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Human Uromucoid

I. Quantitative Immunoassay

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Summary. Uromucoid, the urinary glycoprotein, consists of an immunoelectrophoretically distinct low molecular weight protein and a macromolecular component that can be precipitated with sodium chloride. Uromucoid purified by salt precipitation had chemical and physico-chemical properties similar to those reported previously. The high molecular weight fragment gives a precipitin line in a ring test using an anti-uromucoid serum, but there is a visible reaction by the Ouchterlony technique only after it has been cleaved with sodium dodecyl sulfate. The molecules obtained by the latter method are related immunologically to native uromucoid and are completely identical to the urinary low molecular weight protein. A method is described in detail for the quantitative determination of uromucoid in human urine by single radial immunodiffusion and electroimmunoassay.

Key words: Urinary glycoprotein, characterisation of uromucoid, quantitative determination of uromucoid, radial immunodiffusion, electroimmunoassay.

In addition to serum proteins, urine contains proteins produced by the kidney (1). A characteristic member of the latter group is the mucoprotein first isolated and described in 1950 by Tamm and Horsfall (2), and variously called T- and H- glycoprotein (3, 4, 5, 6), or uromucoid (7, 8, 9), according to the method of isolation. The immunological identity of all these substances leads us, in agreement with others (7, 9, 10), to consider that there is really only one mucoprotein despite slight differences in the chemical analyses. Accordingly, in the present study the term uromucoid has been used throughout.

The classical method of isolating uromucoid is precipitation by 0,58 M NaCl, but a number of other techniques have also been developed (11, 12, 13), all of which have given variably impure products. Uromucoid obtained by the classical method is a filamentous glycoprotein (14) with a carbohydrate content of 28% (3, 15, 16, 17), and a molecular weight of ca. 7 million. (18). It consists of many subunits of 80 000 molecular weight (19, 20, 21), that are attached linearly to one another by non-covalent bonds (21).

At present, the biological functions of this mucoprotein are unknown, in part perhaps, because of the lack of a reliable method for its estimation. Uromucoid is known to inhibit viral haemagglutination (2, 18); and it binds calcium ions and may facilitate the formation of kidney stones (16, 22). The methods used so far for its assay have depended on precipitation of uromucoid and either gravimetric or colorimetric analysis of the material obtained (12, 16, 23, 24); the techniques are capricious and the results highly variable.

An earlier study reported a method for the semi-quantitative immunoassay of uromucoid (25). The purpose of the present experiments was to devise a method for its exact quantitative immunological estimation after dissociation into subunits.

Materials and Methods

Purification of uromucoid

Uromucoid was isolated by the method of Tamm and Horsfall (18). 50,4 l urine at pH 6.5 was diluted with an equal volume of distilled water and then displaced with 0.58 M NaCl per liter was dissolved in it. The mixture was left for 48 h at a temperature of 4 - 6 °C for optimal sedimentation of the floccular precipitate.

The clear supernatant was siphoned off and the sediment was spun in a centrifuged at 3.000 rpm. After washing with 10 l of a cold 0.58 M solution of NaCl, the precipitate was dissolved in 10 l of distilled water and reprecipitated by addition of 0.58 M NaCl at 4 - 6 °C. Washing and precipitation were repeated once more and the sediment redissolved in water. The chloride ions were removed by dialysis against distilled water leaving a colourless, viscous solution.

Determination of dry weight

After dialysis, 3 ml aliquots were lyophilized in glass containers and weighed to give the absolute concentration of the uromucoid solution. The solution was then placed in a high vacuum at 70 °C, over P_2O_5, and thoroughly dried until a constant weight was reached. The final solution contained 0.66 g of uromucoid/100 ml.

210 ml of uromucoid solution were produced from 50.4 l of urine. It contained 0.66 g/100 ml and yielded 1.386 g of dried substance, which corresponds to a yield of 26.6 mg uromucoid per liter of urine.

Production of concentrated urine

Sodium azide was added to fresh 24 h urine. The solution was ultrafiltered until a protein content of 2.7 % was obtained (26). Dialysis and lyophilisation were not used in order to prevent aggregation.

Gel filtration

Urine concentrates (10 ml) were fractionated by gel filtration on a Sepharose 6 B column using an 0.01 m tris-HCl-buffer (pH 7.0) containing 0.05 % sodium azide. The column measured 5 x 100 cm. The fractions were collected in 20 ml portions, and the uromucoid content of each was measured.

Ultracentrifugal analysis

The sedimentation rate of isolated uromucoid was measured in a Beckman Spinco ultracentrifuge, model E. in water, at protein concentrations of 0.2, 0.15, 0.10 and 0.05 g/100 ml, by measurement of direct photographic records of Schlieren patterns with a Leitz measuring microscope UWM II; calculations were done by an IBM 705 computer. The sedimentation constant S^o was estimated by extrapolation to a concentration c = 0, of the regression line calculated by an Olivetti Programma 101. Sedimentation coeffi cients were also measured in a similar fashion after uromucoid had been dissociated into subunits by SDS treatment of aqueous solutions of initial protein concentration 0.4, 0.3, and 0.1 g per 100 ml.

Polyacrylamide gel electrophoresis

This was done in a 5.5 % gel of cyanogum, in a mixed-buffer containing tris, citrate and borate, at pH 8.6; field strength 8 - 10 V/cm (27).

Carbohydrate analysis

The content of hexose (orcinol H_2SO_4), hexosamine (Elsen-Morgan-Reaction), neuraminic acid (Bial's-Reaction) and fucose (cystein-H_2SO_4) were determined by the methods described by Schultze et al. (28).

The nitrogen content of uromucoid was determined by the Kjeldahl technique, with the extinction coefficient set at 280 nm, in an aqueous solution at pH 7.2.

Immunological analysis

The rabbit anti-human and anti-uromucoid sera used were purchased from Behringwerke AG. The uromucoid fraction obtained by triple NaCl precipitation was used as the antigen for immunizing rabbits.

a) Qualitative methods

1. Immunoelectrophoresis was done by the micro-method of Scheidegger (29) on slides spread with a 1.5 % agar gel.
2. Double diffusion was done by the Ouchterlony technique (30) in 1.5 % agar.
3. Ring test: the antigen solution was added to a constant quantity of antibody.

b) Quantitative methods

1. Single radial immunodiffusion after Mancini (31). 2 % agarose slides, 2.35 mm thick and containing 3 % anti-serum were used. The titre of the anti-uromucoid serum, calculated by the method of Becker (32), was 0.88 mg/ml. The charge volume was 20 μl, and diffusion was allowed to occur for 3 - 5 days. Antigen concentrations of 25.0, 12.5, 6.25 and 3.125 mg per 100 ml dissolved in a 0.3 % solution of SDS, were used for the standard curve. Urine specimens were concentrated twofold for analysis. Under these conditions the diameters of the precipitates were 6.2 - 11.7 mm.

2. Electroimmunoassay according to Laurell (33). The electrophoresis apparatus manufactured by Dansk Laboratorieudstyr, Copenhagen (Behringwerke AG) was used. Analysis was performed on glass slides, 10 x 10 cm, coated with 20 ml of a mixture of 1.5% agarose and 2.0% anti-uromucoid serum; (0.02 M-barbital buffer, pH 8.6, 10 V/cm; duration 3 h). The volume of antigen applied was 8 μl.

Antigen concentrations of 25.0, 12.5, 6.25, and 3.125 mg/ml, dissolved in 0.25% SDS were used for the standard curve. After use the gel was washed to remove excess antiserum and the slides stained with Coomassie Brilliant Blue.

Preparation of urine for quantitative immunological determination

To ensure the immunological specificity of the uromucoid reaction it was necessary to dialyse the urine and to concentrate it to half its original volume. Dialysis and ultrafiltration were carried out in collodion tubes, manufactured by Sartorius-Membranfilter GmbH, Göttingen. The outer glass container had a volumetric capacity of 400 ml. 5 ml of urine was measured into a collodion tube and the level of the liquid marked. The urine was dialyzed over night against distilled water at a temperature of 4 - 6 °C. The following morning,

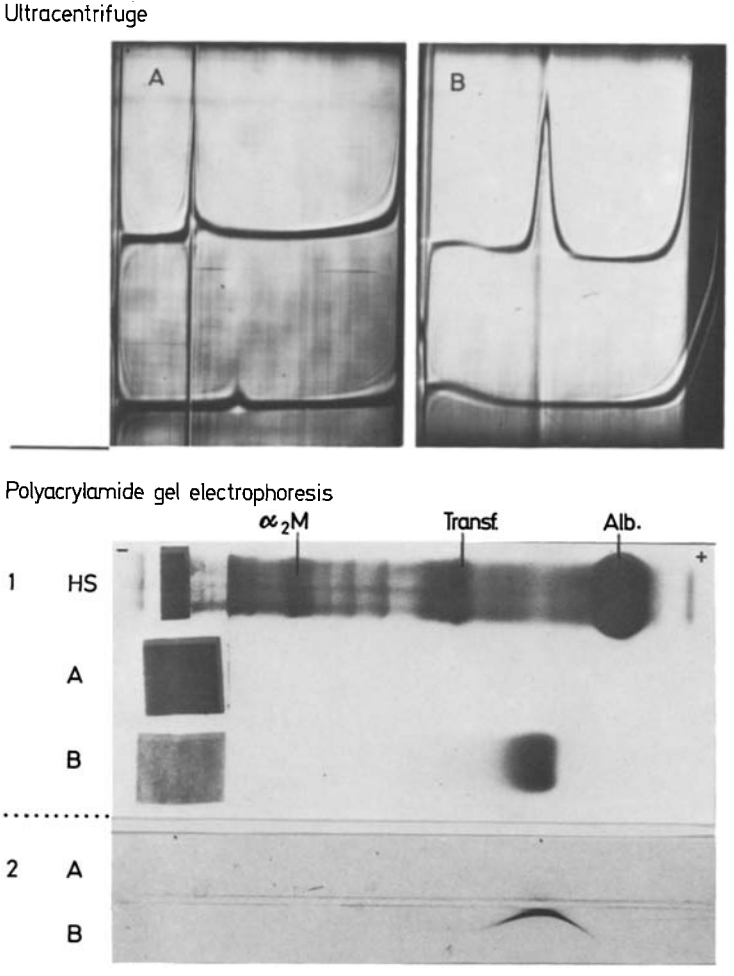

Fig. 1. Characterization of native uromucoid (A) and uromucoid treated with sodium dodecyl sulfate (B) by ultracentrifugation and polyacrylamide gel electrophoresis. - Ultracentrifuge A: above: Wedge cell, uromucoid in water (0.2%); below: normal cell, uromucoid in water (0.05%) 14 min, 59780 rpm, S = 28.3. - Ultracentrifuge B: above: Wedge cell, uromucoid (0.5%) in 0.5 per cent SDS; below: normal cell, sodium dodecyl sulfate (0.5%) 135 min, 59780 rpm, S = 3.8; Polyacrylamide gel electrophoresis: 1. Amido Black staining: HS = human serum, A = uromucoid, B = uromucoid in 0.5 per cent SDS; 2. Immunological reaction with anti-uromucoid serum: A = uromucoid, B = uromucoid in 0.5 per cent SDS

the volume, which had increased, was reduced to 5 ml by vacuum suction. The outer fluid was replaced by an 0.3% solution of sodium dodecyl sulphate (SDS), 0.3 ml of 5 % SDS was pipetted into the inner compartment and its volume reduced to 2 ml. This solution was mixed carefully and its volume was measured and made up to 2.5 ml with 0.3% SDS.

Results and Discussion

1. Isolation

Purified uromucoid was obtained from normal urine by the method described above involving precipitation three times with sodium chloride.

2. Characterization of uromucoid

a) Physico-chemical properties. The uromucoid isolated had a uniform sedimentation pattern on ultracentrifugal analysis; Fig. 1 A shows the typical Schlieren pattern of this material. The sedimentation rate, was high and was dependent on the concentration; a sedimentation constant of 28.3 S was computed from the sedimentation coefficients in solutions of graduated concentrations.

Chemical analysis of the purified uromucoid showed a carbohydrate content of 28.3%, comprising: hexose 9.6% (referred to Gal/Man 1:1), fucose 0.9%, acetyl hexosamine 11.0%, acetyl neuraminic acid 6.8%. The nitrogen content of uromucoid was 12.2%, and its extinction coefficient $E^{1\%}_{280.1\ cm}$ = 11.5. Uromucoid did not penetrate the 5.5% polyacrylamide gel because of its high molecular weight.

All these results are in agreement with data already published (3, 15, 17, 18, 34, 35).

b) Immunological properties. The uromucoid isolated did not react with homologous anti-uromucoid serum on immunoelectrophoresis, because its high molecular weight prevented it from penetrating the pores of the agar gel (Fig. 5). These findings agree with those of Mc Queen (36) and Marr et al. (37), who analyzed uromucoid from animals (rabbits) and man, and are contrary to the observations of others (7, 8, 10, 17, 20). The ring test showed that isolated antigen reacted strongly with antiuromucoid serum. The isolated uromucoid did not contain contaminating serum proteins.

3. Characteristics of uromucoid in urine

Immunoelectrophoretic analysis of concentrated urine using an anti-uromucoid serum showed a typical arched line of precipitation of uromucoid in the position of the α_1- and α_2-globulins (38). According to Heremans et al. (9, 39), and McQueen (36), this line persists if the urine concentrate were reexamined after the 0.58 M NaCl-precipitable component of uromucoid had been removed. This was confirmed by the present studies, as is clearly shown in Fig. 2. However, this technique does not permit visualisation of any reaction between the NaCl-precipitable high molecular weight uromucoid component and its homologous antiserum, as described above. Uromucoid in urine is present in two different forms, which differ in molecular weight and in their precipitability by sodium chloride.

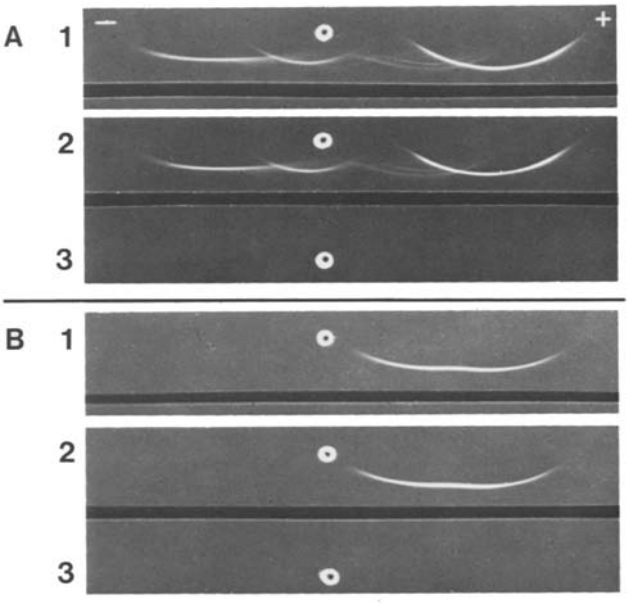

Fig. 2. Immunoelectrophoretic analysis of concentrated urine before (1) and after (2) removal of the uromucoid precipitated by 0.58 M NaCl, and the Tamm-Horsfall mucoprotein (3), against anti-human serum (A) and anti-uromucoid serum (B)

In order to substantiate these findings, urine concentrated by ultrafiltration was subjected to gel filtration on Sepharose 6 B. Testing of these fractions confirmed the results of immunoelectrophoresis (Fig. 3). By Ouchterlony double diffusion uromucoid could only be detected in the position before the albumin peak. However, in the ring test, positive reactions were observed before the albumin peak and also in the macromolecular region. Only

the high molecular weight fragment could be almost completely part is precipitated by NaCl. The strength of the reaction in the ring test indicated that the high molecular weight fraction was the main component of uromucoid in urine.

4. Dissociation into subunits

According to Fletcher et al. (21), the uromucoid precipitated by NaCl consists of sub-groups, of about 80 000 molecular weight, held together by non-covalent bonds. The sub-

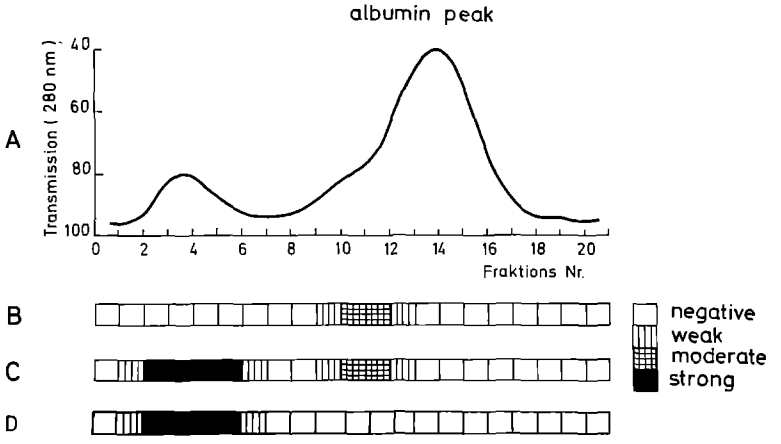

Fig. 3. Behaviour of uromucoid on gel filtration of a urine concentrate through Sepharose 6 B; A) elution pattern, B) identification by Ouchterlony double diffusion, C) identification by the ring test, D) precipitation with 0.58 M NaCl

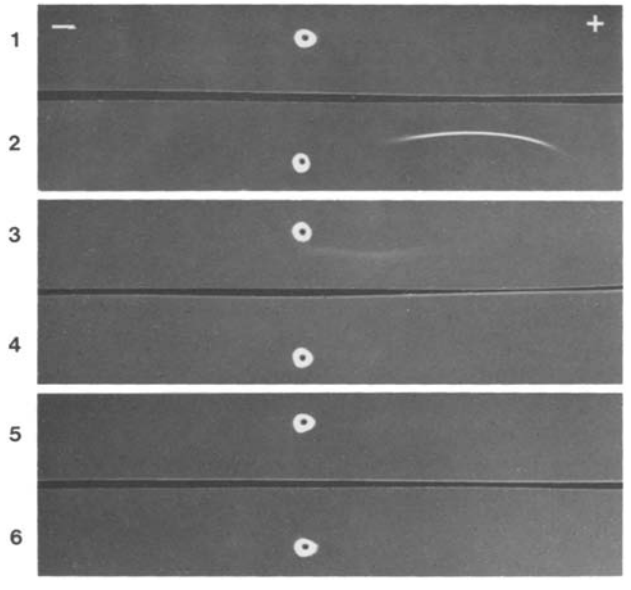

Fig. 4. Immunoelectrophoretic analysis of the effects of different detergents on the cleavage of uromucoid. 1. uromucoid (native) 3.3 mg/ml in water, 2. uromucoid 3.3 mg/ml in 0.25% sodium dodecyl sulfate, 3. uromucoid 3.3 mg/ml in 1.0% sodium caprylate, 4. uromucoid 3.3 mg/ml in 2.0% Genapol^{R}, 5. uromucoid 3.3 mg/ml in 1.0% Tritison X-100, 6. uromucoid 3.3 mg/ml in 2.0% Tween 80, trough: anti-uromucoid serum

units can be produced by dissociating agents and can be detected immunologically in agar gel (37). Several of these compounds were examined for their activity in producing subunits. Fig. 4 shows the effect of various detergents in dissociating uromucoid, as revealed by immunoelectrophoresis. Sodium dodecyl sulphate seemed suitable for fragmenting uromucoid; the subunits produced by its action remained immunologically reactive. Analysis in the ultracentrifuge showed that a 0.25% solution of SDS sufficed fully to dis-

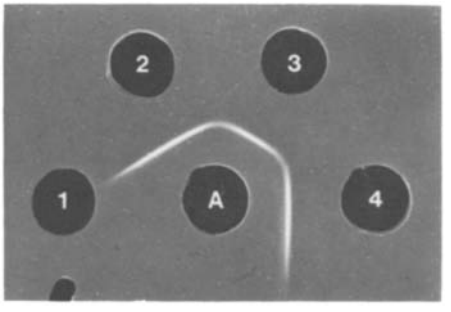

Fig. 5. Demonstration of immunological identity. A. anti-uromucoid serum, 1. high molecular weight uromucoid (NaCl-precipitation), 2. high molecular weight uromucoid + SDS, 3. low molecular weight uromucoid (Sepharose fraction), 4. low molecular weight uromucoid (Sepharose fraction) + SDS

sociate uromucoid in concentrations up to 0.1 g/100 ml. Higher protein concentrations required more SDS.

The uniform molecular size of the uromucoid fragments produced was demonstrated by ultracentrifugation and by polyacrylamide gel electrophoresis (Fig. 1B). It was possible, therefore, to determine the immunological relationship between the high and low molecular weight uromucoids, separated from concentrated urine by gel filtration. Both preparations were tested with and without the addition of SDS in Ouchterlony plates. The reaction of complete immunological identity was found between the high molecular weight component treated with SDS and the low molecular weight fragments, whether or not they had been split with SDS (Fig. 5).

5. Quantitative immunological assay

The suitability of using uromucoid fragments produced with SDS as the standards in quantitative immuno-assays was examined by single radial immunodiffusion (31) and by

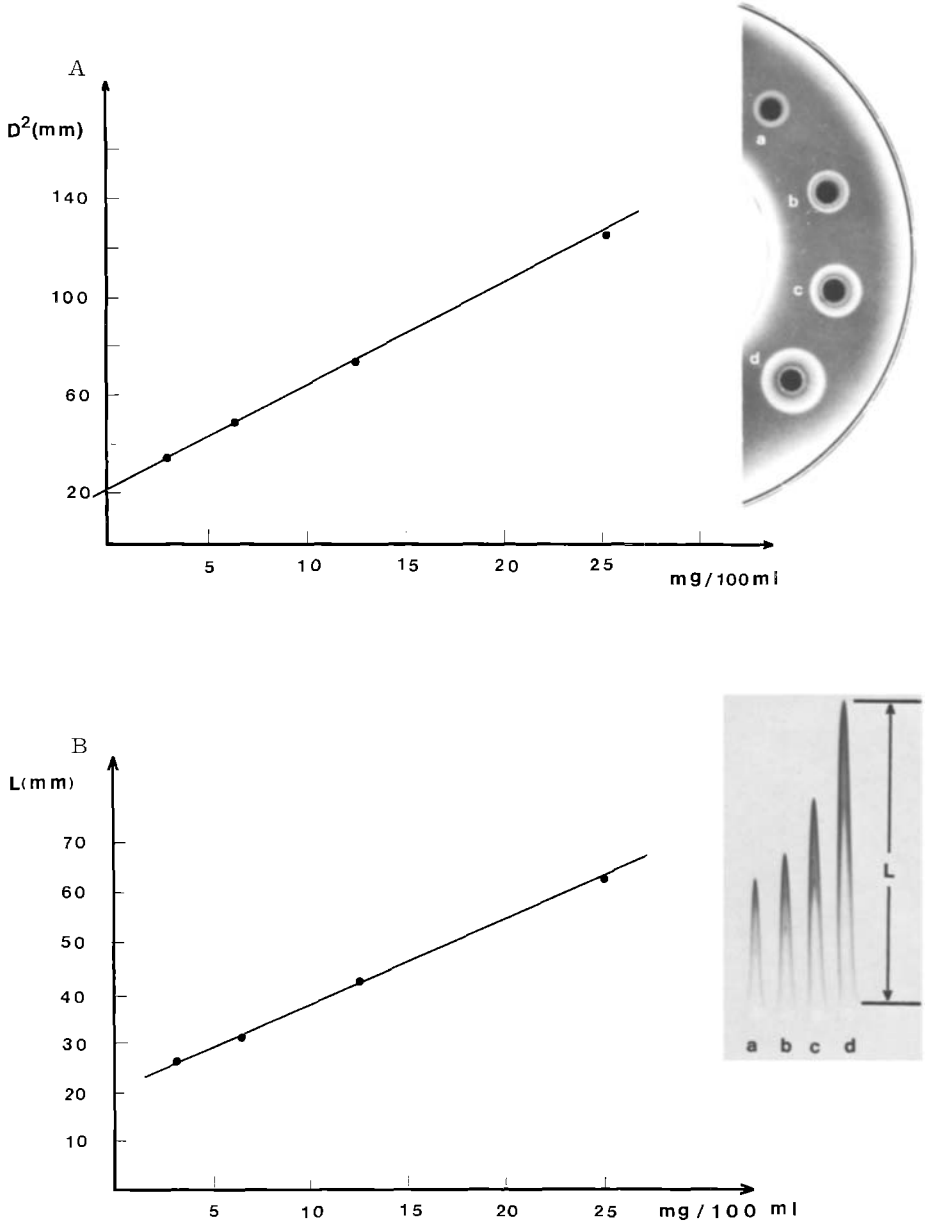

Fig. 6. Proof of the suitability of uromucoid fragments as standards for the quantitative determination of uromucoid. A) single radial immunodiffusion by Mancini's method; B) electroimmunoassay by the Laurell technique, standard: a) 3.1 b) 6.2 c) 12.5 d) 25.0 mg/100 ml

electroimmunoassay (33). As confirmed by ultracentrifugal analysis, an 0.2 - 0.3% solution of SDS afforded the most suitable condition for the cleavage of uromucoid; and, in both methods, a linear relationship was found between the parameters measured and the protein concentration (Fig. 6). The effect of higher concentrations of SDS differed in the two methods. The Laurell technique showed a consistent increase in the heights of the peaks, which did not affect the quantitative results. A second ring of precipitation was observed in single radial immunodiffusion plates that was based on a reciprocal interaction between the excess free SDS and the immunoglobulins of the anti-serum. If the reaction occurred within the antigen-antibody precipitate ring, the quantitative relationships remained unchanged, but if the free SDS penetrated the immunoprecipitate, the ring of the latter was displaced outwards and then no quantitative measurements could be obtained (Fig. 7). Therefore, it was decided to employ a 0.3% solution of SDS for all further studies.

In the light of these results the suitability of the method for assaying urine samples was tested. Urine collected from 8 normal subjects was dialyzed in order to remove electrolytes, especially potassium, which forms an insoluble precipitate with SDS and so alters the concentration of the latter. The dialyzed urine samples were mixed with an 0.3% SDS solution, as described above, and concentrated twofold by ultrafiltration. They were analyzed by comparison with standardized solutions by both immunological techniques (Fig. 8). The results are listed in Table 1. Both methods gave similar values for each urine sample, although there was a large range of variation. The results cannot be regarded as representative of normal man, both because of the small number of subjects and, in particular, since

Table 1. Quantitative immunological determination of uromucoid in normal subjects (n = 8)

Subjects	Electroimmuno assay (mg/100 ml)	Single radial immunodiffusion (mg/100 ml)
Thi I	5.9	5.8
Bi I	5.9	5.6
Schm	0.9	---
Di	11.5	12.7
Ha	1.4	1.7
We	5.4	6.5
Thi II	4.9	5.1
Bi II	6.3	6.3
$\bar{x}$ = 5.3		$\bar{x}$ = 6.2
S = 3.3		S = 3.3
VK = 61.7		VK = 52.75

$\bar{x}$ = mean, S = standard deviation
VK = coefficient of variability
range = 1 - 12 mg/100 ml

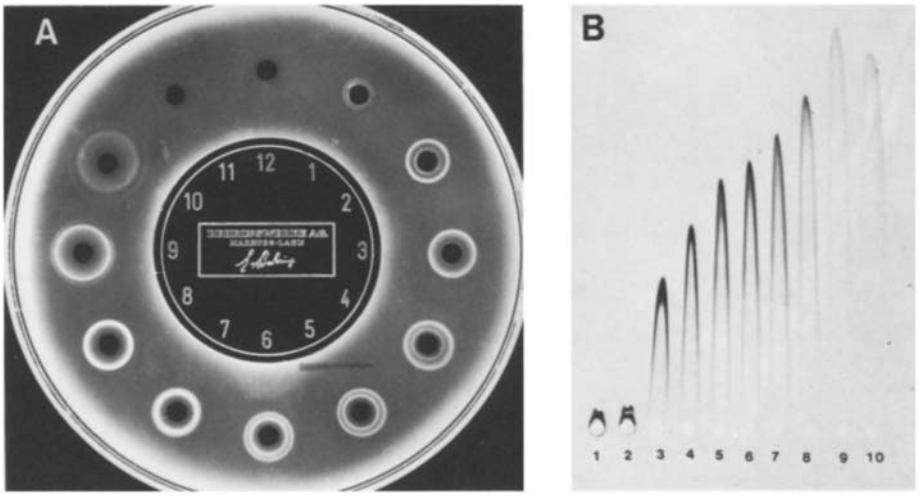

Fig. 7. Effect of different concentrations of SDS. A) single radial immunodiffusion method, B) electroimmunoassay, 13.2 mg/100 ml uromucoid in 1. 0.05 2. 0.1 3. 0.2 4. 0.4 5. 0.6 6. 0.8 7. 1.2 8. 2.0 9. 4.0 10. 8.0% SDS

urine samples and not 24 h urines were analyzed.

Differing quantities of uromucoid were subjected to the urine preparation procedure to check its reliability and quantitative analyses were done by both immunological techniques. The amounts of uromucoid recovered are shown in Table 2; both methods gave results that were in almost complete agreement with each other. In no case was the discrepancy greater than 10% of the amount of uromucoid added, a value within the expected margin of error.

The results of this study show that it is possible to determine the total amount of uromucoid in urine with considerable reliability and reasonably rapidly. The daily excretion of uromucoid by a large number of normal subjects as well as by patients suffering from urolithiasis, is now being measured, and the results will be reported later.

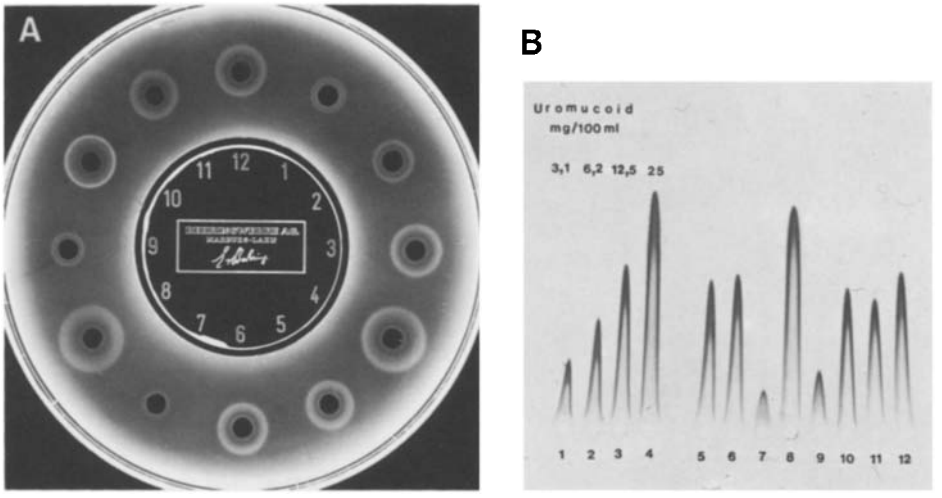

Fig. 8. Quantitative estimation of uromucoid in normal human urine. A) single radial immunodiffusion method, B) electroimmunoassay, 1. - 4. uromucoid standard, 5. - 12. . twofold concentrate of normal urine (Table 1)

Table 2. Reliability of the quantitative immunological determination of uromucoid

Uromucoid added	Uromucoid recovered			
	Electroimmuno assay	deviation	Single radial immunodiffusion	deviation
(mg/100 ml)	(mg/100 ml)	%	(mg/100 ml)	%
2.0	2.0	---	2.0	---
3.0	2.9	3.3.	3.0	---
4.0	3.6	10.0	4.1	2.5
5.0	5.0	---	5.0	---
6.0	5.8	3.3	6.0	---
7.0	6.9	1.4	6.4	5.7
8.0	7.9	1.3	8.1	1.3
10.0	10.3	3.0	10.9	9.0

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