[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE P_H-STABILITY REGIONS OF SERUM ALBUMIN AND OF SERUM GLOBULIN

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In a previous communication[1] a report has been given of the determination by ultracentrifugal methods of the molecular weights of serum albumin and serum globulin at their isoelectric points. It was found that both these proteins when isolated in an appropriate manner are homogeneous with regard to molecular weight. Serum albumin gave the value 67,500 ± 2000 and serum globulin 103,800 ± 3000. For serum albumin the sedimentation constant at 20° is 4.21×10^{-13} and for serum globulin 5.66×10^{-13}. The corresponding diffusion constants are 6.10×10^{-7} and 5.41×10^{-7}. Serum albumin has practically the same molecular weight, the same sedimentation constant and the same diffusion constant as hemoglobin, while serum globulin resembles as regards these constants *r*-phycocyan at a P_H of 6.8. A few preliminary experiments were performed in order to determine the molecular weights of the products obtained from serum globulin by fractional precipitation, *viz.*, the so-called euglobulins and pseudoglobulins. The data obtained seemed to indicate that the originally homogeneous globulin was partially decomposed during the process of fractionation. It was therefore concluded that euglobulin and pseudoglobulin most probably represent artificial products not originally present in the blood serum.

The eminent importance of the serum proteins makes it desirable to extend the determination of their molecular weights to a wider range of P_H and also to study more thoroughly the question of the homogeneity of serum globulin.

Preparation of Material.—The protein material was prepared from horse blood. Serum albumin was isolated from the serum as described under the head "serum albumin II" of the previous communication. The stock solution which served for the determinations in the middle P_H region was kept at a P_H of 4.8 (0.008 *M* in acetic acid and 0.012 *M* in sodium acetate). The stock solution for determinations in extreme acid and extreme alkaline solutions was kept in electrolyte-free condition.

Serum globulin was isolated from the serum as described under the head "serum globulin II" of the previous communication. The main part of the stock solution was kept at a P_H of 5.5 (0.19 *M* in KH_2PO_4 and 0.009 *M* in Na_2HPO_4). For the determinations on the acid side of the isoelectric point a stock solution was prepared in the following way. The material was precipitated three times with ammonium sulfate, as before, dissolved in acetate buffer of P_H 4.6 (0.1 *M* in acetic acid and 0.1 *M* in sodium acetate), and dialyzed against the same buffer until the outer liquid was free from sulfate and chloride.

[1] T. Svedberg and B. Sjögren, THIS JOURNAL, **50**, 3318 (1928).

Pseudoglobulin and euglobulin were prepared from the serum globulin stock solution by dialyzing at 5° for a week against distilled water saturated with toluene. After this treatment, followed electrodialysis with a current density of 0.5 milliampere per sq. cm. for thirty-six hours. After filtering, the pseudoglobulin solution was brought to a P_H of 5.5 by making it 0.019 M in KH_2PO_4 and 0.001 M in Na_2HPO_4. The euglobulin precipitate formed during the dialysis and the electrodialysis was washed with distilled water, saturated with toluene until the filtrate was found to be free from protein and then dissolved completely in phosphate buffer of P_H 5.5 (0.19 M in KH_2PO_4 and 0.009 M in Na_2HPO_4) and dialyzed against the same buffer for a week.

Light Absorption.—The measurements were made with the Judd-Lewis spectrophotometer in 20 mm. cells. Serum albumin was measured in 0.10% solution, serum globulin, pseudoglobulin and euglobulin in 0.05 and 0.10% solutions. In Tables I and II the data for serum albumin and serum globulin are given.

TABLE I

LIGHT ABSORPTION OF SOLUTIONS OF SERUM ALBUMIN

Solvent				P_H of	Wave length of maximum	Wave length of minimum	Extinction coeff.
Subs.	M	Subs.	M	soln.	in $\mu\mu$	in $\mu\mu$	ϵ/c of max.
HCl	0.046	KCl	0.054	1.4	278	250	5.0
HCl	.007	KCl	.093	2.2	278	254	4.8
HAc	.018	NaAc	.002	3.6	280	252	4.8
Na_2HPO_4	.032	NaOH	.002	9.4	278	250	5.2
Na_2HPO_4	.026	NaOH	.012	10.4	278	252	7.4

TABLE II

LIGHT ABSORPTION OF SOLUTIONS OF SERUM GLOBULIN

Solvent				P_H of	Wave length of maximum	Wave length of minimum	Extinction coeff.
Subs.	M	Subs.	M	soln.	in $\mu\mu$	in $\mu\mu$	ϵ/c of max.
HCl	0.072	KCl	0.005	1.3	285	255	18.0
HAc	.023	NaAc	.023				
HCl	.025	KCl	.025	2.5	280	255	18.5
HAc	.023	NaAc	.023				
NaOH	.023						
KH_2PO_4	.026	Na_2HPO_4	.174	7.5	284	254	18.5
KH_2PO_4	.002	Na_2HPO_4	.003	12.5	282	253	23.0
NaOH	.048	NaCl	1%				

The measurements show that within the limits of error the position of the maximum and the minimum does not change with P_H. The extinction at the maximum is independent of P_H except at high alkalinity, where it increases slightly for serum albumin as well as for serum globulin. In the previous communication the maximum extinction coefficient of isoelectric serum globulin was found to be 22. The lower value of 18 now found is probably due to difference in the protein material. A slight change in the chemical composition would in all probability not affect the molecular weight but might very well make itself visible in the light absorption. The study of the sedimentation constant has shown that the serum albumin molecule is stable within the P_H region 4.0–9.0 and the serum globulin molecule in the

region 4.0–8.0. The sedimentation equilibrium determinations indicate that the first stage in the decomposition of both these proteins consists in the disintegration into submolecules of approximately the mass one-half of the original molecule in the case of serum albumin and probably one-third in the case of serum globulin. This disintegration (being proved to be reversible) will in all probability not affect the chemical or optical properties of the protein very much and this accounts for the constancy of the light absorption. It is of interest to compare this behavior of the serum proteins with that of Bence-Jones protein.[2] In the case of this latter protein the first stage in the decomposition consists in the breaking down of the protein unit itself and this is accompanied by a strong increase in light absorption.

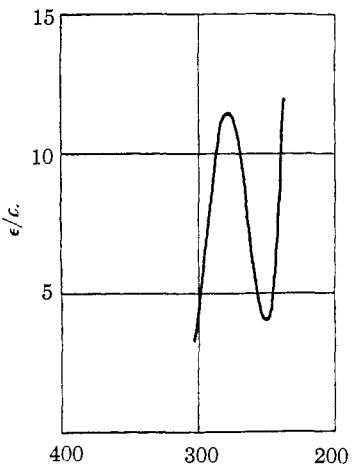

Fig. 1.

The light absorption of pseudoglobulin was measured both in electrolyte-free condition and in phosphate buffer at P_H 5.5 (solution 0.019 M in KH_2PO_4 and 0.001 M in Na_2HPO_4). The position of the maximum was found to be 279 $\mu\mu$ and of the minimum 252 $\mu\mu$. The extinction coefficient of the maximum was 11.5. In Fig. 1 the absorption curve is traced. The absorption of pseudoglobulin is markedly lower than the absorption of the original serum globulin.

Euglobulin was also examined with regard to light absorption. It was found to resemble very much the original serum globulin, being thus quite different from pseudoglobulin.

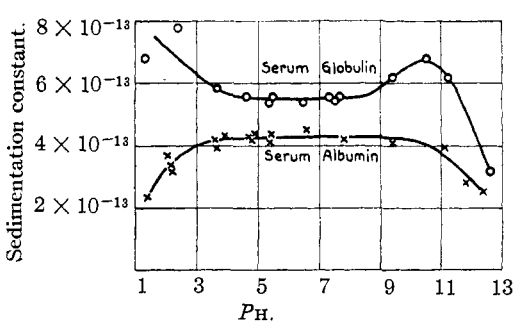

Fig. 2.

The P_H-Stability Region of Serum Albumin and of Serum Globulin.—A number of sedimentation velocity runs were made in the oil-turbine ultracentrifuge[3] at 40,000–45,000 r. p. m. and 20° in the cell. The time of centrifuging varied from three to five hours. The solutions were brought to the desired concentration and P_H immediately before starting the run.

[2] T. Svedberg and B. Sjögren, THIS JOURNAL, **51**, 3594 (1929).

[3] T. Svedberg, "Colloid Chemistry," 2d ed., Chemical Catalog Co., Inc., New York, **1928**, p. 153.

TABLE III

SERUM ALBUMIN, SEDIMENTATION VELOCITY MEASUREMENTS

Solvent				P_H of soln.	Concn. of protein, %	$s_{20°} \times 10^{13}$
Subs.	M	Subs.	M			
HCl	0.046	KCl	0.054	1.4	1.10	2.33
HCl	.007	KCl	.093	2.15	0.25	3.20
HCl	.007	KCl	.093	2.08	1.10	3.67
HCl	.007	KCl	.140	2.16	0.25	3.38
HAc	.018	NaAc	.002	3.60	1.10	3.98
HAc[a]	.018	NaAc	.002	3.64	0.25	4.16
HAc	.016	NaAc	.004	3.98	0.25	4.32
HAc	.008	NaAc	.012	4.8	0.25–1.25	4.21[b]
HAc	.008	NaAc	.012	4.8	0.25	4.34
HAc	.008	NaAc	.012	4.8	0.25	4.35
HAc	.008	NaAc	.012	4.8	0.71	4.40
HAc	.003	NaAc	.017	5.41	1.07	4.38
KH_2PO_4	.095	Na_2HPO_4	.005	5.46	0.18	4.12
KH_2PO_4	.050	Na_2HPO_4	.050	6.67	0.20	4.52
KH_2PO_4	.012	Na_2HPO_4	.188	7.82	1.10	4.21
NaOH[a]	.002	Na_2HPO_4	.032	9.40	1.10	4.01
NaOH[a]	.002	Na_2HPO_4	.032	9.40	1.10	4.05
NaOH[a]	.012	Na_2HPO_4	.026	11.10	0.70	3.99
NaOH[a]	.025	Na_2HPO_4	.017	11.80	0.55	2.84
NaOH	.050	NaCl	2%	12.40	0.55	2.54

[a] 1% in NaCl.

[b] Mean value of previous determinations.

TABLE IV

SERUM GLOBULIN, SEDIMENTATION VELOCITY MEASUREMENTS

Solvent						P_H of soln.	Concn. of protein, %	$s_{20°} \times 10^{13}$
Subs.	M	Subs.	M	Subs.	M			
HCl	0.072	KCl	0.005	HAc	0.023	1.3	0.73	6.80
				NaAc	.023			
Same soln. after standing at 5° for 20 days								9.43
HCl	.025	KCl	.025	HAc	.023	2.46	0.75	7.93
NaOH	.023			NaAc	.023			
HAc	.180			NaAc	.020	3.67	0.75	5.94
HAc	.10			NaAc	.10	4.54	0.50	5.64
KH_2PO_4	.190			Na_2HPO_4	.009	5.5	0.12–1.00	5.66[a]
KH_2PO_4	.190			Na_2HPO_4	.009	5.41	1.08	5.37
KH_2PO_4	.140			Na_2HPO_4	.060	6.50	0.05	5.44
KH_2PO_4	.044			Na_2HPO_4	.156	7.27	.64	5.51
KH_2PO_4	.026			Na_2HPO_4	.174	7.57	.10	5.56
KH_2PO_4	.026			Na_2HPO_4	.174	7.57	.10	5.44
KH_2PO_4	.003	NaOH	.005	Na_2HPO_4	.031	9.41	.08	6.22
KH_2PO_4	.005	NaOH	.017	Na_2HPO_4	.026	10.56	.08	6.92
KH_2PO_4	.003	NaOH	.042	Na_2HPO_4	.017	11.22	.06	6.19
KH_2PO_4	.002	NaOH	.048	Na_2HPO_4	.003	12.52	.04	3.22
		NaCl	1%					
Same soln. after standing at 5° for 6 days								2.32
Same soln. after standing at 5° for 14 days								2.55

[a] Mean value of previous determinations.

The determinations of the sedimentation constant $s_{20°}$ are summarized in Tables III and IV and in the diagram, Fig. 2. For serum albumin the sedimentation remains constant from P_H 4 to 9 and for serum globulin from 4 to 8. These P_H ranges therefore represent the stability regions of serum albumin and serum globulin. The behavior of these proteins outside the stability regions will be discussed later.

The diffusion was found to be normal only in the vicinity of the isoelectric point. Both on the acid and the alkaline side of this point the diffusion was considerably depressed, indicating gel formation.[4]

The Sedimentation of Pseudoglobulin and Euglobulin.—Two different preparations of pseudoglobulin were studied. The runs were made in the same way as in the case of serum albumin and serum globulin and at a P_H of 5.5 (solutions 0.019 M in KH_2PO_4 and 0.001 M in Na_2HPO_4). The first material gave for the sedimentation constant $s_{20°} = 7.72 \times 10^{-13}$ in 0.13% solution, the second material $s_{20°} = 6.44 \times 10^{-13}$ in 0.10% solution and 6.52×10^{-13} in 0.60% solution. The diffusion constant showed considerable drift with time, indicating that the protein in question is a mixture of different molecules. The solutions also contained from 5–10% of non-centrifugible substance.

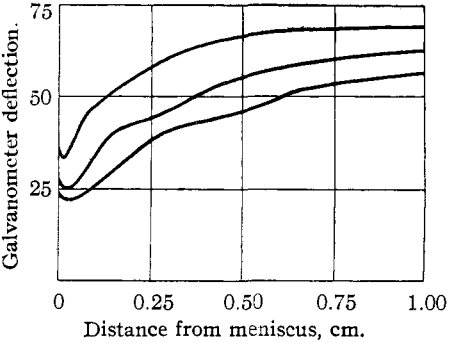

Fig. 3.

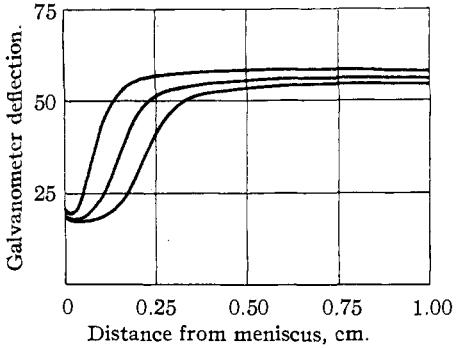

Fig. 4.

Euglobulin was centrifuged at a P_H of 5.5 and in a phosphate buffer 0.190 M in KH_2PO_4 and 0.009 M in Na_2HPO_4. Two runs were made, one in 0.12%, the other in 0.63%, solution. Both runs gave sedimentation curves of a very peculiar shape, indicating that the protein material in question is a mixture of different kinds of molecules. In Fig. 3 the euglobulin sedimentation curves for the times thirty, fifty and seventy minutes after the start are given. For the sake of comparison Fig. 4 gives the corresponding serum globulin curves. In the case of serum globulin the shape of the curves is that of a simple molecular species, while the

[4] Compare T. Svedberg and B. Sjögren, THIS JOURNAL, 51, 3594 (1929).

euglobulin curves show that this protein contains two different main constituents and probably some other component in lower concentration.

The splitting up of serum globulin into pseudoglobulin and euglobulin is not a reversible process. The sedimentation of a mixture of pseudoglobulin and euglobulin showed that the native serum globulin cannot be synthesized from these products by mixing them in the same proportion in which they are formed by dialysis of serum globulin.

The Molecular Weights of the Decomposition Products of Serum Albumin and of Serum Globulin.—The change in the value of the sedimentation constant at P_H 4 and 9 in the case of serum albumin and P_H 4 and 8 in the case of serum globulin indicates that at these hydrogen-ion concentrations the molecules of the serum proteins begin to disintegrate. Because of the anomalous diffusion it was not possible to calculate the molecular weights of the decomposition products from sedimentation velocity measurements. Earlier experiments have shown that sedimentation equilibrium determinations give the right value of molecular weight even in cases where the diffusion is depressed owing to gel formation or forces acting between the protein molecules. Sedimentation equilibrium runs were therefore made on serum albumin and serum globulin in the acid decomposition region. In the alkaline region the formation of non-centrifugible decomposition products renders such determinations uncertain.

Sedimentation equilibrium runs were further made on the isoelectric pseudoglobulin material studied above.

In Table V the equilibrium measurements are given.

TABLE V

DECOMPOSITION PRODUCTS OF SERUM ALBUMIN AND SERUM GLOBULIN. SUMMARY OF SEDIMENTATION EQUILIBRIUM MEASUREMENTS

Protein	Solvent	P_H of soln.	Mol. wt.
Acid serum albumin	0.007 M in HCl 0.093 M in KCl	2.2	60,000 to 21,000
Acid serum albumin	.007 M in HCl .093 M in KCl	2.2	54,000 to 20,400
Acid serum globulin	.025 M in HCl .025 M in KCl	2.5	59,000 to 23,000
	.023 M in HAc .023 M in NaAc		
	.023 M in NaOH		
Isoelectric pseudoglobulin	.019 M in KH_2PO_4	5.5	99,300 to 53,200
	.001 M in Na_2HPO_4		

The data of Table V confirm the conclusion drawn from the sedimentation velocity measurements. The acid serum albumin and serum globulin as well as the isoelectric pseudoglobulin are mixtures of molecules of different weight. Euglobulin was not studied in sedimentation equilibrium because the sedimentation velocity measurements had conclusively shown this protein to be a mixture of different molecular species.

The molecular weights of the acid serum albumin and serum globulin range from about 60,000 to about 20,000, those of the isoelectric pseudoglobulin from about 100,000 to about 50,000.

The Reversibility of the Acid and Alkaline Decomposition.—It is of considerable interest to state whether the decomposition of the serum albumin and serum globulin molecules in acid and alkaline solutions is reversible or not. In order to test the reversibility, solutions of serum albumin and serum globulin were brought to acid or alkaline reaction and left in this condition for a short time (from one hour to one day). The solutions were brought back into the stability region either by direct addition of the suitable reagents or by dialysis at low temperature against a buffer of the desired PH. Both procedures gave the same result. The sedimentation constant was found to have become normal again, which proves that the change is reversible. In Table VI the data are summarized.

TABLE VI

SERUM ALBUMIN AND SERUM GLOBULIN, REVERSIBILITY OF DECOMPOSITION

Subs., %	First solvent	Second solvent	PH of first soln.	PH of second soln.	$s_{20°} \times 10^{13}$ First soln.	$s_{20°} \times 10^{13}$ Second soln.	$s_{20°} \times 10^{13}$ Mean normal value
0.25 Serum albumin	0.007 *M* in HCl 0.093 *M* in KCl	0.008 *M* in HAc 0.012 *M* in NaAc	2.2	4.8	3.50	4.27	**4.21**
0.25 Serum albumin	0.007 *M* in HCl 0.093 *M* in KCl	0.008 *M* in HAc 0.012 *M* in NaAc	2.2	4.8	3.50	4.44	**4.21**
0.30 Serum albumin	0.017 *M* in Na_2HPO_4 0.025 *M* in NaOH	0.017 *M* in Na_2HPO_4 0.017 *M* in KH_2PO_4 0.025 *M* in NaCl	11.8	6.8	3.05	4.46	4.21
0.10 Serum globulin	0.017 *M* in Na_2HPO_4 0.025 *M* in NaOH	0.190 *M* in KH_2PO_4 0.009 *M* in Na_2HPO_4	11.8	5.5	5.05	5.77	5.66

Discussion of Results

The ultracentrifugal study of serum albumin and serum globulin at different hydrogen-ion concentrations has shown that the sedimentation constant is independent of PH within the range 4–9 for serum albumin and 4–8 for serum globulin. These PH ranges therefore represent the stability regions of these proteins. On the acid side of PH 4 and on the alkaline side of PH 9 the sedimentation of serum albumin decreases rapidly. Addition of potassium or sodium chloride does not effect any change in the sedimentation constant, showing that the Donnan potential is sufficiently eliminated. The diffusion is depressed, probably owing to gel formation, but there is not much drift in the diffusion constant with time. The ma-

terial is therefore not very unhomogeneous. The sedimentation equilibrium runs in the acid region show that there are molecules of different weights present. The trend of the equilibrium curves (variation of molecular weight with distance from the center of rotation) indicates that the bulk of the material probably has a molecular weight between 30,000 and 40,000. It is therefore quite possible that the first stage in the acid (and alkaline) decomposition of serum albumin consists chiefly in disintegration into molecules of half the normal weight.

On the acid side of P_H 4 the sedimentation constant of serum globulin increases with increasing acidity and also with time. The diffusion is depressed but also shows a considerable drift with time, indicating that the material is very unhomogeneous. The rapid sedimentation indicates aggregation of part of the decomposed protein. On the other hand, the sedimentation equilibrium run in the acid region shows that there is present a considerable amount of material with a molecular weight between 30,000 and 40,000. On the alkaline side of P_H 8 the sedimentation at first increases with increasing alkalinity, reaches a maximum at a P_H of about 10.5 and then decreases rapidly. The aggregation, therefore, is confined to a narrow P_H region.

It is of interest to note that various investigators[5] have found that the salting out effect of, say, ammonium sulfate on the serum proteins is independent of P_H from about 4 to about 9 and increases rapidly outside this acidity range. Thus Csapó and v. Klobusitzky[5c] found the salting out to be constant between P_H 4.9 and 7.5 and a strong increase at 3.9 and at 9.3. Geill[5e] found constancy in the range 5.0–7.6 and a strong increase at 4.0. The region of constant salting out effect therefore corresponds fairly well with the P_H stability region as determined by means of the ultracentrifugal analysis.

With regard to the nature of pseudoglobulin and euglobulin, the ultracentrifugal analysis has shown that both of these proteins are mixtures of molecules of different weight. The sedimentation constant of pseudoglobulin is considerably higher than that of native serum globulin. The diffusion constant shows a considerable drift with time, indicating that the material is very unhomogeneous. The equilibrium run gave values of molecular weight between 100,000 and 50,000. The heavier molecules which must be present to account for the high value of the sedimentation constant were probably too close to the bottom of the cell in the sedimentation equilibrium run to be noticed.

Euglobulin is still more unhomogeneous than pseudoglobulin. It is

[5] (a) A. Homer, *Biochem. J.*, **11**, 21 (1917); **13**, 278 (1919); (b) St. Rusznyák, *Biochem. Z.*, **140**, 179 (1923); (c) J. Csapó and D. v. Klobusitzky, *ibid.*, **151**, 90 (1924); (d) D. v. Klobusitzky, *ibid.*, **209**, 304 (1929); (e) T. Geill, Dissertation, Kopenhagen, 1928.

characterized by the presence of very large particles settling rapidly during centrifuging. The sedimentation curves (Fig. 3) are of a peculiar shape, indicating the presence of two distinctly different groups of molecules. A mixture of pseudoglobulin and euglobulin is unhomogeneous with regard to sedimentation and is, therefore, not identical with the native serum globulin, which is quite homogeneous (Fig. 4). The opinion put forward by the present writers in the previous communication, *viz.*, that pseudoglobulin and euglobulin are but laboratory products which do not exist in the blood, is strongly supported by the above measurements.

The expenses connected with these experiments have been defrayed by a grant from the foundation "Therese och Johan Anderssons Minne."

Summary

1. The ultracentrifugal methods have been applied to the study of the P_H stability region of serum albumin and serum globulin and to the study of the molecular weights of pseudoglobulin and euglobulin.

2. Serum albumin is stable within the P_H range 4–9 and serum globulin within the range 4–8.

3. Outside the stability region the molecules of these proteins break up into smaller units. The first stage of the disintegration of the serum albumin molecule probably consists in the formation of molecules of half the normal weight. The breaking up of the serum globulin molecule is at first accompanied by the formation of aggregation products and then proceeds with the formation of products of lower molecular weight.

4. Pseudoglobulin and euglobulin are not homogeneous with regard to molecular weight, as is the case with both serum albumin and native serum globulin. Euglobulin is more unhomogeneous than pseudoglobulin and contains aggregates of large mass. It is not possible to regain the original serum globulin by mixing pseudoglobulin and euglobulin. The two latter proteins are to be regarded as laboratory products and are not present in the blood.

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