

CCA03209

## Fragmentation and polymeric complexes of albumin in human urine

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(Received December 14th, 1984; revision March 7th, 1985)

*Key words: Albumin; Polymer; Fragment*

### Summary

Analysis of urine proteins of some individuals with proteinuria by SDS–PAGE and silver staining revealed protein bands in urine which did not appear to be present in plasma. The bands migrated with apparent molecular weights of 260 000, 180 000, 110 000, 45 000, 40 000, 30 000, 24 000, 18 000 and 11 000. These bands were shown to be albumin polymer and fragments by using a polyclonal antibody to (a) immunoprecipitate radiolabelled urine proteins, and (b) identify bands blotted from SDS–PAGE gels onto nitrocellulose paper. The specificity of the polyclonal anti-albumin antibody was confirmed by using two mouse monoclonal antibodies raised against human albumin which, between them, recognized the same protein bands on nitrocellulose paper as did the polyclonal antibody. The results of these studies of albumin in human urine confirm that albumin exists as polymer and also show that albumin fragmentation occurs in urine. Fragmentation occurs by proteolysis of the albumin molecule both at sites within and outside disulfide loops. The predominant cleavage site appears to be approximately two-fifths of the distance from one end of the albumin molecule to produce disulfide-linked fragments of about 45 000 and 30 000 molecular weight.

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### Introduction

Electrophoretic analysis of proteins in urine on the basis of protein electrical charge is a useful clinical tool. Recently analysis of urine protein on the basis of size

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by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has shown that polymers of albumin exist in urine of individuals with nephrotic syndrome [1–3]. However, these studies have also shown that polymerization of albumin occurs mainly as a consequence of freezing urine samples from those individuals taking glucocorticoids [4,5]. The mechanisms responsible for polymerization of albumin are complex and appear to require both modification of the albumin molecule itself as well as the presence of a low molecular weight dialyzable substance in the urine [4,5]. In this study, evidence is provided for the existence of proteolytic modification of albumin in urine.

## **Materials and methods**

### *Urine samples*

Urine was obtained from individuals with proteinuria visiting adult or pediatric outpatient clinics. Urine was centrifuged for 5–10 min on a bench top centrifuge. The supernatant was then removed and either analyzed immediately or frozen in a 1.5-ml plastic tube at  $-70^{\circ}$  for assay.

### *Monoclonal antibody production*

Monoclonal antibodies against human albumin were prepared. BALB/c mice were immunized with human albumin on three occasions over a 3-mth period. Spleen cells were fused with NS-I mouse myeloma cells according to Galfre et al. [6]. Clones were screened using an ELISA assay system in which albumin was bound to the bottom of 96 well Immulon plates (Costar, Cambridge, MA, USA). Bound antibody was detected with alkaline phosphatase conjugated to rabbit antimouse antibody (Cappel Laboratories, Cochranville, PA, USA). Clones producing antialbumin antibody were subcloned by limiting dilution and then further screened for their ability to detect albumin which had been blotted from SDS-PAGE onto nitrocellulose paper. Clones 9G7 and 2D7 were selected for growth characteristics and because they recognized albumin blotted onto nitrocellulose paper following analysis by SDS-PAGE under both reducing and nonreducing conditions. Ascites fluid was obtained from pristane-pretreated mice into which clone 9G7 or 2D7 cells had been injected i.p. For the experiments described both spent culture medium and ascites fluid were used interchangeably.

### *SDS-PAGE and blotting*

SDS-PAGE was performed using a micromethod employing the agarose drop technique [7] for loading samples, and a Laemmli buffer system [8]. A discontinuous polyacrylamide gradient was employed capable of separating proteins in the range of 900 000–9 000 mol wt. Protein was detected by the silver stain technique [9]. Proteins were blotted onto nitrocellulose paper by electrophoretic transfer using a Bio-Rad Transblotting cell (Bio-Rad Laboratories, Richmond, CA, USA). The transfer buffer was Tris (25 mmol/l), glycine (192 mmol/l), methyl alcohol (20% v/v), pH 8.3. The proteins were transferred at 110 V for 1–2 h in a refrigerated recirculating water bath. Following the transfer the nitrocellulose paper (cut exactly to the size of the

gel) was soaked overnight on a rotating mixer in phosphate-buffered saline containing 3 g/100 ml bovine serum albumin (BSA) and 100 ml/l goat serum. Rabbit antialbumin antiserum (at a 1 : 400 dilution) (Cappel) or mouse monoclonal antihuman albumin antibody was then added to the buffer for 1 h. The paper was then washed five times in PBS to remove unbound antibody. Peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit antibody (Cappel) at a final dilution of 1/100 in the 3 g/100 ml BSA, 100 ml/l goat serum in PBS was incubated with the nitrocellulose blot on the rotating mixer at room temperature for 1 h. Following successive washes in PBS the nitrocellulose blot was developed with 5,5'-diaminobenzidine (1 mg/5 ml in 0.1 mol/l Tris, pH 7.4) containing 0.02% H<sub>2</sub>O<sub>2</sub>. The reaction was stopped by washing out the reactants with deionized water.

#### *Radiolabelling and immunoprecipitation*

Urine samples were dialyzed against 0.1 mol/l phosphate-buffered saline, pH 7.4. The dialyzed urine sample (100  $\mu$ l) was mixed with 20  $\mu$ l of <sup>125</sup>I (60 mCi/ml, New England Nuclear, Boston, MA, USA). To this mixture were added two iodobeads (Pierce Chemical Company, Rockford, IL, USA) for 15 min. The beads were removed and the iodinated proteins separated from free iodine by centrifugation through Sephadex G25 (Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, NJ, USA) to give <sup>125</sup>I-labelled protein which was approximately 70–90% precipitable by 30% trichloroacetic acid and with a spec act of about 1  $\mu$ Ci/ $\mu$ g. The <sup>125</sup>I-labelled protein (10  $\mu$ l urine plus 2  $\mu$ l <sup>125</sup>I-urine protein ( $1.6 \times 10^6$  cpm)) was then mixed with 10  $\mu$ l rabbit anti-albumin antiserum for 10 min at 37°C followed by 30 min at +4°C in a 1.5-ml plastic centrifuge tube. To this mixture was added 1 ml 0.1 mol/l Tris buffer, pH 7.4, containing 0.15 mol/l NaCl. Following centrifugation at 20000  $\times$  g for 10 min the supernatant was removed and discarded. The above washing step was repeated. The immunoprecipitate was then suspended in 100  $\mu$ l 10% SDS containing 8 mol/l urea and boiled for 4 min. The samples were divided into two, one portion of which was boiled with 5% beta-mercaptoethanol. The proteins in these mixtures were then separated by SDS-PAGE (using a 12% gel). Molecular weight markers (myosin heavy chain, phosphorylase, albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme) were run on the same gels and identified by Coomassie blue staining. The gels were then dried onto blotting paper and bands of radioactivity were detected by autoradiography on Kodak X-Omat AR film (Eastman Kodak Company, Rochester, NY, USA).

## **Results**

Analysis of urine samples (1  $\mu$ l unconcentrated urine) from nephrotic individuals by SDS-PAGE under non-reducing conditions showed that there were major protein bands in urine that did not have corresponding bands in plasma (Fig. 1). These bands were present at 260 000, 180 000, 110 000, 45 000, 40 000, 30 000, 18 000 and 11 000 mol wt. Analysis of urine samples showed that reduction of some urine samples with multiple bands at high molecular weight caused these bands to disappear and to leave a major band running at 67 000 mol wt comigrating with

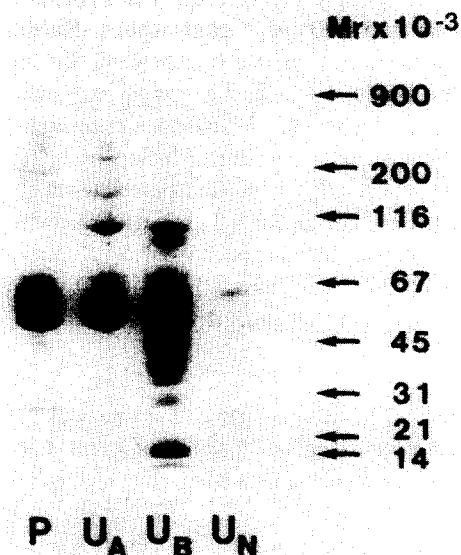


Fig. 1. SDS-PAGE gel run under nonreducing conditions stained for protein by the silver method showing plasma (P), normal urine (U<sub>N</sub>), and urine from two individuals with nephrotic syndrome (U<sub>A</sub> and U<sub>B</sub>). Note the protein bands which are easily seen in U<sub>A</sub> and U<sub>B</sub> but which are not seen in plasma or normal urine.

albumin (Fig. 2, sample U<sub>1</sub>). These results suggested that the high molecular weight bands might be polymers of albumin held together by disulfide bonds. However, in other samples reduction produced bands at lower molecular weight than albumin. In some samples (e.g. Fig. 2, sample U<sub>4</sub>) under reducing conditions only a minor band was seen at the albumin position whereas the majority of protein was seen at lower molecular weights (45 000, 18 000 and 11 000). There were also intermediate examples (as shown in Fig. 2, samples U<sub>3</sub> and U<sub>4</sub>). These results, therefore, suggested that not only was albumin polymerizing but might also be undergoing fragmentation. Therefore, a series of studies were undertaken to determine whether the protein bands observed by SDS-PAGE analysis of urine under reducing and non-reducing conditions were indeed albumin polymer and fragments.

The initial approach used a commercially available polyclonal antialbumin antibody to immunoprecipitate <sup>125</sup>I-radiolabelled urine proteins. The results of one such study are shown in Fig. 3. In one urine sample (lane A), three major radiolabelled proteins are seen by SDS-PAGE under reducing conditions. However, following immunoprecipitation by anti-albumin antibody only one band is seen at 67 000 mol wt corresponding to albumin (lane B). The anti-albumin serum, therefore, precipitated only <sup>125</sup>I-protein with the characteristics of native albumin. In contrast the result for a second sample is different. Under reducing conditions in the radiolabelled total urine protein (lane C) major bands were seen at 45 000, 40 000 and 11 000 mol wt suggesting that these proteins were not albumin. However, following

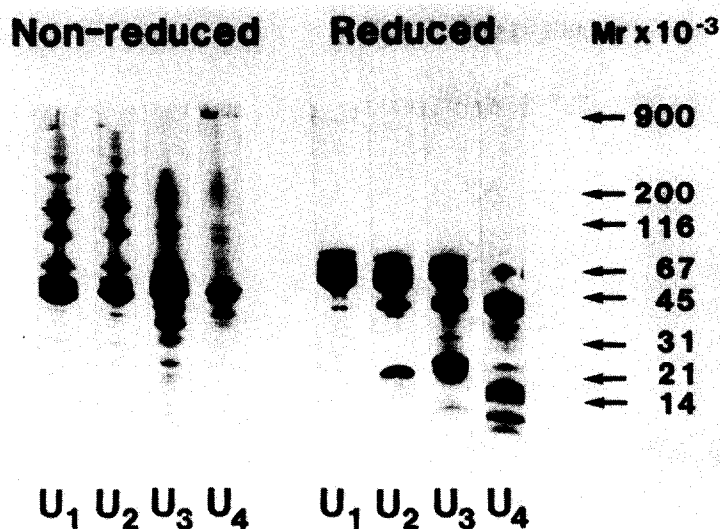


Fig. 2. SDS-PAGE gels stained for protein by the silver method run under both reducing and nonreducing conditions on the same gel. Urine U<sub>1</sub> contains multiple high molecular weight bands which upon reduction disappear to leave a single major band at 67 000 mol wt. Urine U<sub>4</sub> under nonreducing conditions has a very high molecular weight band (> 900 000) in addition to a band at 60 000 mol wt which comigrates with albumin. Upon reduction of U<sub>4</sub> major bands are seen at 45 000, 18 000 and 11 000 mol wt. Urines U<sub>2</sub> and U<sub>3</sub> also contain high molecular weight bands which disappear on reduction, leaving major bands at the level of albumin (67 000) in addition to bands at 45 000, 30 000 and 24 000 mol wt.

immunoprecipitation with anti-albumin anti-serum the same major bands were seen (lane D). These results suggested that the material immunoprecipitated might indeed be fragments of albumin. However, two other possibilities existed. a. The anti-albumin anti-serum might have recognized other proteins apart from albumin in spite of apparently being monospecific as judged by immunodiffusion criteria. b. The lower molecular weight bands seen might have represented non-albumin proteins disulfide-linked or otherwise attached to albumin which were precipitated along with albumin or albumin fragments by the anti-albumin serum. To determine whether the bands seen were indeed fragments of albumin a different approach was taken. A series of mouse monoclonal antibodies were raised against human albumin (see 'Materials and Methods'). Two antibodies were chosen to verify that the polyclonal antibody was indeed recognizing fragments of albumin. The proteins in urine samples were first separated by SDS-PAGE, then blotted onto nitrocellulose paper, and finally the protein bands were identified using either the polyclonal or the monoclonal antibodies. Binding of antibodies to antigen on the nitrocellulose paper was identified by using peroxidase-labelled anti-mouse or anti-rabbit antibodies (see 'Materials and Methods' for details). The results of these studies are shown in Fig. 4 for two different urine samples (lanes A, B, and C in one case, and lanes D, E, and F in the other case). As can be seen in Fig. 4 the polyclonal rabbit anti-serum (A) recognized bands at 40 000–50 000 mol wt which were recognized by monoclonal

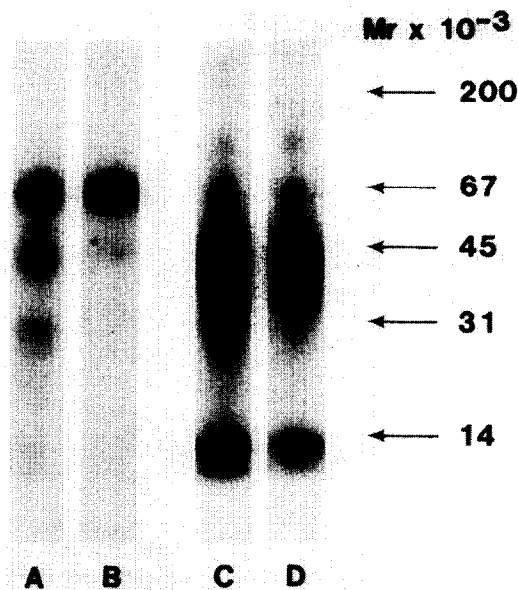


Fig. 3. Autoradiograms of  $^{125}\text{I}$ -urine proteins from two individuals analyzed by SDS-PAGE under reducing conditions. Lane B shows the protein immunoprecipitated by antialbumin serum from the total urine protein shown in lane A. The single band immunoprecipitated by antialbumin serum migrates at 67000 mol wt as expected (lane B). In contrast, in another case lane D shows the bands immunoprecipitated from the total radiolabelled urine protein shown in lane C. In this second case antialbumin antiserum precipitated major proteins at 40000–45000 and 11000, but not at 67000 mol wt.

antibody 2D7 (B) but not by antibody 9G7 (C). On the other hand, antibody 9G7 (C) recognized bands at 31000, 16000 and 11000 which were not seen by antibody 2D7 (B) but were seen by the polyclonal antibody (A). In a second example, the 40000–45000 mol wt bands seen by the polyclonal antibody (D) were again seen by antibody 2D7 (E) but not by antibody 9G7 (F), whereas antibody 9G7 (F) again recognized the lower molecular weight bands seen by the polyclonal anti serum (D). Bands at molecular weight higher than albumin monomer were recognized by both monoclonal antibodies as well as the polyclonal anti-serum. In these and in other blotting studies of urine samples, plasma and serum all the bands recognized by the polyclonal anti-serum were recognized by either antibody 2D7 or antibody 9G7. We can therefore conclude: (a) that the polyclonal antibody was indeed specific for albumin, (b) that the lower molecular weight bands seen by SDS-PAGE in urine under reducing and non-reducing conditions are fragments of albumin, (c) that the monoclonal antibodies 2D7 and 9G7 recognize different parts of the albumin molecule, and (d) that albumin appears to undergo proteolysis at a site approximately two-fifths of the way from one end of the molecule to produce one family of fragments of about 40000–50000 mol wt and one family of fragments of about 30000 mol wt and smaller. The 30000 mol wt fragment could apparently undergo further cleavage to produce 18000, 14000 or 11000 mol wt fragments which were

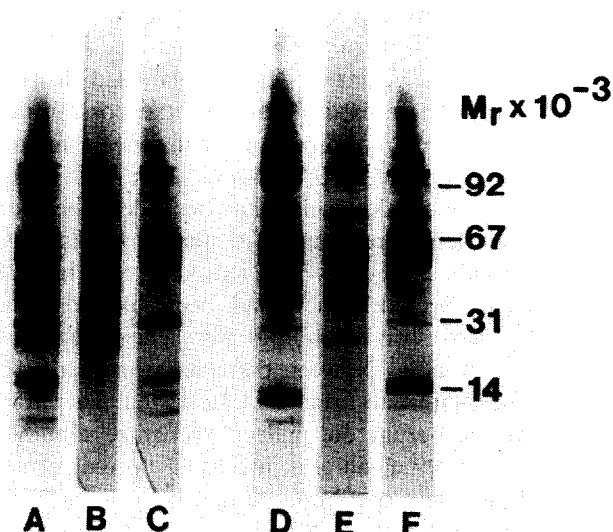


Fig. 4. Nitrocellulose blots from SDS-PAGE gels run under nonreducing conditions. Protein bands were developed by soaking gels in buffer containing antibody, washing free antibody away, and using peroxidase labelled anti-IgG to identify areas of specific antibody binding (see Methods). Blots from two individuals are shown (A, B, C and D, E, F). Lanes A and D use rabbit anti human albumin polyclonal antiserum. Lanes C and F use mouse anti human albumin monoclonal antibody 9G7. Lanes B and E use mouse anti human albumin monoclonal antibody 2D7. Note that all the bands are identified by either monoclonal 9G7 or 2D7.

still recognized by antibody 9G7. Antibody 2D7 recognized the family of fragments of around 40 000–50 000 mol wt which could also be cleaved to smaller fragments of 28 000–30 000 mol wt. In summary, using two different methods, the same conclusion was reached, namely that both fragmentation as well as polymerization of albumin in urine had occurred.

## Discussion

Several studies have described polymers of albumin in urine of individuals with nephrotic syndrome [1–5]. In general polymer formation appears to be related to both glucocorticoid treatment and to freezing of urine samples prior to analysis [2,4]. In the pediatric population with nephrotic syndrome polymer formation has been suggested to herald steroid responsiveness. This is not true for the adult population [5].

This study has focused on albumin fragmentation in urine of individuals with proteinuria. The study was initially done because large amounts of low molecular weight protein (< 67 000) on SDS-PAGE analysis of urine is generally thought to indicate that proteinuria is the consequence of renal tubular dysfunction [10]. When it became apparent that the low molecular weight protein was probably albumin studies were done to prove that this was indeed the case. In the first set of studies a

commercially available polyclonal anti-albumin antibody was used to immunoprecipitate radiolabelled urine proteins which were subsequently analyzed by SDS-PAGE. The results showed that low molecular weight radiolabelled protein were immunoprecipitated by the anti-albumin antibody. However, the possibilities remained that (a) the anti-albumin anti-serum recognized protein other than albumin, or (b) that other proteins were non-specifically precipitated, or (c) that other proteins were disulfide-linked to albumin molecules such as has been described for IgA, IgG, IgM,  $\alpha_1$ antitrypsin,  $\alpha$ -lipoprotein, prealbumin or serum amyloid A [11-16]. Therefore monoclonal anti-albumin antibodies were raised against human albumin and used to identify bands of protein which had been blotted onto nitrocellulose paper from SDS-PAGE gels. Two antibodies were chosen from a panel of antibodies raised. These two antibodies between them recognized all the bands identified by the polyclonal anti-serum in all urine samples tested. Therefore we can conclude that the polyclonal anti-serum did indeed recognize albumin fragments in urine. Furthermore, in many urine samples, including normal urine, albumin fragments were present. Fragmentation of albumin might have come from several different sources. Albumin fragments might have been present in blood and passed through the glomerular filter into urine [17]. They may have been partially broken down in proximal tubular cells by cathepsins and exocytosed, or they may have been generated by proteolytic enzymes present in formed urine. These studies do not discriminate between the above possibilities.

The results of the studies using monoclonal antibodies show that albumin fragmentation in urine occurs about two fifths of the way along the polypeptide chain to yield fragments of about 45 000 and 30 000 mol wt. These fragments are usually disulfide linked since they are seen when the albumin molecule is reduced (see Fig. 2). However, some fragmentation is also seen in the absence of reducing agents (see Figs. 1 and 4). The smaller 30 000 mol wt light chain could apparently undergo further proteolysis into smaller fragments of 16 000 and 11 000 mol wt. The larger 45 000 molecular weight fragment may undergo minor modification to form a family of 40 000-45 000 molecules weight fragments. Thus albumin in urine (particularly frozen urine samples) should be considered as a heterogeneous family of proteins including polymers, monomers and also various albumin fragments which may or may not be disulfide-linked. Techniques which are used to study urine proteins and to measure albumin in urine must take these variations into account.

### Acknowledgements

We are grateful to Ms. Catherine Corson for typing the manuscript, to Mrs. M. Marshall for the artwork, and to Mr. Craig Biddle and Mr. Ed Burns for photography. This work was supported by a grant-in-aid from the American Heart Association and the Michigan branch of the American Heart Association and the USPHS Grant AM 30673. Dr. Roger Wiggins is an Established Investigator of the American Heart Association. Dr Wilson is supported by a Research Cancer Development award (PHS Grant No. 5K04 CA 00845-02) from the National Cancer Institute.



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