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Temperature Behaviour of Human Serum Albumin

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Structural alterations of albumin, their dependence on concentration and the role of free –SH groups at thermal denaturation, as well as the reversibility of thermally induced structural changes, were studied. Application of various physical methods provides information on a series of structural parameters in a major concentration range. Apart from changes of the helix content, heat treatment gives rise to β structures which are amplified on cooling and which are correlated with the aggregation of albumin. With rising temperature and concentration the proportion of β structures and aggregates increases.

At degrees of denaturation of up to 20% complete renaturation is possible in every case. The structure content is concentration-dependent even at room temperature. It may be that intermolecular interactions induce additional α-helix structures which are less stable, however, than the ones stabilized by intramolecular interactions. Unfolding of the pocket containing the free –SH group of cysteine-34 enables disulphide bridges to be formed leading to stable aggregates and irreversible structural alterations. Through binding of *N*-ethylmaleimide to free –SH groups, which blocks the formation of disulphide bridges, it is possible to prevent aggregation and irreversible conformational changes. At temperatures below 65 – 70 °C, oligomers are formed mainly via intermolecular β structures.

In the preparation of human serum albumin for clinical purposes its behaviour at different temperatures is of importance. The albumin is treated at 60 °C for about 10 h to inactivate the hepatitis virus. The structure should be largely retained of course in this treatment. Sodium octanoate or sodium octanoate + acetyltryptophan may be used as stabilizer. We distinguish in general two stages in the heat treatment of albumin. The first stage includes reversible structural alterations, the second one includes irreversible structural alterations, which may not necessarily result in a complete destruction of the ordered structure [1 – 3]. Although a number of investigations are available on the problem of the thermal exposure of albumin, there are still open questions to be answered concerning in particular the nature of structural alterations, the limits of reversibility, the influence of concentration and environmental conditions and the molecular mechanism of the action of the stabilizers and their relative effectiveness under various conditions. Experiments performed on horse serum albumin by Zimmermann and Dittmar [4] showed that higher molecular weight components will be increasingly formed after heat treatment of 15 min at 100 °C. If the time of exposure is 60 min or more, the aggregation products will decompose into low-molecular-weight fragments which are serologically inactive. They may still cause, however, severe shock reactions in anaphylaxia experiments. The authors suggest that species despecifications and desantigenization may only be obtained by a specific splitting of peptide bonds and not by changing the secondary structure. Aoki et al. [5] studied the termal denaturation of bovine serum albumin in the alkali pH range. The results obtained by gel electrophoresis show various components after the heat treatment at 65 °C. Brand and Anderson [6] described the influence of heating and fatty acids on the aggregate formation.

The changes in the physicochemical properties of albumin, which have already been described in the literature, are partly contradictory. The reason is that on the one hand the results obtained imply method-specific information, for instance on the aggregational behaviour and the development of β structure [2, 6 – 10], and on the other hand the con-

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Abbreviations. $H \rightarrow {}^2H$ exchange, hydrogen – deuterium exchange; ESR, electron spin resonance; CD, circular dichroism; MalNEt, *N*-ethylmaleimide.

centration dependence of the effects have been largely neglected.

We have, therefore, attempted to obtain more profound information on the changes in the physicochemical properties of albumin during thermal treatment, in particular on the influence of the concentration and of the free $-SH$ groups of albumin by the use of various physicochemical methods which also permit the carrying out of investigations in a larger concentration range. A later communication will report on possibilities of influencing structural alterations in the thermal treatment of albumin.

MATERIALS AND METHODS

Human serum albumin (commercial product, lyophilized) from the Institut für Impfstoffe (Dessau, G.D.R.) was used for the investigations. If not indicated otherwise, 0.1 M phosphate buffer, pH 6, containing 0.2 M NaCl, served as solvent. The determination of concentration was performed by spectral photometry with $\lambda = 280$ nm on the basis of an absorption coefficient of $A_{1\,\mathrm{cm}}^{1\,\%} = 5.8$ [10]. The samples used for electron spin resonance (ESR) measurements had been filtered through gel (Sephadex G-150). Mercaptalbumin was chromatographically prepared by the separation of albumin molecules by DEAE-Sephadex A-50 [11]. The electrophoretic analysis showed, in agreement with sedimentation measurement, that the mercaptalbumin contained monomers and only a low content of dimers. The labels 2,2,4,4-tetramethyl - 1,2,3,4, - tetrahydro - 5,6 - benzo - γ - carboline-3-oxyl(I) and 3-maleimido-2,2,5,5-tetramethylpyrrolidine-1-oxyl(II) have been used for the ESR investigations. Label I, being non-covalently bound to albumin, was added to the albumin solutions 10 min before the beginning of measurements. A 0.02 M ethanol solution of the label was prepared for that purpose in advance. The conditions of titration were chosen so that the ethanol concentration was 1% or less.

For the covalent labelling of Cys-34 with label II the protein solution was incubated with a 50-times label excess at 15 °C for 30 min. Then the unbound label molecules were separated on Sephadex G-25. The number of $-SH$ groups labelled could be determined by double integration of the ESR spectra obtained after labelling. Disc electrophoresis with polyacrylamide gel was used for gel electrophoretic investigations.

The circular dichroism (CD) measurements were performed on a dichrograph CD 185 (Roussel-Jouan). The measurement of temperature dependence was carried out in a hermetically sealed cell after achieving equilibrium, i.e. when a change in CD could no longer be found (this was the case after heating for 1 h).

The accuracy of temperature measurements was ± 0.2 °C. The structure contents (α-helices, β structures and residual structures ϱ) were calculated according to the curve-fitting method [12]. The CD basis spectra of Chen et al. [13] were used with the calculations. The $-SH$ groups were determined by means of Ellman's reagent [14]. The ESR spectra were recorded with a Varian E3 spectrometer. An on-line computer (KRS 4200) served for the spectral analysis [15]. The maximum sucrose concentration was 37.5 w/v in the determination of the rotational correlation time.

The viscosity was measured by a rotating cartesian-diver viscometer and an Ubbelode viscometer.

The infrared spectra of the solutions were measured by the Perkin-Elmer 180 infrared spectrometer 180. The albumin samples dissolved in 0.05 M ^2H_2O phosphate buffer (pH 7.4) were heated in a thermostated cell and kept at the specified temperature until no further spectral change could be demonstrated in the range of amide I band (about 1 h). Infrared spectroscopic measurements on films were performed in connexion with the $H \rightarrow {}^2H$ exchange measurements.

$H \rightarrow {}^2H$ exchange kinetics were observed on thin films at a temperature of 25 °C and a relative humidity of 97% from the decrease in intensity of the amide A band. The intensities of the amide A band were subsequently normalized to the amide I band. The instrumentation used for the measurements has been described elsewhere [16]. Films of heated albumin solutions (0.5 – 1 h at 80 °C) and untreated albumin solutions were studied. For the film preparation salt-free solutions were used.

The sedimentation behaviour of the albumin samples was studied in an analytical ultracentrifuge Spinco E with ultraviolet absorption optics, monochromator, and photo-electric scanner in the speed range of 17000 to 40000 rev./min at 20 °C. The albumin solutions of different concentrations were first heated to the specified temperature for 1 h and brought to a concentration of 1.4 mg/ml, if required, by dilution with phosphate buffer after cooling down. The concentration gradients were recorded using monochromatic light at $\lambda = 280$ nm. The sedimentation coefficients were calculated from the time shift of the gradients and corrected to standard conditions (water, 20 °C). The concentration portions of monomers and oligomers were calculated from the absorbance ratios of the gradients.

RESULTS

THERMAL DENATURATION

CD Measurements

There are four peaks presents in the CD spectra: at $\lambda = 262$ nm and 268 nm (optically active transitions

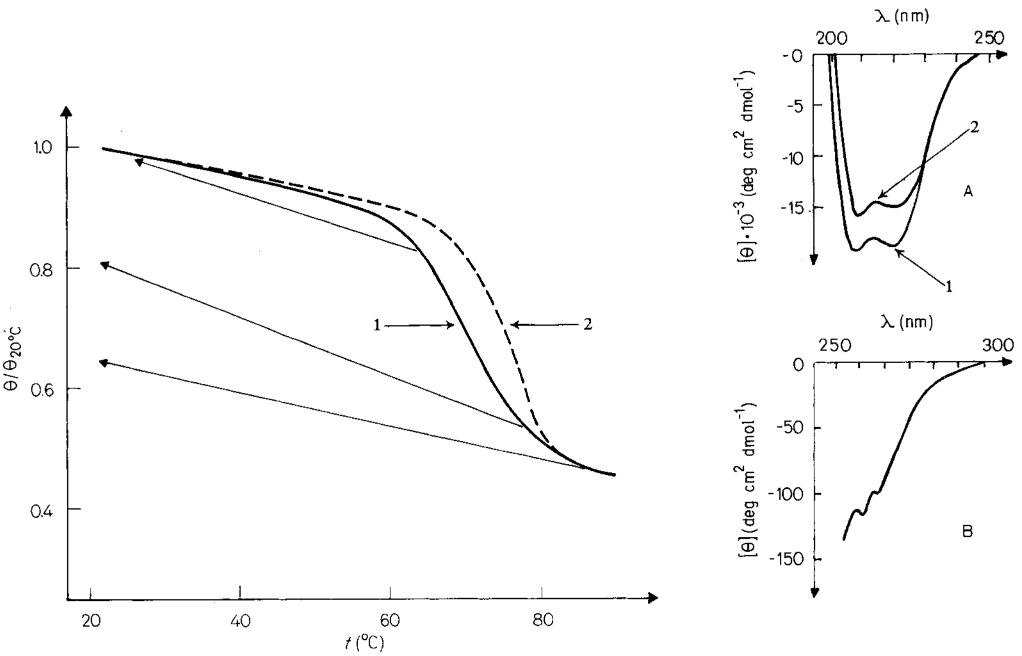

Fig. 1. *Normalized CD melting curves of albumin using* $[\Theta]_{220}$. (1) $c = 0.5$ mg/ml; (2) $c = 0.05$ mg/ml. The degree of renaturation after heating to $t = 65$ °C; $t = 75$ °C; $t = 90$ °C and recooling to 20 °C is indicated by arrows. Insert (A): CD spectrum of albumin in the peptide chromophore region. (1) $c = 0.5$ mg/ml; (2) $c = 0.05$ mg/ml; path lengths = 1 mm and 10 mm respectively. Insert (B): CD spectrum of albumin in the region of aromatic chromophores. $c = 2$ mg/ml; path length = 10 mm

of the aromatic amino acids and disulphide bonds) and at $\lambda = 208$ nm and 220 nm (peptide absorption range) (Fig. 1).

The amplitude of these peaks do not show any major difference in their dependence on temperature. Below 60 °C the melting curves measured at $\lambda = 262$ nm and 268 nm have a somewhat more pronounced decrease than those measured at $\lambda = 208$ nm and 220 nm, where the measurements in the temperature range are reflecting those changes on the albumin molecule which do not yet have any influence on the helix content and are reversible. The CD peak at 220 nm has been analyzed mainly for the present investigaton as the amplitude of this band is correlated with the helix content of the protein.

We could find only a slight decrease of the CD amplitude up to 60 °C; then a further more pronounced reduction follows between 65 °C and 80 °C. There is a degree of denaturation of about 50% at 80 °C (Fig. 1). While the changes in the CD spectrum which may be obtained up to 65 °C are completely reversible, the samples heated to 80 °C will be subject to an irreversible denaturation of about 40% (Table 1).

The structure contents of the renaturated samples heated to $t_D = 65$ °C correspond approximately to those of the original values. The denaturation at 75 °C results in an increase in the residual structure contents at the cost of the α-helix content. The β structure contents will remain nearly constant, but increase, however, during cooling down at the cost of the α-helices (Table 1).

ESR Measurements

The spin labels I (non-covalently bound) and II (covalently bound on the cysteine-34) were used to characterize the structural changes. The advantage of label I and label II as compared to other labels is that I [2] as well as II [17] has only one binding site on the albumin. When labelling with II one obtains an ESR spectrum consisting of two components (A and B in Fig. 2) ($c_{\text{albumin}} = 5$ mg/ml). There is 98% of the bound spin labels in a structured environment (in Fig. 2 marked with A) at 20 °C. The remaining 2% (B) correspond to an unfolded environment of the spin labels. From the temperature dependence of the ratio between the components A and B in the ESR spectrum the beginning of a more pronounced unfolding of the albumin molecule from 55 °C can be seen (Fig. 3). This unfolding will be still reversible up to a temperature of 68 °C.

It may be concluded from the expansion of the (+ 1) component of the spectral content A and the increasing distance of the components of A ($2\,A'_{zz}$ in Fig. 2) by 0.5 G as compared to the spectrum at 20 °C that particles of different molecular weight will be formed (increase of the rotational correlation time by the formation of larger particles). The t_m value of 73 °C (50% content in an immobilized environment, Fig. 3) is in relatively good agreement with the values obtained from CD data. That means that the increase in label mobility by unfolding of the pocket containing the free $-$SH group of cysteine-34 [18] and the con-

Table 1. *Degree of denaturation* D_t *(at the temperature of denaturation* t_D*), degree of irreversible denaturation* I_t *(measured after heating to the temperature of denaturation* t_D *and subsequent cooling down to 20 °C), and structure contents (*α, β, ϱ*) at different temperatures* t_D *with the mean standard deviations of the curve-fitting results*
D = denatured; R = renatured

Substance	t_D	D_t	I_t	α_D	β_D	ϱ_D	α_R	β_R	ϱ_R
	°C	%							
Albumin	20	–	–	61.1 ± 0.4	22.2 ± 1.5	16.7 ± 1.4	–	–	–
Albumin + MalNEt	20	–	–	69.9 ± 0.5	24.9 ± 1.9	5.3 ± 1.9	–	–	–
Albumin	65	12	0	54.9 ± 0.5	16.3 ± 2.0	28.8 ± 1.9	59.6 ± 0.3	24.7 ± 1.2	15.8 ± 1.2
Albumin + MalNEt	65	10	0	60.0 ± 0.6	18.0 ± 2.2	22.5 ± 2.1	66.7 ± 0.5	21.5 ± 2.0	11.8 ± 2.0
Albumin	75	45	30	33.6 ± 0.4	22.6 ± 1.5	43.8 ± 1.4	40.8 ± 0.4	28.1 ± 1.7	31.0 ± 1.6
Albumin + MalNEt	75	30	15	50.8 ± 0.5	21.8 ± 2.1	27.4 ± 2.0	61.8 ± 0.5	29.4 ± 2.0	8.7 ± 2.0
Mercaptalbumin	20	–	–	66.3 ± 0.5	11.0 ± 2.4	22.7 ± 2.3	–	–	–
Mercaptalbumin + MalNEt	20	–	–	68.3 ± 0.6	7.0 ± 2.4	24.7 ± 2.4	–	–	–
Mercaptalbumin	65	12	0	60.0 ± 0.8	7.2 ± 2.9	32.8 ± 2.8	68.5 ± 0.9	3.3 ± 3.6	28.1 ± 3.5
Mercaptalbumin + MalNEt	65	10	0	62.8 ± 0.8	7.0 ± 3.2	30.3 ± 3.1	68.1 ± 0.6	11.9 ± 2.2	20.2 ± 2.1
Mercaptalbumin	75	38	22	43.6 ± 0.7	10.4 ± 2.9	46.0 ± 2.8	53.4 ± 0.6	18.8 ± 2.2	27.8 ± 2.2
Mercaptalbumin + MalNEt	75	20	0	57.0 ± 0.8	6.5 ± 3.2	36.5 ± 3.1	68.6 ± 0.6	10.9 ± 2.4	20.5 ± 2.3

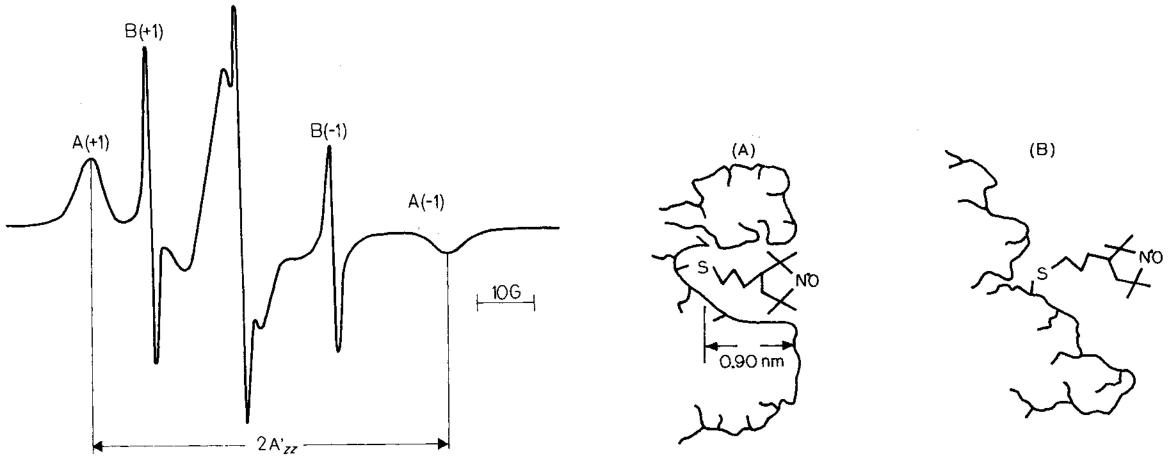

Fig. 2. *ESR spectrum of albumin labelled with label II.* A, strongly immobilized spectral components; B, weakly immobilized spectral components. Insert: schematic drawing of the different environments of label II bound to albumin according to A and B

formation contents measured by CD will take place in parallel, the relative final values obtained, however, being different. A degree of denaturation of the protein of about 45% taken from CD measurements (Table 1) corresponds to an unfolding rate of about 90% near the label. The cooling down of samples to 20 °C after heating to temperatures of 68 °C or more with incubation times of 5 and 20 min will result in different degrees of irreversible structural changes (Fig. 3). In addition the temperature effect was also observed by means of the probe I (Fig. 3). The mobile spectral content of 2% at 20 °C is due in this case to the spin label molecules being free in solution.

The increase in the content of free spin label molecules, which may be found with a temperature rise, can be explained by a decreasing structurization of the binding area of the label on the protein. The same t_m value of 73 °C may be obtained when using the probe I and the label II as well (Fig. 3).

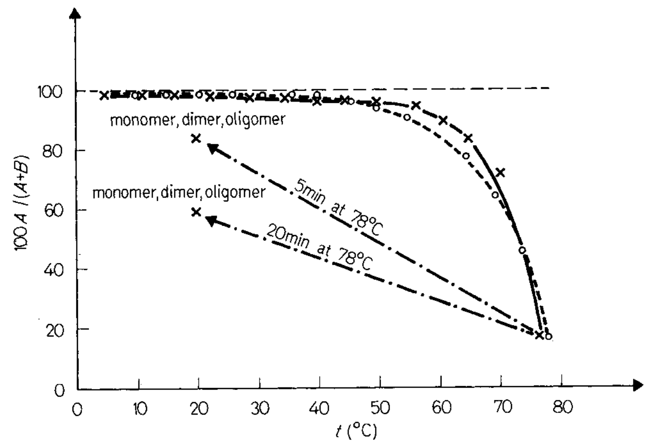

Fig. 3. *Temperature dependence of the ESR spectral component A (%) of label II covalently bound to albumin (×).* The arrows indicate the reversibility after different heating times. Temperature dependence of the ESR spectral component A of label I (%) noncovalently bound to albumin (○)

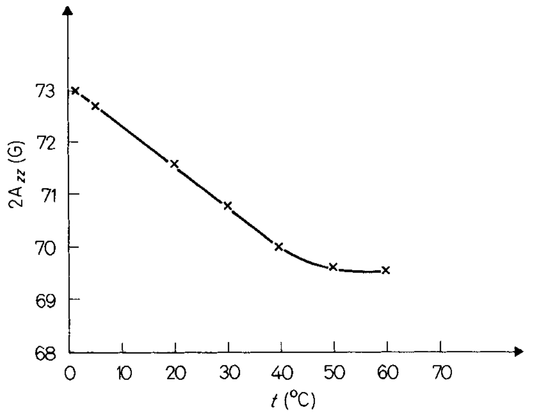

Fig. 4. *Determination of the* A_{zz} *values (2* A_{zz}*) at different temperatures, using McConnell's method [19]*

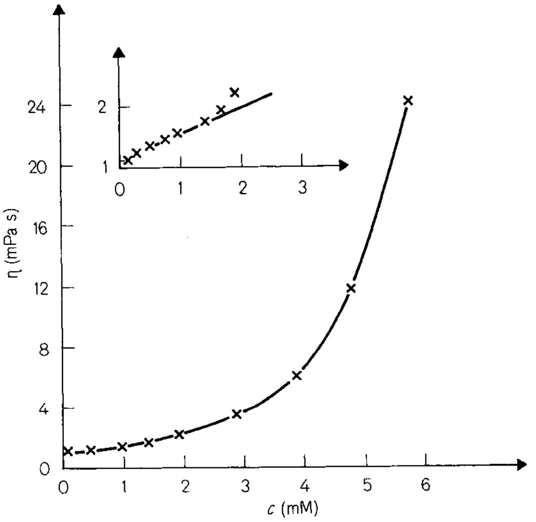

Fig. 5. *Dynamic viscosity* η *of albumin as a function of protein concentration.* Insert: linear region for low protein concentrations

The change in polarity around the NO was observed by means of label I (Fig. 4). With rising temperature the interaction between the spin label and the albumin molecule (according to the van der Waal's interaction) becomes more intensive and the NO group comes into stronger interaction with apolar areas. At 60 °C the line width changes as the rotational movement grows faster so that a further determination of this parameter is no longer possible.

INFLUENCE OF ALBUMIN CONCENTRATION ON THE TEMPERATURE BEHAVIOUR

CD Measurements

The t_m value for the lower concentration (0.05 mg/ml) is higher than that for the higher concentration (0.5 mg/ml) (Fig. 1). The difference (Δt_m) is 5 °C. Albumin concentrations of 0.05 mg/ml and 0.5 mg/ml yield different CD curves (Fig. 1). The structure contents are also different for both the concentrations. We determined the following structure contents at 20 °C and an albumin concentration of 0.05 mg/ml: α-helices = 52%; β structures = 6%; residual structures $\varrho = 41\%$. The α-helix content with $c_{\text{albumin}} = 0.05$ mg/ml is about 15% lower than with $c_{\text{albumin}} = 0.5$ mg/ml.

After heating to 90 °C (we may heat up to 90 °C at $c_{\text{albumin}} = 0.05$ mg/ml without any visible turbidity) and cooling down to 20 °C we have: $\alpha = 41\%$; $\beta = 12\%$ and $\varrho = 48\%$.

It appears to be essential that in addition to the helix contents the β structure contents are also lower than with $c_{\text{albumin}} = 0.5$ mg/ml. This is certainly due to a lower degree of aggregation with less intermolecular β structure contents (see Table 1). While no turbidity of the solution could be found with albumin concentrations ≤ 0.5 mg/ml at a temperature of 75 °C, a turbidity and precipitation were observed at the same temperature with $c_{\text{albumin}} = 5$ mg/ml, so that ultraviolet optical investigations in this range of concentrations at such high temperatures are no longer possible. This can be demonstrated by CD measurements at $\lambda = 268$ nm when a higher concentration can be used because of the relatively low absorption. With $c_{\text{albumin}} = 2$ mg/ml, after decreasing, the CD values begin to increase at 78 °C, indicating increasing turbidity. At 82 °C an apparent CD value will be a attained which corresponds to the original value at 20 °C.

The degree of irreversible denaturation does not exceed 40% in any case investigated. When stabilizers are used (M. Zinke, M. Becker, R. Wetzel, unpublished results) the same final result will also be obtained. Gel electrophoretic investigation then shows only monomers and higher aggregates.

Oligomers of different sizes could be found at temperatures of 60 °C or more (but less than 80 °C) and $c = 0.5$ mg/ml.

Mercaptalbumin, however, will precipitate by 85 °C even when 2 mM sodium octanoate is used as stabilizer (M. Zinke, M. Becker, R. Wetzel, unpublished results), as measured by gel electrophoresis.

Without addition of sodium octanoate the samples with $c_{\text{albumin}} = 5.0$ mg/ml and $c_{\text{albumin}} = 50$ mg/ml will precipitate at a temperature of 75 °C.

Viscometric Investigations

We have to distinguish between a linear range of the concentration dependence in the viscometric behaviour up to 65 mg/ml (approximately 1 mM)

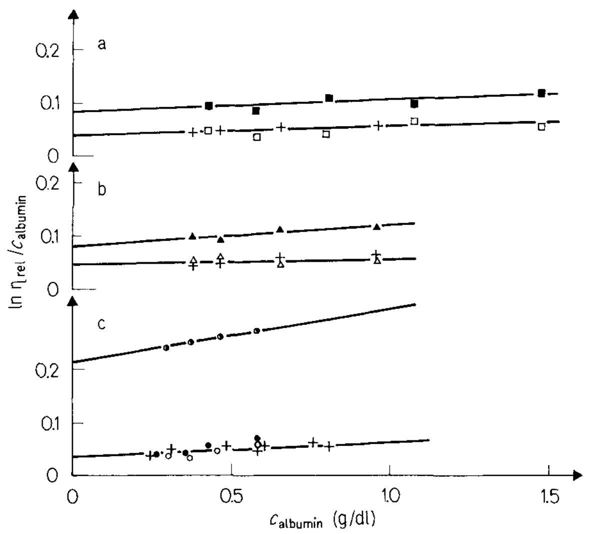

Fig. 6. *ln $\eta_{rel}/c_{albumin}$ versus $c_{albumin}$ after treatment at several temperatures.* The extrapolation to $c_{albumin} = 0$ gives the intrinsic viscosity $[\eta]$. As commonly used $[\eta]$ is given in dl/g. The concentrations of albumin used in the solutions subjected to heat treatment before dilution were: (a) 14.8 mg/ml; (b) 9.6 mg/ml; (c) 5.8 mg/ml. Temperature of the treatment: (+) 20 °C; (○, △, □) 60 °C; (●, ▲, ■) 70 °C and (◑) 80 °C. Albumin in the concentrations 9.6 mg/ml and 14.8 mg/ml changes after treatment with 80 °C to a non-measurable gel-like state

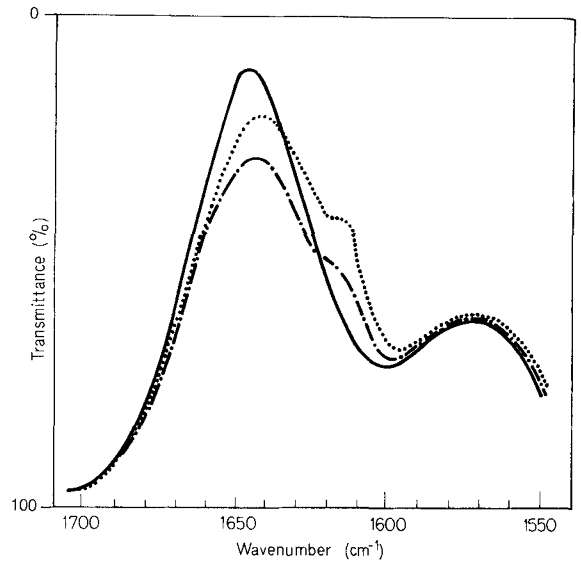

Fig. 7. *Infrared spectra at different temperatures of albumin.* The development of the shoulder in the amide I absorption band indicates the development of β structures. After recooling the effect increases. $t_D = 75$ °C; $c = 10$ mg/ml; $d = 0.05$ mm. (——) 20 °C; (–·–·–) 75 °C; (––––) 20 °C (after recooling)

and an exponential one at higher concentrations (Fig. 5). The linear range was used to study the temperature influence. The instrinsic viscosity $[\eta]$ of heat-treated albumin was determined with concentrations of 5.8, 9.6 and 14.8 mg/ml. The heat treatment was performed by an incubation of 60 min at the respective temperature. The measurements were performed on the renatured albumin at 20 °C ± 0.1 °C (Fig. 6). For the determination of the intrinsic viscosity the quotient of the natural logarithm of the relative viscosity η_{rel} and the albumin concentration c ($\ln \eta_{rel}/c$) was plotted as function of the albumin concentration c (Fig. 6). The intrinsic viscosity remains constant in the range of concentrations studied up to a temperature of 60 °C and is consistent with the value found in the pertinent literature, $[\eta] = 0.037$ dl/g [20], within the error range (± 15%).

Infrared Spectroscopic Investigations

The results of the CD measurements (Table 1) show the increase in the β structure contents of the albumin after heating and subsequent cooling down. The development of those β structures may be observed also by means of the infrared spectroscopy with higher concentrations. The shoulder in the amide I absorption band at 1615 cm^{-1} (Fig. 7) after heat treatment indicates these changes in the secondary structure of albumin.

The development of β structures in the thermal denaturation of albumin is dependent on the concentration. The shoulder in the absorption band can be found with a concentration of 50 mg/ml at a temperature of 70 °C, with $c_{albumin} = 1.4$ mg/ml at 80 °C. The CD measurements performed with $c_{albumin} = 0.5$ mg/ml did not indicate any increase in the β structure contents at $t = 75$ °C. An increase in the β structure contents could be found only after cooling down to room temperature (Table 1). The result obtained by the CD measurements, i.e. the further increase in the β structure contents upon cooling down after heating, could be verified by the infrared measurements. With a concentration of 10 mg/ml, where the development of β structures would be expected at lower temperatures than with the concentrations of 0.5 mg/ml used for the CD measurements, no shoulder was visible in the infrared absorption band at 66 °C except after cooling down the samples.

Infrared spectroscopic investigations on albumin films produced from heated and untreated solutions also show a heat-induced β structure development. The amide A band of films produced from heated albumin solutions (80 °C) has a peak at 3285 cm^{-1}. It indicated a low-frequency shift of 15 cm^{-1} relative to the peak at 3300 cm^{-1} in the film of untreated samples and thus the development of β structures. A heat-induced development of β structures may also be derived from H→^{2}H exchange measurements on

Table 2. *Influence of the original concentration* c_0 *and temperature* t *on the development of aggregates and* β *structures of albumin*

c_0	t	Monomers 4.0–4.5 S	Oligomers ($M_r > 10^6$) 26–36 S	β form in the infrared spectrum (0 = not measured) (1615 cm^{-1})
mg/ml	°C	%		
1.4	20	100	0	–
1.4	65	100	0	–
7.0	65	100	0	0
50.0	65	61	39	–
10.0	68	52	48	0
50.0	68	0	100	0
1.4	70	100	0	–
7.0	70	69	31	0
10.1	70	39	61	+
7.0	73	32	68	0
10.0	73	19	81	0
1.4	75	50	50	–
1.4	78	44	52	0
0.46	80	52	48	–
1.0	80	40	60	+
1.3	80	37	63	0
1.4	80	36	64	+
7.0	80	9	91	
10.1	80	0	100	+

films. Films of the albumin samples heated to 80 °C show an exchange rate markedly delayed as could be expected in the case of a development of β structures [21].

Sedimentation Measurements

Albumin will sediment in the neutral pH range with sedimentation coefficients of 4.0–4.5 S. If the samples are heated to more than 60 °C and cooled down again there will be found in addition to the native protein a faster sedimenting but heterogenous fraction with sedimentation coefficients of 26–36 S. The content of these aggregates in the solution will depend not only on the temperature but also on the concentration of the heated samples (Table 2).

The tendency to develop these high molecular weight complexes or oligomers of the albumin increases obviously with rising temperature and concentration. Assuming that the heated solutions will only contain monomers and oligomers or aggregates, so the conditions (t, c) where both components are present in a portion of 50% may be taken from Fig. 8.

Plotting the partial concentration c_i of monomers and oligomers of the samples heated to 80 °C as a function of the total concentration c_0 in a log-log system clearly reveals a linear increase in the oligomer content with the concentration. We can calculate from this relation, with the temperature and concentration given, not only the contents of oligomers formed but also those of the monomers as shown in Fig. 9.

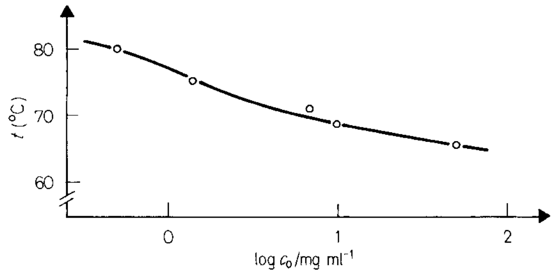

Fig. 8. *Influence of temperature and albumin concentration* (c_0) *on the equivalent monomer/oligomer ratio*

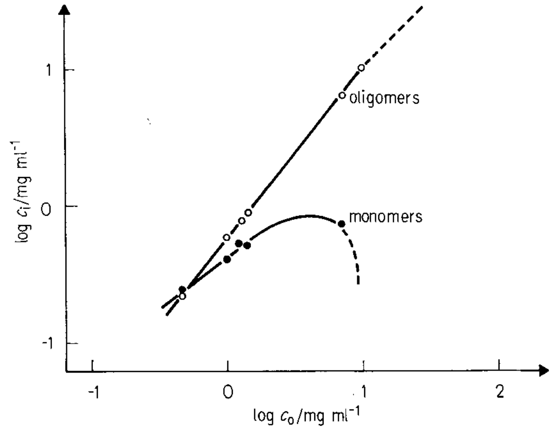

Fig. 9. *Influence of albumin concentration* (c_0) *on the partial concentrations* (c_i) *of oligomers* (○) *and monomers* (●). (----) calculated values. Sample pretreatment at 80 °C for 1 h

The analytic results of the monomeric and oligomeric components are without doubt still dependent on the concentration at which the sedimentation behaviour was tested. As can be seen from Fig. 10 the content of monomeric albumin in a solution with a concentration of 10.1 mg/ml, which has been heated to 70 °C for 1 h and diluted after cooling down, will further increase with decreasing concentration. That possibly means that monomeric molecules are bound to the aggregates partly by adsorption, and this interaction will be reduced by dilution. A complete dissociation of the oligomers with dilution has to be excluded as the formation of aggregates will probably occur while forming covalent disulphide bridges but also hydrophobic interactions and β structures.

INFLUENCE OF THE FREE –SH GROUPS ON THE TEMPERATURE BEHAVIOUR

It has been found [22], by using a series of spin labels of attachement groups with different lengths,

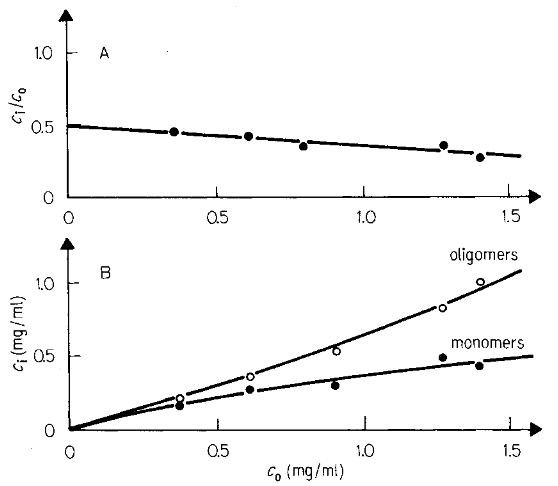

Fig. 10. *Influence of dilution on the monomer/oligomer ratio of albumin.* Dependence of partial concentration of the (A) monomers (●) and (B) monomer (●) and oligomer (○) levels on albumin concentration. Albumin (10.1 mg/ml) was previously heated at 70 °C for 1 h

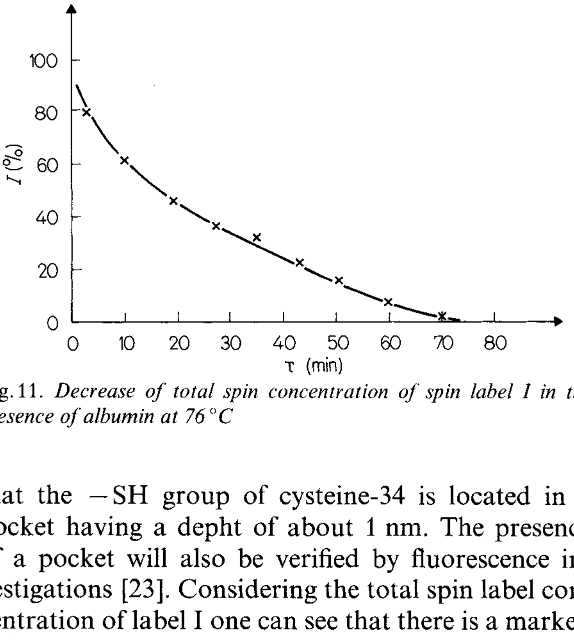

Fig. 11. *Decrease of total spin concentration of spin label I in the presence of albumin at 76 °C*

that the —SH group of cysteine-34 is located in a pocket having a depht of about 1 nm. The presence of a pocket will also be verified by fluorescence investigations [23]. Considering the total spin label concentration of label I one can see that there is a marked reduction of the NO group of the spin labels beginning at temperatures of more than 60 °C. For that reason we did not perform any renaturations tests with the spin label I. Fig. 11 shows the course of the reduction of the NO group of label I at a temperature of 76 °C. The same reduction test (Fig. 11) was performed with the —SH group blocked by MalNEt to clarify whether the —SH group exposed to more than 60 °C reduced the NO group of spin label I. There was no reduction found. That is another finding that the —SH group is hidden in a pocket at temperatures of 60 °C or less. The width of that pocket may be estimated on the basis of the dimensions of I and II to 0.7 nm. There is an increasing unfolding of the pocket containing the free —SH group of cysteine-34 during the thermal denaturation process. It may be supposed that the formation of intermolecular disulphide bridges is an increasingly likely cause of the formation of dimers and a further aggregation by unfolding of the environment of the —SH group.

The solvents used for the isolation of albumin (methanol and ethanol) will cause a loosening of the environment surrounding the —SH group even with 20% mixtures. This may be a starting point for the occurrence of dimers, which could not be found in blood plasma (except for bisalbumeria). The comparison of albumin and especially prepared mercaptalbumin did not show any essential difference in the temperature behaviour as with the label II, indeed only the albumins which have a —SH group which may be labelled could be covered. On the other hand a rotational correlation time of 32 ns for the albumin is obtained by the label II being strongly immobilized by the protein [24]. If the mercaptalbumin is labelled, however, we can find a value of 16 ns for the rotational correlation time. This value is not in the range of 32—63 ns, the values possible for an elongated ellipsoid with an axial ratio of 3.5:1 [25].

A possible explanation for the observed decrease of rotational correlation time could be that fatty acids will be eliminated in the preparation of mercaptalbumin. This could result in a movement of the three globular domains of the albumin molecules relative to each other. To study the possibility that conformational changes of the protein are connected with disulphide bridges, we performed comparative CD measurements with albumin (0.45 free —SH group/molecule) with mercaptalbumin (0.9 free —SH group/molecule) and with albumin and mercaptalbumin using —SH groups blocked by MalNEt (Table 1).

The degree of denaturation at 75 °C as well as the degree of irreversible denaturation (after recooling to 20 °C) will be reduced by the binding of MalNEt. With mercaptalbumin + MalNEt even a complete renaturation will be obtained. The degree of denaturation at $t_D = 75\,°C$ is only 20%. If this value was not exceeded, a complete renaturation could be obtained with all the samples studied. Aggregates could not be demonstrated in this case by gel electrophoretic investigation either; without MalNEt aggregates could be found. If the aggregation through the formation of disulphide bridges is inhibited by the addition of MalNEt, the conformational properties will be retained, i.e. the structure contents do not change.

A difference between mercaptalbumin and albumin is worth noting: it may be seen from gel electrophoretic measurements that the mercaptalbumin does not yet form aggregates at $t = 65\,°C$ and 70 °C, but the albumin does. That correlates with the somewhat lower content of β structures of the mercaptalbumin (Table 1) and could mean that the aggregation takes place in this temperature range mainly through the formation of intermolecular β structures. Then, after unfolding of the pocket ($t \geq 75\,°C$), the formation of aggregates through disulphide bridges will start to develop representing irreversible changes. This will be obviously inhibited at 75 °C ($c_{\text{albumin}} = 0.5$ mg/ml) in the case of the free –SH group being blocked by MalNEt (mercaptalbumin + MalNEt). The α-helix content of the renatured samples also corresponds to that of the original values. With albumin + MalNEt the α-helix content of the renatured samples is almost 90% of the original value. The difference induced by MalNEt in the α helix content is in all cases almost 20%. With samples where the MalNEt was bound to the free –SH group the α-helix content also decreases at $t = 75\,°C$ far less than in the absence of MalNEt. It may be supposed, therefore, that a change of the α-helix content will be caused by the formation of disulphide bridges.

During the temperature rise from 20 °C to 75 °C no significant change of the β structure content could be measured by CD for any sample, i.e. these values do not depend on the binding of the MalNEt to the free –SH groups. However, an essentially lower content of β structures could be found in the mercaptalbumin samples at 20 °C than with the albumin samples. The β contents increase somewhat while the samples cool. The contents of residual structures increase with rising temperatures, the smallest increase occurring with mercaptalbumin + MalNEt, where the minimum decrease of the α-helical content could also be found. After recooling ϱ will be reduced where the contents of residual structures (except for mercaptalbumin + MalNEt are still higher than the original values. In general a decrease of the α helix content with increasing residual structures can be found during the denaturation.

With a more intense aggregation (without MalNEt), where the formation of disulphide bridges does certainly play a significant role, a more profound decrease of the α-helix content could be found as compared to the samples the free –SH groups of which were blocked by MalNEt.

DISCUSSION

The CD measurements with a albumin concentration of 0.5 mg/ml gave a t_m value of 71 °C. This value is dependent on concentration. The measurements with an albumin concentration of 0.05 mg/ml surprisingly yielded up a t_m value 5 °C higher, although the α-helix content is lower than with $c_{\text{albumin}} = 0.5$ mg/ml. A possible explanation could be that additional α-helix structures will be induced with the higher concentration by intermolecular interactions. These structures are less stable than those stabilized by intramolecular interactions and will, therefore, melt earlier and cause a lower t_m value in total for the samples of higher concentration.

An obvious influence of intermolecular interactions could also be found for the concentration dependence of the intrinsic viscosities. There are differences between solutions of different concentrations at temperatures of 60 °C or more. A value corresponding approximately to the untreated sample could still be obtained with a concentration of 5.8 mg/ml for 70 °C. A five-fold increase in the intrinsic viscosity for 80 °C can be found. By 70 °C the value has already doubled with concentrations of 9.6 and 14.8 mg/ml. The measurement of the samples incubated at 80 °C was inhibited by a visible turbidity.

A comparison of these values with the ESR data measured (Fig. 3), where the protein concentration was 10 mg/ml, indicated a good agreement of the transitional temperatures. The ESR investigations gave a t_m value of 73 °C, but we should note that this value relates to the unfolding of molecules around the labels and does not include the total conformation of the albumin molecule, as was the case with the CD investigations. No significant dependence of the unfolding on the concentration could be found in the concentration range of 5–20 mg/ml covered by the ESR spectroscopic investigations. Turbidity could not be observed even at 80 °C. It may be concluded, therefore, that the spin labels stabilize the albumin molecule and that the change of the intrinsic viscosity is connected with the aggregation, i.e. with an intermolecular interaction.

We find a β structure content of 22% with an albumin concentration of 0.5 mg/ml from the CD curves measured at room temperature. Geisow and Beaven [7] determined a β structure content of albumin and larger fragments of the protein of 10–20% ($c_{\text{albumin}} = 0.2$ mg/ml) from CD measurements under comparable conditions. The development of β structures with heating of albumin and high concentrations was demonstrated by Lin and Koenig as well as by Palm in Raman and infrared spectroscopic investigations [2,8,9]. Aggregation and β structure development are associated in the thermal denaturation of albumin. The development of β structures is evident in the infrared spectrum only at higher temperatures and concentrations and oligomer contents > 50%. Raman spectroscopic investigations with bovine serum albumin by Chen et al. [26] do not indicate the presence of β structure contents at room temperature either. However, β structure contents (Table 1) were also calculated from the CD data with lower albumin concentrations and at room temperature. The different results are possibly due to different sensitivities of the methods mentioned to particular β structure properties. According to Woody [27] the rotational strength of the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the peptide chromophore are different for parallel and antiparallel β structures and decrease with increasing sheet size. It may be that in the present case intermolecular β structures of increasing sheet size will develop during aggregation, which become evident in the infrared spectrum, while the increase in the CD values will be reduced with increasing β sheet size (see Table 1).

A connexion between the development of β structures and the aggregation of albumin in the thermal denaturation will be seen from sedimentation measurements. The content of aggregations will increase with rising temperature and concentration. In albumin solutions with a concentration of 1.4 mg/ml aggregates may be found in detectable amounts after heating to 75 °C and subsequent cooling down to 20 °C. They may be found with $c_{\text{albumin}} = 10.1$ mg/ml and 50 mg/ml at 70 °C and 65 °C respectively (Table 2). The formation of intermolecular disulphide bridges by free $-$SH groups plays an essential role in the aggregation process. The irreversible thermal denaturation may be inhibited by the binding of MalNEt to the free $-$SH group. The inhibition of the formation of intermolecular disulphide bridges will also prevent the occurrence of irreversible conformational changes. As the capability to develop a S$-$C bond will not be lost by the linking of the MalNEt ring with the pyrrolidine ring, a stabilization of the albumin by the spin label II could also be expected. The label I, which is sterically rather bulky because of its planar structure, may have functions similar to the fatty acids, its apolar ring system being similarly important for the thermal stabilization [7].

In the range of the reversible denaturation ($t_D < 70$ °C) the aggregation is mainly due to the development of intermolecular β structures measured by CD. The complete blocking of the free $-$SH groups inhibits the irreversible denaturation of serum albumin after heating to 75 °C and excludes to a large extent any aggregation. Aggregates which are due to the formation of S$-$S bridges, will be obviously highly irreversibly. A partial removal of fatty acids, as may occur in the preparation of the mercaptalbumin, will possibly enhance the accessibility of the MalNEt to the free $-$SH groups, reducing the possibility of forming S$-$S bridges and aggregates.

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