10⁻³ M). It is therefore considered that the urine from normal animals has PA activity. On the other hand, the urine from nephritic animals showed lytic activities of both the standard and the heated fibrin plates and these lytic activities were also suppressed by EACA (preliminary experiment). This indicates the presence of P-like activity in the urine of the nephritic group. Renal cortex in the normal and nephritic groups exhibited lysis in the case of the standard fibrin plate. However, the lytic activity was completely suppressed by heat treatment of the standard fibrin plate or EACA (1 × 10⁻³ M) (preliminary experiment). Therefore, the FL activity observed in the renal cortex was ascribed to PA. In the nephritic rats, changes in P-like activity in the urine ran almost in parallel with changes seen in PA activity.
in the renal cortex during the course of the disease. With regard to the source of the urinary P, Nakabayashi et al. (19) demonstrated that plasminogen in blood was converted to P in glomeruli or in urine through the GBM. All these findings taken together suggest that increase in the urinary P-like activity in the nephritic groups may be due to the increases in the PA released from injured cells in the cortex. Detailed investigations on the fibrinolysis system as related to coagulation in induced-glomerulonephritis are ongoing in our laboratory.

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