# MECHANISM OF DENATURATION OF HUMAN SERUM ALBUMIN BY UREA

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Received January 5th, 1987

The mechanism of denaturation of human serum albumin by urea was examined by polarography, polarimetry, circular dichroism, UV-spectrophotometry, gel chromatography, and polyacrylamide gel electrophoresis. Comparison of the results obtained by these methods shows that this reaction is a complex process which cannot be described by a two-state denaturation model. It has been demonstrated that the different states which denaturation produces arise under different denaturation conditions. The different behavior of various species of human serum albumin (monomer, mercaptalbumin and nonmercaptalbumin) during denaturation by urea was examined. As a result the following probable denaturation scheme was proposed: The denaturation of human serum albumin by urea is regarded as a stepwise process involving one stable intermediary product at least (demonstrated electrophoretically). After the rapid initial change of the ordered helical structure extensive hydrophobic domains of the molecule remain folded. Cystine residues are gradually liberated from these domains. Denaturated mercaptalbumin has the conformation of a random coil in which the pairing of native disulfide bonds has been altered because of SH - S—S interchange reactions; in contrast native disulfide bonds are retained in nonmercaptalbumin.

The examination of the denaturation of proteins by various reagents is an efficient tool of studies on protein structures. The denaturation of proteins, however, is a complex process which cannot be described in terms of one property of the protein to such a degree which could characterize completely the type and extent of the change which the structure of the protein molecule has undergone. Each physicochemical method describes a part of the conformation change only; the information provided by each individual method should therefore be carefully valued.

The denaturation of proteins has been described by a two-state model. A two-state transition from the native (N) to the denatured (D) protein is characterized by the presence of these two states during the denaturation process only; no other conformation exists in such a concentration which could not be neglected with respect to states N and D. The discrepancies observed during kinetic analysis of protein denaturation based on the two-state model led to the prediction of the existence of intermediates[1] demonstrated experimentally later[2,3].

A two-state transition as a pathway of HSA denaturation by urea is not probable; it has been postulated that the denaturation involves a series of intermediary products[4,5]. Even though there are numerous reports on serum albumin (SA) denaturation by urea the nature of this process has not been elucidated completely. Moreover, the results yielded by different methods appear contradictory: SA denaturation is shown to be either a very rapid process (by optical rotation[6,7], spectrophotometry[8]) or a fast reaction in the beginning followed by a considerably slower reaction (by viscosimetry[6,9]) or a gradual process (by electrophoresis[10,11]).

Even though SA is synthesized in vivo as one species it can undergo various changes effected by other compounds present in the organism, changes which will manifest themselves during detailed studies on SA characteristics and structure as "microheterogeneity"[12]. A marked feature of the SA heterogeneity are the different properties of the species containing a free SH-group (mercaptalbumin) and of species whose SH-group is linked by a disulfide bond to some other compounds, such as, e.g. cysteine, glutathione (nonmercaptalbumin). The results of studies on thermal denaturation have shown that nonmercaptalbumin is more stable than mercaptalbumin which moreover is practically completely aggregated under the conditions of thermal denaturation[13-16].

In this study we investigated the mechanism of denaturation of human serum albumin (HSA) by urea by polarography, polarimetry, circular dichroism, UV-spectrophotometry, gel chromatography and polyacrylamide gel electrophoresis. On the basis of the results obtained we examined the mechanism of HSA denaturation by urea as regards the existence of stable intermediates and also compared the behavior of the HSA monomer, mercaptalbumin, and nonmercaptalbumin during urea denaturation. The aim of this study was to design a denaturation scheme corresponding to the results obtained by us and also to the results recorded in literature.

## EXPERIMENTAL

### Chemicals

HSA was a product of Imuna, Šarišské Michaľany, which was purified by the method of Chen[17] modified by Sogami and Foster[18]. HSA was dissolved in distilled water (to a 10% solution) at 23°C. Washed and dried granulated charcoal (Imuna, Šarišské Michaľany) was added to the solution (1 g per 1 g of protein). The pH was then decreased to pH 3·0 by the addition of 0·2 mol . . $dm^{-3}$ HCl. The stirred solution was kept at 0°C for 1 h. Charcoal was then separated by 30 min centrifugation (15 000 *g*) at 2°C. The supernatant was neutralized to pH 7·0 by the addition of 0·2 mol $dm^{-3}$ NaOH. Since the preparation had undergone the above operations during which HSA aggregates might have formed the sample was treated further[16] as follows. Sodium chloride was added to the HSA solution to a final concentration 0·1 mol $dm^{-3}$. A NaCl solution of equal concentration (pH 5·6) was used for HSA elution from a Sephadex G-200 column. The course of the separation was monitored spectrophotometrically at 280 nm (Fig. 1). The sample used in the subsequent experiments was a pool of fractions with elution volumes in the range 900—1 000 ml.

The HSA solution was then dialyzed 48 h against distilled water with constant stirring. The homogeneity check on the molecular weight of the sample obtained was performed by polyacrylamide gel electrophoresis[19,20]. The fraction obtained by the above procedure contained the monomer only.

Preparation of mercaptalbumin and nonmercaptalbumin: Commercial HSA was dissolved in 0·05 mol $dm^{-3}$ of phosphate buffer, pH 7·0, and dialyzed 24 h against the same buffer. The HSA fractionation was effected on a DEAE-Sephadex A-50 column. Linear gradients of phosphate buffers (0·05 to 0·2 mol $dm^{-3}$) at pH 7·0 were used for the elution[16]. The course of the separation was monitored spectrophotometrically at 280 nm (Fig. 2). Mercaptalbumin was obtained by pooling fractions with elution volumes 1 730 to 1 920 ml, nonmercaptalbumin by pooling fractions emerging in 2 400—2 600 ml. Both samples were then dialyzed 48 h against distilled water and lyophilized. The thiol group content of both fractions was determined by amperometric titration[21].

Sephadex was from Pharmacia, Uppsala. Chemicals for electrophoresis: N,N,N′,N′-tetramethylethylenediamine, N,N′-methylene-bis(acrylamide), and acrylamide were from Serva, Heidelberg. Urea (reagent grade, Lachema, Brno) was recrystallized from aqueous ethanol. The remaining chemicals were analytical grade preparations of Lachema, Brno.

## Methods

The polarographic measurements were carried out in Polariter $PO_4$ Polarograph (Radiometer, Copenhagen) using the Kalousek vessel with a saturated calomel electrode (SCE) as reference in relation to which all the values of potentials are expressed. The experimental details were the same as described earlier[22].

The sulfhydryl group content in various HSA species was determined in the same polarograph using amperometric titration with methyl iodide and mercuric chloride at —1·3 V.

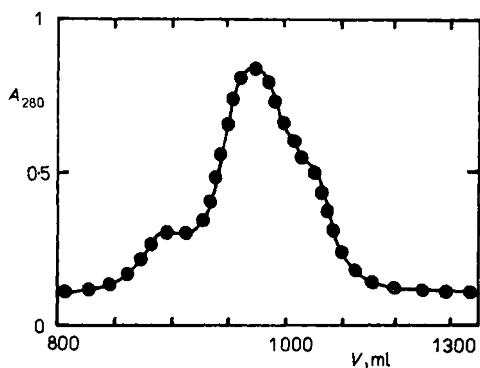



FIG. 1

Chromatogram of commercial HSA on Sephadex G-200 (4 × 200 cm column) at 280 nm, eluent 0·1 mol $dm^{-3}$ NaCl (pH 5·6), flow rate 0·22 ml/min

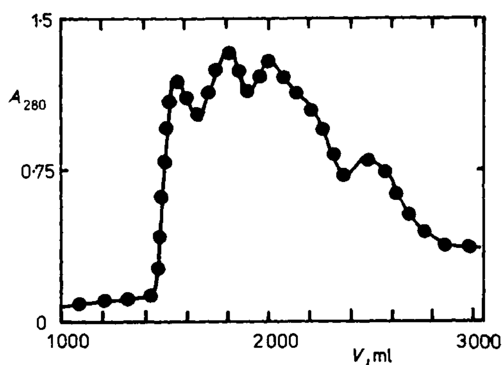



FIG. 2

Separation of mercaptalbumin and nonmercaptalbumin on DEAE-Sephadex A-50 (column 3·5 × 40 cm). Flow rate 0·33 ml/min, linear gradient of 0·05 to 0·2 mol $dm^{-3}$ phosphate (pH 7)

The spectrophotometric measurements in the UV range were carried out in Specord UV-VIS Spectrophotometer (Carl Zeiss, Jena). A four-cell arrangement was used and the optical path of quartz cells was 10 mm.

Optical rotatory dispersion (ORD) measurements were made in the instrument manufactured by Jasco (Model ORD/UV 5) equipped with a quartz cell of optical path 1 mm. The change in ORD was read off at 233 nm (minimum of Cotton effect).

Optical rotation measurements at 589 nm were performed in ETL-NPL Automatic polariter type 143 A (British Physical Laboratories, Radlett).

Circular dichroism spectra (CD) were measured in the instrument manufactured by Jasco (model ORD/UV-5) equipped with the CD accessory and a quartz cell of 1 mm optical path. The CD spectra were measured over the range of 200–300 nm and the value was read off at 222 nm.

Polyacrylamide gel electrophoresis according to Davis[19] was carried out at 200 V and 3 to 5 mA per one tube for 2 h. A detailed description of the electrophoretic measurements has been given elsewhere[20].

### Denaturation

A defined volume of the protein solution was added to the urea solution so that the final HSA concentration in the denaturation mixture might be $7 \cdot 0 . 10^{-5}$ mol $dm^{-3}$ in the case of polarographic, polarimetric, chromatographic, and CD measurements, $3 \cdot 5 . 10^{-5}$mol $dm^{-3}$ in the case of spectrophotometric measurements, and $10^{-4}$ mol $dm^{-3}$ in the electrophoretic experiments. The urea concentrations varied between 0 and 8 mol $dm^{-3}$. The samples were shaken vigorously and immediately analyzed. In the case of polarographic measurements the sample was analyzed after 20 s, in other cases after 1 min.

For polarographic, electrophoretic, and chromatographic measurements aliquots of the denaturation mixture were withdrawn at regular time intervals and added to a medium in which the analysis itself was carried out (with simultaneous decrease of urea concentration). In the remaining assays the changes in protein conformation were examined directly in the denaturation mixture.

All experiments were carried out at 22°C.

## RESULTS AND DISCUSSION

The interpretation of HSA denaturation is complicated by the fact that the monomer contains two components (mercaptalbumin and nonmercaptalbumin), which according to recorded data differ in behavior when exposed to denaturing effects and reagents[13–15].

For this reason the first part of our work was devoted to the preparation and characterization of the indiviual HSA components. The preparation of the monomer, mercaptalbumin, and nonmercaptalbumin was carried out by conventional procedures[16]. The products were characterized by UV-spectrophotometry, polarography, amperometric titration, and polyacrylamide gel electrophoresis. The data obtained by spectrophotometric and polarographic measurements correspond to values for native HSA. The electrophoretic measurements (Fig. 3*a*–*c* at zero urea concentration) show that all three samples contain one electrophoretic component. According

to amperometric titration the content of SH-groups per one component molecule is 0·7 for the monomer, 1 for mercaptalbumin and 0 for nonmercaptalbumin. The composition of nonmercaptalbumin was not studied in more detail. The results of the denaturation experiments with nonmercaptalbumin are identical with the results obtained by the denaturation of a sample prepared from mercaptalbumin by labeling its SH-group with iodoacetamide[23].

In the next part of our study we investigated the behavior of mercaptalbumin, nonmercaptalbumin, and the HSA monomer during denaturation. Fig. 4 shows the polarographically measured time profiles of denaturation of all three species in 8 mol $dm^{-3}$ urea. These profiles can be interpreted in terms of stepwise release of buried cystine residues from the interior of the HSA molecule[24,25]. This release takes place "quantumwise". The individual "quanta" are proportional to numbers of quaternaries of half-cystine residues[22], in accordance with the primary structure of HSA in which 32 out of 35 half-cystine residues exist in 8 quaternaries[26–28]. A comparison of the time profiles with the transition denaturation curves after 24 h of urea treatment shows (Fig. 5) that the values for mercaptalbumin and the HSA monomer are close to each other whereas the values for nonmercaptalbumin are slightly lower.

The effect of various urea concentrations (after 24 h) on the electrophoretic behavior of mercaptalbumin, nonmercaptalbumin, and the HSA monomer shows an essential difference among these species. Mercaptalbumin (Fig. 3*a*) adopts in 1 to

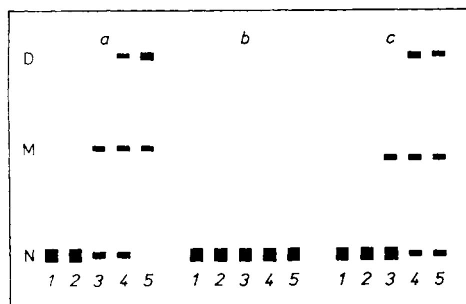



FIG. 3

Electrophoretic behavior of mercaptalbumin (*a*), nonmercaptalbumin (*b*), and HSA monomer (*c*) denaturated by urea 24 h. Urea concentrations, mol $dm^{-3}$: *1* 0 (native protein); *2* 2; *3* 4; *4* 6; *5* 8

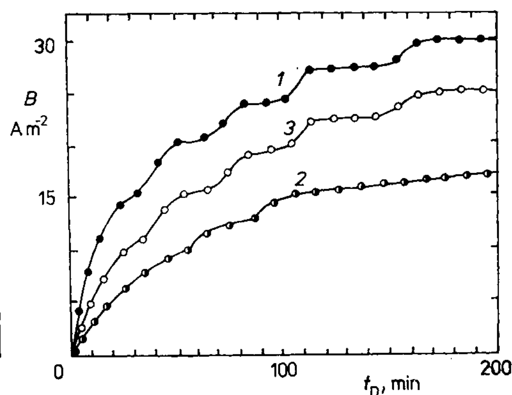



FIG. 4

Polarographic assay of kinetics of denaturation of mercaptalbumin (*1*), nonmercaptalbumin (*2*), and HSA monomer (*3*) by 8 mol . . $dm^{-3}$ urea

4 mol $dm^{-3}$ urea the M conformation of lower electrophoretic mobility than that of the native protein. Another conformation, D, of even lower mobility than that of M is observed at higher urea concentrations. The formation of these two conformations is paralleled by the decrease of the native protein N; this component practically disappears in 8 mol $dm^{-3}$ urea.

The denaturation of nonmercaptalbumin (Fig. 3*b*) does not give rise at the urea concentrations examined (0 to 8 mol $dm^{-3}$) to an electrophoretically different component. There may be 3 reasons for this phenomenon: *1*) the protein is not denaturated in urea, no conformationally different component is formed, *2*) the denaturation gives rise to conformations which do not possess a different electrophoretic mobility, *3*) the process is reversible, i.e., the products formed during denaturation are transformed, because of dilution of the sample in the electrophoretic experiment (a decrease of urea concentration) to a conformation which has the same electrophoretic mobility as the native protein.

Since we know from polarographic studies of nonmercaptalbumin denaturation by urea that conformational changes taking place during this process are paralleled by an increase of the number of half-cystine residues accessible to the electrode reaction, we cannot eliminate the possibility of formation of a conformationally different component. Since the polarimetric (at 589 nm) and spectrophotometric measurements do not show, within experimental error, a difference between the denaturing effect of urea on mercaptalbumin and nonmercaptalbumin it is not likely that the products of mercaptalbumin denaturation could possess an electrophoretic mobility essentially different from that of the products of nonmercaptalbumin denaturation (both species differ in one amino acid residue only). The different electrophoretic behavior of the two species can be explained by the reversibility of denaturation which is due to dilution of the denaturation mixture (as observed

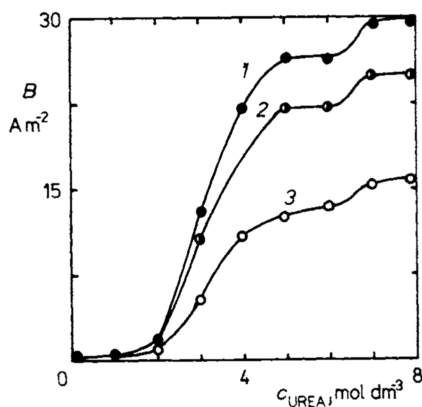



FIG. 5

Transition curves of denaturation of mercaptalbumin (*1*), HSA monomer (*2*), and nonmercaptalbumin (*3*). Plot of Brdička current at −1·60 V after 24 h of denaturation versus urea concentration

during polarographic, chromatographic, and electrophoretic measurements). In contrast, if the denaturation measurement is carried out directly in the denaturation mixture (without changing urea concentration, i.e., by CD, polarimetric, and spectrophotometric measurements), no essential difference is observed in the behavior of the two species. This explanation is supported by the fact that there was a big difference in the renaturation behavior of mercaptalbumin and nonmercaptalbumin when the renaturation process was examined polarographically[23].

The monomer forms components during denaturation which in mobility correspond to conformations M and D, and, to a small degree, components of lower electrophoretic mobility than that of component D. They are most likely dimers and higher *n*-mers of HSA. Even in 8 mol $dm^{-3}$ urea there remains a marked amount of the component which has the same mobility as the native protein. It can thus be said that the electrophoretic behavior of the HSA monomer corresponds to that of a mixture of mercaptalbumin and nonmercaptalbumin (Fig. 3c).

The relatively small difference in the behavior of mercaptalbumin and HSA monomer during the polarographic assay of denaturation can be explained by the presence of a more than two-fold excess of mercaptalbumin over nonmercaptalbumin in the HSA monomer (according to the results of amperometric titration the HSA monomer contained 70% of mercaptalbumin and 30% of nonmercaptalbumin) and by the short renaturation period (<1 min).

The results of optical methods, gel chromatography, and polyacrylamide gel electrophoresis are in agreement with the recorded data[5-11]. It should be noted that the time necessary for the release of all cysteine residues (about 200 min in 8 mol . . $dm^{-3}$ urea) is identical to the time necessary for complete unfolding of SA in 8 mol $dm^{-3}$ urea during viscosimetry measurements[9].

A comparison of all the above results shows that after the rapid disappearance of ordered structures (mostly α-helical) there still remain large parts of the HSA molecule folded. These are most likely the hydrophobic regions[29] in which the cystine residues are buried[30]. These regions become unfolded during the subsequent denaturation which is paralleled by an increase in the polarographic current intensity (Fig. 4) as a result of the fact that additional disulfide groups become available for the electrode reaction.

*Proposal of Denaturation Scheme*

Since no conclusions can be made on the mechanism of denaturation from the results of measurements in which two components with different behavior during denaturation participate, we will consider in this section predominantly the denaturation of mercaptalbumin. The transition curves characterizing mercaptalbumin denaturation after 24-h treatment with urea and measured by optical methods are summarized in Fig. 6.

Unlike during the polarographic measurements (Fig. 4) no gradual change was observed during the spectrophotometric, polarimetric (at 589 nm), and CD measurements yet the magnitudes assayed reached their limiting values immediately after the addition of urea and remained unchanged for 24 h. The results of ORD measurements showed that the value of mean residual rotation $[m']_{233}$ significantly decreased

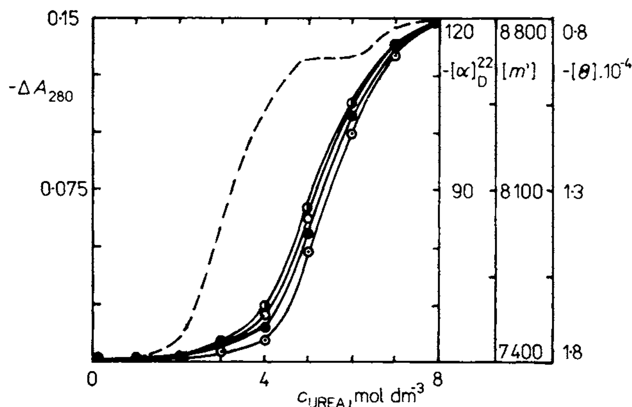



Fig. 6

Transition curves of mercaptalbumin denaturation after 24-h treatment with urea: (●) spectrophotometry at 280 nm, (⊙) cricular dichroism at 222 nm, (○) polarimetry at 589 nm, (◑) optical rotatory dispersion at 233 nm, ----- polarography

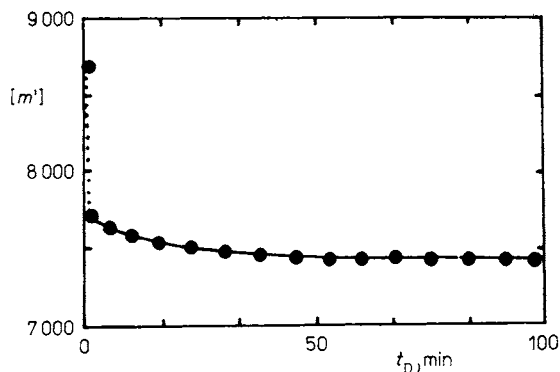



Fig. 7

Time profile of changes of mean residual rotation $[m']_{233}$ of mercaptalbumin during denaturation by 8 ml dm$^{-3}$ urea. The part of curve marked ...... designates a great change of $[m']_{233}$ before the first measurement possible

even before the first measurement could be made. This marked decrease was followed by a smaller change whose time profile is shown in Fig. 7. It follows from the results of electrophoretic measurements (Fig. 8) that in the process of denaturation by 8 mol $dm^{-3}$ urea conformation M is formed as the first one and that conformation D is formed only later. Since the denaturation was discontinued by a six-fold dilution of the denaturation mixture with distilled water it is obvious that our results are influenced by the renaturation of the protein in the medium of low urea concentration. This holds true also for the results of gel chromatography on Sephadex G-200 which are shown in Fig. 9. In spite of that we may conclude from these measurements that the denaturation of mercaptalbumin by urea is a stepwise process.

As demonstrated before[24], the results of the kinetic test[31] applied to the polarographic measurement of denaturation of the HSA monomer by urea show that the denaturation is not a two-state process yet a process which involves one stable intermediate at least. The same results were also obtained with mercaptalbumin. A comparison of the transition curves (Figs 5 and 6) also shows that HSA denatu-

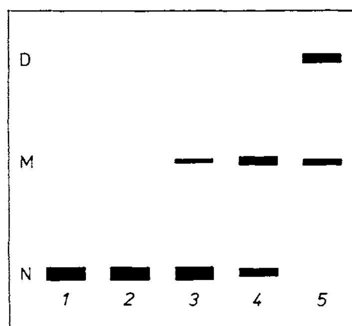



FIG. 8

Electrophoretic investigation of kinetics of denaturation of mercaptalbumin by 8 mol . . $dm^{-3}$ urea. *1* native mercaptalbumin, *2*, *3*, *4*, *5* mercaptalbumin denaturated 1 min, 30 min, 75 min, and 24 h

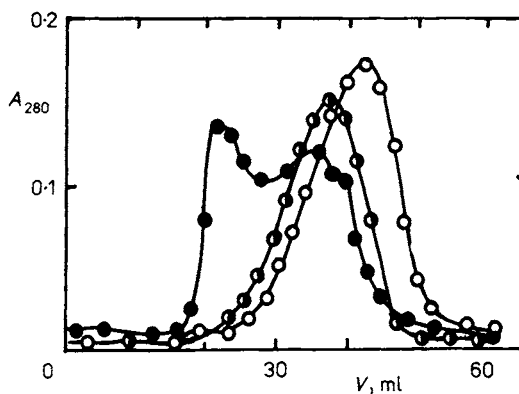



FIG. 9

Gel filtration of native and denatured HSA monomer on Sephadex G-200 (column 1·8 × 20 cm). Flow rate 0·11 ml/min, 0·05 mol $dm^{-3}$ phosphate buffer (pH 8·4), $\lambda = 280$ nm. (○) native HSA, (◑) HSA denatured 5 min by 8 mol $dm^{-3}$ urea, (●) HSA denatured 24 h by 8 mol $dm^{-3}$ urea. The electrophoretic analysis of this sample showed that the fraction emerging in 32 to 40 ml contains a homogeneous component corresponding in mobility to the native protein

ration by urea is not a two-state process since if the two-stage denaturation model were valid the transition curves obtained by various methods would be identical[31].

According to Wallevik[32] the denaturation schemes designed for small proteins are not applicable to serum albumin. This protein behaves as a big macromolecule and its denaturation can be interpreted as a sum of independent unfoldings of the various parts (domains[33]) of its molecule. For this reason an approximate scheme of denaturation only can be proposed which is based on the concept of the cluster model of protein folding[34–36]. Our results show that different states of HSA conformation arise under different conditions of denaturation (cf. Figs 3, 4, 5, 8, and 9); the differences are caused both by differences in urea concentrations and also by differences in the length of the denaturation periods. The completely unfolded conformation D (minimal electrophoretic mobility and minimal content of ordered structures, maximal accessibility of the tyrosyls to the solvent and of the cystine residues to the electrode reaction) is formed in high urea concentrations whereas the incompletely unfolded states (higher electrophoretic mobility, several cystine residues remaining buried) arise either under milder conditions of denaturation (urea concentration below 4 mol $dm^{-3}$) or during the initial stages of denaturation by higher urea concentrations.

As shown above, HSA denaturation by urea is not a two-state process but a gradual process involving one stable intermediate at least. Immediately after the addition of the HSA solution to urea the hydrogen bonds are disrupted (state $X_1$); this state is characterized by a change in optical rotation, CD and UV spectra. The individual subunits of the molecule move away from one another fast immediately afterwards[33] (state $X_2$); this state is paralleled by a substantial increase in viscosity[2,4]. During the subsequent course of denaturation the individual domains

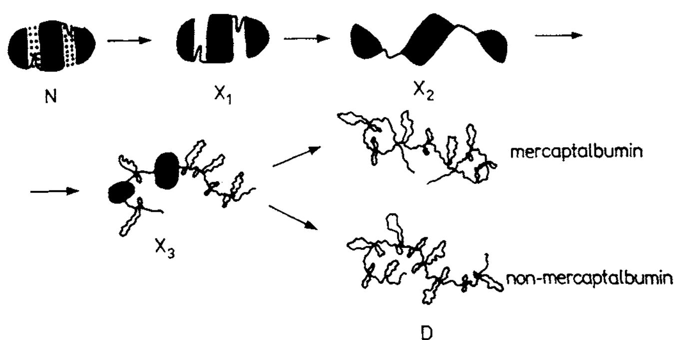
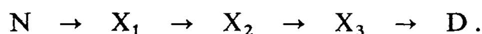



FIG. 10
Scheme of HSA denaturation by urea. The details and the explanation of the symbols are given in the text

are unfolded. This phase can be investigated by polarography, viscosimetry[2,4], and ORD. The application of the kinetic test to the results of polarographic examination of the kinetics of denaturation showed that this reaction involves one stable intermediate, $X_3$, at least. The existence of the stable intermediate was proved experimentally by electrophoretic measurements (state M). The unfolding of the subunits then continues and the final state D (at high urea concentrations) has the conformation of random coil with uninterrupted disulfide bonds; their native pairing, however, can be altered in mercaptalbumin by SH–S—S interchange reactions. The presence of the original disulfide bonds can be predicted from the results of gel chromatography (Fig. 9) and electrophoretic analysis in the case of nonmercaptalbumin. Our model of HSA denaturation by urea can thus be described as follows:



$$N \rightarrow X_1 \rightarrow X_2 \rightarrow X_3 \rightarrow D .$$

It is represented schematically in Fig. 10.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Translated by V. Kostka.