HUMAN OROSOMUCOID: DISSOCIATION OF TYROSINES IN THE SYSTEMS WATER–LOW ALIPHATIC ALCOHOL

Vladimir KARPENKO[a1], Jana HORALKOVA[a] and Milan KODICEK[b]

[a] *Department of Physical and Macromolecular Chemistry, Charles University, 128 40 Prague 2, Czech Republic; e-mail:* [1] *karpenko@prfdec.natur.cuni.cz*

[b] *Department of Biochemistry and Microbiology, Prague Institute of Chemical Technology, 166 28 Prague 6, Czech Republic; e-mail: milan.kodicek@vscht.cz*

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The influence of methanol, ethanol, propan-1-ol, and propan-2-ol on the dissociation of tyrosines of human serum orosomucoid (acid α_1-glycoprotein) was studied. The content of alcohols was chosen so that their solutions had the same value of the relative permittivity. Then, the contribution of electrostatic effects was equal in all cases and the observed differences between aqueous and alcoholic solutions reflected other, nonelectrostatic effects in the studied systems. Analysis of the spectrophotometric titration curve revealed three kinds of tyrosine groups (n_1, n_2, n_3), but only the values pK_1 and pK_2 could be calculated. The observed differences of pK values in aqueous and mixed media are discussed and compared with the structural changes of the orosomucoid molecule.

Key words: Orosomucoid; Tyrosine dissociation; Alcohols effects; UV-spectroscopy; Isopermittivity conditions.

Blood serum orosomucoid (acid α_1-glycoprotein; ORS throughout this paper) is a glycoprotein of the α_1-globuline fraction first isolated independently by Weimer *et al.*[1], and Schmid[2] in 1950. The excellent solubility in water and extraordinary stability made it possible to study ORS thoroughly. Due to the high content of acidic constituents, *i.e.*, glutamic, aspartic, and sialic acid, its isoelectric point varies between 1.8 and 2.7, according to a buffer used[3,4]. This dispersion of pI values is a sign of strong interaction of ORS with inorganic anions in acidic solution that was confirmed for chlorides, bromides, and iodides[5].

The ORS molecule consists of one chain of 181 amino acids; two disulfide bonds are formed by cysteines 5–147, and 72–164. Primary structure of this glycoprotein is characterized by a high number of possible amino acid substitutions, at 21 sites[6]. Five heteropolysaccharide units representing approximately 40% of the total molecular weight of this protein are attached to asparaginyl residues. Distribution of hydrophobic residues is uneven in the ORS molecule; there are marked hydrophobic regions in the first half of its molecule, while the C-end is strongly hydrophilic, from the residue 160. Although ORS was successfully crystallized as the Pb^{2+} salt, the attempts to analyze its

three-dimensional structure failed due to the thermal movement of the peripheral residues[7]. Circular dichroism spectra of ORS were repeatedly investigated; the results have, however, broader distribution[8–10]. The most recent experiments[10] confirmed high content of β-sheet and low of α-helix. The attempts to predict the secondary structure of ORS led to results that deviate from the experimentally found values[11,12]. This protein presumably possesses a compact spatial arrangement characterized by numerous masked groups, approximately 10 carboxyls[13], 5–7 tyrosyls[14,15], 2 tryptophyls[14,16], and almost all phenylalanyls[17]. The dissociation of tyrosines in ORS was thoroughly studied in aqueous solutions and these residues were divided[18] into three groups with pK values 9.9, 11.0, and 11.8.

In the last years stability of the ORS molecule has been studied in mixed solvent systems water–low aliphatic alcohol (methanol[10,12] and ethanol[17]). Behavior of proteins in alkaline solutions is strongly influenced by the dissociation of tyrosines; since there were no reliable data about this process in mixed solvents, the present work is an attempt to fill this gap.

EXPERIMENTAL

Materials

Human serum orosomucoid was isolated from Cohn's Fraction VI of human blood serum (IMUNA, Sarisske Michalany, Slovak Republic), desalted on a Sephadex G-25 column, using fractionation on CM-cellulose as developed in our laboratory[19]. Orosomucoid prepared in this way did not contain any significant amount of impurities as checked by polyacrylamide gel electrophoresis and immunoelectrophoresis; its isoionic pH was 3.67 at the concentration 2.4 wt.%. Concentration of the protein solutions was determined from absorbance at 280 nm ($E_{1\%,280}$ = 8.9; ref.[3]). For the molecular weight of this protein the value 41 000 was accepted[20].

Aliphatic alcohols, all of reagent grade purity, were products of Lachema, Brno; their content in the solutions is given as a volume fraction φ (%) throughout this paper. Experiments were performed with following alcohol contents: methanol (MeOH) 55%, ethanol (EtOH) 44%, propan-1-ol (PrOH) 36%, and propan-2-ol (iPrOH) 36%. The relative permittivity of all these solutions is 57 according to Åkerlof[21].

Inorganic reagents used in this work were of analytical grade purity; volumetric solutions of KOH were freshly prepared carbonate-free.

Methods

Determination of pH. In our work a pH-meter PHM 93 (Radiometer, Copenhagen) equipped with the combined microelectrode pHC 4400 of the same manufacturer was used. In mixed solvents pH-meter readings should be corrected for the effects of the solvent on both the activity coefficient of H_3O^+ and the electrodes, as discussed in detail by Bates *et al.*[22]. This correction was done as described previously[12] and the results for methanolic and ethanolic systems were compared with the literature data[23]. Using this experimentally found dependence, pH-meter readings were corrected so that pH values given in this work correspond to that expected in the aqueous solution.

Spectrophotometric titrations. These experiments were carried out with a spectrophotometer Specord M 40 (Zeiss, Jena) using 1 cm quartz cells tightly closed to prevent evaporation of the solvent.

Temperature was maintained at 25 ± 0.5 °C. Our investigation[15] of tyrosine dissociation in ORS as well as the data[24] with the model compound *N*-acetyl-L-tyrosine ethyl ester (NAcTyr throughout this paper) have shown that the most reliable data on the dissociation of its phenolic hydroxyl are obtained at 244 nm in both aqueous and alcoholic media. The contribution of two disulfide bridges in ORS was found[25] to be negligibly small in the pH range 6 to 11. Therefore, the wavelength 244 nm was chosen for the present experiments with ORS.

The ionization of the phenolic groups was studied by difference spectroscopy; preliminary, both continuous and discontinuous mode were tried. In the discontinuous mode, tandem cells[26] were used and a new solution was prepared for each point of the titration curve. Since the results did not differ in both approaches, further experiments were performed in the continuous mode with a single cell. In these experiments the programmed spectral width adjustment optimalizing the signal to noise ratio was used, with detector gain 5, and integration time 1 s. In a typical continuous experiment the measured sample was a $1 \cdot 10^{-5}$ mol dm^{-3} ORS solution in an appropriate mixed solvent; the ionic strength was maintained at 0.2 by addition of solid KCl. As the relative premittivity of our mixed solutions was above 40 (57), the value of the ionic strength remains basically unchanged in the presence of organic solvent[27]. The reference sample contained ORS of the same concentration dissolved in 0.2 M phosphate buffer pH 7.0. Volumetric solution of KOH was added to the first cell and the same aliquot of buffer to the reference cell. Absorbance was measured immediately after each addition of KOH; pH value of the solution did not change with time. This experimental approach was based on the experimentally proved assumption that no absorption of UV-light by alcohols or phosphate buffer interfere at the wavelength used. Correction for volume changes was done in all experiments.

Calculation of the titration results. Each titration curve was usually constructed from approximately 60 values obtained in at least three separate experiments. The curve plotted as the difference absorbance (ΔA) *versus* pH can be expressed for a group of n_i tyrosines with one intrinsic dissociation constant pK_i by the equation[26]:

$$\Delta A = cl \sum_{i=1}^{N} \frac{n_i \, \Delta\varepsilon_i \, 10^{-pK_i}}{10^{-pH} + 10^{-pK_i}} \ , \qquad (1)$$

where c is the protein concentration, l is the optical path, N is the number of groups (i) of tyrosines with the pK_i value, and $\Delta\varepsilon_i$ is the differential molar absorption coefficient of the dissociated group of the type i.

The pK_i values were determined by two approaches:

– the equation (*1*) was solved using the least-squares non-linear method on the assumption that there are no differences within an i-th group;

– the computer program Origin for sigmoidal curves was applied directly on the experimental data.

A preliminary analysis of the titration curve was done using the classical equation of the titration curve of a polyelectrolyte molecule containing n_i identical and independent groups of the type i:

$$pH - \log \frac{x_i}{n_i - x_i} = pK_i - 0.868 \, wZ \ . \qquad (2)$$

In this equation x_i is the number of groups in deprotonated state at a given pH, pK_i is their intrinsic dissociation constant. At this pH a polyelectrolyte molecule carries the net charge Z, and the electrostatic interactions are expressed by the parameter w defined as

$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) . \qquad (3)$$

Here, e is the elementary charge, D the relative permittivity, and k is the Boltzmann constant; b is the radius of a polyelectrolyte molecule (supposed spherical in this model), a is the closest distance of approach of the center of a neighboring small ion. The reciprocal distance of the ionic atmosphere is denoted κ in this equation.

Calculation of the buried surface. To estimate the accessibility of individual tyrosines, the average fraction of buried surface of individual amino acids was calculated using the values given by Rose *et al.*[28]. For this calculation a sliding average with the window 7 was used and the resulting data were compared with the average buried surface of one amino acid calculated over the whole molecule of ORS.

RESULTS

As shown in Fig. 1, the titration curves of ORS obtained in the presence of alcohols differed significantly from those in the aqueous solution. Simultaneously, the curves in the presence of both MeOH and EtOH were almost identical; not so for the two other alcohols that formed a separate group in this respect. In all mixed media, titration curves of ORS were reversible up to pH 12.5; the values of the molar absorption coefficient (ε) of ORS are summarized in Table I.

The first attempt to analyze these data using Eq. (*1*) in terms of one type of tyrosines (all having the same intrinsic p*K*) did not lead to reasonable results: all p*K*'s were shifted to unacceptably high values. The same was found with Eq. (*2*) which, however,

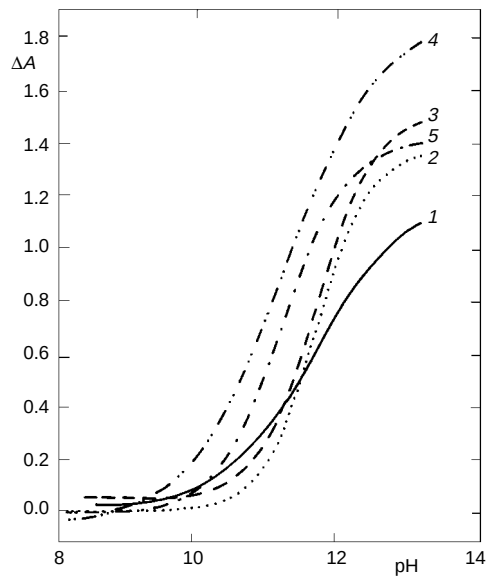

FIG. 1
Spectrophotometric titration curves of ORS at 25 °C (differential absorbance Δ*A* *vs* pH) in: *1* water, *2* MeOH, *3* EtOH, *4* PrOH, *5* iPrOH. Experimental points (approximately 60 for each curve) omitted

yielded much better results when tyrosines were divided into two groups. On closer examination of details of titration curves, a small irregularity in their shape was really detected, as shown in Fig. 2. This observation supported the conclusion drawn from Eq. (*2*) and, therefore, further calculations were done for two types of tyrosines (groups n_1, n_2) that differ in their p*K* values. This approach was in agreement with the literature data about distribution of tyrosines in ORS molecule from the point of view of their accessibility[18]. The third type (n_3), also mentioned in the literature[14,15], could not be included in this calculation, because p*K* of these groups is too high, and their dissociation continues into the region of irreversibility of the titration curve. The final results of this analysis performed with Eq. (*1*) and checked by the program Origin are summarized in Table II.

Calculation of the buried surface of tyrosine residues in ORS as sliding average led to the distribution shown in Table III. The mean fraction of buried surface of one amino acid averaged over the whole ORS molecule was 0.715.

TABLE I
Molar absorption coefficient ε_{244} ($mol^{-1}dm^3\ cm^{-1}$) of ORS in water and mixed solvents at pH 7

Solvent	$\varepsilon \cdot 10^{-5}$
H_2O	1.08
H_2O–MeOH	1.36
H_2O–EtOH	1.46
H_2O–PrOH	1.62
H_2O–iPrOH	1.41

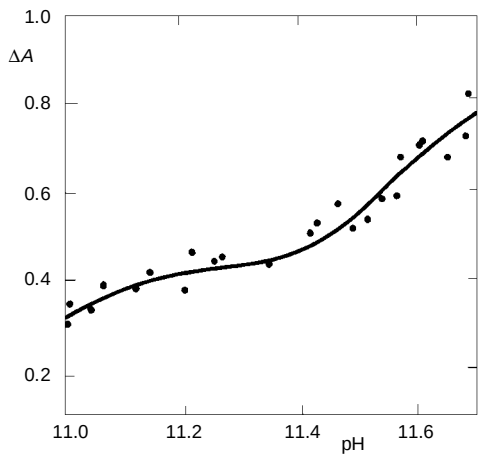

FIG. 2
The detail of the spectrophotometric titration curve of ORS (differential absorbance Δ*A* *vs* pH) in the system H_2O–EtOH

DISCUSSION

The influence of small aliphatic alcohols on aqueous solutions of proteins is a complex process resulting from three main contributions:

1. lowering of the relative permittivity of the solution that enhances the electrostatic interactions;

2. the change of water structure caused by alcohols that can exert effect on the hydration layer of a protein molecule;

3. noncovalent binding of alcohols to a protein molecule.

TABLE II
The numbers (n) of individual groups i of tyrosines in ORS and their intrinsic pK_i values

Solvent	pK_i			n		
	pK_1	pK_2	pK_3	n_1	n_2	n_3
H_2O	10.4	12.0	>12.5	3.0	6.7	2.3
H_2O–MeOH	10.8	11.7	>12.5	2.4	7.0	2.6
H_2O–EtOH	10.9	12.0	>12.5	3.1	7.1	1.8
H_2O–PrOH	10.2	11.4	>12.5	3.3	7.5	1.2
H_2O–iPrOH	10.6	11.5	>12.5	3.2	7.0	1.8

TABLE III
Distribution of tyrosine residues according to the average buried surface calculated as the sliding average with window 7; the tyrosine residue in question was the central one in the window. The residues located in a predicted α-helix are denoted (α), those in supposed β-sheet (β); square brackets denote residues with possible substitutions

The average value of buried surface	Tyrosine residue (No.)
0.770–0.789	50 (β)
0.750–0.769	27 (β), 157
0.730–0.749	74, 78, 91 (β), [110] (β), [115] (β), 142 (α)
0.710–0.729	127
0.690–0.709	65 (β)
0.670–0.689	
0.650–0.669	
0.630–0.649	37

Any discussion of the data obtained in this kind of mixed solvents should take in consideration all three effects. Yet, only one of them, the contribution of electrostatic interactions, can be quantified. In the present discussion the problem will be dealt with in three blocks concerning the number of dissociable tyrosines, their identity, and, eventually, their properties as reflected by the p*K* values.

As far as the first point in question is concerned, tyrosines exhibited similar behavior in water and in mixed solvents; their numbers in both kinds of groups (n_1 and n_2) were almost the same in all these media. This demonstrates that conformational changes of the ORS molecule observed in CD spectra in alcoholic media do not change the molecule substantially when compared with the aqueous solution. As found previously[10] in 30% MeOH, the only difference compared with the aqueous solution was a small increase in the content of β-sheet and β-turns.

The distribution of tyrosines among different groups ($n_1 \approx 3$, $n_2 \approx 7$) found in the present work was in agreement with our previous experiments[15]. For aqueous medium, the present data can be confirmed by the analysis of the titration curve[13] of ORS according to which approximately 21 groups dissociate between pH 10 and 12. Taking into consideration p*K* value of lysines (9.70–10.45, depending on the ionic strength) and the fact that there are no masked residues of this amino acid in the ORS molecule, then, lysines account for approximately 13 charges out of the 21 mentioned above. Thus, in the pH region in question, further 9 groups dissociate; this number corresponds well with the sum $n_1 + n_2$ found in the present work. On the other hand, chemical modification[14] revealed different distribution of accessible tyrosines ($n_1 = 5$, $n_2 = 3$). This is a further illustration of the observation that it is difficult to compare the results obtained by the methods as different as chemical modification of a protein and its titration are.

The attempt to determine on the basis of the calculation of the buried surface which individual tyrosine residues dissociated, led to unconvincing results. There are just three residues (Tyr 37, 65, and 127) with the average value of buried surface lower than the value found as average for the whole molecule of ORS (Table III). On the other hand, if hydrophobicity is calculated on binary scale[12], low values are found for tyrosine residues 37, 65, 74, 110, and 157. It can be thus concluded that Tyr 37 and 65 belong very probably to the group n_1, whereas the other two residues of this group most exposed to the solvent cannot be determined in this way with sufficient reliability. The most accessible residues, as judged from their average buried surface, were Tyr 27 and 50. As in chemical modification experiments[14] residues 65, 74, 110, and 142 have been found buried, the most probable exposed residue is Tyr 37.

Because of the method of pH correction used in this work, all p*K* values given in the Table II were related to aqueous solution. As is obvious, the values obtained in the present set of experiments for the aqueous medium were slightly higher than our data reported previously[15]. This difference arises from the method used for the evaluation of

the titration data. Previous results were calculated from Eq. (*2*) and thus included the effect of the net charge of a protein molecule. In our recent work in mixed solvents Eq. (*2*) cannot be used reliably due to the lack of the precise data on the net charge of ORS molecule in these conditions. Both sets of p*K*'s can be thus compared for aqueous solutions. In the inflection point of the titration curve [$x_i = n_i - x_i$], Eq. (*2*) is simplified:

$$\mathrm{p}K_i = \mathrm{pH} + 0.868wZ \,. \qquad (2a)$$

Then, for example at pH 10, the net charge found previously[13] was $Z = -23$, and $w = 0.03$. The electrostatic correction of Eq. (*2*) is thus $0.868wZ = -0.6$. Indeed, the pK_1 value found in the present set of experiments was by approximately 0.5 units higher than corresponding p*K* found previously. This calculation documents the agreement between the previous and present results in the aqueous solution.

The lack of data concerning the net charge *Z* of the ORS molecule in the mixed media was the reason why Eq. (*2*) could be used only for preliminary decision about probable distribution of tyrosyl residues. In further calculations p*K* values were determined directly from the spectrophotometric titration curve, without electrostatic correction based on *Z*. It is, however, important to compare the differences between p*K* in the aqueous and mixed solvents, and the data in the present work, evaluated by a curve-fitting procedure directly from titration curves, were in this respect comparable. Simultaneously, however, they were shifted to higher values due to the lack of electrostatic correction as mentioned above.

More important was the examination of the p*K* values from the electrostatic standpoint with stress laid on the differences between them. These constants are in the aqueous solution (pK_w) and in the mixed solvent (pK^*) related according to the equation[29]

$$\mathrm{p}K^* = \mathrm{p}K_w + \gamma_e \,, \qquad (4)$$

where the electrostatic contribution to observed changes is expressed as the activity coefficient γ_e. This coefficient is a function of the size of a dissociable group, of temperature, and of relative permittivity of surrounding medium.

Should solely electrostatic effects act, then, because of the same value of the relative permittivity of all mixed solvents used in our experiments, the difference $\Delta\mathrm{p}K \equiv \log \gamma_e = \mathrm{p}K^* - \mathrm{p}K_w$ should also be the same in all the cases studied. As is obvious from Table IV, this was not the case. The expected effect was calculated for the phenolic group and the relative permittivity 57 as given elsewhere[30], and the obtained value of $\log \gamma_e = +0.33$

was compared with the experimental data. Further comparison can be done with our previous data about NAcTyr dissociation in alcoholic media[24].

Then, following conclusions can be drawn:

1. With the exception of PrOH, ΔpK values are positive for both NAcTyr and n_1 groups of ORS. These values are, particularly in MeOH and EtOH, smaller in ORS than in NAcTyr, and are closer to the calculated log γ_e. The latter observation applies also to n_1 groups in the presence of iPrOH.

2. For n_2 tyrosyls in ORS ΔpK changes are opposite in sign as compared with the model compound. It shows that dissociation of these groups is easier in mixed solvents than in aqueous solution.

3. When we compare the ΔpK caused by different alcohols, the differences between MeOH and EtOH on one hand, and PrOH and iPrOH on the other hand are more pronounced for NAcTyr than for ORS.

Tyrosines dissociate in the alkaline region where the negative net charge of a protein is the main driving force of conformational changes which lead eventually to irreversible denaturation. These changes can be followed as a decrease in the content of the regular secondary structures. Their absolute content in the ORS molecule is a matter of discussion, therefore, only the relative decrease in α-helix content between pH 9 and 12 for aqueous and mixed media is used here. These data are summarized in the Table V, and the α-helix content is expressed in percents when the reference value (100%) is helix content at pH 9. The changes in alcoholic solutions are markedly smaller than in water. At the same time, a slight deviation from this observation is apparent in EtOH which at the concentration given here (40%) exhibits anomalous behavior[17].

Conclusions. Previous discussion can be summarized in several observations which, however, cannot be quantified at the present state of knowledge. First, as follows from pK_1 values, the dissociation of n_1 tyrosines of ORS is dominated by electrostatic effects arising from the change of permittivity of the solvent. Second, the effects of alcohols

TABLE IV
The difference $\Delta pK \equiv \log \gamma_e = pK^* - pK_w$ for NAcTyr (ref.[24]) and ORS

Alcohol	NAcTyr	ORS	
		n_1	n_2
MeOH	+1.1	+0.4	–0.3
EtOH	+1.2	+0.5	0.0
PrOH	+0.5	–0.2	–0.6
iPrOH	+0.7	+0.2	–0.5

manifested by p*K* changes are "smoothed" in the protein as compared with the behavior of NAcTyr which has higher Δp*K* values[24]. Exceptional in this respect is the influence of PrOH; it can be only speculated that in the presence of this alcohol hydrophobic interactions contribute to a greater extent to the stability of ORS molecule. It seems that it is the linearity of the molecule of this alcohol which is advantageous in this respect, as compared with iPrOH.

Except for EtOH, dissociation of n_2 tyrosines is significantly easier in the presence of alcohols in spite of the fact that, as shown in Table V, the loss of structure is slower in these alcohols. It is obviously a picture of combined denaturation, alcoholic and alkaline. With increasing pH the growing negative net charge destabilizes the protein molecule as a whole, but, simultaneously, low aliphatic alcohols stabilize helical formations[31,32]. High negative value of Δp*K* is a sign of the prevailing effect of lower permittivity which enhances electrostatic repulsion. The resulting effect is breaking of bonds keeping the native structure of ORS.

TABLE V
The α-helix content of ORS in alkaline media compared with the values at pH 9 (data from refs[10,17,33]) taken as 100%

Solvent	pH		
	10	11	12
H_2O	90	70	50
MeOH (55%)	100	100	90
EtOH (40%)	90	85	
PrOH (36%)	100	95	75
iPrOH (36%)	95	85	75

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REFERENCES

1. Weimer H. E., Mehl J. W., Winzler R. J.: J. Biol. Chem. *185*, 561 (1950).
2. Schmid K.: J. Am. Chem. Soc. *72*, 2816 (1950).
3. Schulze H. E., Heremans J. F.: *Molecular Biology of Human Plasma Proteins*, p. 203. Elsevier, Amsterdam 1966.
4. Jeanloz R. W. in: *Glycoproteins* (A. Gottschalk, Ed.), 2nd ed., Chap. 6, Sect. 1. Elsevier, Amsterdam 1972.
5. Karpenko V., Kalous V.: Collect. Czech. Chem. Commun. *40*, 2131 (1975).

6. Schmid K., Kaufmann H., Ysemura S., Bauer F., Emura J., Motoyama T., Ishiguro H., Nanno S.: Biochemistry *12*, 2711 (1973).
7. Schmid K. in: *Alpha$_1$-Acid Glycoprotein* (P. Baumann, C. B. Eap, W. E. Müller and J.-P. Tillement, Eds), p. 7. Alan R. Liss, New York 1989.
8. Yamagami K., Schmid K.: J. Biol. Chem. *242*, 4176 (1967).
9. Schmid K., Kamiyama S.: Biochemistry *2*, 271 (1963).
10. Kodicek M., Infanzon A., Karpenko V.: Biochim. Biophys. Acta *1246*, 10 (1995).
11. Aubert J.-P., Loucheux-Lefebvre M. H.: Arch. Biochem. Biophys. *175*, 400 (1976).
12. Karpenko V., Sinkorova L., Kodicek M.: Collect. Czech. Chem. Commun. *57*, 641 (1992).
13. Karpenko V., Kalous V.: Collect. Czech. Chem. Commun. *42*, 45 (1977).
14. Schmid K., Chen Li-chuang H., Occhino C., Foster J. H., Sperandio K.: Biochemistry *15*, 2245 (1976).
15. Svobodova X., Karpenko V., Kalous V.: Collect. Czech. Chem. Commun. *42*, 1742 (1977).
16. Kalal P., Kalous V.: Collect. Czech. Chem. Commun. *49*, 165 (1984).
17. Janackova L., Karpenko V.: Collect. Czech. Chem. Commun. *59*, 2190 (1994).
18. Yamagami R., Labat J., Pandey R. S.: Biochemistry *7*, 2873 (1968).
19. Karpenko V., Pavlicek Z., Kalous V.: Biochim. Biophys. Acta *154*, 245 (1968).
20. Haupt H.: Private communication.
21. Akerlof G.: J. Am. Chem. Soc. *54*, 4125 (1932).
22. Bates R. G., Paabo M., Robinson R. A.: J. Am. Chem. Soc. *67*, 1833 (1963).
23. De Ligny C. L., Luykx P. F. M., Rehbach M., Wieneke A. A.: Rec. Trav. Chim. *79*, 699 (1960).
24. Karpenko V., Horalkova J., Kodicek M.: Collect. Czech. Commun. *61*, 1261 (1996).
25. Karpenko V.: Unpublished results.
26. Donovan J. W. in: *Physical Principles and Techniques of Protein Chemistry* (S. J. Leach, Ed.), Part A, p. 101. Academic Press, New York 1969.
27. Izmailov N. A.: Elektrokhimiya rastvorov, p. 617. Izd. Kharkovskogo Gosudarstvennogo Universiteta, Kharkov 1959.
28. Rose G. D., Geselowitz A. R., Lesser G. J., Lee R. H., Zehfus M. H.: Science *29*, 834 (1985).
29. Cohn E. J., Edsall J. T.: *Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions*, p. 105. Reinhold, New York 1943.
30. Karpenko V., Skrabana R.: Collect. Czech. Chem. Commun. *58*, 267 (1993).
31. Herskovits T. T., Gadegbeku B., Jaillet H.: J. Biol. Chem. *245*, 2588 (1970).
32. Vorobjev Y. N., Scheraga H. A., Hitz B., Honig B.: J. Phys. Chem. *98*, 10940 (1994).
33. Cerna-Horalkova J.: *Thesis*. Charles University, Prague 1995.