Urinary Enzymes and Low-Molecular-Mass Proteins as Indicators of Diabetic Nephropathy

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We measured the excretion rates of six urinary enzymes that either originate from the proximal renal tubule, like alanine aminopeptidase (EC 3.4.11.2), alkaline phosphatase (EC 3.1.3.1), γ-glutamyltransferase (EC 2.3.2.2), and N-acetyl-β-D-glucosaminidase (EC 3.2.1.30), or that are typical low-molecular-mass proteins, like lysozyme (EC 3.2.1.17) and pancreatic ribonuclease (EC 3.1.27.5). These rates were compared with those of total protein and albumin in urine of 36 insulin-dependent diabetic men and 30 healthy men. Seventeen of the diabetics had "clinical proteinuria," defined as excretion of more than 7.5 g of protein per mole of urinary creatinine (group B). Group A comprised the 19 diabetics without proteinuria. Except for γ-glutamyltransferase, the excretions of enzymes and proteins were significantly higher in diabetics than in controls and were greater in group B than in group A. N-Acetyl-β-D-glucosaminidase was the analyte most often increased in group A (89%), followed by albumin and alkaline phosphatase (each 32%). All patients in group B showed increased excretion of N-acetyl-β-D-glucosaminidase. We conclude from the comparative data that this enzyme may be useful as an early predictor of diabetic nephropathy.

Additional Keyphrases: diabetes · proteinuria · N-acetyl-β-D-glucosaminidase

Increased excretion of urinary albumin is one of the earliest indicators firmly associated with later-developing diabetic nephropathy (1–3). It precedes the appearance of "clinical proteinuria" (defined as proteinuria of more than 0.5 g/24 h) or of increased values for serum creatinine (3). Some studies (e.g., 4–8) have also shown increased urinary enzymes or low-molecular-mass proteins in urine of patients suffering from diabetic nephropathy. Increased excretion of albumin is generally assumed to result from changes in the glomerular basement membrane, whereas increased excretion of urinary enzymes or of low-molecular-mass proteins supposedly indicates tubular dysfunction. However, few comparative investigations have been performed to characterize the validity of these analytes, and the underlying different biochemical mechanisms they reflect, as possible predictors of diabetic nephropathy (8, 9). Detection of reliable predictors of diabetic nephropathy is desirable because this complication may be reversed or delayed by strict glycemic control and treatment of hypertension in the early stages of nephropathy (10). Therefore, in this paper we evaluate the excretions of the tubular enzymes alanine aminopeptidase (AAP), alkaline phosphatase (AP), γ-glutamyltransferase (GOT), N-acetyl-β-D-glucosaminidase (NAG), and the low-molecular-mass proteins lysozyme (LYS) and pancreatic ribonuclease (RNase) in comparison with the excretions of total protein and albumin as the recognized criteria of diabetic nephropathy.³

Materiales, Methods, and Subjects

Apparatus: We used a DU-8 spectrophotometer (Beckman Inc., Fullerton, CA), photometer PCP 6121, analyzer ACP 5040, and pipettes (all from Eppendorf Gerätebau Netheler & Hinz, Hamburg, F.R.G.), a microplate reader Multiscan MCC and digital multichannel pipettes (all from EFLAB Oy, Helsinki, Finland), a refrigerated centrifuge (Model K23; VEB Zentrifugenbau, Leipzig, D.D.R.), and 25 cm × 1 cm (i.d.) chromatographic columns.

Reagents: 4-Nitrophenyl phosphosphate, Tris, peroxidase, polycytidilic acid, and Nephur®-tests (test strips for proteinuria) were obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G.; alanine-4-nitroanilide, diethanolamine (distilled before use), Amido Black 10B and lantanum nitrate from E. Merck, Darmstadt, F.R.G.; 4-nitrophenyl-N-acetyl-β-D-glucosaminidase, Brij 35, and bromocresol green from Serva Feinbiochemica, Heidelberg, F.R.G.; Sephadex G50 (medium) and Phadezym®-tests from Pharmacia Fine Chemicals AB, Uppsala, Sweden; human albumin from VEB Impfstoffwerke, Dessau, D.D.R.; and test combinations for the measurement of GOT activity from Lachema, Brno, Czechoslovakia. Other chemicals of analytical grade were from VEB Labochemie, Apolda, D.D.R., or from E. Merck.

Procedures: We determined AAP, AP, GGT, NAG, and RNase activities at 37°C as described previously (11, 12), with the following concentrations in the reaction mixtures (per liter): 2 mmol of alanine-4-nitroanilide and 50 mmol of Tris (pH 7.80) for AAP; 10 mmol of 4-nitrophenyl phosphosphate, 0.5 mmol of MgCl₂, and 1 mol of diethanolamine (pH 9.80) for AP; 4 mmol of γ-glutamyl-4-nitroanilide, 101 mmol of NaCl, and 101 mmol of glycylglycine (pH 8.20) for GGT; 2 mmol of 4-nitrophenyl-N-acetyl-β-D-glucosaminidase and 100 mmol of citrate buffer (pH 4.40) for NAG; and 0.3 mmol of polycytidilic acid, 200 mmol of imidazole/HCl buffer (pH 7.0), and 50 mmol of NaCl for RNase. For quantification of LYS, we used a heterogeneous enzyme immunoassay as direct immunometric assay (13).

To determine total protein in urine, we used a method with Amido Black 10B with which 10 mg of protein per liter was detectable (14). Urinary albumin was assayed by mixing equal volumes of gel-filtered urine with bromcresol green reagent and measuring the absorbance at 623 nm after 15 s. Final concentrations in the reaction mixture were, per liter, 1.44 mmol of bromcresol green, 25 mmol of acetate buffer (pH 4.2), and 3.2 g of Brij 35. The detection limit for this method was 8.5 mg/L.

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³ Nonstandard abbreviations (and systematic names of enzymes): AAP, alanine aminopeptidase (microsomal aminopeptidase; ω-aminocacyl-peptide hydrolase (microsomal), EC 3.4.11.2); AP, alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1]; GGT, γ-glutamyltransferase [(γ-glutamyl)peptidase-amino-acid γ-glutamyltransferase, EC 2.3.2.2]; NAG, N-acetyl-β-D-glucosaminidase (N-acetyl-β-D-glucosaminidase N-acetylglucosaminidase, EC 3.2.1.30); LYS, lysozyme (peptidoglycan-N-acetylmuramoylhydrolase, EC 3.2.1.17); RNase, pancreatic ribonuclease (ribonuclease I, EC 3.1.27.5).
\(\beta_2\)-Microglobulin was measured with the Phadezym test kit according to the suppliers' instructions. Creatinine was determined by a kinetic method (15), glucose by an enzymatic method (16), and hemoglobin A\(_1\) by a colorimetric method (17).

**Subjects and specimens:** To exclude sex-related differences in urinary enzyme excretion and to facilitate the interpretation of results, we studied only male subjects. The control group consisted of healthy hospital staff members. The 36 insulin-dependent diabetics were divided into two groups, one without (group A) and the other with (group B) diabetic nephropathy (Table 1). Diabetic nephropathy was assumed when persistent proteinuria (3)—defined as more than 7.5 g per mole of urinary creatinine and positive protein readings with Nephur-test or sulfosalicylic acid—had already been established in previous examinations at our outpatient department. No patient in group A, and all but two subjects in group B, showed diabetic micro-angiopathy, as characterized by diabetic retinopathy diagnosed by ophthalmoscopy. None had urinary tract infection or other renal diseases and none was being treated with antibiotics. There were differences between the groups with regard to systolic blood pressure, with nine of the 17 patients in group B receiving antihypertensive treatment.

Untimed urine specimens were collected from all subjects between 07:00 and 11:00 hours, centrifuged (1800 \(\times\) g, 10 min), and subjected to gel filtration. Urinary enzymes were measured no more than 4 h later (11). Simultaneously collected blood samples were centrifuged, and the sera were stored at \(-20^\circ\)C until analysis (no longer than two weeks after collection).

**Calculation:** Results for AAP, AP, GGT, NAG, and RNase were expressed in units (U), 1 U corresponding to the liberation of 1 \(\mu\)mol of 4-nitroaniline, 4-nitrophenol, or acid-soluble nucleotides per minute at 37 °C. Results for LYS were given in mass units (\(\mu\)g or mg), with purified human LYS being used as calibration material (13). The excretions of all analytes were related to urinary creatinine (11, 18). This approach permits the use of untimed urine specimens, a method also feasible in outpatient clinics.

Statistical differences were tested by the U-test according to Mann and Whitney and by the \(\chi^2\)-test (contingency table). Correlation coefficients were calculated as rank correlation coefficients according to Spearman. The Kolmogorov–Smirnov test was used to test the distribution of analytes in the control group.

**Results**

We divided our diabetic patients into two groups according to the diagnosis of diabetic nephropathy (Table 1). We found normal values for creatinine (<106 \(\mu\)mol/L) and \(\beta_2\)-microglobulin (<2.8 mg/L) in the serum of all patients. Table 2 lists the values for excretion of enzymes, low-molecular-mass proteins, and albumin. Except for GGT, diabetic patients excreted more enzymes and proteins than did healthy persons. Patients in group B showed higher

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**Table 1. Characteristics of the Control Group and the Groups of Insulin-Dependent Diabetics without and with Nephropathy**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Diabetic group A (without nephropathy)</th>
<th>Diabetic group B (with nephropathy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>30</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Age, y</td>
<td>32 (10)</td>
<td>24 (3)</td>
<td>40 (11)</td>
</tr>
<tr>
<td>Duration of diabetes, y</td>
<td>—</td>
<td>2.3 (1.8)</td>
<td>22.9 (8.8)</td>
</tr>
<tr>
<td>Insulin doses, unit/kg</td>
<td>—</td>
<td>0.46 (0.17)</td>
<td>0.75 (0.27)</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>n.d.</td>
<td>80 (8)</td>
<td>83 (11)</td>
</tr>
<tr>
<td>Diastolic</td>
<td>n.d.</td>
<td>121 (9)</td>
<td>138 (14)</td>
</tr>
<tr>
<td>Systolic</td>
<td>n.d.</td>
<td>11.1 (2.1)</td>
<td>10.2 (1.8)</td>
</tr>
<tr>
<td>Hemoglobin A(_1), %</td>
<td>n.d.</td>
<td>74 (14)</td>
<td>82 (21)</td>
</tr>
<tr>
<td>Serum creatinine, (\mu)mol/L</td>
<td>1.96 (0.42)</td>
<td>1.81 (0.86)</td>
<td>2.06 (0.57)</td>
</tr>
<tr>
<td>Serum (\beta_2)-microglobulin, mg/L</td>
<td>1.55 (172)</td>
<td>86 (78)</td>
<td></td>
</tr>
<tr>
<td>Urinary protein, g/mol 24 h</td>
<td>4.55 (1.5)</td>
<td>3.43 (1.37)</td>
<td>149 (217)</td>
</tr>
</tbody>
</table>

*Diabetic nephropathy was diagnosed when proteinuria was more than 7.5 g per mole of urinary creatinine and results of protein test reagent strips were positive.

**Table 2. Urinary Excretions of Enzymes, Low-Molecular-Mass Proteins, and Albumin in Controls and Diabetic Subjects**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Excretion, mean (and SD), per mmol of urinary creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
</tr>
<tr>
<td>AAP, U</td>
<td>1.05 (0.32)</td>
</tr>
<tr>
<td>AP, U</td>
<td>0.59 (0.27)</td>
</tr>
<tr>
<td>GGT, U</td>
<td>3.57 (1.33)</td>
</tr>
<tr>
<td>NAG, U</td>
<td>0.37 (0.15)</td>
</tr>
<tr>
<td>Albumin, mg</td>
<td>1.83 (0.98)</td>
</tr>
<tr>
<td>RNase, kU</td>
<td>3.72 (1.76)</td>
</tr>
<tr>
<td>LYS, (\mu)g</td>
<td>1.96 (1.09)</td>
</tr>
</tbody>
</table>

*Statistical comparisons were performed with the U-test between the control group and the two diabetic groups (\(^a\) \(P < 0.01\); \(^c\) \(P < 0.001\)) as well as between both diabetic groups (\(^{a,c}\) \(P < 0.05\); \(^{a,d}\) \(P < 0.01\); \(^{a,f}\) \(P < 0.001\)).
values for AAP, AP, NAG, LYS, and albumin than those in group A. In group A, there were no significantly different excretions of urinary enzymes and low-molecular-mass proteins between patients with normal and increased excretions of albumin (P < 0.05).

Because mean values hardly allow one to assess the clinical significance of the different analytes, we calculated percentages of increased values in both groups, using the 97.5 percentiles of the control group as upper reference limits for this calculation (Table 3). Because all analytes except LYS in the control group were normally distributed (Kolmogorov–Smirnov test), we calculated these limits on the basis of the gaussian distribution. In the control group, values for all analytes, including LYS, were below these limits. Especially in group A it became evident that abnormal NAG activities occur more often than those of other enzymes, low-molecular-mass proteins, and albumin (χ²-test; P < 0.001). All patients in group A with increased excretion of albumin also had increased NAG activities. Moreover, low-molecular-mass proteins were more seldom increased in both diabetic groups than albumin (χ²-test; P < 0.05, e.g., for RNase).

To discover possible relations between excretion rates and clinical conditions in diabetics, we made some correlation studies. Considering all patients together, we did not find any correlation between excreted analytes and urinary glucose, hemoglobin A₁, or blood pressure.

Discussion

When clinical proteinuria occurs in insulin-dependent diabetics, renal changes are irreversible and diabetic nephropathy progresses to chronic renal insufficiency (19, 20). Thus, indicators are needed to predict an incipient diabetic nephropathy which may be reversed by strict glycemic control (10). Such indicators should fulfill the following diagnostic and analytical criteria:

- They should be accessible by non-invasive, simple tests.
- They must point out changes before total protein excretion is abnormal.
- Their determination should be reliable and valid.
- They should possibly not be interfered with by preanalytical factors.

Characteristic structural changes are found in the kidneys of diabetics, both in the glomerular basement membrane and in the tubular cells (21). Consequently, it can be supposed that glomerular and tubular functions reflect these metabolic and structural changes in the early stages of diabetes. We therefore measured urinary analytes that are altered in their excretion behavior in case of glomerular (total protein, albumin) and tubular dysfunction (enzymes from proximal tubular cells, low-molecular-mass proteins). The enzyme NAG is found in lysosomes of the proximal renal tubule, whereas AAP, AP, and GGT are located in the brush-border membrane of the nephron. When the tubular cells are damaged, they release these enzymes into ultrafiltrate and thus the enzyme activities in urine increase. On the contrary, LYS and RNase are typical low-molecular-mass proteins, which are freely filtered through the capillary wall of the glomerulus, then almost completely reabsorbed and catabolized by the proximal tubular cells. Injury to the tubular cells diminishes these functions of tubular cells and causes increased excretion of these proteins. Thus the increased excretion rates seen for all these analytes investigated here reflect general cell damage; however, different pathobiochemical mechanisms underlie these increased rates.

From the diagnostic point of view, we have to conclude that only albumin and NAG fulfill the above-mentioned conditions as suitable predictors because they increase even before total protein excretion is persistently elevated (Table 3, group A) and their excretion rates are sufficiently higher when clinical proteinuria occurs (Table 3, group B).

Hitherto, some studies have shown that the "microalbuminuria"—i.e., an increased excretion of albumin before total protein is increased and a dipstick-positive result is obtained—can be considered to be a good predictor of the later development of diabetic nephropathy (1–3). Similar assumptions have been made for urinary NAG activity (6, 7, 22). Our investigations confirm these data. A recent report (8) published after we analyzed our data recommends determination of AAP rather than NAG. However, these authors (8) substantiated their decision by the evaluation of means. They did not mention the frequencies of abnormal values for both enzymes, which have to be calculated for such a conclusion.

If we consider microalbuminuria as the criterion of an incipient diabetic nephropathy (22), the higher rate of abnormal NAG activity as compared with albumin excretion that we observed does not contradict the clinical usefulness of NAG activity determination for that purpose. Because the detection limit of the method used for the determination of albumin is 8.5 mg/L and thus below the limit of microalbuminuria (1, 23), and because our calculated cutoff point for the albumin/creatinine ratio corresponds very well with data determined by enzyme immunoassays (24), insufficient sensitivity of our method can be excluded as the reason for the different frequencies of abnormal values of both analytes. On the contrary, we believe that these different frequencies indicate different biochemical changes and provide different information.

Defects of charge-selectivity and size-selectivity in the glomerular basement membrane, together with the increase in transglomerular filtration pressure, are responsible for an increased excretion of albumin (19, 20), whereas an increased NAG activity in urine suggests tubular dysfunction (6, 9). In any case, the view that tubular function is not affected in diabetic subjects in the early stages of diabetic nephropathy has to be revised (23). The conclusion that tubular function is undamaged was drawn from earlier measurements of β₂-microglobulin (23), but recent results

### Table 3. Percentages of Increased Values of Urinary Enzymes and Proteins in Diabetic Subjects

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP</td>
<td>21</td>
<td>82</td>
</tr>
<tr>
<td>AP</td>
<td>32</td>
<td>71</td>
</tr>
<tr>
<td>GGT</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>NAG</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td>Total protein</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Albumin</td>
<td>32</td>
<td>94</td>
</tr>
<tr>
<td>RNase</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>LYS</td>
<td>11</td>
<td>18</td>
</tr>
</tbody>
</table>

*We used the following percentiles of the control group as upper reference limits for this calculation: 1.68, 1.12, 6.18, and 0.96 U per millimole of creatinine for AAP, AP, GGT, and NAG, respectively; 7.5 and 3.75 g per mole of creatinine for the excretion of total protein and albumin, respectively; 7.17 kU per millimole of creatinine for RNase, and 3.19 mg per mole of creatinine for LYS.
achieved with other low-molecular-mass proteins bring this assumption into question (4, 5). Our findings of increased LYS and RNase support this view. However, our data also show that the reabsorptive–digestive function of tubular cells is not essentially disturbed. The high metabolic capacity and the overall renal hypertrophy of the kidney in the early stages of diabetes could explain this scarcely altered function (24).

Other authors found increased NAG activity in urine as a function of the concentration of hemoglobin $A_1$ and blood pressure (7, 25). We could not confirm these relationships.

In summary, we suggest that measurement of NAG activity in urine may provide helpful information in the follow-up study of diabetics for the early diagnosis of diabetic nephropathy. A long-term study should be performed in order to show its diagnostic relevance as compared with albumin. Commercial test combinations for determination of urinary NAG activity are available now (26, 27). The test is simple, does not require gel-filtered urine, and offers the opportunity of measuring this enzyme also in small outpatient clinics. In addition, urine samples can be stored for several months at $-25^\circ C$ with no decrease in NAG activity.

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References