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Gel Filtration of Albumin on Sephadex G-200 in Urea*

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Human serum albumin has been subjected to gel filtration on Sephadex G-200 in different molarities of urea. In 0, 1, and 3 M urea, peaks corresponding to the monomer and dimer of albumin appeared. At 4.0, 4.5, and 5 M urea, one fourth to one half of the albumin seemed to unfold to a volume with a Stokes radius of about 6.3 mμ while the rest was unchanged. At 7 M urea a more severe unfolding occurred.

From theoretical calculations and experimental unfolding curves, it has been shown that the unfolding of some proteins in urea occurs stepwise, so that stable intermediate states between the native and the totally unfolded conformation exist.[11] The effect of urea is probably due to an increase in the solubility of the nonpolar groups buried in the interior of the protein molecules.[8] This causes an unfolding of the structure and an increase in volume, the packing of the polypeptide organization becoming less compact.

As gel filtration separates molecules according to size, it was thought that this fractionation method might be used to demonstrate and to give information on the increase in volume of proteins caused by urea, especially regarding the occurrence of stable intermediate unfolded states. For this purpose Sephadex G-200, having a working range from 5000 to 800 000 was used.[5] Human serum albumin was chosen as a model substance, because its molecular weight (69 000) is well below the upper separation limit of the Sephadex gel, but high enough for several regions in the molecule to unfold independently at denaturation.

EXPERIMENTAL

Freeze dried human serum albumin was supplied from Behringwerke (Marburg an der Lahn). It was labelled with ^{125}I by the iodine monochloride method.[7] Care was taken that less than one atom of iodine was incorporated into each molecule of albumin, and that the specific activity did not exceed 5 μCi per mg of protein. The purity of the radioactive labelled preparations was controlled by autoradiography of the immunelectro-

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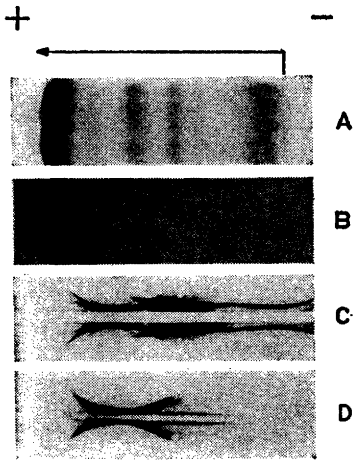

Fig. 1. A. Paper electrophoresis of a mixture of ^{125}I-labelled albumin and normal human serum. B. Autoradiography of A. C. Immunoelectrophoresis of a mixture of ^{125}I-labelled albumin and normal human serum. D. Autoradiography of C. The ^{125}I-activity is found only in the albumin fraction.

phoresis and the paper electrophoresis (Fig. 1). The Sephadex G-200 gel * was allowed to swell for at least 7 days in 0, 1, 3, 4, 4.5, 5, and 7 M urea, buffered with 0.4 M sodium phosphate pH 7.5. Columns of 65 or 100 to 115 cm × 2.3 cm were packed by gravity and were equilibrated with the same buffers at a flow of 4 ml/h for 3 days before use. To avoid crystallization of the urea because of evaporation, the air leading to the reservoirs was saturated with water by bubbling through distilled water. Protein concentrations were measured at 280 mμ in a Beckman DU spectrophotometer. ^{125}I and ^{22}Na-activity were measured in a well-type scintillation counter with an automatic sample changer. The background was 30 and 60 cpm, respectively.

About 0.1 μCi ^{125}I-labelled albumin and 0.01 μCi ^{22}NaCl were mixed with 500 μl human serum and 40 % sucrose to a total volume of 550 μl, and the sample was applied by gravity through a teflon tube to the top of the column through the buffered urea. Fractions of 2 ml were collected by an automatic fraction collector at a constant flow rate sustained by a pump. The experiments were performed at 20 to 22°C.

Stokes radius is expressed by the equation

$$\frac{V_e - V_0}{V_i} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\,\frac{a}{r} + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right]$$

given by Ackers.[1]

V_e is the effluent peak volume of a molecule; V_0 the void volume of the column, in this case protein peak I; V_i the liquid volume within the gel grains, *i.e.* the difference between the elution volume for ^{22}NaCl and V_0; a is the Stokes radius and r the effective pore radius for the gel. In each experiment, r was calculated from the $(V_e - V_0)/V_i$ and the Stokes radius for albumin monomer, *i.e.* 3.61 mμ,[1] and the value obtained for r was used to calculate the unknown radius.

RESULTS

0, 1, and 3 M urea. As seen in Fig. 2, three protein peaks are obtained, while the ^{125}I-activity emerges in a major peak (VI) of albumin monomer comprising about 92 % of the activity and a minor peak (V) of albumin dimer with a Stokes radius of about 5.5 mμ (Table 1).

* Pharmacia Fine Chemicals, Uppsala, Sweden.

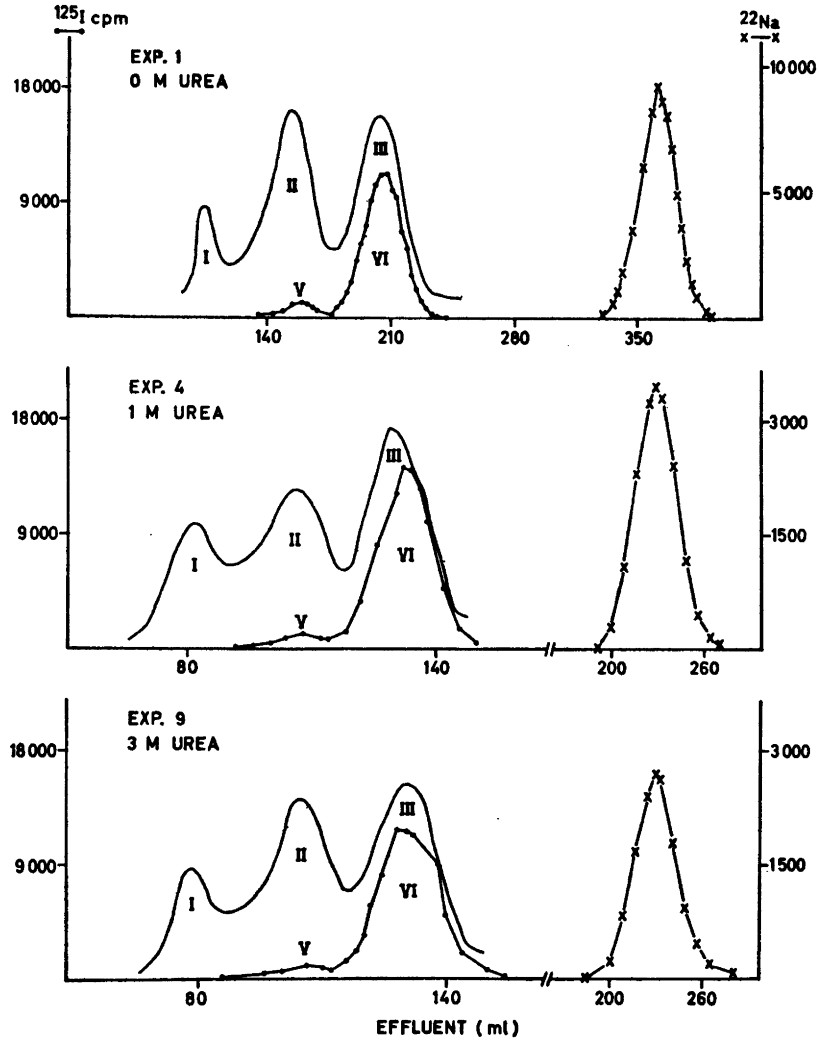

Fig. 2. G-200 Sephadex gel filtration of a mixture of human serum (D_{280}: ————), ^{125}I-labelled albumin (●————●) and ^{22}NaCl in 0, 1, and 3 M urea. The void volumes of the columns are equivalent to the elution maxima for protein peaks I. The inner volumes of the columns were calculated by subtracting the void volumes from the elution maxima for ^{22}NaCl.

4, 4.5, and 5 M urea (Fig. 3). Three protein peaks are still seen but the separation is less distinct. Instead of the small peak of albumin dimer seen in the 0 to 3 M urea columns, a peak called IV comprising 26 to 43 % of the ^{125}I-activity appears between protein peaks I and II. The Stokes radius for this peak is about 6.3 mμ (Table 1).

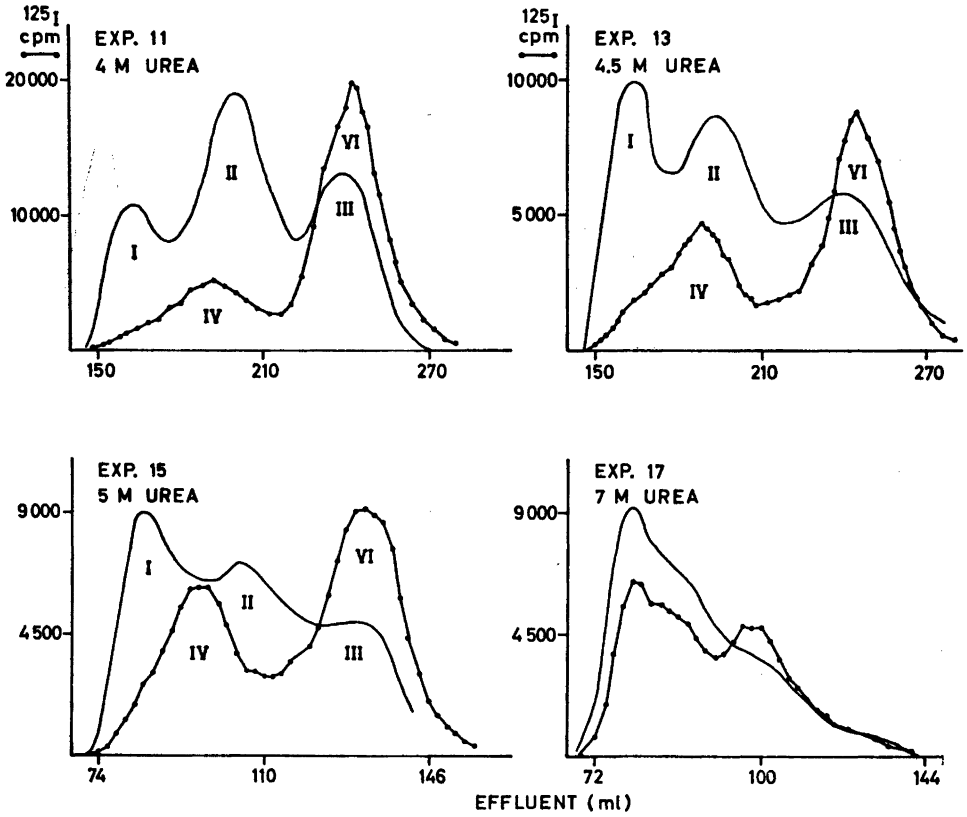

Fig. 3. G-200 Sephadex gel filtration of a mixture of human serum (D_{280}: ————) and ^{125}I-labelled albumin (●————●) in 4, 4.5, 5, and 7 M urea. The ^{22}NaCl peaks have not been included in the drawings.

When ^{125}I-labelled albumin is dialysed *versus* 5 M urea and then run on a 5 M urea column, the same diagram is obtained as for an undialyzed sample, 40 to 45 % of the activity being eluted between protein peaks I and II with a Stokes radius of 6.6 mμ (Fig. 4, Expt. 29).

When peak VI (albumin monomer) from a 5 M urea gel filtration experiment is rerun on a 1 M urea column all the ^{125}I-activity appears as albumin monomer (Fig. 5, Expt. 22). Rerun on a 5 M urea column, the main part reappears as albumin monomer and a minor fraction comprising about 12 % of the activity, appears as a peak IV with a Stokes radius of 6.1 mμ (Fig. 5, Expt. 26).

When peak IV (Stokes radius 6.3 mμ) from a 5 M urea column (Expt. 30) is dialyzed *versus* 1 M urea for 8 days, and run on a 1 M urea column, about 40 % of the activity appears as albumin monomer, the remaining activity being eluted as a peak IV (Fig. 5, Expt. 19). Rerun on a 5 M urea column the

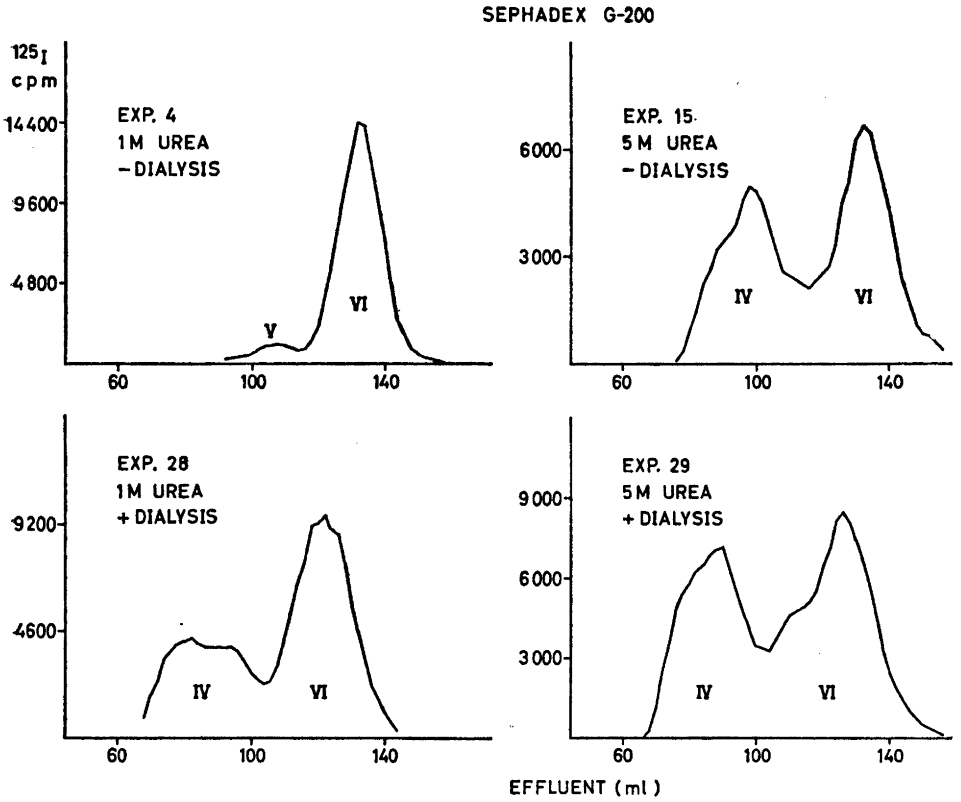

Fig. 4. G-200 Sephadex gel filtration of ^{125}I-labelled albumin in 1 and 5 M urea, without and with previous dialysis *versus* 5 M urea. Only the ^{125}I-radioactivity curves are depicted.

main part of peak IV reappears as peak IV (Fig. 5, Expt. 23). The minor fraction, comprising less than 10 % of total, being eluted later, may be due to contamination with peak VI material in Expt. 30.

7 M urea (Fig. 3). At this molarity the elution of serum protein appears as one peak with two small shoulders on the rear part; the ^{125}I-activity curve shows two poorly separated peaks, the major one being eluted with the void volume of the column.

DISCUSSION

The present studies show that the denaturing effect of urea on human serum albumin can be recorded by Sephadex gel filtration. Above 3 M urea (pH 7.5) an increase in volume is seen for part of the molecules. It is probably due to unfolding or isotropic swelling and occurs stepwise, the first step giving

molecules with a Stokes radius of about 6.3 mμ. At 7 M urea unfolding to above the working range for Sephadex G-200 occurs.

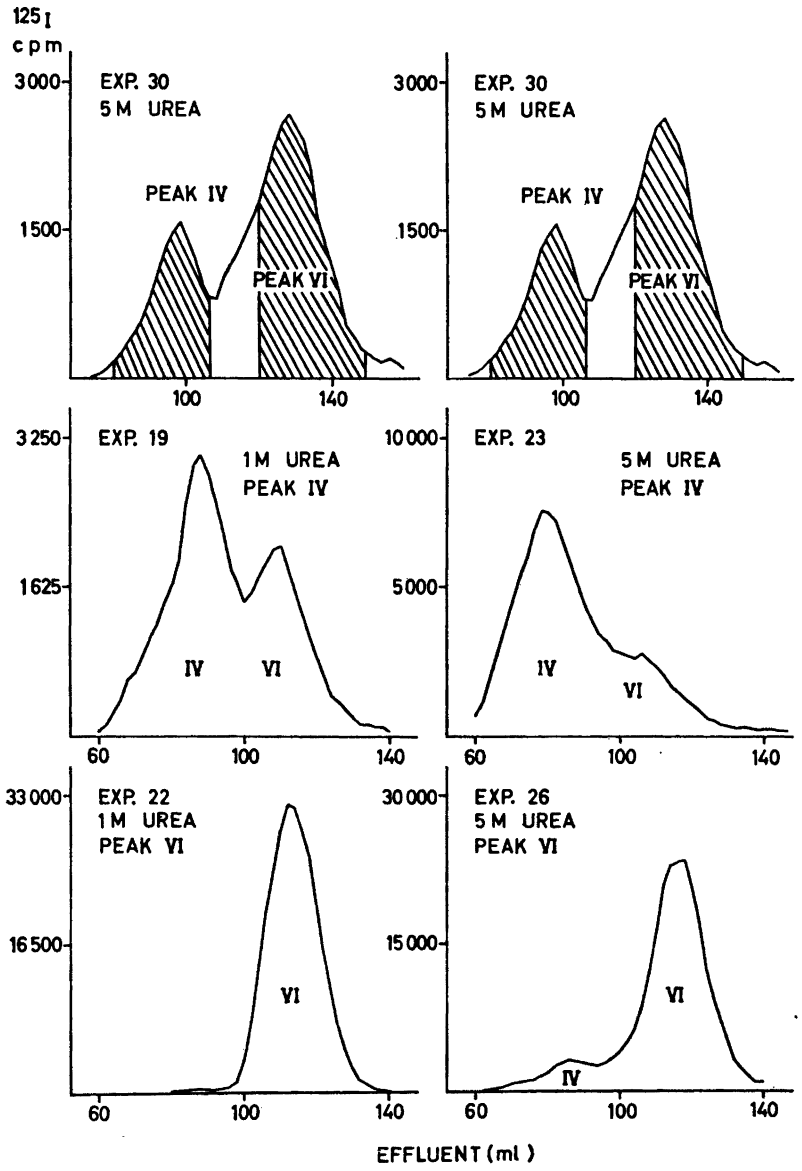

Fig. 5. Partially unfolded ^{125}I-labelled albumin from G-200 Sephadex gel filtration in 5 M urea (Peak IV) rerun in 1 M (Expt. 19) and in 5 M (Expt. 23) urea. *Not* unfolded albumin (peak VI) rerun in 1 M (Expt. 22) and in 5 M mole (Expt. 26) urea. Only the ^{125}I-radioactivity curves are depicted.

Table 1. Gel filtration data of human serum albumin on Sephadex G-200 in urea.

Expt. No.	Urea conc. M	^{125}I-labelled albumin monomer (Peak VI) $\frac{V_e-V_0}{V_i}$	a/r [b]	r [c] (mμ)	% of total ^{125}I-activity	^{125}I-labelled albumin dimer (Peak V) $\frac{V_e-V_0}{V_i}$	a/r [b]	a [a] (mμ)	% of total ^{125}I-activity	^{125}I-labelled albumin (Peak IV) $\frac{V_e-V_0}{V_i}$	a/r [b]	a [a] (mμ)	% of total ^{127}I-activity
1	0	0.385	0.1983	18.20	91.5	0.208	0.2994	5.45	8.5				
2	0	0.396	0.1932	18.68	88.7	0.198	0.3069	5.73	11.3				
3	0	0.415	0.1848	19.53	90.6	0.218	0.2923	5.71	9.4				
4	1	0.354	0.2131	16.94	92.4	0.184	0.3179	5.39	7.6				
5	1	0.345	0.2175	16.60	93.2	0.176	0.3241	5.38	6.8				
6	1	0.363	0.2087	17.30	91.5	0.175	0.3248	5.62	8.5				
7	3	0.374	0.2034	17.75	91.9	0.187	0.3155	5.60	8.1				
8	3	0.350	0.2150	16.79	91.6	0.181	0.3200	5.37	8.4				
9	3	0.350	0.2150	16.79	92.3	0.185	0.3171	5.32	7.7				
10	4	0.346	0.2170	16.64	73.8								
11	4.5	0.346	0.2170	16.64	69.1					0.135	0.3620	6.02	26.2
12	4.5	0.346	0.2170	16.64	62.6					0.116	0.3827	6.37	30.7
13	5	0.331	0.2247	16.07	57.2					0.116	0.3827	6.37	37.4
14	5	0.302	0.2400	15.04	56.2					0.101	0.4009	6.44	42.8
15	5	0.330	0.2252	16.03	60.9					0.090	0.4158	6.25	43.1
										0.093	0.4115	6.60	39.1

[a] Stokes radius.
[b] Obtained from Table III in Ref. 1.
[c] For the calculation of r a Stokes radius of 3.61 mμ for human serum albumin was used.

While the effective pore radius, r, in columns without urea in experiments 1, 2, and 3 (mean 18.8 mμ) was the same as found by Ackers [1] on the basis of gel filtration data from Rogers *et al.*,[10] a moderate decrease occurred on addition of urea to the buffer. This did not affect the Stokes radius found for albumin dimer (mean 5.5 mμ, Table 1).

By optical rotatory dispersion Callaghan and Martin [2] found a linear progression of unfolding of human serum albumin in from 2 to 9 M urea at pH 5.5 to 6. These authors suggest that a smooth transition occurs from the rigid helical structure to the open random coil. However, this method measures the average changes and cannot distinguish between a partial or total unfolding of one molecle, or changes effecting all or only some molecules.

The results in the present study indicate that the denaturation of albumin occurs stepwise and further that human serum albumin is a microheterogeneous protein.

The existence of intermediate states for the unfolding of globular proteins has been suggested by Tanford [11] from calculations of the standard free energy of unfolding in urea. Thus for ribonuclease, three regions with the ability to unfold independently were suggested. In accordance with this, gel filtration on Sephadex G-100 [6] shows that the elution volume for ribonuclease decreases on exposure to 4 and 8 M urea. Whether more than one partial unfolded state, as found in the present study, is possible for human serum albumin, has to await the availability of a gel filtration material with a higher separation limit than Sephadex G-200. The occurrence of stable, partially unfolded intermediate states on denaturation indicates that the bondings being responsible for the tertiary conformation of the peptide chains are interdependent.

While the ribonuclease studied by Llosa *et al.*[6] remained homogeneous and showed only one peak of elution at 0, 4, and 8 M urea although the elution volume decreased, two well-separated peaks were obtained for human serum albumin in 4 to 5 M urea in the present study. This cannot be due to an equilibrium between native and denatured molecules as this would tend to give one out-flattened peak or two poorly separated peaks. It seems more likely that more than one type of albumin molecules is present having different sensitivities to the denaturing effect of urea.

Microheterogeneity of albumin has been suggested by Pedersen [9] on the basis of the skewed elution curve for human serum albumin on Sephadex gel filtration. Similarly changes affecting only part of the molecule on denaturation of bovine albumin have been found by Ferris and Katz.[3] At exposure for 24 h at pH 5.0 to urea molarities of 4 or more, components with electrophoretic mobilities ranging from 0 to 30 % of the fastest component were produced. The transformation to an unfolded form of part of the molecules increased the frictional resistance causing a reduction in electrophoretic mobility. Differences in the susceptibility to the action of urea combined with Sephadex gel filtration may prove useful for the fractionation of proteins showing similar patterns in standard fractionation procedures.

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