THE INTERACTION OF OXYGENOUS ANIONS WITH HUMAN SERUM ALBUMIN

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The interactions of anions SO_4^{2-}, NO_3^- and HCO_3^- with human serum albumin have been studied. The numbers of anions bound to a molecule of the protein were determined by measuring the electric conductance; the data were evaluated in the form of Scatchard's plot. The curves for SO_4^{2-} and NO_3^- suggest that bivalent ligands may be bound through one valency. The degree of their binding to albumin was much lower than that calculated from the change of pH of isoionic solution with increasing concentration of the added salt. With the ions HCO_3^- the dominating phenomenon was hydrolysis of the salt, with the consequential shift of pH to the alkaline region.

The interaction of serum albumin with various inorganic anions have been devoted much attention[1-5]. The protein was found to bind readily anions of different sizes mainly through its basic groups, *i.e.* amino, imidazole and guanidine groups[6-8]. However, most experiments dealt with only the quantitative aspect of the problem (number of bound ions per molecule of the protein). The number of the binding sites and the corresponding association constants were not determined.

The present paper describes the binding of anions NO_3^-, HCO_3^- and SO_4^{2-}. In the case of the anions NO_3^-, whose binding was studied previously[9], we have attempted to determine the number of the binding sites and the firmness of the linkage. The ions HCO_3^- were chosen as another type of univalent anions, whereas the ions SO_4^{2-} as more complex anions, carrying two elementary charges.

EXPERIMENTAL

Human serum albumin was a commercial product of Imuna, lot 18/1–69. Electrophoresis in polyacrylamide gel showed a very low content of the dimer. The experiments were carried out with the isoionic protein (pH 5·20), prepared by desalting the commercial preparation on a column of Amberlite MB-3 (Serva). The concentration of the solution (0·5 to 1%) was determined by drying *in vacuo* at 105°C over P_2O_5. The solutions of the selected salts, $NaNO_3$, $NaHCO_3$, Na_2SO_4 and NaCl, were prepared from A. R. chemicals. The distilled water, in which all the experiments were performed, had a conductivity of (2 to 6). $10^{-6}\ \Omega^{-1}\ cm^{-1}$. The values of pH were measured with an apparatus Radiometer, pHm 4c, employing a glass electrode G-202 B and a calomel electrode K-100. The number of anions bound per molecule of albumin was calculated from the electric conductance as previously described[10]. The conductance was measured with a conducto-

meter OK-102 (Radelkis). All the conductance and pH measurements were carried out in a thermostated vessel at 25°C. Disc electrophoresis was carried out according to Davis[11] at a voltage gradient 7 V/cm; the protein in the discs of the polymer was stained with a 1% solution of amidoblack 10 B in 7% acetic acid. Gel chromatography on Sephadex G-200 was carried out in a column 1·8 × 23 cm at a flow rate of 14 ml per hour. The eluted protein was collected in 4-ml fractions, evaluated spectrophotometrically for protein concentration at 280 nm (Zeiss spectrophotometer VSU-1).

RESULTS AND DISCUSSION

Fig. 1 shows that $NaNO_3$ and Na_2SO_4 (in some cases even $NaHCO_3$) added to an isoionic solution of albumin bring about more marked pH shifts than halides. Our data for nitrate anions, especially at higher concentrations, differ from those reported by Scatchard[9] (curves *3* and *4*, Fig. 1). Since this discrepancy might be due to a difference in the properties of the albumins we also measured pH in relation to the concentration of Cl^- ions. As can be seen from Fig. 1 the data were identical with the reported ones[9].

The curve for ions HCO_3^- is not included in Fig. 1, since in this case the pH changes were too high. At 1M concentration of HCO_3^- the pH shift was 3 units. In view of this great shift the calculation of the average number of ions bound to a molecule of albumin ($\bar{\nu}$), from conductance

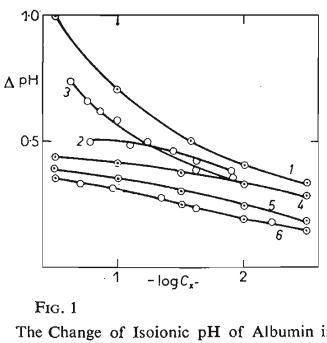


FIG. 1

The Change of Isoionic pH of Albumin in Relation to Concentration of Free Anions in the Solution, C_x-(mol cm^{-3})

○ Our measurements, ⊙ data from reference 9, *1* NaCSN, *2* Na_2SO_4, *3* $NaNO_3$, *4* $NaNO_3$, *5* NaBr, *6* NaCl.

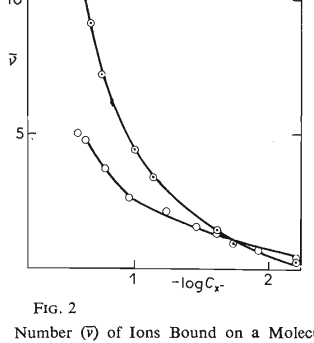


FIG. 2

Number ($\bar{\nu}$) of Ions Bound on a Molecule of Albumin, in Relation to Concentration of these Ions in the Solution, C_x-(mol dm^{-3}) ○ SO_4^{2-}, ⊙ NO_3^-.

data, had to include the correction for the change in the number of the bound protons. This correction was read off from the titration curve of albumin[12]. The value of $\bar{\nu}$ was calculated from the equation[10]

$$\bar{\nu} = \frac{(\varkappa_1 - \varkappa_2)(V_0 + v) - n\bar{h}l^0_{OH^-}}{An[l^0_{x^-} - (B_1 l^0_{x^-} + B_2)\sqrt{C_{x^-} \cdot (1 + \sqrt{C_{x^-}})^{-1}}]}, \qquad (1)$$

where $\varkappa_1$ and $\varkappa_2$ designate the conductivities of the solution without and with albumin respectively (Ω^{-1} cm^{-1}), V_0 the starting volume of the solution (cm^3), v the volume of the added solution of salt (cm^3), n the number of mol of the protein in a given volume of the solution, $\bar{h}$ the average number of bound protons after a given pH change, $l^0_{OH^-}$, $l^0_{x^-}$ (Ω^{-1} cm^2 mol^{-1}) the mobilities of hydroxyl anions and the investigated anions (X^-), C_{x^-} the concentration of free anions of the investigated salt (mol dm^{-3}); in the given system of units the values of B_1 and B_2 for an aqueous solution at 25°C were 0·2300 and 60·65, respectively. The empirical constant A for albumin was previously found[10] to be 7.

The calculation for ions HCO_3^- revealed that the two terms in the numerator of equation 1, *viz.* $(\varkappa_1 - \varkappa_2)(V_0 + v)$ and $n\bar{h}l^0_{OH^-}$, have the same value. Consequently, the measured change of pH is caused by the OH^- ions arising from hydrolysis of $NaHCO_3$, and not by the binding of anions HCO_3^-. These anions might be bound at lower pH values (in the isoelectric region), but with the addition of $NaHCO_3$ the pH is soon shifted to between 7 and 8, where the binding of ions HCO_3^- could hardly occur.

In the case of ions NO_3^- equation (*1*) was employed also. The results were compared with those calculated from the non-corrected equation, lacking the term $n\bar{h}l$. Comparison of the two sets of data revealed that the differences did not exceed 1% of the values calculated from equation (*1*). The calculation was based on the assumption that in the isoelectric region no Na^+ ions were bound on serum albumin[1]. The curves relating the average number of anions bound on a molecule of albumin ($\bar{\nu}$) to the logarithm of concentration of the free ions in the solution (Fig. 2) approach limit

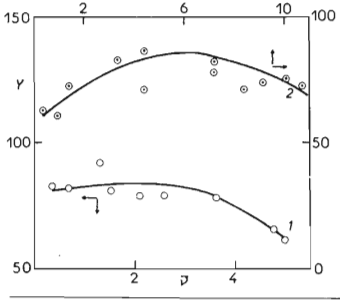
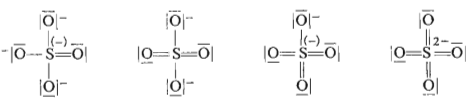


FIG. 3

The Quantity $Y = \bar{\nu}\, e^{2w\bar{\nu}Z}/c\,\gamma_{\pm}$ in Relation to the Number of Bound Ions, $\bar{\nu}$
1 SO_4^{2-}, *2* NO_3^-.

values. With SO_4^{2-} ions the limit value of $\bar{\nu}$ is 5 to 5·5, with NO_3^- ions it is 11·5 to12, but in the latter case the approach to the limit value is less apparent. The curves were further analysed in terms of Scatchard's plot. The quantity $\bar{\nu}\, e^{2w\bar{\nu}Z}/c\gamma_\pm$ was plotted *vs* $\bar{\nu}$ (Z is the charge of the ions studied). In this case the electrostatic factor w was obtained directly from the titration curves of albumin at different ionic strengths of the solution. Within the range of the concentrations employed it attained values between 0·025 and 0·05. The values of the mean activity coefficients, $\gamma_\pm$, for different concentrations of the salts were reported by Izmajlov[13]. The dependence of the quantity $\bar{\nu}\, e^{2w\bar{\nu}Z}/c\gamma_\pm$ on $\bar{\nu}$ for Na_2SO_4 and $NaNO_3$ (Fig. 3, curves *1* and *2* respectively) took courses rather different from the usual type[1,2], since neither the number of binding sites (n) of the anions, nor the corresponding association constant (k) could be read off from them. The curves exhibiting peaks, obtained by our calculation, occur with association–dissociation equilibria of proteins[14]. In order to ascertain whether or not the association occurred we investigated electrophoresis in a polyacrylamide gel, containing albumin and the studied salt, and gel chromatography on Sephadex G-200, equilibrated with a 0·1M solution of the salt. Neither method demonstrated association of the albumin molecules. An alternative interpretation of the anomalous plots of $\bar{\nu}\, e^{2w\bar{\nu}Z}/c\gamma^{\pm}$ *vs* $\bar{\nu}$ could be sought in the idea of a bivalent ligand bound through one valency[14]. In the case of the bivalent ions SO_4^{2-} this possibility is quite probable. These ions have a tetrahedral character, their structure being a resultant of the four extreme variants[15]



The rather high entropy of hydration[13], −52·5 el.u., suggests a bulky hydration envelope of these ions. This might be a steric hindrance, causing the ions to be orientated with an apex to the binding site. In this connexion it can be pointed out that it is near the apices that the negative charge resides. It is also possible that the binding sites of albumin are so remote that an ion SO_4^{2-} cannot be attached to two at the same time.

The structure of the ion NO_3^- is known to be a flat one. In addition to the negative charge on one of its oxygen atom, a small negative charge is supposed to occur on another oxygen atom, this charge being counterbalanced by a small positive charge on the central nitrogen[15]. Considering the flat structure of the ion, with oxygen atoms on its periphery, an electrostatic linkage might arise between a positively charged site on albumin and one of the two negatively charged oxygen atoms of the nitrate anion. From this point of view the NO_3^- ions are similar to the SO_4^{2-} ions, which might account for the peak on the plot of $\bar{\nu}\, e^{2w\bar{\nu}Z}/c\gamma_\pm$ *vs* $\bar{\nu}$.

TABLE I

A Survey of $\bar{\nu}_{theor}$, $\bar{\nu}_{exp}$ and ΔpH for Different Concentrations of NO_3^- and SO_4^{2-}

C_{x^-}, mol dm^{-3}	ΔpH	w	$\bar{\nu}_{theor}$	$\bar{\nu}_{exp}$
		NO_3^-		
0·01	0·35	0·05	8·15	0·5
0·05	0·48	0·035	15·0	2·5
0·1	0·57	0·026	25·2	4·5
0·13	0·80	0·025	36·9	11
		SO_4^{2-}		
0·01	0·35	0·05	4·08	0·5
0·05	0·46	0·035	9·47	2·0
0·1	0·49	0·026	14·1	2·5
0·13	0·50	0·025	19·2	5·0

Another anomaly in the behaviour of anions SO_4^{2-} and NO_3^- in their interactions with albumin is apparent from Fig. 1. The pH shifts due to their presence were greater than those effected by chloride or bromide anions, although the number of Cl^- ions bound to a molecule of albumin was greater than that of the NO_3^- ions, and about twice as great as the number of the SO_4^{2-} ions. This observation raised the question of what part of the pH shift was caused by the binding of the ions. We employed the equation[16]

$$\Delta pH = 0{\cdot}868 w \bar{\nu} Z , \qquad (2)$$

valid for the isoionic point, where Z is the charge of the ion and w the electrostatic factor, whose value was calculated from the titration curves of albumin. Substituting the experimental values for ΔpH in this equation we calculated the values of $\bar{\nu}_{theor.}$, which proved to be essentially higher then the actual values, $\bar{\nu}_{exp.}$, determined by conductance measurements (Table I). This means that the binding of anions is not the only cause of the measured pH shifts. The conformational adaptability of albumin in its interactions with organic anions[17,18] suggests that even the bulky inorganic ions affect the conformation in such a way that more protons are bound to other functional groups, which manifests itself by pH shifts to the alkaline region.

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