BEHAVIOUR OF HUMAN BLOOD SERUM OROSOMUCOID (ACID α_1-GLYCOPROTEIN) IN THE PRESENCE OF SMALL ALIPHATIC ALCOHOLS

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Dedicated to Professor Otto Wichterle on the occasion of his 80th birthday.

The influence of both methanol and ethanol on the stability of orosomucoid molecule was studied over a broad range of alcohol concentrations (volume fraction 0 – 70%), and pH between 7 and 12. The data obtained by optical methods were compared with information from prediction studies which were focused on the structural features and physicochemical parameters of amino acids in the orosomucoid molecule. From this analysis the following conclusions can be drawn: (i) ethanol exerts a more pronounced effect on α-helix formation than methanol does, (ii) at higher pH the electrostatic contribution is the crucial effect in the destabilization of the orosomucoid molecule, (iii) out of 12 phenylalanines in the orosomucoid molecule only 1 – 2 are exposed to the solvent; of the residues which are not subject to substitution appears to be Phe 141, (iv) its neighbor, Tyr 142, appears to be a key residue, the dissociation of which destabilizes one of two longer helical segments of the orosomucoid molecule, (v) the limits of applicability of the fourth-derivatives of UV spectra with respect to phenylalanines were examined; analysis of the data is recommended on a more precise mathematical basis, taking into consideration the bandwidth of the spectral peaks.

Orosomucoid (acid α_1-glycoprotein; ORS throughout this paper) is a glycoprotein of the α_1-globuline fraction of human blood serum. This protein has attracted attention since the first successful isolation, when its rather remarkable properties came to light. They include, in the first place, high thermal stability and excellent solubility in water; from the physicochemical point of view the very low isoelectric point, varying between 1.8 and 2.7, is striking (for detailed information see reviews by Schulze and Heremans[1], and by Jeanloz[2]). The ORS molecule is made up of one chain consisting of 181 amino acids, with substitutions possible at 21 sites. Five heteropolysaccharide units are attached to asparaginyl residues in the first half of this chain[3]; two disulfide bonds[4] are formed by cysteines 5 – 147, and 72 – 164. Hydrophobic residues are distributed unevenly along this chain where marked hydrophobic regions between residues 1 – 15, 85 – 105, and 140 – 147 are observed, while the C-end from the residue 160 is strongly hydro-

philic[5]. The three-dimensional structure of ORS seems to be a compact one, which results in a rather high number of masked residues: 9 tyrosyls[6], 10 carboxyls[7] and two tryptophyls[8] in the native state. Prediction[5,9] of secondary structures based on primary structure suggests 21% α-helix and 21% β-sheet, the first value being in good agreement with the data from CD spectra of this protein[5]. As found previously the α-helix content increases in the presence of methanol, with additional masking of 1 tyrosine residue[5]. There are 12 phenylalanines in the ORS molecule, but six of them are in positions with possible substitution[3]. The aim of the present work was to continue the study of the ORS molecule in the presence of methanol and ethanol in alkaline media. At the same time an attempt was undertaken to find general rules for the application of the fourth-derivative spectra.

EXPERIMENTAL

Materials

Human serum orosomucoid was isolated from Cohn's Fraction VI of human blood serum (IMUNA, Šarišské Michaľany, The Slovak Republic) using a fractionation method on CM-cellulose developed in our laboratory[10]. Prior to this isolation the Fraction VI was desalted on a Sephadex G-25 column. The protein prepared in this way did not contain a significant quantity of impurities, and was homogeneous as checked by polyacrylamide gel electrophoresis and by immunoelectrophoresis. Redistilled water was used in all the experiments. The concentration of the protein solutions was determined from absorbance at 280 nm ($E_{1\%,280} = 8.9$; ref.[1]), or by drying a small sample of given solution to the constant weight (in vacuo at 105 °C above P_2O_5). The value 41 000 was accepted for the molecular mass of ORS (ref.[11]). *N*-Acetyl-L-tyrosine ethyl ester (NATyrE, Serva, Heidelberg), and L-phenylalanine (Phe, Sigma, Deisenhofen), all of analytical grade purity, served as model compounds. In spectroscopic measurements methanol and ethanol of spectroscopy grade were used; throughout this paper their content is given as a volume fraction ϕ (%). All inorganic reagents were of analytical grade purity as well.

Methods

Fourth-derivative spectrophotometry (FD spectrophotometry). This method, described by Padros et al.[12], was examined for water–methanol systems in order to get a picture of the possible applications of this technique to weaker chromophores, like phenylalanines (for theory see below). FD spectra were recorded with a Philips PU 8800 spectrophotometer over wavelengths ranging from 240 to 350 nm; the optical path was 1 cm. The concentration of ORS was $5 \cdot 10^{-5}$ mol dm^{-3}, of the model compounds: NATyrE $3 \cdot 10^{-4}$, and Phe $5 \cdot 10^{-3}$ mol dm^{-3}. All the FD-spectra were taken at 25 °C.

Temperature perturbation difference spectrophotometry (TPD spectrophotometry). These spectra were recorded with a Specord M 40 spectrophotometer (Zeiss, Jena); temperature was maintained by a thermostat or by a temperature regulating block TSA 1 (Zeiss, Jena). Temperature was varied over the range 20 – 40 °C with the precision ±0.1 °C; the reference sample was kept at 20 °C. Concentrations of the model compounds were as follows: NATyrE $1.1 \cdot 10^{-6}$, Phe $3.0 \cdot 10^{-4}$ mol dm^{-3}.

The number of exposed chromophores was calculated in the usual way using a system of two equations, both of the following general form:

$$x\left(\frac{\Delta\varepsilon_\lambda}{\Delta T}\right)_{PHE} + y\left(\frac{\Delta\varepsilon_\lambda}{\Delta T}\right)_{TYR} = \left(\frac{\Delta\varepsilon_\lambda}{\Delta T}\right)_{ORS}. \quad (1)$$

In these equations x and y denote the numbers of exposed phenyalanines and tyrosines, respectively. For calculation of the number of exposed residues, the values of $\Delta\varepsilon_\lambda$ at the wavelength λ_1 were substituted into the first equation, at λ_2 into the second one. In this calculation tyrosine residues were chosen as a kind of reference since their exposed number had been determined previously by the same method[5,13]. Comparison of our results with these data allowed us to decide whether the values calculated in this series of experiments were reasonable. Correction of the TPD spectra for the thermal increment of absorption was made according to Demchenko and Zyma[14].

CD Spectrometry. These experiments were carried out in the same way as described previously[5] using a Jasco ORD/UV-5 spectropolarimeter with a CD module. Estimation of the α-helix content was made with the aid of the equation given by Greenfield and Fasman[15].

Determination of pH. pH was measured with an OP-265 pH-meter (Radelkis, Budapest) using a combined electrode OP-0808 P made by the same manufacturer. In mixed solvents pH-meter readings should be corrected for solvent effects on the activity coefficient of H_3O^+ ions as well as for its direct influence on the electrodes. In this work correction of the pH-meter readings was made as described previously[5].

Prediction Methods

In order to estimate the probability of a certain properties or of a certain behaviour of individual residues attempts were made to predict these parameters on the basis of the primary structure of ORS. Several sets of data characterizing the physicochemical properties of individual amino acids were chosen so as to get information about hydrophobicity and the degree of exposure of these residues.

Mathematical treatment of these data was based on the calculation of a sliding average (SA7; the number indicates the length of the window) and a weighted sliding average (WSA). The second type of calculation was applied because the influence of more distant residues upon the central one decreases with distance. Since no detailed knowledge of the structure of the ORS molecule is available, except for helical regions and β-sheets[5,9], in this calculation the simplest possibility was chosen. For the central residue 100% of the parameter was taken, for the residues +1 and −1 only 75% of the original value, for the residues +2, −2, 50%, and eventually for +3 and −3 a contribution of 25% was considered. In this calculation the window was thus always 7.

This method of calculation must be used with necessary care since the most reasonable information can be obtained in the regions where classical secondary structures (helix, sheet) are not expected. In structures of this kind the contribution of other neighboring groups should be considered.

Theoretical Introduction to the Fourth-Derivative Spectra

Papers[16,17] describing the basic principles of the fourth-derivative spectra of proteins have focused on the fact that an important role in the interpretation of these curves is played by the bandwidth of the individual peaks. No further discussion or mathematical treatment of this problem was presented. As found in our experiments, however, this issue can be of particular importance.

In a general approach an isolated absorption band can be approximated by a Gaussian curve[18] (Fig. 1*a*)

$$A_\lambda = A_{\lambda_0}\left[\frac{-(\lambda-\lambda_0)^2}{b}\right], \quad (2)$$

where λ_0 is the wavelength of the absorption maximum, A_λ and A_{λ_0} are absorbancies at λ, and λ_0, respectively; the parameter b is derived from the half-width d of an absorption band:

$$b = \frac{d^2}{4} \ln 2 . \tag{3}$$

The substitution of $x = \lambda - \lambda_0$ into Eq. (2) leads to the following expression

$$A_\lambda = A_{\lambda 0} \exp\left(- \frac{x^2}{b} \right), \tag{4}$$

the fourth-derivative of which is

$$A_\lambda^{(4)} = A_{\lambda_0} \left[\left(\frac{2}{b} \right)^4 x^4 - 6 \left(\frac{2}{b} \right)^3 x^2 + 3 \left(\frac{2}{b} \right)^2 \right] \exp\left(- \frac{x^2}{b} \right). \tag{5}$$

The fourth-derivative of the curve shown in Fig. 1*a* has a more complicated shape (Fig. 1*b*) which, nevertheless, makes it possible to calculate the value of A_{λ_0} as

$$A_{\lambda_0} = \frac{h_2 d_{12}^4}{48 (5 - \sqrt{10})^2} = 6.17 . 10^{-3} h_2 d_{12}^4 \tag{6}$$

or

$$A_{\lambda_0} = \frac{h_2 x_1^4}{192 \ln^2 2} = 9.87 . 10^{-2} h_2 x_1^4 . \tag{7}$$

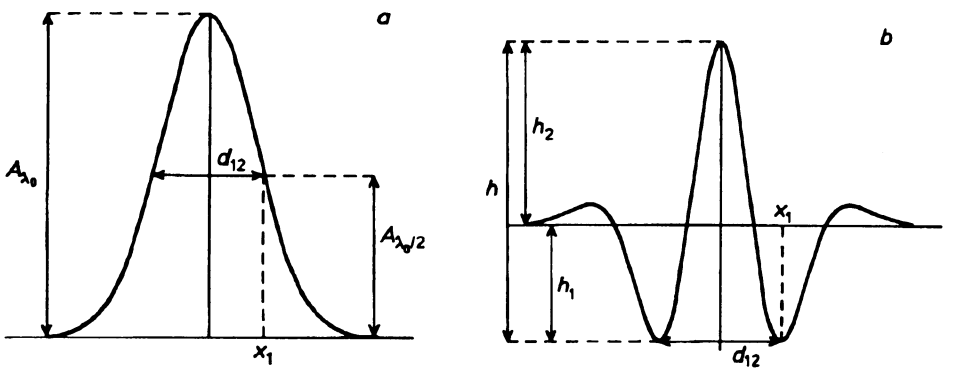

FIG. 1
The Gaussian curve (*a*) and its fourth-derivative (*b*)

In this approach the value h_2 (the height of the maximum above the zero line) must be determined. For practical reasons it is more suitable to estimate the total height h (Fig. 1*b*). Then

$$A_{\lambda_0} = 6.1 \cdot 10^{-2} h x_1^4 . \qquad (8)$$

Equation (*8*) was therefore used in this work. It is obvious that the main difficulty of this approach consists of the precise estimation of x_1 since this value has a decisive effect on the resulting A_{λ_0}. The Eq. (*8*) can generally be used in all cases where the bandwidth varies, a rather common feature in protein spectra.

RESULTS

Fourth-Derivative Spectra

The advantage of this method lies in its ability to reveal individual absorption bands, even weak ones such as that of phenylalanine, as compared with other UV-chromophores, tyrosine and tryptophan. For a model compound, phenylalanine, a typical spectrum was observed, similar to that described by Padros et al.[16], with three main peaks. Their position, however, was not significantly influenced by methanol. Only a small red shift of the order of 1 nm was observed which appeared at alcohol contents higher than 30%.

In ORS the picture was quite different (Fig. 2): in the region 245 – 270 nm only one main peak at approximately 250 nm was observed, while the second, a very small one, was located between 260 and 270 nm. Sometimes a small shoulder appeared on the longer- wavelength side of the main peak. As shown by Ichikawa and Terada[19] tyrosine and tryptophan do not exert any influence on the second derivative spectrum of phenylalanine over the range of 245 to 270 nm. There is no reason to expect any different behaviour in FD spectra.

The position of the peak at 250 nm did not change at pH of 5 and 7 over the range of methanol content ϕ between 0 and 70%, while the peak at 294 nm of the same spectra, originating from both tyrosine and tryptophan, was blue-shifted by 1 – 2 nm, indicating a slightly higher interaction of these residues with the more polar solvent, in this case with the water–methanol system as compared with the less polar interior of the protein molecule.

Analysis with regard to the shape of the peaks made it apparent that any calculation of the precise values of A_{λ_0} would involve a certain degree of error. The analysis was therefore limited to a comparison of individual curves; values on the *y*-axis were expressed in arbitrary units.

Because of the differences in the total height of the FD peaks on their left and right side (Fig. 2), two sets of values were used whenever possible: h_L (left side), h_R (right side), and correspondingly A_L, and A_R. For phenylalanine the peak at 250 nm was

examined in order to obtain data comparable with the corresponding peak in the spectrum of ORS. As shown in Fig. 3, h_L as well as A_L calculated from the Eq. (*8*) yielded curves of identical shape, the curves for h_R and A_R differed only slightly from each other. This result leads to the conclusion that in this particular case the height h is a sufficient parameter for characterization of this peak, since no band broadening occurs. There is, however, a small maximum on all the curves (Fig. 3) which appears at methanol contents ϕ between 40 and 60% and is more pronounced for h_L and A_L. Its coincidence with the extreme on the dependence of the excess enthalpy of mixing for the water–methanol system on the alcohol concentration (see Discussion) indicates that the structural changes of the solvent are reflected in this way by the spectrum.

With ORS the picture differed substantially. Only the right-hand side values of h could be used, since on the left side a strong peptide absorption band at shorter wavelengths distorted the shape of the FD curve to a significant extent. When the values of h_R for the peak at 250 nm were plotted as a function of methanol content (Fig. 4), no change was observed at pH of 7 or 10, while at pH 12 the curve was shifted to higher values with a marked maximum at methanol contents ϕ between 50 and 60%. Calculation of the same data using Eq. (*8*) produced different results – only the curve for pH 7 was similar to the previous plot (Fig. 5). The other two curves, for pH of 10 and 12,

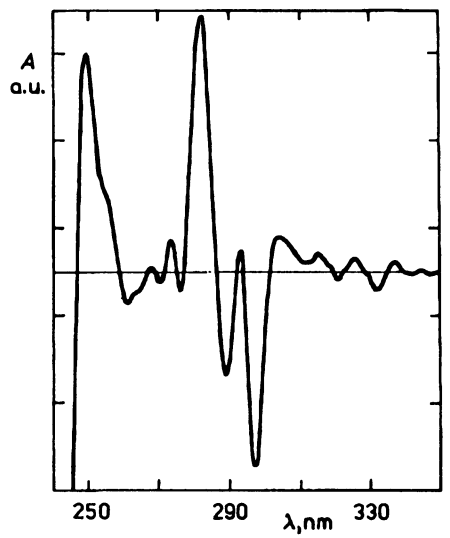

FIG. 2
The fourth-derivative of the absorption spectrum of ORS in water at pH 10

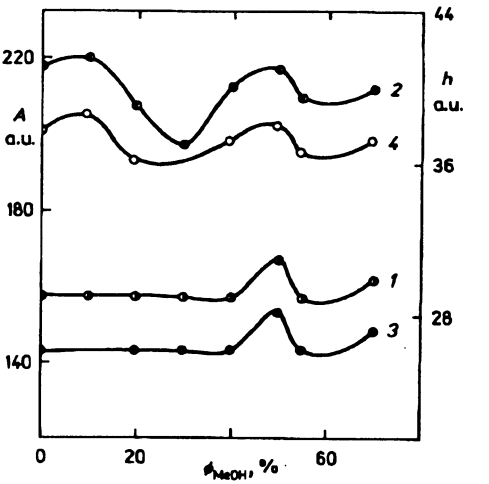

FIG. 3
The values of h_L, h_R, resp. A_L, A_R for the absorption maximum of phenylalanine at 250 nm in the presence of methanol at pH 12: *1* h_L, *2* h_R, *3* A_L, *4* A_R

exhibited a sharp maximum located again at methanol contents ϕ between 40 and 60%. If the two figures (Figs 4 and 5) are compared it is obvious that only at pH 7 the height h can be used directly. At pH 10 the peak of the ORS spectrum at 250 nm was broadened just in the abovementioned concentration range of methanol. Interpretation of the curves in both figures with respect to pH 12 is difficult and rather speculative. The most probable explanation is that this effect results from a simultaneous increase in absorbance and broadening of the peak.

Temperature Perturbation Difference Spectra

An attempt was undertaken to compare the abovementioned results with the data obtained by a different method. The number of exposed chromophores can be determined by TPDS, but for phenylalanines this method is not as sensitive as for the other two aromatic chromophores, tyrosine and tryptophan. It is difficult to find suitable wavelengths for the determination of exposed phenylalanines as is apparent just from the FD of the ORS spectrum, since in the region where both phenylalanine and tyrosine absorb, their absorbance is very weak. In the region of the main peak at 250 nm the contribution to absorbance originates from phenylalanines only; the low peak between 260 and 270 nm appeared as the sole possibility.

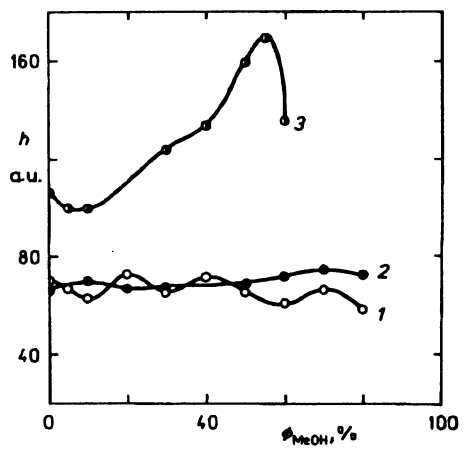

FIG. 4
The values of h_R of the maximum at 250 nm of the fourth-derivative spectrum of ORS in water–methanol system at various pH: *1* 7, *2* 10, *3* 12

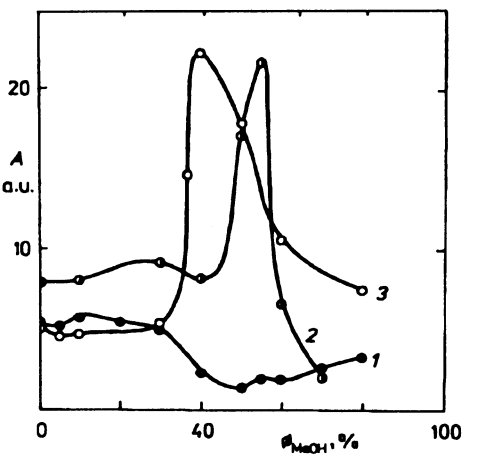

FIG. 5
The values of A_R of the maximum at 250 nm of the fourth-derivative of the spectrum of ORS in water–methanol system. pH: *1* 7, *2* 12, *3* 10

In the course of this work several combinations of wavelengths were tried and their effectivity was judged by comparison with the number of exposed tyrosines found previously[5] in combination with tryptophans. In this way the wavelength pairs of 262/266 nm and 262/269 nm were found to be the most suitable. Even with these sets, only the data for pH 7 led to reasonable values of exposed phenylalanines (Table I).

In ethanol, a similar attempt was undertaken with the resulting number of exposed phenylalanine residues varying between 0.5 and 1.5. In both cases a slight increase in this number was observed in the presence of alcohol.

CD Spectra

In the presence of ethanol ORS yielded principally the same picture as with methanol[5]: an increase in α-helix content with increasing alcohol concentration. This increase was observed from ϕ_{ETOH} approximately 20%, i.e. from a value slightly lower than in methanol, and appeared at each pH examined, i.e. 7, 10, and 12. The study of pH-effects on ORS at constant ethanol content yielded a rather unexpected result: in spite of the accuracy of all the measurements, the data from the CD spectra at ϕ_{ETOH} 20% could not be evaluated due to broad scattering of results. Thus at this ethanol content no estimation of α-helix content was made, and further experiments are necessary to explain this anomaly, which will be discussed below.

For the remaining concentrations of ethanol (ϕ_{ETOH}), 40 and 70%, the α-helix content of ORS exhibited similar pH dependence – a rather broad maximum at pH 9. In 70% ethanol, however, after a decrease in the α-helix content between pH of 9 and 10.5 a further increase was observed (Fig. 6).

The influence of methanol and ethanol on the α-helix formation was compared (Fig. 7) at alcohol concentrations which are close to the extreme on the dependence of excess enthalpy of mixing of particular alcohol with water[20]. It is apparent that the helix-forming ability increases from methanol to ethanol, and, at the same time, pH extreme is more pronounced in ethanol. For both alcohols this maximum was shifted to higher pH with respect to water.

TABLE I
The number of exposed phenylalanine residues at pH 7 at different methanol content

Methanol content ϕ, %	The number of residues
0	0.3
30	1.2 – 1.3
55	1.5 – 1.7
70	1.8

Prediction Studies

The aim of these studies was the critical consideration of experimental data concerning the behaviour of phenylalanines. In any discussion of this particular problem attention should be turned to the fact that phenylalanines are the only amino acids in ORS with a rather high possibility of substitutions. It is thus advantageous to divide them into two groups. In Group I are residues which are single in their positions, while Group II consists of residues where a substitution by other amino acids is possible. So Group I consists of residues 26, 48, 49, 51, 61, and 141; Group II of residues 32, 73, 98, 112, 114, and 126 (according to ref.[3]). It is immediately apparent that five out of the six residues of Group I are localized in the first third of the ORS chain. This region is generally rather hydrophobic[5].

The prediction approach was chosen for the calculation of the buried surface using the data given by Rose[21]. Both SA7 and SAW yielded seemingly very similar results, yet their expression in a tabular form (Table II) showed a marked shift of SAW to the higher values.

Concerning the ORS molecule, out of Group I only Phe 141 is located in a helical region; of Group II, Phe 98, and to a certain extent Phe 32 located just on one end of a probable short helix (consisting obviously of one turn).

The same calculation was performed using the data on octanol/water distribution of amino acids according to Fauchère and Pliška[22], and using the solvation energies given

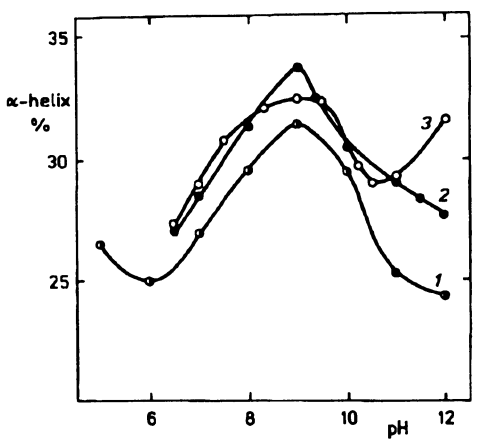

FIG. 6
α-Helix content (%) of ORS as a function of pH; ethanol content ϕ_{ET} (vol.%): *1* 0, *2* 40, *3* 70

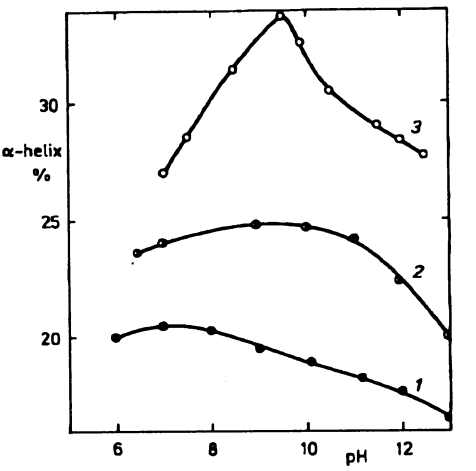

FIG. 7
α-Helix content (%) of ORS as a function of pH in different solvents: *1* water, *2* methanol (ϕ 55%), *3* ethanol (ϕ 40%)

by Eisenberg and McLachland[23]. In all these procedures a marked difference was observed for those phenylalanines in Group I which are not located in expected helical regions: the weighted values of the parameters expressing their hydrophobicity and the values of the solvation energies were always higher than the values of the usual sliding average. With regard to the distribution of individual phenylalanines these results were in very good agreement with the data on the average buried surface given in Table II.

DISCUSSION

In examining the behaviour of proteins in water–small aliphatic alcohol systems, several points must be taken into consideration. The most important of them are: the structure of water–alcohol mixtures, the hydration of proteins, and the binding of alcohols to proteins. In spite of recent intensive investigation of these problems not much can be said; the majority of data is still on a rather qualitative level. The structure of water–alcohol mixtures has been discussed at length by Franks[24], and later by Franks and Desnoyers[25]. Typical of these systems is the excess enthalpy of mixing (ΔH_{ex}), which exhibits a minimum for both methanol and ethanol[20], for methanol a rather broad one reaching its lowest value at the molar fraction $x \approx 0.35$ (ϕ 55%); for ethanol this minimum is sharper, situated at $x \approx 0.15$ (ϕ 40%). In our work particular attention was paid just to these values. The critical concentration x_c of alcohols is an important parameter: below this value alcohols interfere destructively with the low density domains in water, above this value a clustering of alcohols prevails[25]. For ethanol this concentration was found[25] to be $x_c \approx 0.08$ (ϕ 23%). Concerning methanol solutions, Tanaka et al.[26,27] pointed out the water–water interactions in these solutions. Hydration of amino acids and proteins has been discussed repeatedly[28 - 30], the results, however, are comparable on a qualitative level only. The same applies to the data on the binding of small alcohols to proteins[31].

In light of these facts our data should be discussed as a result of multifactorial influence where the contribution of individual factors cannot be estimated with the

TABLE II
Average buried surface of phenylalanine residues in orosomucoid (numerical values according to Rose et al.[32]; bold numbers denote the Group I of phenylalanines)

The fraction of buried surface	Sliding average; window 7	Weighted sliding average; window 7
>0.79		**48**, **49**, 112
>0.76	**26**, **48**, **49**, **51**, **61**, 112, 114, 126	**26**, **51**, **61**, 73, 98, 114, 126
>0.73	73, 98, **141**	32, **141**
>0.70	32	

necessary precision. For both methanol and ethanol their binding to ORS by hydrogen bonds as well as via hydrophobic interactions should be expected. Changes in the water structure together with decreasing permittivity represent the second contribution to the final picture observed on the experimental level.

Generally, the effect of ethanol is the same as that of methanol – i.e. an increase in α-helix content with increasing alcohol concentration. As apparent from Fig. 7 ethanol has even greater helix-forming ability. In both alcohols there are concentrations which appear to elicit extreme behaviour. In methanol it is the region of ϕ_{MEOH} between 30 and 50% (ref.[5]); in the present work a marked deviation from normal behaviour was observed for $\phi_{ETOH} \approx 20\%$. This value coincides fairly well with $x_c \approx 0.08$ (23%), the concentration when the structure of water is significantly changed by ethanol. According to Franks and Desnoyers[25], at $x_{ETOH} \approx 0.06$ this alcohol acts on water in the same manner as does a rise in the temperature. These changes in bulk water structure are obviously reflected to a certain extent by the hydration layer of proteins with a subsequent loss of stability. This mechanism yields an explanation of why the CD spectra of ORS in 20% ethanol did not yield reproducible results. Anomalous behaviour of proteins in the presence of 20 vol.% ethanol was observed in β-lactoglobulin–retinol system[32].

The FD spectra examined in this work were shown to be of use provided that band broadening is included in their mathematical treatment. Though changes in the behaviour of chromophores can then be enhanced, the interpretation of these data is possible only jointly with other methods. In our work the FD spectra at pH 12 in 70% ethanol together with the CD spectra yielded one important result. As apparent from Fig. 6 the curves for water and for water–ethanol systems are of very similar shape with a maximum at pH approximately 9. This value should be a matter of further investigation. The only difference is in ϕ_{ETOH} 70% when the second increase in α-helix content is observed at higher pH, approximately above 10.5. This is obviously the effect of an alcohol denaturation, a process occurring when a protein is destroyed to a greater extent by the alkaline medium and assumes a more randomly coiled structure, but, at the same time, segments of this coil form helical structures under the influence of alcohol[33]. This observation is supported by FD curves which are anomalous under exactly the same experimental conditions.

One general conclusion can be drawn from all the experiments, that anomalous behaviour of ORS coincides with extremes of ΔH_{ex} vs alcohol concentration curves. This observation only supports the hypothesis that the hydration layer of proteins is highly influenced by alcohols. At present, though no detailed scheme of this mechanism can be given, there are, nevertheless, signs that in the case of ethanol, competition between this alcohol and the water of the protein hydration layer takes place[34]. A further question to be solved in the future is the effect of higher alcohols (1-propanol, 1-butanol) from the point of view of their increasing hydrophobic portion. The prevailing hydro-

phobic interaction could have exerted an effect on the conformational changes of the protein as observed in HSA (ref.[35]).

In terms of the stability of the protein molecules, electrostatic interactions are of particular importance because of their long-range effects. In the presence of alcohols these interactions are enhanced due to a decrease in the permittivity of the solvent. From this point of view the influence of alcohols on the formation of α-helix as a function of the net charge Z of the protein molecule is worth examining (Fig. 8). Although the permittivities of both alcohol solutions were almost the same ($\varepsilon_{MEOH} \approx 60$, $\varepsilon_{ETOH} \approx 63.5$; both at 20 °C, ref.[36]), the curves differed substantially from the original ones shown in Fig. 7. For the interpretation of Fig. 8 it was necessary to make a simplified assumption – the net charge Z was supposed to be the same in water and in the water–alcohol solution since no data are available on the proton binding to ORS in mixed solvents of this kind. Therefore the data from the titration curves of ORS in water were used[7].

In water and water–methanol systems a decrease in the α-helix content was almost linear from $Z \approx -28$. A gradual loss of the secondary structure is obviously the result of the stronger electrostatic repulsion due to the increasing net negative charge. In the presence of ethanol a similar decrease was observed, yet not as linear as in water or methanol; at higher Z this decrease was slightly slower. This effect can be ascribed to the additional stabilizing influence of ethanol (alcohol denaturation, ref.[33]). The maximum α-helix content was in the presence of ethanol over an extremely narrow range, with Z approximately between −22 and −25. Compared with the data from Fig. 7 this region corresponds to pH ≈ 9.5. According to our previous results[6] there are three tyrosine residues with pK 9.9 in the ORS molecule. Their dissociation could have been the

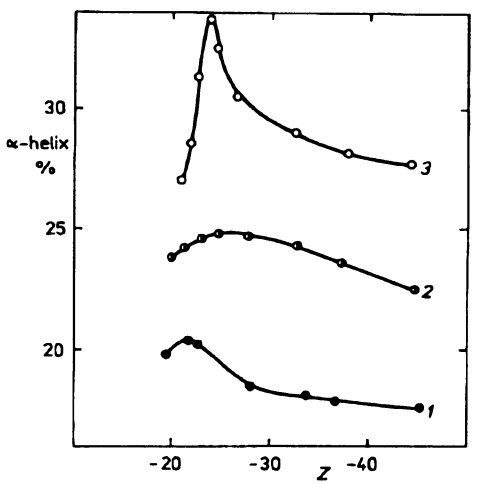

FIG. 8
α-Helix content (%) as a function of the net charge Z of ORS molecule in different solvents: *1* water, *2* methanol (ϕ 55%), *3* ethanol (ϕ 40%)

crucial moment in α-helix formation and destruction. On a closer look at a probable helical formation[5] in the ORS molecule between residues 135 – 146, the tyrosine residue in question could be Tyr 142. In this segment of the predicted helix are residues Glu 140, Phe 141, Tyr 142, and Glu 143. At alkaline pH glutamic acid residues are negative so that the dissociation of Tyr 142 further increases this charge. Electrostatic repulsion is enhanced simultaneously since the permittivity is lowered in the presence of alcohol.

In this work particular attention was paid to phenylalanine residues. The results of TPDS lead to the conclusion that the majority of them are masked in the ORS molecule, and FD spectra indicate that the state of the phenylalanines is not significantly influenced by the presence of methanol or ethanol. Taking into consideration the lower sensitivity of spectral methods with respect to phenylalanines the number of these residues exposed to the solvent varies between one and two.

For further discussion of the state of phenyalanine residues in ORS prediction studies must be consulted. In all of them Phe 141 is the only residue from both Group I and Group II which exhibits extreme values, be it low hydrophobicity or high exposure to the solvent as compared to the rest of the phenylalanines. Since this residue is located in an expected helical segment, the values of SW7 should be considered to be more reliable. The higher exposure of Phe 141 to the solvent is in accord with the fact that its direct neighbour, Tyr 142, could have been the residue which, by its dissociation, exerts an important influence on the destruction of this helical formation. In other words this tyrosine residue obviously is not in the masked group. Then Phe 141 can be expected to be the one phenylalanine exposed to the solvent. The other is very probably Phe 32, which however, is located in a position with a possible substitution.

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