Studies of Diabetic Nephropathy. I. Effects of Storage Time and Temperature on Microalbuminuria

Fumiko Hara,*a Kyomi Nakazato,a Kiyoko Shiba,a Junko Shimoda,c Tomie Koijima,c Yukihito Fukumura,c and Isao Kobayashid

Department of Medical Technology, College of Medical Care and Technology, Gunma University,* 3–39–15 Showa-machi, Maebashi, Gunma 371, Japan, School of Allied Health Sciences, Faculty of Medicine, Tokyo Medical and Dental University, 1–5–45 Yushima, Bunkyo-ku, Tokyo 113, Japan and Department of Laboratory Medicine and Clinical Laboratory Center, Gunma University School of Medicine, 3–39–15 Showa-machi, Maebashi, Gunma 371, Japan. Received February 2, 1994; accepted May 27, 1994

The effect of storage time and temperature on the immunological turbidimetric measurement of a low concentration of albumin in urine was investigated. In storage at −20°C, the albumin level decreased, but the rate of this decrease differed considerably among specimens. However, under storage at room temperature for 2 weeks, or at 4°C for 5 weeks albumin levels did not show significant changes. At −40°C and −80°C there were only slight decreases. At −40°C decreases were slightly greater than at −80°C. Therefore, −80°C was found to be the optimal temperature for long-term storage of urinary albumin. Some of the specimens showed a 50% decrease in albumin level after storage for 9 weeks at −20°C, but remained unchanged after storage for the same period at −80°C. A pair of specimens preserved at −20°C and −80°C were isolated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). After electrophoresis, urinary proteins were stained by silver staining to observe bands, and albumin content was determined by immunoblotting. A decrease in albumin concentration was also observed by densitometric detection.

Keywords: diabetic nephropathy; microalbuminuria; sample storage; immunological turbidimetry

Few would argue that the value of albumin level determination in urine is an indispensable tool in the early detection of diabetic nephropathy. Various studies have focused on temperature and storage time of urine specimens for the determination of microalbuminuria. Odagiri and Demura reported no change in albumin after storage at −20°C, while several other investigators noticed a decrease after such storage. However, all the research performed so far has focused exclusively on changes in albumin level at −20°C and has determined only mean values. Using immunological turbidimetry, we examined changes in albumin level in individual urine specimens after storage for up to 9 weeks at room temperature, at 4°C, −20°C, −40°C and −80°C.

Specimens showing changes due to temperature were isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Urinary proteins were stained by silver staining, and albumin was determined by immunoblotting. These two methods were used to observe changes in proteins and albumin fraction. In addition to this experiment, we investigated adsorption of albumin on the inner surface of tubes.

MATERIALS AND METHODS

Samples: Twenty-nine diabetic outpatients at our hospital were selected as subjects. Random urine specimens (negative for protein by a urine test paper) were collected and pipetted into Assist Tubes (Sarstedt Co., West Germany) for freezing in aliquots of 0.5 ml. The tubes were sealed and kept at room temperature (20–28°C), at 4°C, and in a deep freezer at −20°C, −40°C and −80°C until measurement. Storage times were one, two, five or nine weeks. Frozen specimens were thawed at room temperature before assay.

Methods of Determination: Albumin Concentration: Using the Microalbumin HA Test (Wako Pure Chemical Industries, Osaka Japan) as a reagent, specimens were analyzed using a Model 7150 automated analyzer (Hitachi Co., Tokyo). Linearity was 5 to 200 μg/ml. Two different urine samples were used to study reproducibility. The within-run CVs were 1.84% (n = 10, x = 20.8 μg/ml) and 1.43% (n = 10, x = 48.0 μg/ml).

SDS-PAGE: Using the Rapidus-Duplex Minislab Electrophoretic System (Atto, Tokyo), urinary protein was isolated by SDS-PAGE with 7.5% gel according to a modified version of Laemmli’s technique. Urinary proteins were stained with silver staining using the reagents that we prepared according to Morrissey’s technique.

Immunoblotting: Immunoblotting was performed following the method of Towbin et al. After electrophoresis, proteins separated on gel were transferred to Immobilon membrane (Millipore Co., U.S.A.) at 10 V/cm for 120 min using semi-dry Horize Blot AE-6670 (Atto, Tokyo). Blocking was done overnight at 4°C using Block Ace (Snow Brand Milk Product Co., Tokyo). The membranes were washed 3 times with 10 mM Tris–154 mM NaCl buffer solution (pH 7.4) for 10 min each, immersed in reaction mixture with the anti-human albumin-rabbit serum (Dakoas-produk, Denmark) diluted 1:1000 for 30 min at room temperature, washed 3 times with 10 mM Tris–154 mM NaCl buffer solution (pH 7.4) for 20 min each, and finally stained with Immunostain (Konica Co., Tokyo). We detected albumin using the immunological technique to check whether it adsorbed to the inner surface of the tubes. After removing the urine, the inner surface of the tubes was washed 3 times with 10 mM Tris–154 mM NaCl buffer solution (pH 7.4). The immunological technique we choose was the above immunoblotting method.
RESULTS

Temperature and Storage Time  The specimens were divided into 3 groups by protein concentration: low (5 to 10 μg/ml; N = 4), medium (10 to 30 μg/ml; N = 12) and high (30 to 100 μg/ml; N = 13). Changes in albumin level with storage period were examined (Fig. 1). Excluding the specimens stored at −20 °C, most specimens in all groups were fairly stable for up to two weeks, except one from the low concentration group at −40 °C. At −20 °C, some specimens were fairly stable, and the greatest change in albumin level with storage time was observed 3 groups.

Two specimens from the low concentration group showed a remarkable decrease in albumin level, down to near zero, after one or two weeks. Others from either the medium or high concentration groups also showed decreased albumin level with storage time.

Table 1 shows the number of specimens whose albumin level changed by more than 20% during storage in the three specimen groups. In the low concentration group, 4 specimens showed no change after five weeks of storage at room temperature. After nine weeks, a decrease was observed in 1 of the 4 specimens. At 4 °C, changes were seen in 2 specimens, one of which showed a decrease after five weeks of storage. At −20 °C all specimens showed a decrease, 2 of them after one week of storage.

The medium concentration group showed fewer temperature-dependent changes than the lower concentration group, with similar values at 4 °C, −40 °C and −80 °C. At −20 °C, 6 of the 12 specimens showed decreases.

The high concentration group showed similar findings to those of the medium concentration group.

Comparison of the three groups revealed a marked difference in the low concentration group, including specimens which were below the detectable limit. In storage at room temperature, the value increased slightly. At 4 °C, the value was stable for up to five weeks, and at −20 °C, the low concentration group showed a slight decrease at nine weeks. Compared with −40 °C, −80 °C specimen storage resulted in more stable albumin levels for a longer period.

Concentration Changes in Individual Specimens  Figure 2 shows changes in albumin concentration of 5 specimens with storage time at −20 °C and −80 °C. All five showed

---

Fig. 1. Changes in Urine Albumin Due to Storage Temperature
4 specimens were in the low-concentration group (A), 12 in the middle concentration group (B), and 13 in the high-concentration group (C). W = weeks; RT = room temperature.
a decrease of 50% or more by the ninth week of storage at 
−20 °C. Specimen 1 decreased from 22.2 to 5.46 µg/ml; specimen 2 from 35.2 to 18.7 µg/ml; specimen 3 from 15.4 to 8.2 µg/ml; specimen 4 from 92.1 to 42.1 µg/ml; and specimen 5 from 28.4 to 12.3 µg/ml.

While none of the specimens showed changes for nine weeks at −80 °C, they showed fairly large decreases with time at −20 °C. In all 5 specimens, pH was acidic with values of 5.3, 5.1, 5.3, 5.4 and 4.9, respectively, and mean pH value was 5.2.

Findings of SDS-PAGE After storage for nine weeks at −20 °C or −80 °C, the 5 specimens underwent electrophoresis using SDS-PAGE, followed by silver staining. Figure 3A shows electrophoretic patterns for specimens stored at −20 °C and −80 °C (number of specimens circled) in comparison with standard materials (molecular weight of albumin = 67000). The amount of sample loading at −20 °C and −80 °C was the same, yet the albumin fractions at −20 °C showed narrower bands. Densitometry was carried out to confirm this. Storage at −20 °C resulted in sharper bands than those at −80 °C, indicating decreases in albumin fractions.

Immunoblotting patterns showed only one band at the albumin fraction for all specimens (Fig 3B) and the bands

| Table 1. Number of Specimens Showing a 20% Change in Value from That at Time of Urine Collection |
|-----------------|-----|-----|-----|-----|-----|
| µg/ml           | Week| RT  | 4 °C | −20 °C | −40 °C | −80 °C |
| 5 ≤ ~ < 10      |     |     |     |       |       |       |
| 1W              | 0/4 | 0/4 | 2/2 | 1/1/4 | 0/4   |       |
| 2W              | 0/4 | 0/4 | 2/2 | 1/1/4 | 0/4   |       |
| 5W              | 0/4 | 0/4 | 2/2 | 1/1/4 | 0/4   |       |
| 9W              | 1/1/4 | 0/4 | 2/2 | 1/1/4 | 0/4   |       |
| 10 ≤ ~ < 30     |     |     |     |       |       |       |
| 1W              | 1/10/12 | 0/12 | 2/2 | 0/12 | 0/12 |       |
| 2W              | 1/10/12 | 1/10/12 | 2/2 | 0/12 | 0/12 |       |
| 5W              | 2/20/12 | 0/12 | 4/4 | 0/12 | 0/12 |       |
| 9W              | 1/10/12 | 0/12 | 6/6 | 1/1/12 | 10/12 |       |
| 30 ≤ ~ < 100    |     |     |     |       |       |       |
| 1W              | 1/1/13 | 1/1/13 | 2/2/13 | 1/1/13 | 1/1/13 |       |
| 2W              | 2/1/13 | 3/1/13 | 5/3/13 | 3/2/13 | 1/1/13 |       |
| 5W              | 1/1/13 | 1/1/13 | 4/4/13 | 1/1/13 | 1/1/13 |       |
| 9W              | 1/1/13 | 0/13 | 6/6/13 | 1/1/13 | 1/1/13 |       |

RT: room temperature. The denominator represents the total number of specimens showing a change, and the figure in parenthesis refers to the number of specimens showing a decrease.

Fig. 2. Weekly Concentration Changes in Specimens at −20 °C (A) and −80 °C (B)

Specimen 1 (○) decreased from 22.2 to 5.46 µg/ml; specimen 2 (■) from 35.2 to 18.7 µg/ml, specimen 3 (□) from 15.4 to 8.2 µg/ml, specimen 4 (■) from 92.1 to 42.1 µg/ml, and specimen 5 (□) from 28.4 to 12.3 µg/ml.

Fig. 3. Electrophoretic Patterns of Urinary Protein and Albumin

(A) Electrophoretic patterns of urinary protein. (B) Electrophoretic patterns of albumin. The specimen numbers are the same for (A) and (B). Circled numbers indicate storage at −80 °C and non-circled numbers storage at −20 °C.
Adsortion of Albumin on the Inner Surface of the Tubes

Four specimens were pipetted into each assist and stored at $-20^\circ$C and $-80^\circ$C for nine weeks. Adsorption of albumin on the inner surface of the tubes is shown in Fig. 4. A higher level of albumin was detected in specimens stored at $-20^\circ$C than at $-80^\circ$C.

DISCUSSION

Various studies have been reported on the determination of urinary albumin depending on temperature and storage time of specimens. At $-20^\circ$C storage, Osberg observed low values from the 2nd week, and Elving at 2 weeks, 2 months and 6 months. Erman reported a decrease from $40.7 \pm 5$ mg/24 h at the time of urine collection in 73 patients, and from $32 \pm 4.3$ mg/24 h after 7 d. Townsend observed a value decrease in specimens from 15 healthy individuals 7 weeks after collection, as well as sediment in 7 of these. However, no consideration was given to the fact that the sediment might have been the direct cause of lower values.

We also found sediment in the bottom of the tubes of several specimens frozen at $-20^\circ$C. However, at $-80^\circ$C the specimens were homogeneously frozen, and no sediment was traced. This suggests that the time required to reach a frozen state had some effect on the specimens. Regardless of the presence of sediment, we centrifuged the specimens at $1000 \times g$ for 10 min, but observed no differences.

As for storage temperature, all concentration groups showed fairly stable values. Townsend observed slight increases after specimens were stored with NaN$_3$ for 2 weeks.

At $4^\circ$C, the values were stable for up to 5 weeks. Osberg observed no changes for 8 weeks, Elving for 2 weeks, Townsend for 6 weeks, and Erman for 7 d.

Compared with $-80^\circ$C, storage at $-40^\circ$C caused only slight decreases in albumin levels. This, for long-term storage, $-80^\circ$C was found to be the optimal condition as shown in Fig. 1.

The greatest decrease in albumin level occurred when specimens were stored at $-20^\circ$C. Considerable differences in the rate of decrease were observed, indicating that this temperature was inadequate for storage of urine specimens.

Changes in pH during storage were reported by Osberg and Townsend and Erman, although these changes were believed to be too slight to affect albumin values. Townsend adjusted urine pH to 7 using 1 mol NaOH or HCl and froze adjusted and non-adjusted specimens. They found that the value decreases ranged between 23 to 91% in intact specimens compared with adjusted pH specimens.

One of the specimens showed a decrease of about 20% by the 2nd week, 8 showed a pH of 4.9—5.8 (a mean of 5.3) and one showed a pH of 6.3 at urine collection. The mean pH of the specimens showing a decrease of more than 50% by the 9th week was 5.2. These findings are consistent with the report of Townsend et al.

Sorensen reported that protein adsorbed to tubes depending on the pH or other storage conditions. We carried out a pilot study to evaluate this phenomenon and detected albumin on the inner surface of the tubes used as containers (Fig. 4). This adsorption had caused a decrease in albumin concentration. We were unable to determine the amount of albumin on the inner tube surface, but we found that the albumin level in urine was higher in tubes stored at $-20^\circ$C than in those stored at $-80^\circ$C. The decrease in albumin level during storage at $-20^\circ$C could not be accounted for merely by tube adherence. Since albumin is apt to be polymerized, we thought that the various factors in the assay methodology for albumin determination might be affected, for example, the degree of albumin polymerization during storage.

Specimen 3 (Fig. 3b), was stored at $-20^\circ$C. Starting at a molecular weight of 67000 and above, a dark stain was seen. The following was hypothesized: if the specific epitope of albumin reacting with monoclonal antibody was hindered by albumin polymerization, the albumin would not be detected. So far, this specimen is the only case available and no definite conclusion could be reached. This point is currently under investigation as well as the question of albumin adherence on tubes. This study did not take patient medication intake into consideration. Future studies will need to investigate how medication can affect results.

CONCLUSION

Conditions for storing urine albumin were investigated. Specimens were stored at room temperature, at 4°C, $-20^\circ$C, $-40^\circ$C, and $-80^\circ$C and assayed at sampling and after one, two, five and nine weeks of storage.

The protein concentration was divided into the following three ranges: low (5—10 µg/ml), medium (10—30 µg/ml), and high (30—100 µg/ml) for comparison. A decrease in albumin level occurred in storage at $-20^\circ$C, especially when pH was acidic at sampling. The variation was attributed to the period of time to reach a frozen satate and the pH of specimens.

Storage of specimens is important for the assay of microalbuminuria. An adequate storage temperature was
thought to be 4°C for a short period and -80°C for a long period.

Two reasons may explain the decrease in albumin fraction: albumin adsorbed to the inner surface of tubes, and assay of albumin might have been affected by the degree of polymerization.

REFERENCES