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ISOELECTRIC FOCUSING IN LOW-DENATURING MEDIA: VISUALIZATION IN RENAL DISEASE OF VARIATION OF THE ISOELECTRIC POINT OF ALBUMIN NOT RELATED TO A REMARKABLE CONFORMATIONAL VARIATION

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SUMMARY

The isoelectric properties of serum and urinary albumin from normal subjects and patients with nephrotic syndrome have been investigated in various conditions of denaturation, obtained by using urea (0—8 *M*) as a support in isoelectric focusing. In normal human serum, albumin is rather acidic (p*I* = 4.7) when focused in glycerol while the denatured form obtained by exposing the protein to 8 *M* urea has a much higher p*I* (6.1). Albumin from nephrotic patients is acidic in glycerol but at very low levels of urea (2 *M*) it shifts from p*I* 4.7 to p*I* 6.1; the same effect has been induced by treating albumin with activated charcoal at low pH. In order to obtain more information on urea-induced changes, we have recorded the circular dichroic spectra of albumin when exposed to the concentration of urea used in gels, and we found that no conformational transition occurs for urea concentrations < 5 *M*. Taken together, these observations reveal that variation of the p*I* of albumin in nephrotic syndrome occurs mainly due to a dissociating effect of urea on charged substances bound to this protein.

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INTRODUCTION

The high-resolution technique of isoelectric focusing (IEF) allows the fractionation of albumin into four to five microheterogeneous bands migrating in a narrow range of isoelectric points (p*I*) from 4.6 to 4.8 [1]. In 1971, Salaman and Williamson [2] reported the p*I* of serum albumin to be higher when the protein was run in denaturing media, such as concentrated urea (6—8 *M*), than in standard conditions. The p*I* of the unfolded molecule was shown to correspond to the isoionic point of the protein as calculated from its known amino acid sequence, and it was then hypothesized that the low p*I* of the native molecule would be explained by abnormally low p*K* of some ionizing groups, caused by peculiarities of the tertiary structure (hydrophobic environment) [2]. The use of high concentrations of urea (6—8 *M*) in IEF gels has therefore become an interesting extension of traditional IEF, suitable for the characterization of albumin in denaturing conditions. Lower concentrations of urea ($\leqslant$ 2 *M*) are, however, thought to have no influence at all on albumin structure, and urea (2 *M*) is widely used (instead of glycerol) as support for IEF gels.

The data presented here show that when albumin from patients with renal disease (nephrotic syndrome and renal insufficiency) is run in IEF gels containing 2 *M* urea, the p*I* of an abundant part (ca. 50%) of the protein shifts to the isoionic point, without any detectable alteration of circular dichroic (CD) spectra. Reasons for considering a dissociating influence of urea towards one or more, hitherto uncharacterized, substance(s) as being causal for this behaviour of albumin are discussed.

EXPERIMENTAL

Chemicals

Acrylamide, N,N'-methylenebisacrylamide (BIS), N,N,N',N'-tetramethylendiamine (TEMED), ammonium persulphate and Affi-Gel Blue were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.); carrier ampholytes (Ampholines) were from LKB (Bromma, Sweden); Silane A 174 and IEF marker proteins were from Pharmacia (Uppsala, Sweden); urea was from Fluka (Buchs, Switzerland); potassium dichromate, silver nitrate, sodium carbonate, formaldehyde and other chemicals of analytical grade were from Merck (Darmstadt, F.R.G.).

Albumin preparation

Albumin was purified from sera and urine of eight patients suffering from nephrotic syndrome (proteinuria > 3.5 g per 24 h) and various grades of renal insufficiency, as defined by their serum levels of creatinine (between 1.5 and 10 mg/dl). Sera were drawn in a fasting state (in the morning); 24-h urine collections were performed at home during periods of normal physical activity. A 1-ml volume of serum diluted in 400 ml of 0.05 *M* Tris—HCl (pH 7) or ca. 10—15 ml of urine with the same buffer was applied to Affi-Gel Blue packed columns (7 × 1.5 cm I.D.) equilibrated with 0.05 *M* Tris—HCl—0.25 *M* potassium chloride (pH 7) buffer. Chromatography was carried out at room

temperature following a modification [3] of the original method of Travis and Pannel [4]. The albumin fraction was thoroughly ultrafiltered with Amicon PM-30 membranes using an inert gas atmosphere. Before use, albumin was analysed by immunoelectrophoresis against mono- and polyspecific antisera in 1% agarose gels according to Grabar and Williams [5].

Defatting of albumin

Albumin was defatted following the charcoal treatment originally described by Chen and Koester [6].

IEF and silver stain

Ultrathin-layer (240 μm) IEF was performed in polyacrylamide slab gels (T = 5%; C = 3%) cast on silanized glass plates [7] (13 × 13 cm). Gaskets were formed with Parafilm rectangles, one layer giving 120 μm thickness. The standard polymerization solution contained 12% glycerol, ammonium persulphate as catalyst, TEMED as accelerator and 2.5% (v/v) carrier ampholytes in a non-linear pH 4—7 range obtained by mixing 50 parts of pH 4—6 and 50 parts of pH 6—8 Ampholines. Some experiments were performed in 2 M urea, in which case the samples were equilibrated at room temperature for 10 min in urea. The pH gradient was evaluated with six pI marker proteins and with an LKB surface electrode. The runs were performed at 10°C by applying 2000 V (13 W) for 6 h. Prefocusing was for 1 h at 500 V (13 W). Electrode solutions were 0.2 M sodium hydroxide at the cathode and 0.2 M orthophosphoric acid—0.1 M glutamic acid at the anode.

Increasing concentrations of urea in a gel were obtained by polymerizing five strips containing increasing amounts of urea (0—2 M).

Silver stain was performed with the photochemical method of Merril et al. [8]. After trichloroacetic acid (TCA) fixation, gels were rinsed three times for 10 min each in a 10% ethanol—5% acetic solution and subsequently treated for 10 min with 3.4 mM potassium dichromate—3.2 mM nitric acid in the dark with gentle agitation. Gels were then placed in a 10 mM silver nitrate solution under a high-intensity uniform light for 30 min. Developer was prepared by mixing 0.28 M sodium carbonate and 0.5 ml of 40% formaldehyde; the reaction was stopped with 1% acetic acid. All steps were carried out at room temperature with freshly made reagents.

Circular dichroic spectra

The CD spectra were recorded with a Jasco J 500 A spectropolarimeter at room temperature using cuvettes of 0.1 cm path length. The spectra were expressed as molar ellipticity (in the far-UV region). Ellipticities $[\theta]$ (deg cm^2/dmol) were calculated by using 113 as the mean residue mass. The instrument was calibrated by using $d(+)$-10-camphorsulphonic acid, which shows a positive CD band at 290.5 nm with a $\Delta\delta$ of +2.36 l/mol cm (the concentration was determined by using 34.5 l/mol cm at 285 nm as extinction coefficient for the anhydrous form). Transition curves between native (N) and unfolded (U) albumin were obtained using various concentrations of denaturant (1—8 M urea) and the percentage of N was calculated according to the formula $([\theta]_{\text{observed}} - [\theta]_{\text{Gu} \cdot \text{HCl}(6M)})/([\theta]_{\text{urea}(0\,M)} - [\theta]_{\text{Gu} \cdot \text{HCl}(6M)})\%$, where in this case θ is the ellipticity at 222 nm; U was obtained by exposing the protein

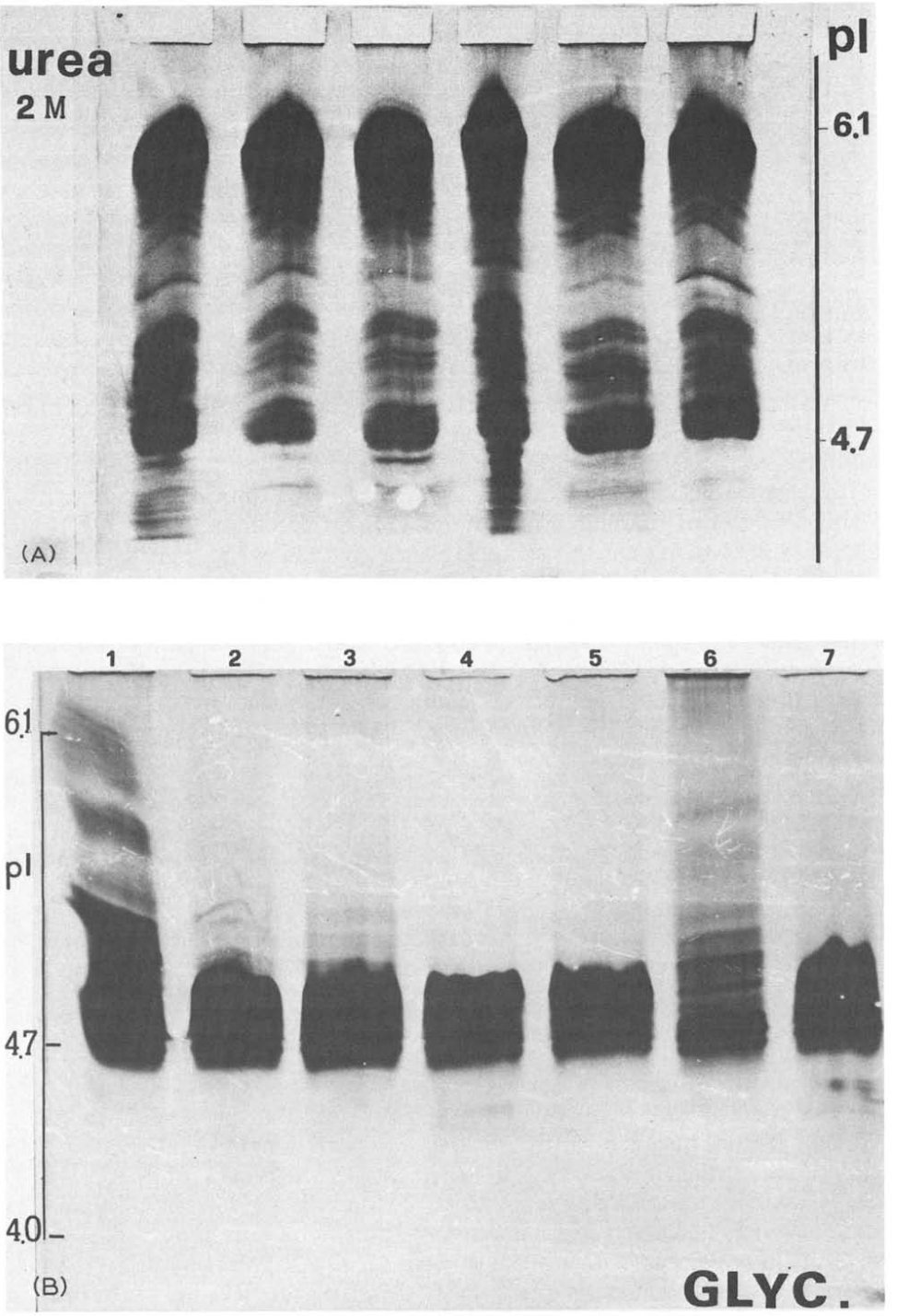

Fig. 1. Urinary albumin from nephrotic patients electrofocused in gels containing different supports: (A) 2 *M* urea, (B) 12% glycerol. Track 7: normal human serum albumin. Silver stain.

to 6 *M* guanidine hydrochloride (Gu · HCl), which is necessary to obtain a complete unfolding of the molecule. In all cases, before CD measurements, the samples were incubated at room temperature for 30 min with various concentrations of denaturant. After 30 min we did not observe any further variation of the value.

RESULTS AND DISCUSSION

Isoelectric focusing

During an extensive study on renal selectivity properties in nephrotic syndrome complicated with renal insufficiency, numerous serum and urinary albumin samples were run in IEF gels containing 2 *M* urea as a support. All albumin samples, under these conditions of electrophoresis, show a particular behaviour which can be summarized as follows: ca. 30% of the protein focuses at a p*I* of 4.7, i.e. the p*I* of normal albumin, another 50% migrates at p*I* = 6.1 and, finally, numerous microheterogeneous bands focus between the two extremes (Fig. 1A). In contrast, when the same samples are run in IEF gels containing glycerol as a support, the pattern is remarkably different (Fig. 1B):

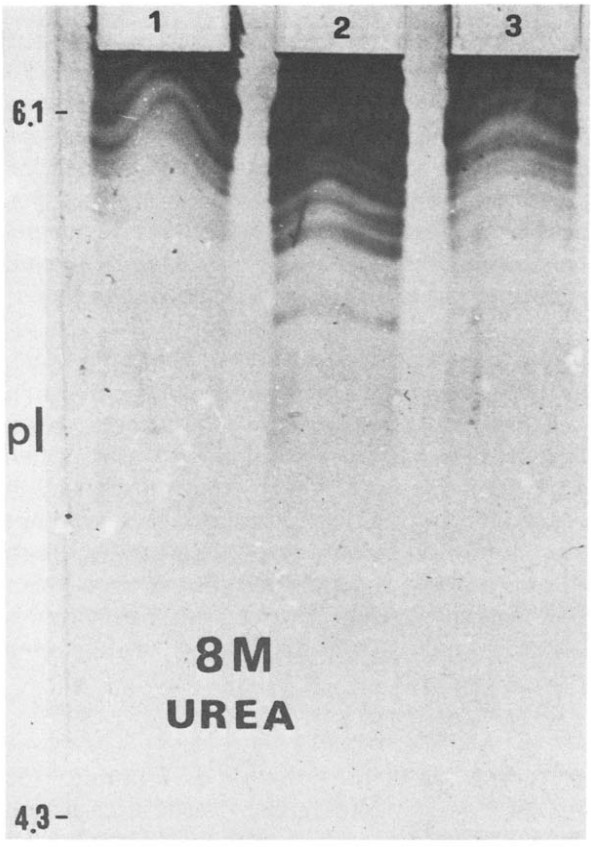

Fig. 2. Normal human serum albumin (track 1) and albumin from nephrotic patients (urinary track 2 and serum track 3) electrofocused in gels containing 8 *M* urea. Silver stain.

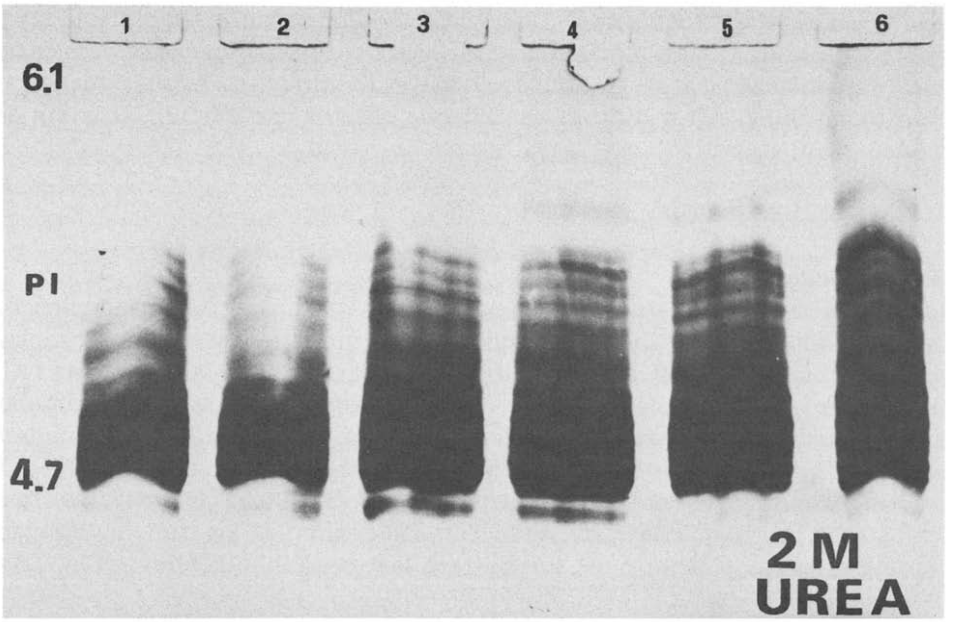

Fig. 3. Normal human serum albumin in IEF gels containing 2 *M* urea as a support. Silver stain.

all the albumin isoforms migrate at their p*I* of ca. 4.7, with the exception of a few more cationic bands visible in the upper part of tracks 1 and 6. When the concentration of urea in the gel is increased up to 8 *M*, the pattern shown in Fig. 2 is observed: all albumin isoforms now migrate at p*I* = 6.1, with only a partial microheterogeneity around this p*I*. Finally, when six samples of normal serum albumin are run either in glycerol or in 2 *M* urea, no difference at all between the two conditions of electrophoresis can be found (Fig. 3).

To determine the urea concentration that induces variation of albumin p*I*, the protein was run in a gel containing increasing amounts of urea (0--2 *M*). The electrophoretic pattern shown in Fig. 4A indicates that a clear increase in p*I* of urinary albumin occurs at urea concentrations of 1.5 *M* or greater, although a few subtle bands with a p*I* higher than the original are detectable in the strip containing 1 *M* urea. At variance, normal serum albumin focused in the same gel (Fig. 4B) migrates as a single line throughout the whole gradient of urea concentration. By increasing the concentration of denaturant up to 6 *M*, the two different pools of albumin show no difference (not shown), with a pattern resembling that of Fig. 2 obtained with 8 *M* urea; this behaviour is explained by a derangement of the intimate structure of the protein (see below).

Circular dichroic spectra

As for any other protein, three factors determine the p*I* of albumin: (1) amino-acid-charged lateral groups; (2) secondary and tertiary structures which create hydrophobic environments; (3) interaction of albumin with small charged ligands.

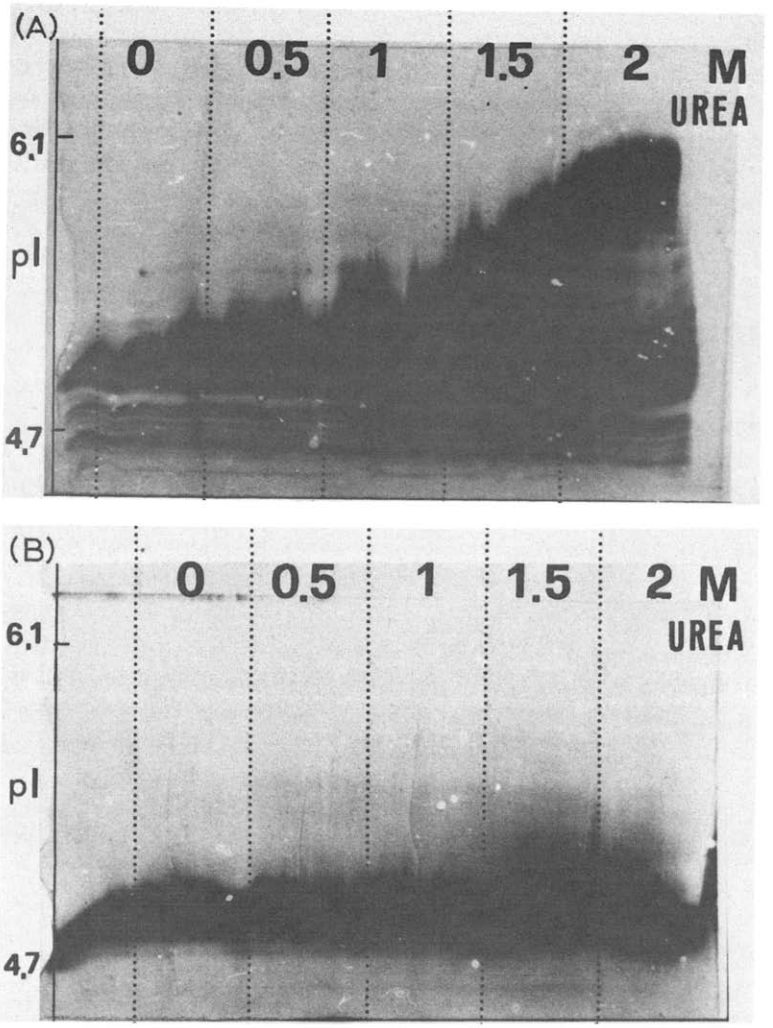

Fig. 4. IEF of albumin in gels containing increasing amounts of urea, from 0 to 2 *M*. (A) Urinary albumin from nephrotic patients; (B) normal serum albumin.

The sum of these three factors determines the p*I* of albumin (which is 4.7), while the true isoionic point calculated from the known amino acid sequence of albumin by using the Linderstrom—Land equation and assuming an average p*K* of 4.5 for carboxyl and 6.5 for histidine is ca. 6.0, which is very close to the p*I* of albumin p*I* in 8 *M* urea. Factor 1 cannot explain the difference of albumin p*I* in 2 *M* urea, since in glycerol the same protein migrates at its p*I*; factors 2 and 3 await further work.

As urea can significantly reduce the α-helix content of any protein by interference with the intra-chain hydrogen bonds that stabilize the secondary structure influencing conformational adaptation (due to S—S bridges), we have attempted to find out whether albumin from nephrotic syndrome was in a denatured state at 2 *M* urea. The CD spectra (at 222 nm) of albumin exposed at various concentrations of urea (0—8 *M*) have been recorded and the transition curve from a native to an unfolded condition has been drawn by plotting the

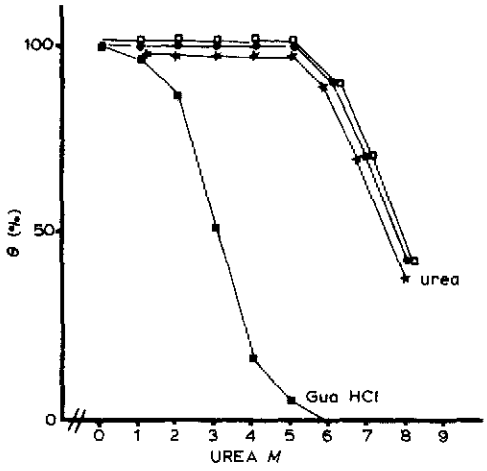

Fig. 5. Circular dichroic spectra (at 222 nm) of albumin from various sources treated for 30 min with various concentrations of urea from 0 to 8 *M*. The total unfolding of albumin was obtained with Gu · HCl (■). (*) Normal human serum albumin; (●) serum albumin from nephrotic patients; (□) urinary albumin from nephrotic patients.

various concentrations of urea against values of ellipticity. By taking as 100% denaturation the CD signal obtained by exposing albumin to 6 *M* Gu · HCl (corresponding to a random coil), it has been calculated that albumin begins to unfold from 5 *M* urea onwards, and that 8 *M* urea is able to induce only a partial unfolding (up to 70—75%) of the molecule. The schematic representa-

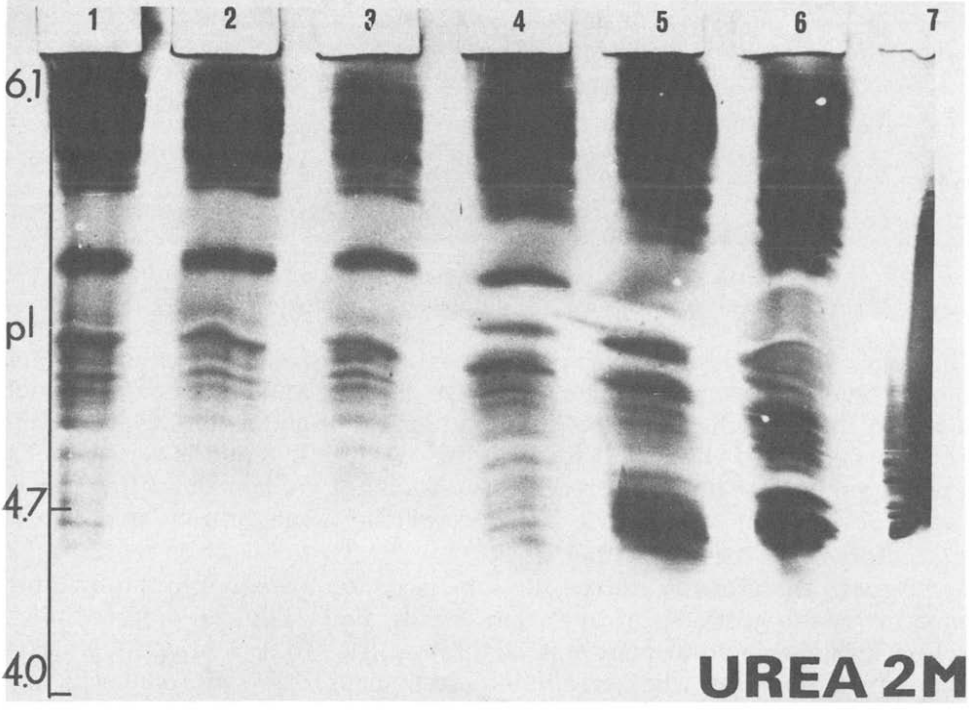

Fig. 6. IEF in 2 *M* urea of defatted albumin from normal donors (tracks 4–6) and from patients with nephrotic syndrome (tracks 1–3). Silver stain.

tion of the curve between the two extremes of urea concentrations is shown in Fig. 5, which represents a sigmoidal adaptation where 100% albumin is in the native condition up to 5 *M*, then the α-helix content begins to fall. Therefore, no conformational variation of albumin from nephrotic patients can explain the altered electrophoretic behaviour.

IEF of defatted albumin

The third possibility at stake is the influence of urea on charged ligands that bind to albumin with a hydrophobic interaction. If this is the case, the charcoal treatment proposed by Chen and Koester [6] for defatting albumin (but which is also suitable for other hydrophobic interactions) should alone produce a variation of the p*I* of albumin, and this variation should be of the same entity as the one induced by urea. We have therefore submitted albumin from nephrotic patients and normal subjects to the charcoal treatment and the treated samples have then been refocused in an IEF gel containing 2 *M* urea. In all cases [both in normal subjects (tracks 4–6) and in nephrotic patients (tracks 1–3)], the treatment produced a shift of the p*I* of albumin to the

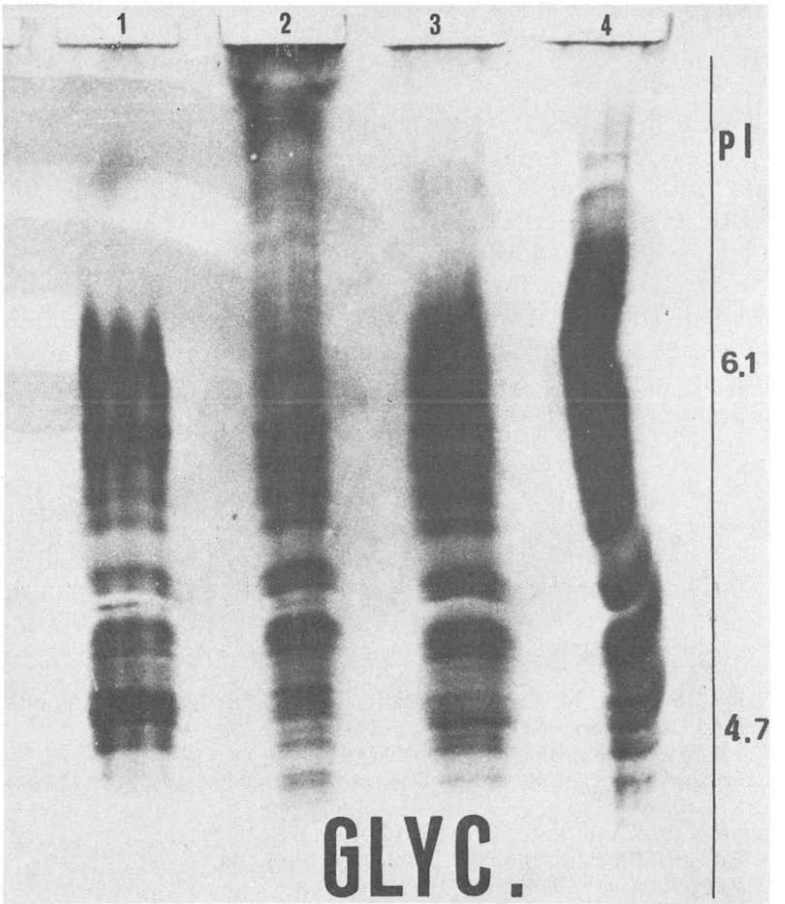

Fig. 7. IEF in glycerol of defatted albumin from normal donors (tracks 1 and 2) and from nephrotic patients (tracks 3 and 4). Silver stain.

isoionic point, along with a few other bands which focused between 4.7 and 6.1. This closely resembles the change of albumin p*I* from nephrotic patients induced by 2 *M* urea (Fig. 6). The same shift of p*I* was also observed when the two pairs of charcoal-defatted albumins (normal and urinary from nephrotic patients) were run in a gel containing glycerol as support (Fig. 7).

Molecular alteration of albumin in nephrotic syndrome

Taken together, these observations indicate that in nephrotic syndrome the linkage of albumin with one or more, hitherto uncharacterized, charged substance(s) is weak and that it can be dissociated by 2 *M* urea. On quantitative grounds, it is possible to estimate that ca. 10 mol of this ligand are transported per mol of albumin (assuming an increase of 0.1 units of p*I* for every H^+ dissociated). Two possibilities are at hand to explain this fact: (1) albumin per se binds weakly charged substances that are normally transported by albumin; (2) the substance in question is the product of the renal disease and is not normally present in human serum. This second possibility clearly implies that the substance in question has a high affinity for albumin (in competition with normally transported molecules). On the basis of IEF analyses of defatted albumin, reported in Figs. 6 and 7, it seems reasonable to suspect that the second possibility is unlikely since both defatted albumins behave in a very similar way.

The pathophysiological involvement of such a phenomenon is not yet clear, however, it is fully possible that it could have important influences on the renal handling of albumin with deleterious effects on kidney equilibrium [9].

In a more laboristic vein, we conclude that urea in low concentrations (up to 2 *M*) cannot be readily used as a support for IEF instead of glycerol; however, it offers a very useful tool for studying the binding of albumin with many substances in vitro. The model will be used to investigate the interaction of albumin with numerous substances that accumulate in chronic renal insufficiency [10] and/or in nephrotic syndrome, whose importance in altering macromolecules has perhaps been underestimated up to now.

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