THE BEHAVIOUR OF NATIVE OROSOMUCOID IN ACID AND ALKALINE MEDIA*

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Hydrogen ion titration curves of human serum orosomucoid have been determined in water (at 15, 25 and 35°C), in 6M guanidine hydrochloride and in water–dioxane systems. The molecule of native orosomucoid has been found to contain 10 masked carboxyl groups that get exposed by the action of 6M guanidine hydrochloride. The titratable carboxyl groups can be classified into two groups, *viz.* with a low and with a higher pK (2·95 and 4·14 at I 0·1). pK's of the functional groups of orosomucoid have also been determined in guanidine hydrochloride and in water–dioxane mixtures. From the titration curves and from orosomucoid–benzene interactions it can be judged that the molecule of orosomucoid contains a hydrophobic core in a solvent-permeable envelope.

Orosomucoid (an acidic α_1-glycoprotein), having the electrophoretic mobility of human serum α_1-globulins, is an interesting substance for physico-chemical investigations[1–3]. This protein is extraordinarily resistant to denaturing conditions and has a very low isoelectric point. Depending on the buffer chosen, its value[1] ranges between 1·8 and 2·7, which testifies to considerable binding of anions of these buffers. Interaction with chloride, bromide and iodide ions was investigated in our laboratory previously[4]. The chloride ions appeared to be bound most extensively, there being two probable mechanisms of binding. The problem of binding of inorganic ions is generally related to acidobasic equilibria of a protein, which can be elucidated on the basis of titration curves. Orosomucoid was first titrated by Popenoe and Drew[5]; they titrated both the native protein and a preparation freed from sialic acid, but the results were not mathematically evaluated. In our laboratory[6] the acid-region part of the titration curve of orosomucoid was partially analysed, but no correction for binding of anions was taken. The objective of this work was a more detailed analysis of the whole titration curve, determined not only in water but also in mixed media, in which the chain uncoils and long-range electrostatic interactions disappear[7] (guanidine–HCl), including media affecting the hydrophobic regions of the molecule[8] (dioxane). Since the primary structure of orosomucoid[9] is known it was possible to confront it in detail with titration data, and thus to reveal the exact numbers of functional groups buried in the molecule.

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EXPERIMENTAL

Orosomucoid was isolated from Cohn's fraction VI of human blood serum (Imuna) by our usual chromatographic procedure[2]. Its purity was tested by immunoelectrophoresis; the content of impurities coloured with Amido Black 10B did not exceed 2%. For all experiments isoionic orosomucoid was employed; it was obtained by desalting on a column of Serdolit MB (Serva).

The chemicals (KCl, HCl, KOH) were A. G. commercial products (Lachema). The volumetric solutions of KOH were carbonate-free and were kept in the cold under nitrogen. For titration 0·2M-KOH and 0·2M-HCl were used. Formaldehyde and dioxane (Lachema) were distilled before use. Dioxane was kept at 0°C. Guanidine HCl (Fluka AG) was purified, then tested for purity by the method of Nozaki and Tanford[10].

Potentiometric titrations were carried out by the continual procedure with 3-ml samples of the solutions. The concentration of orosomucoid was 2 to 3%. Its exact value was determined by drying a small sample of the solution *in vacuo* over P_2O_5 at 105°C until a constant weight was attained. The ionic strength of the solutions was adjusted with KCl to different values up to 0·1. To prevent surface denaturation by nitrogen bubbled through the solution a small amount of n-octanol was added. Titrations in water were carried out at different ionic strengths of the solution at 25°C; at the ionic strength 0·1 also at 15 and 35°C.

Titrations in aqueous formaldehyde were carried out at 25°C and an ionic strength of 0·1, the concentration of Gu HCl was 6M. This titration was carried out on the alkaline side to pH $\approx$ $\approx$ 11, since at higher values of pH the data on the number of dissociated protons are not reliable; GuH^+ ions dissociate also and guanidine itself undergoes chemical reactions[10].

Titrations in aqueous dioxane also refer to 25°C. The volume ratios of water to dioxane were 5 : 1, 4 : 1 and 3 : 1.

Spectrophotometric titrations, to delineate approximately the dissociation of tyrosine residues, were carried out discontinually using a universal spectrophotometer VSU-I (Zeiss, Jena) at a wavelength of 295 nm. The volume of the solutions was 3 ml, the protein concentration was 0·25%. Differential measurements at room temperature were performed.

pH was measured in all experiments with a compensation pH meter Radiometer pHM 4c using a glass electrode G 202B and calomel electrodes K 100 and K 401. The solutions were thermostated with a precision of $\pm 0{\cdot}1$°C. Calibration was carried out with a standard Radiometer buffer pH 6·5 and with standard buffers ÚSOL Prague: phthalate pH 4, phosphate pH 7 and borate pH 9. Where necessary, corrections for the alkaline error were taken from nomograms of the manufacturers of the electrodes.

In non-aqueous media other corrections also had to be introduced. In the water–dioxane systems two correction factors were considered[11]: one concerned the potential of the glass electrode in the partially non-aqueous medium, the other the effect of the non-aqueous medium on the interaction of ions.

The first factor was calculated by Van Uitert's method[12], according to which the stoichiometric concentration of protons, $[H^+]$, equals

$$-\log [H^+] = B + \log U_H , \qquad (1)$$

where B is reading of the pH-meter and U_H a correction factor for the medium, corrected for activity by the equation

$$U_H^0 = U_H \cdot (\gamma_\pm)^{-1} , \qquad (2)$$

where $\gamma_\pm$ is the mean activity coefficient of the electrolyte for a given concentration and com-

position of the solution. With the chosen ratios of water to dioxane 5 : 1, 4 : 1 and 3 : 1 Van Uitert's equation gives the values of log U_H^0 as 0·01, 0·02 and 0·03 respectively.

The other correction factor relates to the apparent activity coefficient of H^+ ions (γ_{H^+}), determined from the equation[11]

$$\mathrm{pH} = -\log C_{HCl}^0 - \log \gamma_{H^+} . \qquad (3)$$

For this purpose the pH readings on the meter corrected according to equation (*1*) were plotted *vs* concentration of the acid (C_{HCl}^0) or the base in the system. The line for $\gamma = 1$ was also drawn in this graph. The differences between the experimental values of pH and the corresponding points on this line give the values of $-\log \gamma_{H^+}$ for the individual pH's. Corrections for both factors were taken for titrations in water–dioxane mixtures; in systems with GuHCl only the correction for the activity coefficient was taken.

Processing of experimental data. Evaluation of the experimental data started from the well-known titration curve of a protein[13].

$$\mathrm{pH} - \log x_i/(n_i - x_i) = (\mathrm{p}K^0)_i - 0{\cdot}868wZ , \qquad (4)$$

where x_i denotes the number of dissociated functional groups i out of a total number of these groups n_i, their internal dissociation constant is K_i^0; the net charge of the protein molecule is Z and the electrostatic parameter w is defined, by the equation[18]

$$w = e^2/2DkT[1/b - \varkappa/(1 + \varkappa a)] , \qquad (5)$$

where b designates the radius of the protein molecule, a the distance of its centre from the centre of a small ion in the closest vicinity, e the elementary charge, T the absolute temperature, D the dielectric constant of the medium, k the Boltzmann constant and $\varkappa$ the reciprocal value of the Debye width.

The measured data were evaluated by the usual graphical method starting from equation (*4*). The obtained values of pK and w were back-inserted into the equation until best agreement with the experimental data was attained.

The titration curves of orosomucoid determined at different temperatures were further evaluated by Wyman's method[14]. The apparent dissociation heats, Q', were calculated from the equation

$$Q' = -4{\cdot}579T_1T_2(\mathrm{pH}_2 - \mathrm{pH}_1)(T_2 - T_1)^{-1} , \qquad (6)$$

where T_1 and T_2 are the absolute temperatures of the titrations ($T_1 > T_2$); pH_1 and pH_2 the corresponding measured values of pH. The calculated values of Q' were plotted *vs* $1/2(\mathrm{pH}_1 + \mathrm{pH}_2)$.

RESULTS AND DISCUSSION

Stoichiometry of Titration Curves

A characteristic feature of the titration curves of orosomucoid is great steepness of their acid sides, which even at low pH's show no distinctly terminated maximum as a limit number of bound protons ($\bar{h}$, Fig. 1). At pH's below 1·5 the changes are usually irreversible, so that calculation of $\bar{h}$ is doubtful.

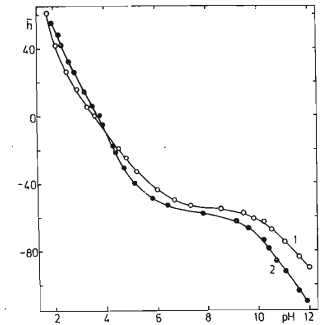

FIG. 1

Titration Curve of Orosomucoid in Water at 25°C

Ionic strength *1*: 0; *2*: 0·1.

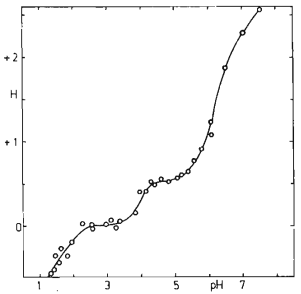

FIG. 2

Dissociation Heat of Orosomucoid, H [kcal/mol], in Relation to pH

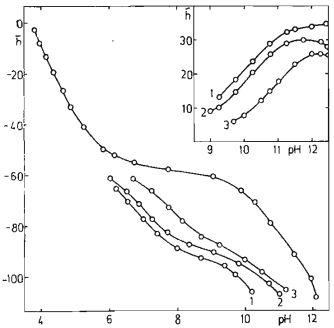

FIG. 3

Titration of Orosomucoid in the Presence of Formaldehyde at 25°C

Molarities of formaldehyde: *1* 3·67, *2* 2·30, *3* 0·68. Framed points: dissociation of ε-amino groups determined from differences between the original curve and curves *1*, *2* and *3*.

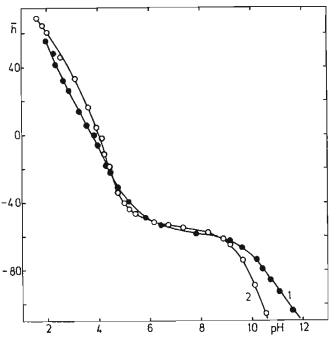

FIG. 4

Titration of Orosomucoid in Water (*1*) and in 6M-GuHCl (*2*) at 25°C

The titrations at the three temperatures confirmed again that in the acid region as far as pH 6 two kinds of functional groups, differing somewhat by their dissociation heats, dissociated (Fig. 2). The dissociation heats of the two kinds of groups, 0 and 0·5 kcal/mol, are within the range corresponding to carboxyl groups according to different authors (Miyamoto[15] ±2 kcal/mol, Cannan[16] ±1 kcal/mol, Mahling[17] ±3 kcal/mol). Beyond pH 6 the dissociation heat increases, which indicates transition to imidazole groups. In this pH region, however, the dissociation heats give no information on the type of the dissociating functional groups. For this reason the range corresponding to dissociation of ammonium groups was delineated by titration in the presence of formaldehyde (Fig. 3). Above a formaldehyde concentration of 3·67M the curve no longer changed, which determined the maximum number of amino groups to be titrated.

Precise evaluation of these data and the data of spectrophotometric titration allowed us to delimitate the dissociation regions of the respective functional groups and to determine their numbers (Table I).

As has been said, it was difficult to determine the maximum of the titration curve of orosomucoid in the acid region, the maximum was chosen as the average of a series of measurements in an extremely acid region around pH 1·5. Irreversible changes in a molecule of orosomucoid in the acid region manifest themselves by a sharp break, whence $\bar{h}$ steeply decreases to the zero order of magnitude.

With the maximum value of $\bar{h}$ thus obtained it was possible to read from the titration curve that 63·2 functional groups dissociated in the studied region of pH. According to analyses[9] a molecule of orosomucoid contains a total of 84 dissociable groups. Consequently, 20·8 of them were not detected by titration. From this number 9 guanidine groups can be subtracted as not dissociating as far as extreme alkalinity and, according to spectrophotometric titration, 2·2 tyrosine residues have not dissociated yet either. Consequently, there is a rest of 9·6 non-dissociated residues.

TABLE I

Dissociation Regions and Numbers of Titrated Functional Groups of Orosomucoid

Group	Dissociation range pH	Number in a molecule	
		titration	analysis[9]
Carboxyl (I)	max—3·75	18·8	47·5
Carboxyl (II)	3·75—6	18·0	
Imidazole	6 —7·5	3·6	3
ε-Amino	7·5 —12	13·6	12·5
Phenolic	8 —12	8·8	11

Except for the carboxyl groups, however, the agreement between the reported and our own data is very good. The difference (discrepancy) by one amino group is probably caused by the terminal α-amino group being involved in the titration of the ε-amino groups. Consequently, this analysis shows that a molecule of orosomucoid contains 10 carboxyl groups that cannot be titrated under normal conditions. It is noteworthy that these groups remained masked even on the irreversible changes occurring in extremely acid media. The values of $\bar{h}$ steeply decreased in this region and did not change with time any longer.

We have attempted to unveil these masked carboxyl groups by exposure to high concentrations of GuHCl, which is known[7,18–20] to unfold proteins. Fig. 4 shows that the titration curve of orosomucoid in 6M-GuHCl goes higher in the acid region than the normal curve and, as calculation suggests, all the carboxyl groups get unmasked by these conditions. However, not even in this case did the titration curve approach a limit value of $\bar{h}$; instead, a rapid decrease of $\bar{h}$ was observed in the region below pH 1·4 – 1·5.

Dissociation Constants of Functional Groups

To evaluate the titration data from equation (*4*) it would be necessary to know the net charge, *Z*, of the protein molecule, which is given not only by the charged groups, but also by the inorganic ions bound from the solution. It was easier to evaluate the data obtained at zero ionic strength, in this case *Z* could be considered to be roughly equal to the number of bound protons, read off from the titration curve. However, most experiments refer to an ionic strength *I* 0·1.

As we have found previously[4], orosomucoid binds a great number of chloride ions at the isoelectric point. Towards the acid side this binding increases. If we considered that the number of Cl^- ions ($\bar{\nu}$) bound to a molecule of orosomucoid at pH 3·8 and I 0·1 is 55, then at pH 2 $\bar{\nu}$ would equal about 220 to 240. This would mean, however, that the net charge of orosomucoid was considerably negative, which deduction is at variance with the electrophoretic migration of this protein under the given conditions. Thus it appears that the data on Cl^- binding obtained by conductance measurements[4] include even chloride ions bound by extraordinarily weak interactions, as was the case elsewhere[21]. These ions do not affect the behaviour of a protein in electrophoresis and cannot, therefore, be involved in *Z*. The net charge of a protein is influenced only by anions attached through rather firm electrostatic bonds to charged binding sites, and our previous experiments[4] indicate that their number is approximately the same for all halide anions. In view of this fact only a rough correction was taken for the bound chloride ions. The starting value was the number of Br^- or I^- ions bound to isoionic orosomucoid (the linkage is purely electrostatic) determined previously[4]; this was applied to the acid side of the titration curve (Karpenko and Kalous[4], Fig. 3). For the sake of comparison we have

TABLE II

pK's of Functional Groups of Orosomucoid at 25°C

Group	Titration in water		GuHCl	Water : dioxane		
	$I = 0{\cdot}0$	$I = 0{\cdot}1$	6M	5 : 1	4 : 1	3 : 1
Carboxyl (I)	2·80	2·95	3·20	3·03	3·07	3·16
Carboxyl (II)	4·25	4·14	3·92	4·54	4·73	4·89
Imidazole	5·65	6·57	6·65	6·5	6·8	6·9
ε-Amino	10·45	9·70	9·95	10·4	10·4	10·2

also determined a series of values for acid pH's that would correspond to the binding of Cl^- ions found by conductance measurements, *i.e.* a series starting with $\bar{\nu} = 55$ for pH 3·8. In a graphical solution of equation (*4*) the values of its left-hand side were plotted *vs* two series of Z values. It appeared that the two series of Z values for a given type of functional group gave points lying on one straight line. This, however, may be a coincidence and in any case it is safer to consider only the lower values of $\bar{\nu}$, corresponding only to the electrostatically bound Cl^- anions. An example is the plot for carboxyl groups (I) in dioxane (Fig. 5); the plot involves the assumption that dioxan has no appreciable effect on the binding of Cl^- ions.

Evaluation of the data to determine pK's of the functional groups of orosomucoid omits the tyrosine residues, which, in aqueous media, were studied previously[22]; in mixed solvents the spectrophotometric titrations have not been performed. Results of a mathematical analysis of the titration curves of orosomucoid are listed in Table II.

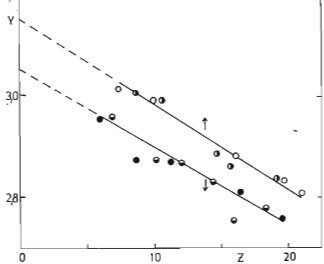

FIG. 5

Graphical Determination of pK of Carboxyl Groups (I) in Dioxane at 25°C and I 0·1. *1* Water to dioxane ratio 3 : 1, with ○ and without ◑ correction for Cl^- binding. *2* Ratio 5 : 1, with ● and without ◒ correction for Cl^- binding. $Y = pH - \log x_i/(n_i - x_i)$.

Out of the pK values found in titration in aqueous media those for carboxyl groups (I) are the most conspicuous as they are much smaller than the lower limit of the usual spread of data[23] for carboxyl groups in various proteins (4·3 to 4·7). So low a value of pK is closest to that reported for sialic acid[24] (pK 2·65). Hence it seems very likely that the carboxyls (I) are largely residues of sialic acid. However, the number of carboxyls (I) determined by titration, 18·8, is higher by 2·8 than the number of sialic acid residues in a molecule of orosomucoid[25]. This means that 2 to 3 more carboxyl groups have anomalously low pK values. A possible explanation is vicinity of basic functional groups, strongly facilitating dissociation of the carboxyl groups. The primary structure of orosomucoid[9] contains 6 glutamic acid residues and 2 aspartic acid residues neighbouring a basic amino acid on either side. Although the spatial arrangement of the polypeptide chain is important, the primary structure suggests that carboxyl groups of anomalously low pK values may be present in the molecule of orosomucoid.

Also the pK values of imidazole groups and ε-amino groups are close to the lower limit of the usual data (Tanford and coworkers[23]: imidazole groups 6·4 – 7·0, amino groups 10·1 – 10·6). To estimate all factors affecting dissociation of these groups in orosomucoid it would be necessary to know the tertiary structure of this protein and to have more information on its saccharide component. It is obvious that the electrostatic forces are not the only factor responsible for the low pK values since the latter remain lower, excepting imidazole groups, than those found with other proteins even in titrations in 6M-Gu.HCl (Table III).

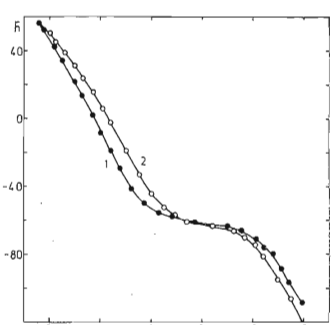

FIG. 6

Titration Curves of Orosomucoid in Water (*1*) and in Dioxane (*2*)

Water : dioxane = 3 : 1, 25°C, I 0·1.

The Electrostatic and Other Effects

The electrostatic parameter w was calculated from equation (*5*). In the investigated range of pH its value was found to vary discontinuously and considerably. The individual kinds of functional groups seem to have their own values of w, but even within the dissociation region of a given kind of groups the fluctuation is often appreciable. At an ionic strength of 0·1 the value of w ranged between 0·008 and 0·130. The moderate decrease in pK of carboxyls (II) with increasing ionic strength indicate that the functional groups of orosomucoid affect one another. In view of the foregoing facts we were interested in the titration in 6M-Gu.HCl, where the long-range electrostatic interactions are known to be suppressed or nonexistent[6]. In this case the term $0{\cdot}868\ wZ$ in equation (*4*) should equal zero. However, evaluation of the data for orosomucoid showed this term to have a non-zero value, in the acid region the value of w being not much different from that in water at I 0·1. For carboxyl groups (II) w equalled approx. 0·06 in either case. Since the value of w determined from experimental data does not involve only the electrostatic interactions[7] we believe that this type of long-range interactions is suppressed by 6M-Gu.HCl, but that strong interactions of another type, probably hydrophobic, remain operative. The most convincing indication of it is considerable binding of benzene to orosomucoid. A 0·6% solution of orosomucoid dissolved[26] 1·28 g of C_6H_6/100 ml, whereas a 1%-albumin[27] only 0·061 g of C_6H_6/100 ml. It is noteworthy that the titration curve of orosomucoid was not affected by the presence of benzene, except that one imidazole group was masked[28]. This suggests that the orosomucoid molecule contains a hydrophobic core.

To assess better the role of non-electrostatic interactions in a molecule of orosomucoid the protein was titrated in dioxan (Fig. 6). This solvent affects the formation of hydrogen bonds and stability of hydrophobic bonds[8]. To characterize the effect of dioxane in more detail we calculated the differences between pK's of functional groups in the presence and in the absence of dioxane I 0·1 (Table III).

TABLE III

Comparison of pK's of Functional Groups of Different Proteins in 6M-GuHCl

Group	Orosomucoid	Lysozyme[19]	Ribonuclease[7]
Carboxyl	3·20	3·9	3·8
	3·92	4·35	4·3
Imidazole	6·65	6·5	6·5
ε-Amino	9·95	10·35	10·35

According to Brandts[8] the effect of dioxan is the resultant of two separate molecular processes. One of them is occasioned by a decrease in the dielectric constant of medium. This change must also affect the value of the electrostatic parameter, *w*. Using the tabulated values[29] of the dielectric constant for systems water–dioxane it was possible to calculate the change in *w* corresponding to this effect only. Since the dissociation constant in a protein, *i.e.* in a system with electrostatic interactions, is generally a function of the parameter *w* (derived, *e.g.*, by Tanford[13]) we could also calculate changes of p*K* corresponding to known changes of the dielectric constant. If the effect of dioxane consisted exclusively in a reduction of the dielectric constant of the medium the p*K*'s of the functional groups of orosomucoid would change by differences $(\Delta pK)_{theor.}$ listed in Table IV. These differences are approximate values, but it is evident that they represent only a smaller part of the total change in p*K*, so that the main effect of dioxane on orosomucoid is not an effect on electrostatic interactions.

Comparison of p*K* changes in titration of orosomucoid in aqueous dioxan with those found with bovine serum albumin[11] shows a qualitative agreement for carboxyl and imidazole groups, *i.e.* an increase in p*K*. In the case of amino groups of albumin a decrease in p*K* was observed. From Table III it is apparent that the resultant effect of dioxane on orosomucoid tends to stabilize the molecule, probably by supporting hydrophobic interactions.

CONCLUSIONS

Evaluation of titration curves of orosomucoid in different media suggests that its molecule is very stable. This stability is probably caused by a compact molecular core, impermeable to solvents. The core seems to contain 10 masked carboxyl groups. The existence of such a core is indicated by several results of our experiments. Firstly, orosomucoid bound much benzene, but the dissociation of functional groups

TABLE IV

Δp*K* in Dioxane and $(\Delta pK)_{theor}$ Corresponding to a Change in the Dielectric Constant of the Medium

Group	Water : dioxane			$(\Delta pK)_{theor}$
	5 : 1	4 : 1	3 : 1	
Carboxyl (I)	0·08	0·12	0·21	0·04
Carboxyl (II)	0·40	0·59	0·75	
Imidazole	−0·07	0·23	0·33	−0·01
ε-Amino-	0·70	0·70	0·50	−0·12

was not appreciably affected by it; secondly, the electrostatic parameter w calculated from the titration curve for the isoelectric region (pH 3·6 to 3·8) ranged between 0·04 and 0·06. These values are in close agreement with those calculated from the binding of Br^- and I^- ions to this protein (*i.e.* anions bound by electrostatic forces only) and testify rather to a model of spherical protein whose envelope as well as core are impermeable to solvents.

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REFERENCES

1. Jeanloz R. W. in the book: *Glycoproteins* (A. Gottschalk, Ed.). Elsevier, Amsterdam 1966.
2. Kalous V.: Biochim. Biophys. Acta *107*, 139 (1965).
3. Schmid K.: Chimia *26*, 405 (1972).
4. Karpenko V., Kalous V.: This Journal *40*, 2131 (1975).
5. Popenoe E. A., Drew R. M.: J. Biol. Chem. *228*, 673 (1957).
6. Karpenko V., Pavlíček Z., Kalous V.: Biochim. Biophys. Acta *154*, 245 (1968).
7. Nozaki Y., Tanford Ch.: J. Amer. Chem. Soc. *89*, 742 (1967).
8. Brandts J. F. in the book: *Structure and Stability of Biological Macromolecules* (S. N. Timasheff, G. D. Fasman, Eds), p. 213. M. Dekker, New York 1969.
9. Schmid K., Kaufmann H., Isemura S., Bauer F., Emura J., Motoyama T., Ishiguro M., Nanno S.: Biochemistry *12*, 2711 (1973).
10. Nozaki Y., Tanford Ch.: J. Amer. Chem. Soc. *89*, 736 (1967).
11. Sun S. F.: Biochim. Biophys. Acta *200*, 433 (1970).
12. Van Uitert L. G., Hass Ch. G.: J. Amer. Chem. Soc. *75*, 451 (1953).
13. Tanford Ch.: *Physical Chemistry of Macromolecules*. Wiley, New York 1966.
14. Wyman J.: J. Biol. Chem. *127*, 1 (1939).
15. Miyamoto S., Schmid C. L. A.: J. Biol. Chem. *90*, 165 (1931).
16. Cannan R. K., Palmer A. H., Kibrick A. C.: J. Biol. Chem. *142*, 803 (1942).
17. Mahling A.: Behringwerk-Mitt., Heft *42*, 167 (1962).
18. Mihalyi E.: Biochemistry *9*, 804 (1970).
19. Roxby R., Tanford Ch.: Biochemistry *10*, 3348 (1971).
20. Rickert W. S., McBride-Warren P. A.: Biochim. Biophys. Acta *371*, 368 (1974).
21. Steinhardt J., Reynolds J. A.: *Multiple Equilibria in Proteins*, Chapter VII. Academic Press, New York 1969.
22. Svobodová X.: *Thesis*. Charles University, Prague 1975.
23. Tanford Ch., Swanson S. A., Shore W. S.: J. Amer. Chem. Soc. *77*, 6414 (1955).
24. Cleave A. J., Kent P. W., Peacocke A. R.: Biochim. Biophys. Acta *285*, 208 (1972).
25. Schultze H. E., Heremans J. F.: *Molecular Biology of Human Proteins*, Part I. Elsevier, Amsterdam 1966.
26. Karpenko V., Yampolskaya G. P.: Unpublished results.
27. Volynskaya A.V., Izmaylova V. N., Pchelin V. A., Yampolskaya G. P.: Vysokomol. Soedin. *11*, 2509 (1969).
28. Karpenko V.: Unpublished results.
29. Conway B. E.: *Electrochemical Data*, p. 42. Elsevier, Amsterdam 1952.

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