# THE BINDING OF CHLORIDES, BROMIDES AND IODIDES TO NATIVE OROSOMUCOID

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The binding of $Cl^-$, $Br^-$ and $I^-$ ions to human serum orosomucoid has been studied by the method of equilibrium dialysis and electric conductance measurements. The quantity of the bound chloride ions was four to five times greater than that of $Br^-$ or $I^-$ ions. In the case of $Br^-$ ions binding sites of 3 types have been distinguished, 1,3 and 20 in number, the respective association constants being 570, 40 and 6.

The paper also describes the changes of pH observed in adding the ions $Cl^-$, $Br^-$ and $I^-$ to an isoionic solution of orosomucoid. These pH changes were very similar and increased in the order $Cl^- < Br^- < I^-$. The study of interactions of $Cl^-$ ions with orosomucoid in acid media suggests that $Cl^-$ ions are bound not only by electrostatic forces, like $Br^-$ and $I^-$ ions, but also by hydrogen bonds to non-dissociated carboxyl groups.

Orosomucoid[1] (an acid $\alpha_1$-glycoprotein[2]) is one of the most studied[3-11] proteins of human blood serum. This protein is relatively well accessible, mainly by the methods of preparative chromatography[12]. The majority of physical and chemical properties of orosomucoid are markedly influenced by the fact that about 40% of its molecular weight belongs to a carbohydrate constituent[13]. Orosomucoid is also the most soluble serum glycoprotein[14]. Its characteristic property is an unusual stability in changes of temperature and pH. This protein is not denatured in boiling water and is stable in a pH range of 2·5 to 11.

Consequently, the structure of orosomucoid seems to be very firm and probably has a compact core. This compactness manifests itself by a large number of masked groups in the molecule. Under the normal conditions only 5 out of the 12 tyrosine residues are freely accessible and out of the 42 carboxyl groups only 36 can be titrated[15]. The tyrosine residues get exposed by the action of 8M urea[14], the carboxyl groups can all be titrated after denaturation of the protein in 6M guanidine hydrochloride[16].

The great number of carboxyl groups in a molecule of orosomucoid is responsible for the extraordinarily low isoelectric point of this protein. The data on its value vary in a rather wide range, from pH 2·7 in a phosphate buffer to approx. pH 1 in trichloroacetic acid[4]. This range indicates a strong interaction of orosomucoid with anions, but there has been no direct attempt to evaluate this interaction qualitatively and quantitatively.

The present paper describes the binding of halide ions (chloride, bromide and iodide) to human serum orosomucoid at the isoionic point. We have attempted to evaluate the measured values in order to determine the number of binding sites and their association constants. An attempt has also been made to study the binding of chloride ions to orosomucoid in acid media.

## EXPERIMENTAL

Orosomucoid was isolated from Cohn's fraction VI of human blood plasma by chromatography on CM-cellulose[16]. The homogeneity of the preparation was verified by disc electrophoresis and immunoelectrophoresis, the impurities detectable by Amido Black did not exceed 3% of the total protein. For all experiments the protein had been desalted on a column of ion exchanger Amberlite MB 3 (Serva) and the concentrations of the orosomucoid solutions were determined by dry weights of aliquot samples, dried *in vacuo* over $P_2O_5$ at 105°C. The chemicals employed, NaCl, NaBr, NaI and HCl, were of A.G. purity. All solutions were prepared with distilled water having a conductivity not greater than $5 \cdot 10^{-6}\ \Omega^{-1}\ cm^{-1}$.

To determine the average number of ions ($\bar{\nu}$) bound to a molecule of the protein two methods were employed:

*The conductometrical method*[17]. The resistance of the solutions was measured with a transistor conductometer, constructed by Hládek[18], connected to a Wheatstone bridge MLL (Metra). The experiments were carried out with 6 ml samples of 1 to 1·5% solutions of orosomucoid at a frequency of 1000 Hz and a potential difference of 2 V. The error of measurement did not exceed 0·5% of the measured value. Solutions (0·1M) of the investigated electrolytes were added from an automatic burette ABU 1c (Radiometer) to the studied system, thermostatted to 25 ± ± 0·1°C.

*Equilibrium dialysis* was carried out analogously to Scatchard's procedure[19]. We used perspex cells, whose two compartments (4·5 ml each) were separated by cellophane. One compartment was filled with an isoionic 1—3% solution of the protein, into the other a solution of the investigated salt of known concentration was pipetted. The filled cells were left standing at 6 to 8°C for 72h, during which time they were occasionally shaken. Then the concentration of the electrolyte was determined conductometrically in the second compartment and, roughly, in the protein compartment, and the volumes of the solutions in the two compartments were measured again. The value of $\bar{\nu}$, *i.e.* the number of anions bound to a molecule of the protein, was calculated from Scatchard's equation[19], involving correction for the Donnan equilibrium. The use of this equation is based on the assumption that ions of one type of charge are bound to orosomucoid, *i.e.* anions in the given case. Prior to the experiments the cellophane membranes were washed 24 h in distilled water. By blank experiments we ascertained the bindings of the investigated anions to these membranes at different concentrations of the salts and the results were used to correct the values of $\bar{\nu}$ measured in the systems with the protein.

*Disc electrophoresis* was performed according to Davis[20] at a potential gradient of 7 V/cm. The protein in the disc of the polymer was stained with a 1% solution of Amido Black 10 B in 7% acetic acid.

*Thin layer chromatography* was investigated in a gel of Sephadex G-150 Superfine, containing 1% NaCl. An apparatus of the firm Pharmacia was employed.

pH *was measured* with a pH-meter pHm 4c (Radiometer), with electrodes G 202 B, K 100 and K 401. The solutions had been thermostatted to 25 ± 0·1°C.

## RESULTS AND DISCUSSION

*Dialysis and conductometry.* In the first series of experiments the binding of chloride ions to orosomucoid was measured by the method of equilibrium dialysis, so that we determined the precise values of $\bar{\nu}$ for $Cl^-$ concentrations 0·01, 0·05 and

0·1M (Fig. 1*A*) and could compare these data with the results of conductance measurements. In this way we determined the value of the empirical constant $A$, occurring in the equation for calculation of $\bar{\nu}$ in the conductometrical method[17]. In the case of orosomucoid $A$ was found to equal 1. When we had determined this value we were able, in another series of experiments, to measure conductometrically the binding of $Cl^-$, $Br^-$ and $I^-$ ions to orosomucoid in a range of free-ion concentrations from $2 \cdot 10^{-3}$ to $1{\cdot}5 \cdot 10^{-5}$M (Fig. 1*A*,*B*). Whereas bromide and iodide ions were bound to about the same extent (Fig. 1*B*), the number of bound chloride ions was four to five times greater. In order to obtain further qualitative data on the binding of the individual ion species the experimental data were evaluated in the form of Scatchard's plot of $\bar{\nu}e^{2w\bar{\nu}}/C\gamma_{\pm}$ *vs* $\bar{\nu}$. In the expression plotted on the axis of ordinates the electrostatic parameter $w$ was set equal, in the nearest approximation, to the value determined from the titration curves of orosomucoid, no correction being taken for the binding of anions[16]. The values of the mean activity coefficients, $\gamma_{\pm}$, of the salts were taken from Izmajlov's[21] monograph. The curves based on the Scatchard plots for $Cl^-$ and $I^-$ ions enabled us to determine neither the number of binding sites, $n_i$, for these ions, nor the corresponding association constants, $k_i$. An example of such a curve is the Scatchard plot for $I^-$ ions in Fig. 2. Evaluation of this type

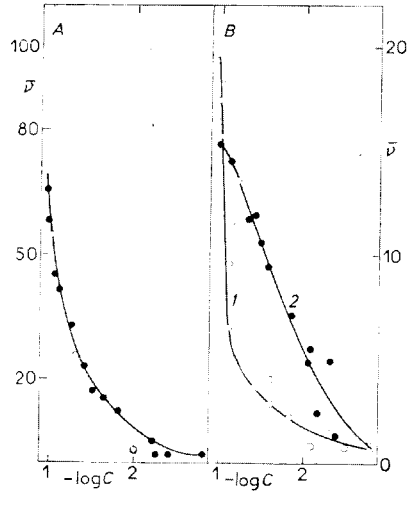


FIG. 1

Binding of Halides to Isoionic Orosomucoid

*A* Binding of $Cl^-$ ions, determined by equilibrium dialysis (○), conductometrically (●). *B* Binding of $Br^-$ ions (○) and $I^-$ ions (●) determined conductometrically.

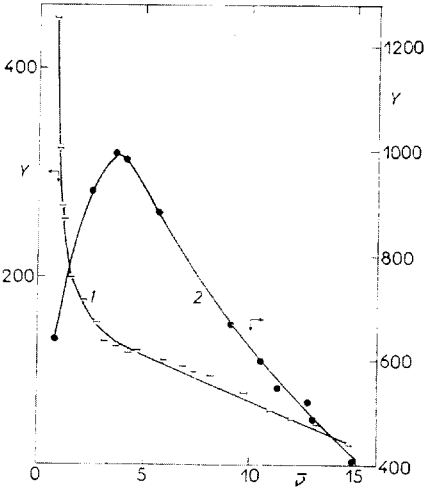


FIG. 2

The Scatchard Plots for the Interactions of $I^-$ (●) and $Br^-$ (∈) ions with Isoionic Orosomucoid; $Y = \bar{\nu}e^{2w\bar{\nu}}/C\gamma_{\pm}$

of plot was possible in the case of bromide ions only (Fig. 2) and the obtained values of $n_i$ and $k_i$ were inserted by the iteration method into the equation

$$\bar{\nu} = \frac{nke^{2w\bar{\nu}}C}{1 + ke^{2w\bar{\nu}}C} \qquad (1)$$

until agreement was reached between the calculated values of $\bar{\nu}$ and the experimental values in Fig. 1B. In equation (*1*) $C$ denotes molar concentration of the free ligand in the solution. The binding sites for $Br^-$ ions on the molecule of orosomucoid can be divided into 3 groups, numbering $n_1 = 1$, $n_2 = 3$ and $n_3 = 20$, the respective association constants being $k_1 = 570$, $k_2 = 40$ and $k_3 = 6$.

*The binding* of $Cl^-$ *and* $Br^-$ *ions.* In the case of $Cl^-$ and $I^-$ ions the form of the Scatchard plot was rather unusual and difficult to interpret. Such a form was observed when the protein was in an association–dissociation equilibrium[22]. However, in the conditions of our experiments association of orosomucoid molecules was very improbable. Owing to the relatively great number of the bound anions the net charge of an orosomucoid molecule was negative and electrostatic repulsion prevented association of the molecules. If we assume the simplest dimerization of the type $2\,A \rightleftharpoons A_2$, Tanford's equation[23] allows us to calculate the contribution of the electrostatic free energy ($\Delta W_{el}$) to the total energy change associated with this reaction. According to this equation

$$\Delta W_{el} = 2^{2/3}\frac{Z^2\varepsilon^2}{DR}\left(1 - \frac{2^{1/3}KR}{1 + 2^{1/3}Ka}\right) - \frac{Z^2\varepsilon^2}{DR}\left(1 - \frac{KR}{1 + Ka}\right), \qquad (2)$$

where $Z$ is the net charge of a protein molecule, $R$ its radius ( a spherical molecule is considered), $a$ the minimum distance between the centres of the molecule and the small ion, $\varepsilon$ the elementary charge, $D$ the dielectric constant of the medium and $K$ the reciprocal value of the Debye width.

If we consider, in this simplification, the molecule of orosomucoid as a sphere of a radius $R = 25$ Å, and the concentration of $Cl^-$ ions is $C = 0{\cdot}001$M ($Z = -1$), the value of $\Delta W_{el}$ results as $+7{\cdot}8$ cal/mol. For a chloride concentration $C = 0{\cdot}1$M ($Z = -50$) $\Delta W_{el} = 16400$ cal/mol. With such a great positive contribution to the total free energy of the considered reaction dimerization of orosomucoid molecules could hardly be energetically preferred.

The fact that no dimerization occurred was confirmed by thin-layer gel chromatography and disc electrophoresis. In either case, at different concentrations of the studied anions, orosomucoid gave a single zone, its mobility corresponding to the monomer. With the present-day insight into the structure of orosomucoid[24] and into the mechanism of protein–anion interactions, curves like the one shown in Fig. 2

are very difficult to interpret. Some light might be cast on this problem by a more detailed study of the orosomucoid variants; these variants differ in some amino acid residues of the polypeptide chain, as was found by Schmid and coworkers[11,25].

*The binding sites.* We have further attempted a closer characterization of the binding sites, particularly those for chloride ions, whose binding is very different from that of the other ionic species. Primarily we took into account the positively charged groups in the orosomucoid molecule. At the isoionic point the number of these groups[11,26] is approximately 24 (9 arg, 3 his, 12·5 lys.). This number is sufficient to explain the binding of $Br^-$ and $I^-$ ions, but not the binding of $Cl^-$ ions, which must also bind to other sites. A possible explanation seems to be the formation of hydrogen bonds to carboxyl groups, *i.e.* —COOH...$Cl^-$. According to Pauling[27] the only possible acceptors of protons are F, O, N and Cl, but other authors[28,29] corroborated the existence of hydrogen bonds between proton donors and halide anions, the strength of the bond having been reported to decrease in the order $Cl^- > F^- > Br^- > I^-$, or $F^- > Cl^- > Br^- > I^-$. In either case the weakest hydrogen bonds are formed by the ions $Br^-$ and $I^-$. Another factor restricting the formation of hydrogen bonds to $Br^-$ and $I^-$ might be the radii of these ions, reported by Pauling[27] to be: $Cl^-$ 1·81 Å, $Br^-$ 1·95 Å, $I^-$ 2·16 Å. The tertiary structure of orosomucoid has not thus far been determined, but it is evident that even sterically the chloride ions are predisposed to the formation of hydrogen bonds. At the isoionic point there are still 16—17 undissociated titratable carboxyl groups, which together

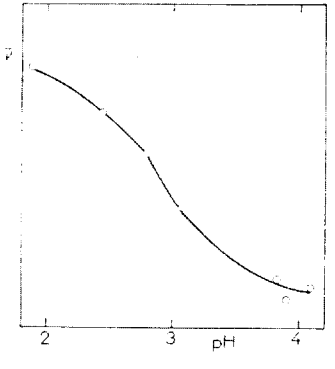


FIG. 3

Binding of $Cl^-$ ions to Orosomucoid from Solutions of 0·1M–NaCl of Different pH Values

With the method employed it was impossible to determine the absolute values of $r$.

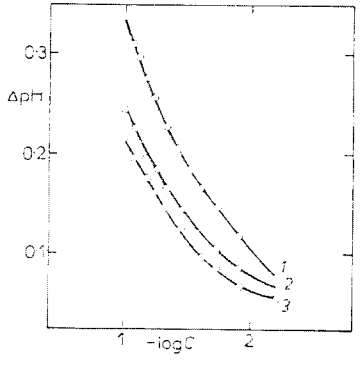


FIG. 4

Changes of the Isoionic Point of Orosomucoid in the Addition of Sodium Chloride *3*, Bromide *2* and Iodide *1*

with the basic groups amount to about 40–41 possible binding sites of $Cl^-$ ions (for experimental data see Fig. 1 and the preceding paper[16]). The potential participation of carboxyl groups in the binding of chloride ions has also been shown by experiments in which HCl was added to a system orosomucoid–NaCl up to pH 2. The data on the binding of chloride ions were evaluated only qualitatively since the exact value of the constant A has not yet been determined by conductance measurements in this case[17]. Nevertheless, Fig. 3 shows close similarity between the curve relating the number of bound $Cl^-$ ions to pH at a constant concentration of free $Cl^-$ ions in the solution (0·1M) and the dissociation curve of an acid with p$K$ between 2·9 and 3·0. The formed curve shows the binding of $Cl^-$ ions only; the calculation has been corrected for the dissociation of the functional groups of orosomucoid. The shape of the curve in Fig. 3 supports our hypothesis that apart from other groups the carboxyl groups of orosomucoid are involved in the binding of chloride ions, since their p$K$ in this protein lies in the given range[16]. If we further consider the 18–19 hydroxyl groups of tyrosine and serine in a molecule of orosomucoid[11] (11 tyr and 7 to 8 ser), undissociated at the isoionic pH, we obtain a total of 58 to 60 possible binding sites of $Cl^-$ ions, which can account for the unusually high interaction with these ions. The hydrogen bond between chloride ions and the hydroxyl groups would probably be weak, but their existence cannot be ruled out.

On the basis of what has been said 2 types of $Cl^-$-orosomucoid bond can be considered: *a*) electrostatic bonds to the positively charged groups, *e.g.* $NH_3^+Cl^-$, *b*) hydrogen bonds to undissociated groups, —COOH...$Cl^-$, —OH...$Cl^-$. The two types of binding of Cl ions must evidently affect the behaviour of $OH^-$ ions produced by dissociation of water.

pH *changes in solutions of the protein.* We followed the pH changes accompanying the addition of a salt to an isoionic solution of the protein. The least changes were observed in the addition of NaCl (Fig. 4), although it was the chloride ions that (out of the three ion species studied) bound to the protein in the largest extent (Fig. 1*A*,*B*). However, the pH changes caused by the individual anions were not very different from each other and increased in the order $Cl^- < Br^- < I^-$. No difference was observed between sodium and potassium salts, which demonstrates that only the anions were responsible for the pH changes. The result illustrated in Fig. 4 can be interpreted if two types of chloride binding are assumed. In the case of the electrostatic bonds (see The binding sites) the positive charge of the functional group is compensated by the charge of a $Cl^-$ ion, so that the hydroxyl anion originally attached by this group gets released into the solution, with a consequent increase of pH. By contrast, the formation of hydrogen bonds entails no liberation of $OH^-$ ions, so that pH remains practically constant.

With bromide and iodide anions only bonds of type *a*) exist and are responsible for the measured changes of pH. On the other hand, $Cl^-$ ions can affect the value

of pH provided they are bound to the protein by electrostatic forces. Since the numbers of ligands bound to orosomucoid electrostatically are comparable in the case of the three anions the observed pH changes (Fig. 4) were not much different.

Further we studied the relation of ΔpH to $\bar{\nu}$. Simple anions obey the well-known equation[30]

$$\Delta\text{pH} = 0{\cdot}868w\bar{\nu} \tag{3}$$

but the values of $w$ calculated from this equation for orosomucoid, using experimental values of ΔpH and $\bar{\nu}$, seemed to be independent of the salt concentration, or rather increased with it in some cases. Since the simple equation (*3*) gave improbable values of $w$ we introduced a correction for dissociation of the functional groups of orosomucoid, accompanying the changes of pH. The isoionic point of orosomucoid lies on the steep part of the titration curve of this protein[14], so that even a slight pH change is accompanied by dissociation of a certain number of functional (here carboxyl) groups. This means that the second term of the equation describing the titration curve manifests itself more markedly. This equation is

$$\text{pH} - \log \frac{x_i}{n_i - x_i} = (\text{p}K_0)_i - 0{\cdot}868wZ\,, \tag{4}$$

where $x_i$ designates the number of functional groups (with the internal dissociation constant $K_0$) that dissociated from the total number of these groups, $n_i$, and $Z$ denotes the net charge of the protein molecule.

The corrected equation (*3*) for anions assumes the form

$$\Delta\text{pH} + \log \frac{x_2(n_i - x_1)}{x_1(n_i - x_2)} = 0{\cdot}868w\,\Delta Z\,, \tag{5}$$

where $x_1$ and $x_2$ are the numbers of dissociated groups $i$ before (*1*) and after (*2*) the addition of a salt, $\Delta Z$ the accompanying change in the net charge. The value of $\Delta Z$ is the resultant of the binding of anions and the dissociation of functional groups.

Table I gives the values of the second term of equation (*5*) (designated as $\log X$) and of the electrostatic parameter $w$ calculated from the same equation.

As can be seen, even the values of $w$ calculated from the corrected equation (*5*) for individual anions are rather different, but it was interesting to compare them with the theoretical values of the electrostatic parameter, $w_1$ and $w_2$, also given in Table I. These values were calculated from the equations[23] for the calculation of the electrostatic free energy of a spherical protein molecule. The value of $w_1$ corresponds to a molecule of a molecular weight 40000 and radius $R = 25$ Å,

TABLE 1

Comparison of the Second Term of Eq. (5) with Experimental Values of the Electrostatic Factor $w$ for Halide–Orosomucoid Interactions

| Halide | $C$, mol/l | $\bar{v}$ | ΔpH | log $X$ | $w$ | $w_1$ | $w_2$ |
|---|---|---|---|---|---|---|---|
| $Cl^-$ | 0·01 | 6 | 0·06 | 0·05 | 0·021 | 0·161 | 0·054 |
| | 0·05 | 26 | 0·15 | 0·14 | 0·013 | 0·111 | 0·021 |
| | 0·1 | 55 | 0·22 | 0·19 | 0·0086 | 0·093 | 0·015 |
| $Br^-$ | 0·01 | 1·6 | 0·07 | 0·08 | 0·108 | 0·161 | 0·054 |
| | 0·05 | 4·5 | 0·18 | 0·15 | 0·086 | 0·111 | 0·021 |
| | 0·1 | 15 | 0·25 | 0·32 | 0·044 | 0·093 | 0·015 |
| $I^-$ | 0·01 | 5·5 | 0·11 | 0·12 | 0·048 | 0·161 | 0·054 |
| | 0·05 | 13 | 0·24 | 0·30 | 0·047 | 0·111 | 0·021 |
| | 0·1 | 16·5 | 0·34 | 0·49 | 0·058 | 0·093 | 0·015 |

the value of $w_2$ to a spherical molecule (partly permeable for the solvent) of the same molecular weight. The radius of the molecule $R_0 = 50$ Å, the radius of the solvent impermeable core $R = 25$ Å. The values of both $w_1$ and $w_2$ refer to 25°C. Comparison of the values of $w$ with those of $w_1$ and $w_2$ reveals that with $Cl^-$ and $I^-$ ions the differences between $w$ and $w_2$ are smaller then those between $w$ and $w_1$. The values obtained with $Br^-$ ions are rather similar to those of $w_1$. Although these data represent a rough approximation only (since the orosomucoid molecule is not a spherical[25] one) it seems justified to regard the orosomucoid molecule as one partly permeable to solvent.

This structure of the molecule would seem to account for some physico-chemical and chemical properties of orosomucoid. If the molecule does have an outer envelope well permeable for the solvent then the parts of the polypeptide chain occurring in it can well interact with water. This interaction would help to account for the unusual good solubility of orosomucoid in water, despite the great number of hydrophobic residues in the molecule[11]. At the same time the considerable number of masked groups could be attributed to the compact, probably hydrophobic core of the molecule.

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REFERENCES

1. Winzler R. J., Dewor A. W., Mehl J. W., Smyth I. M.: J. Clin. Invest. *27*, 609 (1948).
2. Schmid K.: J. Amer. Chem. Soc. *72*, 2816 (1950).
3. Weimer H. E., Mehl J. W., Winzler R. J.: J. Biol. Chem. *185*, 561 (1950).
4. Schmid K.: J. Amer. Chem. Soc. *75*, 60 (1953).
5. Schultze H. E., Göllner L., Heide K., Schönenberger M., Schwick G.: Z. Naturforsch. *10b*, 463 (1955).
6. Popenoe E. A., Drew R. M.: J. Biol. Chem. *228*, 673 (1957).
7. Kalous V.: Biochim. Biophys. Acta *107*, 139 (1965).
8. Eylar E. H., Jeanloz R. W.: J. Biol. Chem. *237*, 622 (1962).
9. Eylar E. H., Jeanloz R. W.: J. Biol. Chem. *237*, 1021 (1962).
10. Kalous V., Poncová M.: This Journal *30*, 737 (1965).
11. Schmid K., Kaufmann H., Isemura S., Bauer F., Emura J., Motoyama T., Ishiguro M., Nanno S.: Biochemistry *12*, 2711 (1973).
12. Bezkorovainy A., Winzler R. J.: Biochim. Biophys. Acta *49*, 559 (1961).
13. Schultze H. E., Heremans J. F.: *Molecular Biology of Human Proteins*, p. 188. Elsevier, Amsterdam 1966.
14. Yamagami K., Labat J., Pandey R. S., Schmid K.: Biochemistry *7*, 2873 (1968).
15. Karpenko V.: Unpublished results.
16. Karpenko V., Pavlíček Z., Kalous V.: Biochim. Biophys. Acta *154*, 245 (1968).
17. Karpenko V., Kalous V., Pavlíček Z.: This Journal *33*, 3457 (1968).
18. Hládek L.: Chem. listy *60*, 238 (1966).
19. Scatchard G., Scheinberg H. J., Armstrong S. H.: J. Amer. Chem. Soc. *72*, 535 (1950).
20. Davis B. J.: Ann. N.Y. Acad. Sci. *121*, 404 (1964).
21. Izmajlov N. A.: *Elektrochimija Rastvorov*, p. 128. Charkov 1959.
22. Vodrážka Z.: Chem. listy *60*, 938 (1966).
23. Tanford C.: *Physical Chemistry of Macromolecules*, Chap. 7. Wiley, New York 1966.
24. Schmid K.: Chimia (Aarau) *26*, 405 (1972).
25. Schmid K., Tokita K., Yoshizaki H.: J. Clin. Invest. *44*, 1394 (1965).
26. Gotschalk A.: *Glycoproteins*, Sec. 4B. Elsevier, Amsterdam 1966.
27. Pauling L.: *Die Natur der Chemischen Bindung*, Kap. 12. Verlag Chemie, Weinheim 1968.
28. West R., Powell D. L., Whatley L. S., Lee M. K. T., von R. Schleyer P.: J. Amer. Chem. Soc. *84*, 3221 (1962).
29. Allerhand A., von R. Schleyer P.: J. Amer. Chem. Soc. *85*, 1233 (1963).
30. Steinhard J., Reynolds J. A.: *Multiple Equilibria in Proteins*, p. 74. Academic Press, New York 1969.

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