Numerous determinations of the amino acid composition of various commercial preparations of the protamine, salmine, have been made (1-6). From several of these determinations (1, 2, 4, 6) minimal molecular weights ranging from 7000 to 10,000 have been calculated on the assumption that the preparations were homogeneous. Analyses of two commercial samples (Krishell and Sharp and Dohme) in this laboratory agree, in general, with the published values mentioned above. However, the molecular weight of the chloride salt of the Krishell sample was determined by sedimentation-diffusion to be closer to 4000 (7). This lower value was confirmed by light scattering which gave a maximal (weight average) molecular weight of approximately 5000. By an end group method, Phillips (8) found an average molecular weight of 3800 for another commercial preparation.

Since no physical heterogeneity could be detected in the Krishell sample (7), it was concluded that the disagreement between the physicochemical molecular weight and the minimal molecular weight from amino acid analysis could be due to a chemical heterogeneity in which molecules of approximately the same size have slightly different amino acid compositions. Such a situation has recently been shown to exist in the case of clupeine by Felix and coworkers (9). Felix (10) has also demonstrated the apparent heterogeneity of two preparations of salmine. One, a commercial preparation (Salmo salar), was heterogeneous by the method of countercurrent distribution and the other (species not given) by paper chromatography. In addition, Phillips (8) has reported a non-uniformity of end groups in the preparation mentioned above.
As pointed out by other authors, the differences noted in various samples of salmine could arise from differences in salmon species, from mixtures of protamine from ripe and unripe sperm cells, or from differences in methods of preparation. These same factors could also be responsible for the chemical heterogeneity of any one sample. It was felt desirable, therefore, to prepare a sample of salmine from the ripe sperm of a single species of Pacific salmon by the mildest method possible. Such a preparation could be used to investigate the microheterogeneity problem and also for physicochemical measurements on what preliminary experiments have shown to be a very interesting compound. The anion-binding ability and the polyelectrolyte viscosity behavior of salmine, for example, have already been reported (11).

The present results give evidence that the lower physicochemical molecular weight is to be favored over the higher ones previously calculated from amino acid analyses and that salmine from a single species, even when prepared under mild conditions, is chemically heterogeneous.

**EXPERIMENTAL**

*Chemical Methods*—Total nitrogen was determined by the micro-Kjeldahl method of Ma and Zuazaga (12).

Reactive arginine in salmine and free arginine in hydrolysates were determined by a modified Sakaguchi reaction. Total arginine after hydrolysis (in 6 N HCl in sealed tubes at 110° for 20 hours) was found to be 1.34 times the apparent content given by direct reaction of the Sakaguchi reagent with the intact protamine. Monier and Jutisz (13) found an average factor of 1.30 for arginine peptides.

Proline was determined by the Chinard reaction (14) on the hydrolysate. Amino acid analysis was performed according to Moore and Stein (15). In accordance with a later unpublished suggestion of Moore and Stein, arginine was also determined on a 15 cm. column of Dowex 50 jacketed at 50° and eluted with 0.4 M citrate buffer at pH 5.0.

Because of the difficulties involved in isolating the dinitrophenyl derivative of proline from a hydrolysate for end group determination (16), a modification (17) of the Sanger procedure was carried out on the intact protamine. A volume of salmine solution between 0.1 and 2.0 ml. and containing approximately 0.6 mg. was used. To this were added 0.2 ml. of 1 per cent tetraborate buffer, pH 9.0, and 5.0 μmoles of 2,4-dinitrofluorobenzene dissolved in 0.1 ml. of absolute ethanol. The mixture was heated at 60° for 1 hour, and, without cooling, enough 1 N HCl was added to bring the final volume to 3.0 ml. The absorption of the acidified solution at 420 mμ was then read immediately in a photometer against a reagent.

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A standard solution of l-proline containing 0.15 μmole was allowed to react with 2,4-dinitrofluorobenzene at the same time.

**Spectrophotometric Analysis**—The ultraviolet absorption spectrum of the salmine was measured at various stages of the preparation in a Beckman model DU spectrophotometer.6

**Ultracentrifugal Analysis**—The sedimentation analysis reported here was performed in a Spinco model E ultracentrifuge7 with schlieren optics including a phase plate diaphragm assembly.

**Electrophoretic Analysis**—This analysis was made in an Aminco-Stern electrophoresis apparatus8 at 1°.

**Dialysis**—Because of its small molecular size, salmine will diffuse through ordinary dialysis tubing. The tubing routinely used in this laboratory for salmine dialysis is prepared by heating 3½ inch Visking seamless cellulose tubing9 at 90° in air for about 24 hours.10

**Collection of Ripe Sperm**—The salmon milt was generously provided by Dr. John E. Halver, Salmon Nutrition Laboratory, United States Fish and Wildlife Service, Cook, Washington. It was collected at the Service Hatchery at Underwood, Washington, from chinook salmon (Oncorhynchus tschawytscha) during the spawning season in late September. Mature sperm were stripped into a beaker and immediately dispersed through a 23 gauge needle into a bath of liquid nitrogen. The fine granules of frozen sperm were then removed and stored under liquid nitrogen until they were packed in dry ice and sent by air express to the Bethesda laboratory. On arrival, the two slabs of dry ice used were still large, and the milt was hard. The milt was kept in a freezer until needed.

**Preparation of Salmine Chloride**—Several different preparations with slight variations in procedure have been made. The results reported below were obtained on a sample prepared as follows: The cells were washed five to six times by centrifugation in the cold with 3 volumes of 0.1 M NaCl interspersed with three washings with water. They were then washed three times with 50 per cent ethanol, twice with 95 per cent ethanol, and once with 50 per cent ether in ethanol. To extract the protamine, 1.5 volumes of cold 1.5 M NaCl in 50 per cent ethanol were added to the washed cells which were stirred vigorously for 15 minutes.11 The protamine extract was recovered by centrifugation, a well packed sediment of nucleic acid being removed. The extract was then dialyzed against cold distilled

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6 Beckman Instruments, Inc., Fullerton, California.
7 Spinco Division, Beckman Instruments, Inc., Belmont, California.
8 -American Instrument Company, Inc., Silver Spring, Maryland.
9 Visking Corporation, Chicago, Illinois.
10 According to a suggestion of D. W. Kupke, personal communication.
11 The salt solution is strong enough to dissociate the nucleoprotamine completely, yet the ethanol prevents solution of the free nucleic acid.
water until the NaCl concentration was about 0.1 M. The dialyzed protamine solution was then filtered through Hyflo Super-Cel\(^{12}\) to remove the turbidity developed during dialysis. Part of the filtered solution was dialyzed again to remove most of the salt and then lyophilized. The rest of the solution was converted to the phosphate and sulfate salts as described below.

**Preparation of Salmine Phosphate from Salmine Chloride**—Salmine in dilute NaCl (0.1 M or less) can be precipitated as the phosphate salt by the addition of 1 mole of potassium phosphate buffer (pH 7) per mole of arginine residue and 2 volumes of ethanol.\(^{13}\) The precipitate thus obtained was dissolved in a minimal amount of water and then reprecipitated with 0.5 mole of phosphate per mole of arginine residue and 2 volumes of ethanol.

**Preparation of Salmine Sulfate**—Salmine chloride can be converted to the sulfate by an analogous procedure, except that 1 volume of ethanol is sufficient for precipitation. To assure complete replacement of chloride by sulfate, a solution was put through an anion exchange resin (IRA-400)\(^{14}\) in the sulfate form. A preparation completely free from inorganic salt for dry weight and nitrogen determination was obtained by two cold precipitations by taking advantage of the large temperature coefficient of solubility of salmine sulfate in water. In the cold, salmine sulfate separates as a clear, immiscible liquid.

### Results

**Yield**—In a typical preparation of the phosphate salt, 100 gm. of frozen milt (12.7 gm. dry weight) gave 3.12 gm. of salmine, expressed as the free base. This is 75 per cent of the total amount of Sakaguchi-reacting material present in the original milt. Of the arginine not recovered, 15 per cent was lost in the washing process and 10 per cent in the succeeding steps, including conversion to the phosphate.

**Purity and Characterization**—Evidence for the physical homogeneity of the preparation is illustrated in Figs. 1 and 2. Fig. 1 shows the pattern obtained in the ultracentrifuge after 89 minutes at 59,780 r.p.m. The absence of any gross contamination by macromolecular species is evident. In addition, the symmetrical salmine boundary demonstrates the relative physical homogeneity of the sample. The sedimentation constant, \(s_{20}^{w}\), is \(0.98 \times 10^{-13}\) cm. sec.\(^{-1}\) dyne\(^{-1}\).

In Fig. 2 are shown the electrophoretic boundaries obtained after 1500 minutes at a voltage gradient of 2.7 volts cm.\(^{-1}\). Again there is a single

\(^{12}\) A product of Johns-Manville, New York, New York.

\(^{13}\) We have not been able to precipitate either the chloride salt or the free base with ethanol.

\(^{14}\) Rohm and Haas Company, Philadelphia, Pennsylvania.
peak. However, in both of the two runs performed, a second peak was noticed about 3 hours after beginning the run. This peak was presumed to be an anomaly, because it did not separate from the leading boundary but formed spontaneously behind it and later disappeared. All electrophoretic boundaries were abnormally sharp. The mobilities in two runs

**Fig. 1.** Sedimentation diagram of 1 per cent salmine phosphate in 0.1 M potassium phosphate buffer, pH 7.2, in synthetic boundary cell taken 89 minutes after reaching operating speed of 59,780 r.p.m.

**Fig. 2.** Schlieren boundaries of electrophoresis run of 0.5 per cent salmine phosphate in 0.1 M potassium phosphate buffer, pH 7.0, after 1500 minutes at 2.7 volts cm.−1. Upper section, ascending boundary; lower section, descending boundary.
at about 0.5 per cent protamine and at pH 7.0 in 0.1 M potassium phosphate calculated for 0° were 2.05 and 2.12 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} for the ascending boundary and 1.79 and 1.92 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} for the descending boundary.

The ultraviolet absorption of the twice precipitated salmine was small and non-specific. The absence of a peak at 260 m\mu is evidence for the absence of nucleic acid, since this substance has a very high extinction coefficient. Because salmine contains no aromatic amino acids (1-6), no peak is expected at 280 m\mu. The flat absorption in this region is a further indication of the freedom from contaminating proteins or aromatic amino acids and peptides. In several, but not all, preparations a plateau or even small maximum in the ultraviolet absorption curve has been noticed in the vicinity of 270 m\mu prior to the phosphate (or sulfate)-ethanol precipitations. Consequently, this step is now used routinely in the preparation procedure. The salmine can be converted back to the chloride salt by use of a strong anion exchange resin in the chloride form.

**Molecular Weights and Arginine Content of Various Preparations of Salmine**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>End group mol. wt. X 10^{-3} (unhydrated base)</th>
<th>Sedimentation-diffusion mol. wt. X 10^{-3}</th>
<th>Moles arginine Mole end group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krishell</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharp and Dohme</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>This work</td>
<td>4.07 ± 0.10</td>
<td></td>
<td>18.7 ± 0.5</td>
</tr>
</tbody>
</table>

* Calculated from the following experimentally determined parameters: $\Theta_{0} = 0.66 \times 10^{-13}$, $D_{20} = 13.4 \times 10^{-7}$, and $\bar{v} = 0.700$. These were measured on solutions of the chloride salt and are significantly different from corresponding values for the phosphate (7).

**Analysis**—Two carefully prepared samples of salmine sulfate, free from inorganic salt, were found to contain 24.7 per cent and 24.8 per cent nitrogen. Various determinations of the end group molecular weight based on this nitrogen value and the assumption that the N-terminal amino acid is proline (cf. (4, 18-20)) give $4.99 ± 0.12 \times 10^{3}$ gm. per mole for salmine sulfate. For this molecular weight, the corrected Sakaguchi reaction indicates $18.7 ± 0.5$ residues of arginine per mole.

The nitrogen content and the molecular weight of the unhydrated “free” base (containing the arginine residue in the form RNH$_2$) may be calculated from that found experimentally for the sulfate salt. By

**Note**—Direct determination of the nitrogen content of the unhydrated base is possible after conversion of a salt of salmine to the free base on a strong base exchange resin, but this is inadvisable since the salmine solution obtained is so basic (pH 12.5 or above) that the protamine is partially hydrolyzed after a short time at room temperature.
subtracting the weight of 18.7 half sulfate groups and 18.7 hydrogen ions from \(4.99 \times 10^3\), one obtains \(4.07 \times 10^3\) for the molecular weight of the base. (A sulfur analysis on another preparation \((S = 5.82\) per cent) justifies this calculation.) This leads to a value of 30.4 per cent nitrogen which compares with 30.7 per cent reported by Phillips (8). In Table I is compared the molecular weight obtained for this preparation with those obtained earlier for two commercial samples.

In Table II are given the number of residues of amino acids per 100,000 gm. of free base and the minimal molecular weight calculated for each amino acid present.

**Table II**

Amino Acid Composition of Salmine

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>No. of residues</th>
<th>Minimal mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>66</td>
<td>1,520</td>
</tr>
<tr>
<td>Proline</td>
<td>68*</td>
<td>1,470</td>
</tr>
<tr>
<td>Glycine</td>
<td>42.6</td>
<td>2,350</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.1</td>
<td>8,260</td>
</tr>
<tr>
<td>Valine</td>
<td>30.9</td>
<td>3,240</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.3</td>
<td>12,000</td>
</tr>
<tr>
<td>Arginine</td>
<td>476 (461)*f</td>
<td>210</td>
</tr>
</tbody>
</table>

* By direct Chinard reaction on unfractionated hydrolysate.
† By direct Sakaguchi reaction on unfractionated hydrolysate.

**DISCUSSION**

One unique step in the preparation method presented here is the direct but mild extraction of the protamine from the washed cells (presumably nuclei; cf. (10)). Earlier published methods involved either an extraction with strong mineral acid (21) or the dissociation of the nucleoprotamine in a concentrated salt solution (22). The latter method necessitates a tedious separation of the protamine from a large volume of highly viscous nucleic acid solution.

Although no identification of the impurities present in the original salmon milt has been made, the washing procedure described removes a yellow substance in the early stages. Most of the supernatant solutions are turbid owing to material which is low in protamine and nucleic acid. (Only at salt concentrations above 0.2 M does the washing solution dissolve an appreciable amount of salmine.) In addition, evaporation of the ethanol-ether wash leaves a small deposit of fatty material.

From the results presented in Table I, it is concluded that the smaller physicochemical value of about 4000 for the molecular weight is to be
favored over the previously accepted higher values calculated from amino acid analyses, since the lower value has been confirmed by a chemical end group method. The end group molecular weight reported here is within the 10 per cent reliability of the value of 3800 given by Phillips (8).

Although several other protamines, notably clupeine, have been demonstrated to be physically and chemically heterogeneous (10, 9), relatively little work has been done on salmine. The sample investigated by Velick and Udenfriend (4), which was electrophoretically homogeneous and showed a single sedimentation boundary, was a commercial preparation from a genus of Pacific salmon. The same apparent physical homogeneity has been found in this preparation. However, an examination of the results of the amino acid analysis in Table II seems to establish beyond doubt that a true chemical heterogeneity exists in the case of salmine, since a reasonable minimal molecular weight cannot be calculated from these data.

Although the type of heterogeneity demonstrated by Felix (10) by countercurrent distribution and paper chromatography may exist in the case of protamine from Pacific salmon, there is also the possibility that European and Pacific species differ in this respect. In previous experiments on commercial samples, we were not able to obtain any chromatographic separation on paper even though there was the same evidence of chemical heterogeneity in these samples as in the present preparation.

SUMMARY

Protamine (salmine) from the ripe sperm of a single species of Pacific salmon (Oncorhynchus tschawytscha) was prepared in high yield by the direct extraction of washed milt with 1.5 M NaCl in 50 per cent ethanol.

The purity of the preparation was established by sedimentation, electrophoresis, and ultraviolet absorption studies. The nitrogen content of the sulfate salt was determined to be 24.8 per cent.

The "free" unhydrated base was found to contain 18.7 ± 0.5 arginine groups per molecule and to have an end group molecular weight of (4.07 ± 0.10) \times 10^3. This molecular weight is in good agreement with the value found previously for a commercial sample of salmine by sedimentation-diffusion, but is considerably below earlier accepted values calculated from amino acid analyses. The amino acid composition presented here can be reconciled with the molecular weight only by assuming a chemical heterogeneity.

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PHYSICAL AND CHEMICAL PROPERTIES OF PROTAMINE FROM THE SPERM OF SALMON (ONCORHYNCHUS TSCHAWYTSCHA): I. PREPARATION AND CHARACTERIZATION

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