Physical and Chemical Properties of Protamine from the Sperm of Salmon (Oncorhynchus tschawytscha)

II. ANION BINDING CHARACTERISTICS

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Since the publication of a short communication (1) 4 years ago on the binding of sulfate and phosphate to salmine, considerably more information concerning the nature of the molecule is available (2-4). We now present the results of a direct determination of the anion-binding characteristics of salmine, and, in light of the physical and analytical properties, a scheme to account for the fact that out of 18 to 19 positive groups there are six binding sites with very high affinity for anions, the remaining groups having much lower binding constants.

EXPERIMENTAL

The salmine used in these studies was purified as the sulfate by the cold precipitation procedure described in Paper I (2). The binding behavior of salmine prepared directly from ripe sperm is identical with that of the two commercial samples we have used (Krishell and Merck, Sharp and Dohme*); hence, these three sources have been used indiscriminately. Salmine chloride was prepared by passing a water solution of the sulfate through an IRA-400 column in the chloride form. The preparation of salmine thiocyanate proved to be more troublesome: passing the sulfate through an IRA-400 column in the thiocyanate cycle gave a product free from sulfate, but instead of the theoretical 21 per cent. Subsequent preparations were made by treating the salmine sulfate with an equivalent amount of Ba(SCN)$_2$, removing the precipitated BaSO$_4$, and passing the remaining solution through the IRA-400 column in the thiocyanate cycle. This gave a product free from sulfate, but it contained a slight, known excess of thiocyanate, which was accounted for in the binding calculations. Table I summarizes the composition of the salmine salts used in this study.

Concentrations were determined by dry weight, Kjeldahl nitrogen, or the refractometric method described by Svensson and Odengrim (5). Salmine concentrations were varied from 0.0014 to 0.24 M.

The results of the measurements are summarized in Table II and the experimental points of Fig. 2. Over a wide range of total anion concentration, the number of anions bound is about six per molecule of salmine; this is true both for mono- and divalent anions. Assuming that these six sites constitute a set, an evaluation of the apparent intrinsic association constant for the binding of anions to this set can be made from the experimental data by the use of the following equation:

$$i_A = \frac{k'_A \Delta a}{1 + k'_A C_A + \frac{1}{k'' A C A}}$$

(1)

The cell used in these measurements was constructed from two lucite blocks (Fig. 1). It differs from cells described previously (6, 7) in having smaller fluid sample compartments, a more convenient filling and rinsing arrangement, and liquid junctions in stopcocks making agar bridges unnecessary. Matching holes of about 1 ml. capacity were milled into the face of each block and additional holes were drilled to serve as connectors to the solution containers and to the three-way stopcocks necessary for flushing the chambers and for connecting by means of liquid junctions to the saturated calomel electrodes. The two blocks were clamped together with the membrane placed between the matching holes to form a two-compartment cell. Potentials were measured with a vibrating reed electrometer connected to a Brown recorder.

With a cell of this type, the activity of the anion of a salmine salt is determined as follows. With a salmine sulfate solution of known concentration in one compartment of the cell, various concentrations of potassium sulfate are rinsed into the other compartment, and the potential difference across the membrane is measured for each known salt concentration. Voltages of the order of 1 to 2 mv. above and below zero are recorded, corrected for the measured cell asymmetry of about 0.1 mv., and then plotted against the logarithm of the known salt concentration. The intercept at zero voltage gives the activity of the sulfate ion in the salmine compartment. A series of measurements is made on successive dilutions of the salmine salt, and also with known quantities of neutral salt added to the salmine solution.

RESULTS

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$$i_A = \frac{k'_A \Delta a}{1 + k'_A C_A + \frac{1}{k'' A C A}}$$

(1)
TABLE I

Analyses of saline salts

<table>
<thead>
<tr>
<th></th>
<th>Calculated</th>
<th>Found</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>S</td>
<td>6.01</td>
<td>5.82</td>
</tr>
<tr>
<td>N</td>
<td>24.90</td>
<td>24.80</td>
</tr>
<tr>
<td></td>
<td>Salmine chloride</td>
<td></td>
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<tr>
<td>Cl</td>
<td>13.96</td>
<td>13.33</td>
</tr>
<tr>
<td>SO₄</td>
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<tr>
<td>N</td>
<td>26.05</td>
<td>26.30</td>
</tr>
<tr>
<td></td>
<td>Salmine thiocyanate</td>
<td></td>
</tr>
<tr>
<td>SCN</td>
<td>21.00</td>
<td>22.30</td>
</tr>
<tr>
<td>SO₄</td>
<td>0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>N</td>
<td>28.97</td>
<td>29.13</td>
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</tbody>
</table>

Table II

Anion distributions in saline solutions

<table>
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<tr>
<th>Salmine concentration</th>
<th>Total anion concentration</th>
<th>Free anion</th>
<th>$f_A$</th>
<th>Net charge on saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloride</td>
<td>Thiocyanate</td>
<td>Sulfate</td>
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<tr>
<td>0.0758</td>
<td>0.00142</td>
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<td>2.05</td>
<td>0.0383</td>
<td>M</td>
<td>M</td>
<td>0.0253</td>
</tr>
</tbody>
</table>

Calculated Found

$\bar{f}_A = \frac{k'_{ACAn}}{1 + k'_{ACa}}$

Curve 1 of Fig. 2 was calculated from Equation 2 with $k'_{ACa} = 3600$ and $n = 6$. The concentrations of chloride ion were corrected with the values of the activity coefficients given in Harned and Owen (9). The same activity coefficients were used for the thiocyanate ion. Thus, the apparent association constant for the chloride and thiocyanate ions has a minimal value of 3600. The constant for the sulfate ion is larger than that for the chloride and thiocyanate ion but cannot be evaluated more precisely from our data. However, the value $n = 6$ for the first set of sites is the same for all three anions.4

With such very large association constants, the binding is effectively the same on a molar basis for all the anions studied. This results in a net charge of +12 for the salmine cation when monovalent anions are used, but only +6 when divalent sulfate is the counter ion. (See last column of Table II.)

There are 18 to 19 arginine residues per 4070 gm. of salmine (2). All of these are potential binding sites for anions. While the chloride and thiocyanate ion data were used to evaluate the

4 We assume that there are six sites comprising the first set for all three anions. The data for the chloride ion indicate that this is reasonable, but there may be more than one set for the thiocyanate and sulfate ions such that the sum of the sites totals six.
binding constant for the first set of six sites, we hoped that the sulfate and thiocyanate data would permit the evaluation of the constants for the next 12 to 13 sites. The solubility of the salmine salts as well as the precision of measurement limited the range of data at higher free anion concentrations. With the limitation of fit that was imposed by the \( \varphi \) values of only 7, the Curves 2 and 3 of Fig. 2 were calculated from Equation 2 for a second set of sites with the values \( n_2 = 12 \) and \( k'_{A_2} = 1 \) and the values \( n_2 = 12 \) and \( k'_{A_2} = 5 \) respectively. The binding of thiocyanate and sulfate to the second set when added to the binding to the first would thus give the value of about 7 in \( \varphi \) found at \( \log c = -1 \) to \(-2.5\). The corresponding constant for the chloride ion appears to be small enough so that the second set would show no binding until the value of \( \log c \) approached zero or greater.

**DISCUSSION**

Let us assume that salmine, known to be heterogeneous, is a collection of closely similar molecules containing 18 to 19 arginine residues and 8 or 9 neutral amino acid residues per mole of 4070 gm. The justification for postulating a collection of molecules deviating very little from the average composition lies only in the fact that numerous attempts at fractionation have been unsuccessful. Perhaps supporting evidence is given by the similar results of amino acid composition determinations on samples from very different sources and methods of preparation.

Given this relatively restricted composition and size which requires some clustering of arginine residues, one of the simplest arrangements of the amino chain to give six strongly binding sites for anions consists of alternating triplets of arginine residues with singlets or pairs of neutral residues. A structural model built on this scheme shows that the guanidinium ions of the three adjacent arginine residues can actually be brought into close approximation and thus would have a very high electrostatic affinity for an anion. Looking at it another way, an anion can be shared by the three guanidinium ions, reducing their affinity for additional anions by a considerable factor. Six such groups of three arginines explain our finding of six sites with very high association constants, the remaining sites with constants ranging in value from about 1 to 5 for the thiocyanate and sulfate ions and with a value for the chloride ion too low to be measured by our technique.

It will be noted that such a model is similar to one proposed by Wilkins (10) on the basis of x-ray diffraction data and the requirement that the distance between arginine residues fit the spacing between phosphates in the nucleic acid double helix. Wilkins suggests that there could be four arginine residues separated by pairs or triplets of neutral amino acid residues. Our model with triplets of arginines separated by single or pairs of neutral residues is dictated by the anion binding results, the amino acid composition, and the results of Monier and Jutisz (11) who report no peptide from salmine containing more than two successive neutral amino acid residues. We have felt it desirable to report on the binding behavior of salmine, since it provides such a remarkable example of specific site binding apparently based on a specific structural arrangement of the charged groups in the molecule.

**SUMMARY**

The salmine cation, with 18 to 19 potential sites for anions, is shown to have six sites with apparent intrinsic association constants of 3600 or more for chloride, thiocyanate, and sulfate. The remaining 12 sites have association constants in the neighborhood of 5 or less. To account for this remarkable example of specific site-binding, a model is proposed which groups the arginine residues in six clusters of three residues each, separated by single or pairs of neutral amino acid residues.

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**REFERENCES**

Physical and Chemical Properties of Protamine from the Sperm of Salmon (Oncorhynchus tschawytscha): II. ANION BINDING CHARACTERISTICS
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