Urinary Proteins Studied by Use of Isoelectric Focusing. I. Tubular Malfunction in Association with Exposure to Cadmium

O. Vesterberg and G. Nise

Isoelectric focusing in thin-layer polyacrylamide gel has been applied to studies of urinary proteins. We could differentiate the following patterns: tubular, glomerular with high and low selectivity, as well as mixed glomerular and tubular patterns, as seen in some cases of chronic pyelonephritis and uremia. The typical tubular-protein patterns—i.e., as developed in association with cadmium exposure—were characterized by elevated excretion of β2-microglobulin. Possibly the severity of renal malfunction can be assessed by the isoelectric focusing technique. To identify the protein zones, we in some cases complemented isoelectric focusing with electrophoresis at right angles in acrylamide gel and also in agarose gel containing antibodies to provide additional information.

Additional Keyphrases: diagnostic acid • β2-microglobulin • environmental hazards • renal disease, tubular and glomerular

The types and relative amount of plasma proteins excreted in urine reflect kidney function, and in some cases also mirror the concentration of certain proteins in plasma. Plasma proteins appear in urine as a result of the combined effects of glomerular filtration and of reabsorption and catabolism in the proximal tubuli (1). More than 30 different plasma proteins, each in very low concentration, have been found in the urine of healthy subjects, by various methods such as immunological ones (2). Most proteins in urine from patients with tubular malfunction are considerably smaller than are most of the proteins in urine from patients with glomerular disease (1–5). Various procedures—such as electrophoresis, gel chromatography, and immunological methods—have been used with varying success to differentiate normal, tubular, and glomerular proteinuria. In particular, paper electrophoresis (5) and gel chromatography patterns (4, 6) have been used to detect tubular malfunction caused by accumulation of cadmium in the kidneys.

We undertook an investigation of the possible use of isoelectric focusing in polyacrylamide gel (7) to obtain increased resolution of urinary proteins for diagnosis of renal malfunction after exposure to cadmium.

Material and Methods

Urine samples were obtained from workers exposed for various times and to various concentrations of cadmium. Occupationally unexposed persons provided control specimens. Samples from patients with glomerulonephritis, systemic lupus erythematosus, pyelonephritis, uremia, and from one patient with the Fanconi syndrome (tubular malfunction) were studied. Sodium azide at a final concentration of 1 g/liter was used as a preservative. To each 10 ml of urine, 0.5 ml of phosphate buffer (0.2 mol/liter, pH 7.5) was added to increase the pH to more than 6.5, because β2-microglobulin is unstable at pH values of less than 5.5 (8). Samples were usually concentrated about 25-fold at reduced pressure in collodion tubes (Membran Filter Gesellschaft, Göttingen, Germany). All the concentrates were then diluted with Tris–acetic acid buffer (2 mmol/liter, pH 7.5) to give a final 20-fold concentration.

Creatinine was determined by a modification of the Jaffé reaction (9).

Total protein was measured according to Piscator (10).

Isoelectric focusing was performed in principle as described earlier (7). However, the “Ampholine” (pH 8–10) was replaced by the same volume of Ampholine (200 g/liter, pH 9–11) to obtain a gradient in pH from 3 to 10. The following mixture was usually used: Ampholine (LKB-Produkter, 169 25 Bromma, Sweden) for pH 3–10, 2.8 ml; for pH 9–11, 0.4 ml; for pH 4–6, 0.2 ml; and for pH 5–7, 0.2 ml; and 10 ml of a stock solution of acrylamide 302.4 g/liter and

From the Chemical Division, Occupational Health Department, The National Board of Occupational Safety and Health, S-100 26 Stockholm 34, Sweden.

Received Mar. 27, 1973; accepted Aug. 17, 1973.

1 An apparatus for this is now available from LKB-Produkter.
bisacylamide 12.5 g/liter, to which was added 46 ml of distilled water in which 7.5 g of sucrose was dissolved. After de-aeration under reduced pressure for 3 min, 0.8 ml of a riboflavin solution (4 mg/100 ml of water) was added. By this means a gel was obtained with $T = 5\%$ and $C = 4\%$ measuring 11.5 x 24.5 cm and 0.2 cm thick. The acrylamide and bisacrylamide were purchased from British Drug House, Poole, England, and without further purification. The stock solutions of acrylamide and riboflavin could be stored for one week and one month, respectively, if kept in the dark at 4 °C. For studies of certain proteins, narrow pH-range gels covering two pH units were prepared. To get a pH-range especially suitable for resolving the proteins focusing close to $\beta_2$-microglobulin, we made the gel with Ampholine, pH 5–7, 3.2 ml, and pH 3–10, 0.4 ml, instead of the Ampholines mentioned above.

Concentrated and unconcentrated urine samples were applied by soaking up to 0.1 ml into pieces of chromatography paper (1.0 x 0.8 x 0.1 cm) and placing them close to the cathode. Amounts of purified $\beta_2$-microglobulin between 0.1 $\mu$g to 5 $\mu$g were applied to gels as standard reference markers.

The potential was increased successively during the experiment such that the electrical load never exceeded 45 W, so that 1000 V was used as the final voltage during the last 30 min (11). By using this procedure, focusing was complete in 80 min for the wide pH range. For the pH range 5–7, 1200 V was used for at least 150 min. Staining with Coomassie blue was performed either as described by Vesterberg (7) or by Söderholm et al. (11). Results were best when the staining solution was prepared the day before use.

After destaining, the gels were stored in sealed polyethylene bags. With experience, good estimates of the amount of $\beta_2$-microglobulin in the urine samples could be made by visual comparison with the standards. This was confirmed by densitometry with a Zeiss Chromatogram Scanner. Not only is it important to determine the amount of $\beta_2$-microglobulin, but also to estimate the amount of albumin and to inspect the general pattern of stained protein zones. The amount of $\beta_2$-microglobulin was expressed per gram of creatinine.

Sometimes a 0.4-cm wide strip was cut from the middle of the protein zones of one sample, from anode to cathode, and applied parallel to and close to the cathode in a narrow groove of equal size in a fresh plate of polyacrylamide gel impregnated with a Tris [tris(hydroxymethyl)aminomethane] buffer (pH 8.9) prepared according to Dale and Latner (12). The electrode solution was a Tris-glycine buffer pH 8.3 (12). A potential of 700 V was applied for 3 h. Staining was performed as described previously (7). By this procedure, unique patterns were obtained.

\[2\%T = g\ of\ acrylamide + g\ of\ bisacrylamide/100\ ml\ of\ solution;\]
\[\%C = 100 \times g\ of\ bisacrylamide/100\ ml\ solution/T.\] [Nomenclature according to Hjertén, S., Arch. Biochem. Biophys., Suppl. 1, 147 (1962)].

Fig. 1. Protein zones stained after isoelectric focusing of urinary proteins in polyacrylamide gel in the pH range 3–10

A centimeter scale (left) shows the distance from the anode. From left to the right the patterns have been classified as: (a) conc. tubular; (b) conc. normal; (c) unconc. sample of d normal; (d) conc. normal; (e) unconc. sample of f normal; (f) conc. sample normal; (g) unconc. sample of h normal; (i) conc. nephrosis urine, showing an advanced glomerular pattern, also with a tubular trait; (k) unconc. sample of j.

Fig. 2. Result of pH measurements at 10 °C on the gel surface from anode to cathode in an experiment corresponding to Figure 1

To identify the protein zones and to obtain an idea of the relative amount of different proteins, we did isoelectric focusing in one dimension, followed by electrophoresis at right angles into an antibody-containing agarose gel. The best results were obtained when acrylamide was added to the agarose gel as described by Skude and Jepson (13).

Results

A typical result of isoelectric focusing in a wide pH range is shown in Figure 1. At least six protein zones could be seen in samples from "normal" occupationally unexposed persons. The pH-course, as estimated with a surface pH electrode (7), is shown in Figure 2. The zone in the urinary patterns corresponding to $\beta_2$-microglobulin was easy to recognize in the gels for high resolution—i.e., covering two pH units—as shown in Figure 4. The pH-course in this type of gel is shown in Figure 5. The best estimate of $\beta_2$-microglobulin was obtained when the zone width in centimeters was multiplied by the maximum absorbance

1180 CLINICAL CHEMISTRY, Vol. 19, No. 10, 1973
I showed that the value of fractions A and B was greater than 100 μl of unconcentrated sample of the case shown in e. In c and e, note the predominant zones of albumin and proteins with somewhat higher isoelectric points (e.g., transferrin) staining most intensely. These patterns are classified as pure glomerular with a low degree of selectivity; (d) 100 μl of conc. urine showing a tubular pattern caused by exposure to cadmium by occupational inhalation of dust during many years. (Note the occurrence of β2-microglobulin in the a and d samples.) (g) conc. and (h) unconc. samples from a patient with lupus-glomerulonephritis (pattern classified as pure glomerular with a low degree of selectivity); (i) conc. and (j) unconc. samples from a patient with pyelonephritis; (k) 5 μg of β2-microglobulin. (The zone on this level in samples i and j has been shown by examination in a narrow pH range to be equal to β2-microglobulin.) (l) 0.4 μg of albumin.

Studies of urine samples obtained on different days from any one person usually showed constant protein patterns.

Care had to be exercised in the interpretation when high concentrations of β2-microglobulin appeared in urine simultaneously with high albumin concentrations and other proteins in cases of nephrotic syndrome, pyelonephritis, and uremia. Especially here, studies in a narrow pH-range were valuable to differentiate the proteins in the β2-microglobulin region. Sometimes β2-microglobulin was also added to a sample to give additional evidence for identity (cf. Figure 4). A summary of typical patterns in different diagnoses is given in Table 1.

The procedure of isoelectric focusing in one dimension and electrophoresis at right angles was also used for identification purposes and increased the resolution, e.g., when proteins have close isoelectric points but differ in size, because if proteins have the same isoelectric point, the larger ones usually show a lower electrophoretic mobility. This technique has also been found useful for other proteins (12, 15).

A blind study has been made on a group of people occupationally exposed to cadmium, where β2-microglobulin was estimated by a radioimmunochemical method and urinary proteins have been separated by conventional paper electrophoresis, as well as by isoelectric focusing. The results of this study will be published elsewhere (14).
Table 1. Summary of Typical Concentration of Proteins in the Patterns from Patients with Various Diagnoses

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>ß₂-microglobulin</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic glomerulonephritis</td>
<td>normal</td>
<td>elevated</td>
</tr>
<tr>
<td></td>
<td>(slightly elevated in some cases)²</td>
<td></td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>normal</td>
<td>elevated</td>
</tr>
<tr>
<td>(glomerular type)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic pyelonephritis</td>
<td>normal or elevated²</td>
<td>elevated</td>
</tr>
<tr>
<td>Cases with significant exposure to cadmium</td>
<td>elevated</td>
<td>normal (slightly elevated in some cases)²</td>
</tr>
</tbody>
</table>

² Especially in cases with much impaired renal function characterized by abnormally high concentrations of creatinine in the serum and diminished creatinine clearance.

Discussion

Urinary proteins have been studied earlier by isoelectric focusing but mainly with respect to pyelonephritis (15, 17). The technique was found to be useful for differentiating normal subjects from those with pyelonephritis (17). The protein pattern in pyelonephritis has been classified as a mixture of tubular and glomerular patterns of low selectivity (18). This is also in accord with our results on some of the cases with chronic pyelonephritis. For this reason it is not sufficient just to measure the lower-molecular-weight proteins in urine, but also necessary to quantify them in relation to other proteins, or at least to get an idea of the general pattern of proteins present.

It would be desirable to determine the amounts of various proteins excreted per 24 h. In practice, however, it is well known that one cannot be certain that 24-h specimens are complete. For this reason it is desirable to correlate the values with the degree of concentration of the urine either by density, osmolality, or with creatinine content. Unfortunately, none of these methods is ideal, but we think that they provide more meaningful information than when no correction at all is used, especially in the case of very dilute urines.

It is easy to recognize “pure” glomerular patterns, characterized by a relative increase in the amount of albumin. Various degrees of selectivity can be judged from the general pattern. More “pure” tubular patterns—e.g., as seen in association with cadmium exposure—are recognizable by the presence of an increased amount of ß₂-microglobulin, the other proteins being present in normal or only slightly increased concentrations. Thus, as with pyelonephritis, the general pattern gives additional information.

It is important to reexamine urines showing a band in the ß₂-microglobulin area in the pH range 3–10 at a higher degree of resolution—i.e., in the range pH 5–7—to identify and quantitate the proper ß₂-microglobulin band. A quite safe way of identifying the ß₂-microglobulin band is to focus side by side a urine sample with and without added ß₂-microglobulin. To assess the significance of ß₂-microglobulin concentrations in tubular malfunction resulting from exposure to cadmium, one must know what level of ß₂-microglobulin is “normal.” To answer this properly, many urine samples would have to be investigated. Data from about 100 normal persons indicate that the average value probably is in the region of 60 to 110 µg/g of creatinine, which is comparable to the value of 80 µg/24 h reported earlier (8). At present, it is not possible to define exactly the borderline between normal and tubular patterns. The persistence of elevated concentrations of ß₂-microglobulin in urine, as well as the concentrations in blood, should also be checked, especially in patients with significantly increased concentrations of serum creatinine.

As we mentioned earlier, some of the cases with chronic pyelonephritis and chronic glomerulonephritis showed patterns characterized by a general increase in protein excretion. Few of them were of the mixed glomerular and tubular type, although the glomerular patterns always predominated. The elevated concentrations of ß₂-microglobulin often seen in the cases are probably partly due to increased concentrations in serum, and decreased or partly inhibited reabsorption of this protein in the primary filtrate from the glomeruli (2). The rare cases with a tubular disorder caused by cadmium and also having a glomerular pattern for other reasons can be differentiated from the cases—e.g., pyelonephritic ones—with a mixed glomerular and tubular pattern by the result of analysis of cadmium in blood and urine, which gives additional evidence for exposure to cadmium. The differentiation is not important from a preventive occupational health point of view, because both persons with definite chronic pyelonephritis and those with advanced renal damage by cadmium should not be exposed to cadmium. Other, more rare, cases of tubular dysfunction have been described in the literature (1, 18); the patterns obtainable by isoelectric focusing with such urines remain to be studied.

Our results show that the amount of useful information and also the resolution obtained by isoelectric focusing is much higher than with earlier methods such as electrophoresis and gel chromatography. Furthermore, the procedure is rapid and many samples can be run in parallel to facilitate comparison. In our opinion, the method is valuable for differentiation of the following urinary protein patterns: glomerular with high and low selectivity, mixed glomerular and tubular, and tubular ones, e.g., as seen in association with cadmium exposure. Furthermore, owing to the high resolution of the technique, further information may be gained by studies on individual protein zones in different samples and diseases.
Thanks are due to Dr. Cyril Smyth for his critical comments and revision of the English text, and to Dr. R. Möllby for photographic assistance. Dr. P.-E. Evrin is especially acknowledged for supplying samples of $\beta_2$-microglobulin, and Dr. K. Kubota for the urine samples from Japan.

References


