# MUTUAL EFFECTS OF ANIONS DURING INTERACTION WITH HUMAN SERUM ALBUMIN

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The simultaneous interaction of anionic pairs $Cl^- + Br^-$, $CNS^- + Cl^-$ and $CNS^- + Br^-$ with human serum albumin was examined. The relationships between the number of bound ions and the concentration of free ions in solution served for determining the number of binding sites of different types and the corresponding association constants. It was found that $Cl^-$ and $Br^-$ affect each other slightly in binding to serum albumin; the pair $CNS^- + Cl^-$ was found to use a considerably smaller number of binding sites. The most pronounced mutual interaction during binding was found for the pair $CNS^- + Br^-$ where binding is inhibited at higher concentrations.

Human serum albumin was among the first proteins to be studied for their ability to bind various inorganic[1-5] as well as organic[6-9] substances, this being due to its exceptional binding properties and its ready availability. In the field of interactions with inorganic ions some attention has been paid to their binding at isoionic pH and in the acid region. In a number of experiments the number of binding sites and the corresponding association constants have been determined[1,2,4]. Attempts at studying the simultaneous interaction of two different substances with albumin are rather rare[4,10], the most common type of experiment being the binding of the acid-salt pair with the same anion, the binding of the anion being expressed as a function of pH. In the present work we designed and developed a method for the case that two salts take part in the interaction, the anions being different, the cation common to both. The anions are known to interact at isoionic pH with albumin while the cations of the salts used are not bound under the conditions. The results of the measurements were evaluated qualitatively and quantitatively.

## THEORETICAL

To determine the number of ions ($\bar{\nu}$) bound to a protein molecule we used the conductometric method described before[11]. The method was found to be adequate for measuring the interaction of various single anions with the protein. To use it also for pairs of anions some further premises had to be made. Whereas in a conventional conductometric estimation a 1M salt solution is added to the protein solution, in the case of anion pairs the solution was prepared with each of the ions in a 0·5M con-

centration (thus *e.g.* 0·5M-NaCl + 0·5M-NaBr) and this solution was taken for a solution of the hypothetical salt NaX at a 1M concentration. For this hypothetical salt the changes of conductivity and of pH were experimentally determined and from these the number of anions $X^-$ bound to the albumin molecule. The experimental values of $\bar{\nu}$ were compared with those calculated from values observed with the various anions alone under the assumption that these ions do not influence each other and that the resulting value of binding is an additive property of the two. Difference between the two types of values then indicates the mutual effects of anions during their simultaneous binding to albumin.

To carry out such measurements with the greatest possible accuracy it is essential that the conductivities of anions used in such a pair be as similar as possible which obviously limits the selection of salts for experiments. To calculate the binding of hypothetical ions $X^-$ we used molar conductivity $l_{X^-}$ which represented an arithmetic mean of the molar conductivities of the two anions examined. Similarly, the mean values of activity coefficients $(\gamma_\pm)$ of the two salts were used[12]. In further text we shall use for simplicity $X_A^-$ instead of $Cl^- + Br^-$, $X_B^-$ instead of $CNS^- + Cl^-$ and $X_C^-$ instead of $CNS^- + Br^-$.

The number of binding sites $(n_i)$ and the corresponding association constants $(k_i)$ were determined for every type of hypothetical anions on the basis of[13]

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

where $[C]$ is the concentration of the free ligand in solution (mol/l), $w$ is an electrostatic factor (calculated from the titration curves of albumin[14]). Values of $n$ and $k$ were determined graphically according to Scatchard's plot of $\bar{\nu}e^{2\bar{\nu}w}/C_{\gamma_\pm}$ against $\nu$. From these the value of $\bar{\nu}$ was computed and the values of $n$ and $k$ were varied until full agreement with the experimental observations was reached.

## EXPERIMENTAL

*Material.* The serum albumin was made by Imuna, n.o. batch 18/1—69. For all the experiments the protein was deionized on a column of Amberlite MB-3 (Serva). The concentration of the solution for estimating ion binding lay between 0·5 and 1·0%. For determining the pH change caused by a change of concentration of the anions present a 1% solution of albumin was used. The protein concentration in solution was determined by drying a known volume *in vacuo* over phosphorus pentoxide at 105°C. As was shown by disc electrophoresis according to Davis[15] the preparation used did not contain appreciable amounts of the dimer.

*Methods.* Measurements were done with individual salts (NaCl, NaBr and NaCNS) and with pairs (NaCl–NaBr, NaCNS–NaBr, NaCNS–NaCl). The salts were all of reagent-grade purity and all the solutions were prepared from distilled water, the specific conductivity of which did not exceed $5 \cdot 10^{-6}\,\Omega^{-1}\,cm^{-1}$. For measuring pH, a pH-meter of the pHm 4c type (Radiometer)

was used, together with a G-202B glass electrode and K-100 and K-401 calomel electrodes. The specific conductivity of the solutions was estimated with a OK-102 Radelkis conductometer. All the solutions were maintained at 25 ± 0·2°C.

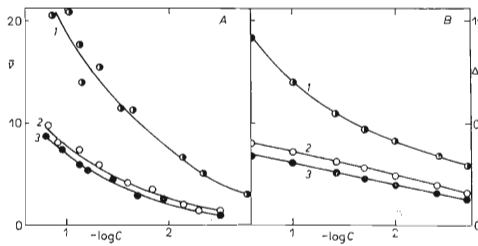


FIG. 1

Interaction of Anions with Human Serum Albumin (● $Cl^-$, ○ $Br^-$, ◑ $CNS^-$)

*A* Number of anions bound to the protein molecule ($\bar{\nu}$) in dependence on the logarithm of concentration [C] of free ions in solution; *B* change of pH of an isoionic protein solution in dependence on the logarithm of concentration of the anions in solution.

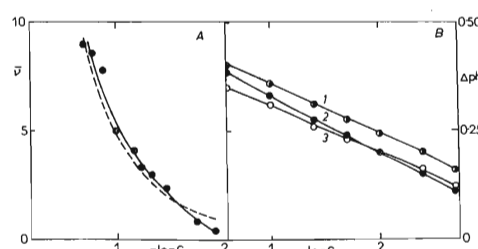


FIG. 2

Simultaneous Interaction of $Cl^-$ and $Br^-$ with Human Serum Albumin

A Number ($\bar{\nu}$) of "anions" $X_A^-$ (●) bound to the protein molecule in dependence on the logarithm of concentration [C] of these free "anions" in solution. The broken curve corresponds to values for $X_A^-$ calculated from the assumption of additivity; *B* change of pH of an isoionic protein solution in dependence on the logarithm of concentration of the anions examined in solution; ○ $Cl^-$, ◑ $Br^-$ (values from Fig. 1) and ● $X_A^-$.

## RESULTS AND DISCUSSION

From experiments with single anions curves were obtained that agreed well with literature data[1,2] (Fig. 1*A*). Likewise, the numbers of binding sites $n_i$ and the corresponding association constants $k_i$ (Table I) are in agreement with the usually reported values.

The first pair to be studied was $Cl^- + Br^-(X_A^-)$ since the two similar anions were not expected to affect each other during interaction. Fig. 2*A* shows the dependence of the number of bound ions $\bar{\nu}$ on their concentration in solution to be practically identical with the curve obtained by calculation. Table I shows the values of $n_i$ and $k_i$ for this case. The number of binding sites of the first type is almost the same as in the case of the binding of individual ions, particularly in the region of higher concentrations. In the case of second-type sites ($n_2$) one may observe a decrease in their number. This difference in $n_2$ for $X_A^-$ and $Cl^-$ and for $X_A^-$ and $Br^-$ is greater than could be accounted for by the experimental error and by the approximation in using average values of molar conductivities and mean activity coefficients. The lower value of $k_1$ (a three-fold decrease) indicates significantly weaker binding.

We attempted therefore to check this finding once more, using an independent method. Changes of pH after addition of the ions examined to an isoionic protein solution were estimated. It is known that in such a case the following equation[16] may be written for simple anions: $\Delta pH = 0{\cdot}868\, w\bar{\nu}$. The pH changes were recorded

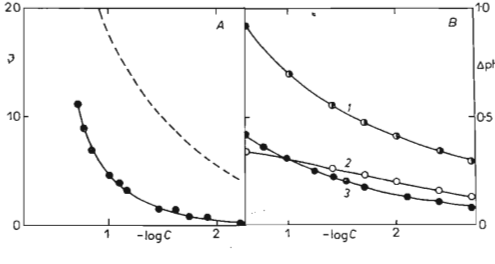


FIG. 3

Simultaneous Interaction of $CNS^-$ and $Cl^-$ with Human Serum Albumin

*A* Number ($\bar{\nu}$) of "anions" $X_B^-$ (●) bound to the protein molecule in dependence on the logarithm of concentration [C] of these free "anions" in solution. The broken curve corresponds to values for the $X_B^-$ calculated from the assumption of additivity; *B* change of pH of an isoionic protein solution in dependence on the logarithm of concentration of the examined anions in solution: ○ $Cl^-$, ◑ $CNS^-$ (values from Fig. 1) and ● $X_B^-$.

first for the individual anions (Fig. 1*B*). The curves obtained are in good agreement with the data of Scatchard and coworkers[5]. The same measurement was then made with the pair $Cl^- + Br^-$ and the values were compared with those for the individual ions. As may be seen in Fig. 2*B* the curve corresponding to the pair $Cl^- + Br^-$ (solid points) is practically indistinguishable from the dependence of ΔpH on $-\log C$ for $Cl^-$ or $Br^-$ alone. On the basis of these results it may be concluded that $Cl^-$ and $Br^-$ interact with the same binding sites of albumin but that in this interaction they mutually weaken the binding which is macroscopically reflected in a decrease of $k_1$.

The observed decrease of binding is much more pronounced with the pair $CNS^- + Cl^-(X_B^-)$. It may be seen in Fig. 3*A* that the true binding of "anions" $X_B^-$ is substantially lower than computed from the assumption of independent interaction. This finding was supported by the measurement of pH of an isoionic solution (Fig. 3*B*) where the curve corresponding to the anions $X_B^-$ resembles markedly the curve for $Cl^-$. Processing of the dependence shown in Fig. 3*A* led to the values of $n_i$ and $k_i$ shown in Table I. With the first type of binding sites two deviations may be seen. The number of these sites $n_i$ dropped well below the values corresponding to the individual anions and, in reference to $CNS^-$, the value of the association constant $k_i$ dropped hundred-fold. Hence it follows that during simultaneous binding to human serum albumin the anions $CNS^-$ and $Cl^-$ influence each other so as to decrease their binding. The experimental arrangement did not permit to determine whether

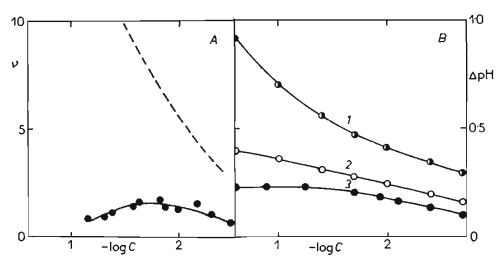


FIG. 4

Simultaneous Interaction of $CNS^-$ and $Br^-$ with Human Serum Albumin

*A* Number ($\bar{\nu}$) of "anions" $X_C^-$ (●) bound to the protein molecule in dependence on the logarithm of concentration [C] of these free "anions" in solution. The broken curve corresponds to values for $X_C^-$ calculated from the assumption of additivity; *B* change of pH of an isoionic protein solution in dependence on the logarithm of concentration of the examined anions in solution: ○ $Br^-$, ◑ $CNS^-$ (values from Fig. 1) and ● $X_C^-$.

TABLE I

Comparison of Binding Parameters of Individual Anions and of Their Pairs

$n_1$, $n_2$ Number of binding sites of first and second type; $k_1$, $k_2$ association constants of binding sites of first and second type.

| Ion type | Molar conductivity $mol^{-1}\ \Omega^{-1}\ cm^2$ | $n_1$ | $k_1$ | $n_2$ | $k_2$ |
|---|---|---|---|---|---|
| $Cl^-$ | 76·35 | 10 | 30 | 44 | 1 |
| $Br^-$ | 78·1 | 10 | 35 | 55 | 1 |
| $CNS^-$ | 66·5 | 10 | 1 000 | 30 | 25 |
| $Cl^- + Br^-$ ($X_A^-$) | 77·2 | 9 | 10 | 30 | 2 |
| $CNS^- + Cl^-$ ($X_B^-$) | 71·4 | 2 | 10 | 20 | 2 |
| $CNS^- + Br^-$ ($X_C^-$) | 72·3 | $\leqq 2$ | — | — | — |

in this interaction one of the two anions was bound preferentially. The binding to second-type sites is also decreased. The decrease is not so pronounced as with the $n_1$ sites. The increase of the $k_2$ constant is not highly significant in view of its absolute value.

The most surprising results were obtained with the pair $CNS^- + Br^-(X_C^-)$. As shown in Fig. 4*A*, the binding of $X_C^-$ increases only slowly with their increasing concentration, much less than predicted by the calculation and attains a maximum at about $X_C^- = 0{\cdot}02$M. With further increase of concentration of the two ions it begins to decrease so that conductometric measurements could be carried out only up to 0·085M. At higher concentrations the difference in conductivities dropped practically to zero.

The dependence of ΔpH on log $X_C^-$ is much lower than the curves corresponding to the individual anions. Fig. 4*B* shows that the curve of this dependence for the two

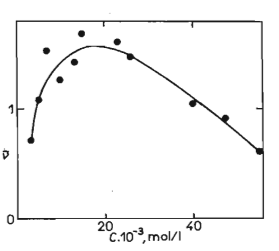


FIG. 5

Dependence of the Number of Bound "Anions" $X_C^-(\bar{\nu})$ on the Concentration of these Free Ions in Solution (*C*)

ions rises gradually approximately up to 0·05M, whereafter the value of pH remains practically constant. This behaviour indicates that at higher concentrations no further ions of the pair are bound to albumin. The binding curve for $X_C^-$ as shown in Fig. 4*A* cannot be evaluated directly quantitatively. From its maximum (for $\bar{\nu} \approx 1{\cdot}6$) it may be concluded that at most two binding sites participate in the binding.

The behaviour of the pair $CNS^- + Br^-$ with increasing concentration can be divided into two phases on the basis of the curves of Fig. 4*A*. During the first phase, at a free ligand concentration of $<0{\cdot}02$M, the usual interaction is found when the anions are bound to the protein molecule even if in a rather limited extent. We are thus dealing with the reaction $P + iX \rightleftarrows PX_i$ $(i = 1, 2)$ where P is the protein and X the ligand. The second phase, in a concentration range of the free ligand of $>0{\cdot}02$M, actually represents the dissociation of the complexes formed through the action of excess free ligand. The drop of $\bar{\nu}$ is much slower than was the increase of this quantity during the first reaction phase which is best seen in a plot of $\bar{\nu}$ *vs* $C_x$ (Fig. 5). The quantitative evaluation of this type of interaction is rather difficult but we attempted at least a rough approximation, when electrostatic effects were disregarded. The first reaction phase is apparently a common interaction when the number of bound anions can be expressed as a function of $C_x$ by a common equation (*1*). To express the decrease in the number of bound anions we introduced the empirical term $K[C_x]^2$. The experimental curve of binding of $CNS^- + Br^-$ shown in Fig. 4*A* can then be expressed by

$$\bar{\nu} = \frac{nk[C_x]}{1 + k[C_x]} - K[C_x]^2 . \qquad (2)$$

If $n$ is set equal to 2 and if it is assumed that both types of binding sites are equivalent, the measured values of $\bar{\nu}$ are met for $k = 200$ and $K = 250$. The relatively high value of the constant $k$ indicate a tight binding of $X_C^-$ to both binding sites but at higher concentrations of these ions the $K$ constant predominates which indicates the dissociation of the complex formed. The mechanism of this dissociation which is connected with a hitherto unknown reaction will be studied in the future.

If the binding parameters of the systems containing $CNS^-$ are compared a single similar value is found, *viz.* the number of binding sites $n_1$. It is possible that the binding of $CNS^- + Cl^-$ and $CNS^- + Br^-$ involves the same first-type site.

The study of three anionic pairs thus showed that even during simultaneous interaction of similar anions, such as $Cl^-$ and $Br^-$, a mutual effect resulting in decreased binding is observed when one of the ions is $CNS^-$. The possibility cannot be excluded that the role of these ions in biological conditions is not in their being bound themselves in great quantities to albumin, thus preventing the binding of other ions, but in their forming a system with other ions, from which only few ions are bound to albumin.

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