CHEMISTRY OF THE WHITEFISH SPERM.

BY VERNON LYNCH.*

(From the Department of Biochemistry, University of Cincinnati, Cincinnati.)

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Preparation.

For the following analyses about 2 kilos of the ripe testicles of the common whitefish (Coregonus albus) of the Great Lakes were employed. This material, which had been packed in ice, was cleaned and passed through a meat grinder, and the resulting fluid mass was filtered through cheese-cloth. As shown by microscopic examination, the tails of the spermatozoa were no longer visible, their disappearance being probably the result of autolysis. After the addition of an equal volume of water to reduce the viscosity, the sperm heads were thrown down as a solid white mass by rapid centrifuging. The supernatant liquid was then drawn off, and the heads were suspended in water and again centrifuged rapidly. After four such washings, the heads are pure white and do not stick together.

This material was added to three volumes of 95 per cent alcohol, after a small portion had been reserved for the determination of the water content. The mass of sperm heads was extracted

* The work of which the results are embodied in the following pages was carried out in my laboratory in the University of Chicago and under my direction two or three years ago. The manuscript was unfortunately mislaid and was not found until after my discharge from the Army. There has consequently been a delay of three years in the publication of the paper. In the meantime the death of the author by accident has lost to American science one of its most talented young men. Dr. Lynch's mind was unusually keen and active, and he possessed a winning personality, marked originality, courage, and vitality. The manuscript is published in the form in which he left it, with a few minor changes and corrections.

The chemistry of the sperm head is from every point of view interesting and important. Practically only three nuclei have been quantitatively analyzed; namely, the sperm head of salmon, that of the herring, and the nuclei of the corpuscles of the blood of geese. It was to secure additional information along these lines that Dr. Lynch undertook the analysis of the sperm head of the whitefish, Coregonus albus. The material was kindly furnished by the Booth Fisheries Company.—Albert P. Mathews.
four times with boiling, redistilled alcohol, and twice with redistilled ether. These extracts were united and evaporated to dryness, but the amount of lipin material obtained was negligible and was not analyzed. In the sperm, at least, the lipins are almost entirely in the cytoplasm, and no nucleus has yet been shown to contain much fatty material.

It is this composition of the nucleus which in part accounts for the fact that the microscopic appearance of the nuclei remains unchanged through the course of this preparation, but the possibility of the loss of material from the nuclei is by no means excluded.

The yield of dried heads was about 100 gm.

Physical Properties.

The heads of these spermatozoa are very heavy and are readily thrown down by centrifuging. This high specific gravity is due to the large content of nucleic acid, which contains much phosphorus, and to the unusually small amount of water which they hold. The water content of some centrifuged heads, from which as much as possible of the adhering water had been removed with filter paper, was found to be 48.7 per cent. As would be expected from such concentrated nuclei, a high index of refraction is observed.

The heads appear to be little affected by a great many strong chemical reagents. They are insoluble in water, ammonia, and solutions of ordinary salts, and are only partially attacked by dilute sulfuric, nitric, hydrochloric, and acetic acids. Dilute sodium and potassium hydroxide dissolve them slowly. Of special interest is the ease with which they are attacked by dilute phosphoric acid and acid phosphate solutions. With these comparatively weak reagents, they swell up, become sticky, agglutinate, and may dissolve. Thus their solution is effected not so readily by a strong reagent as by an adequate one. The ease with which they swell after entering the egg cytoplasm is well known. In general it was noted that substances which had a solvent action upon these dead sperm heads would also agglutinate a suspension of them.
The neutral salt of protamine and nucleic acid exhibits similar properties, but Steudel\(^1\) found evidence of a smaller molecular aggregation in the salt of clupeine and nucleic acid than that present in the sperm head. He found that a solution of the artificial salt of protamine nucleate had a lower viscosity than the same concentration of the dissolved sperm heads and he suggested to account for this that the nucleic acid in the sperm head was polymerized. Some support for this view is given later in the paper where it is shown that 1 molecule of protamine in the sperm head has united with it 4 molecules of nucleic acid.

**Ultimate Analysis.**

The following determinations of carbon and hydrogen in the alcohol-ether-extracted sperm heads were made by Liebig combustions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \text{CO}_2 )</th>
<th>( \text{C} )</th>
<th>( \text{H}_2\text{O} )</th>
<th>( \text{H} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{gm.} )</td>
<td>( \text{gm.} )</td>
<td>( \text{gm.} )</td>
<td>( \text{gm.} )</td>
<td>( \text{per cent} )</td>
</tr>
<tr>
<td>0.3478</td>
<td>0.5378</td>
<td>0.1467</td>
<td>0.1813</td>
<td>0.0201</td>
</tr>
<tr>
<td>0.2456</td>
<td>0.3778</td>
<td>0.1030</td>
<td>0.1309</td>
<td>0.0145</td>
</tr>
<tr>
<td>0.5642</td>
<td>0.8671</td>
<td>0.2365</td>
<td>0.2841</td>
<td>0.0316</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td>5.77</td>
</tr>
</tbody>
</table>

The nitrogen determinations were made by the Gunning-Arnold Kjeldahl method.

Chemistry of Whitefish Sperm

The phosphorus determinations were made by wet ashing as in the Neumann method and weighed as magnesium pyrophosphate.

The results are given in Table I, and beneath are given certain data from analyses of other nuclei for comparison.

The values for carbon and hydrogen are lower than those found in an ordinary protein. This is due principally to the large proportion of nucleic acid in which the values are even lower. The high content of nitrogen is due to the presence of a basic protein, protamine. Where the protein is a less basic histone, the values for nitrogen are lower, as in nucleohistone for example. The high content of phosphorus shows the presence of a large amount of nucleic acid, 70 per cent or more, for, as will be seen below, practically all the phosphorus is in this combination. In nuclei where the protein is less basic, the proportion of nucleic or phosphoric acid to protein is less. The ratio of phosphorus to nitrogen, being about the same in the sperm and in a neutral salt of nucleic acid and protamine, is evidence that the sperm is, in great part, a neutral protamine nucleate.

The nucleic acid nitrogen is calculated from the phosphorus content, employing the percentage formula of Steudel, \( C_{46}H_{12}N_{12}P_{4}O_{30} \). It will be observed that the nucleic acid nitrogen makes about half the total nitrogen. The other half is almost entirely protamine nitrogen.
The ash determination is of little significance. It consists very largely of phosphoric acid, and comparison with the phosphorus percentage shows that some of the phosphoric acid has been lost, presumably by reduction of the phosphorus. It was not possible to free the ash of carbon entirely. The only base determined in the ash was calcium.

<table>
<thead>
<tr>
<th>TABLE I. Analyses of Nuclei.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whitefish.</td>
</tr>
<tr>
<td>Sperm heads.</td>
</tr>
<tr>
<td>Average.</td>
</tr>
<tr>
<td>Herring* sperm.</td>
</tr>
<tr>
<td>&quot;† &quot;</td>
</tr>
<tr>
<td>Clupeine† nucleate.</td>
</tr>
<tr>
<td>Nuclei of corpuscles.‡</td>
</tr>
<tr>
<td>Nucleohistone.</td>
</tr>
</tbody>
</table>

† Steudel.1

The absence of iron is of special interest. No iron could be detected with potassium thiocyanate in the ash obtained either by the Neumann method or in the electric muffle. No organic compounds of iron were present in this extracted nucleus. More-
over, the common text-book statement that nucleoprotein always contains iron is erroneous. It may be doubted if pure nucleoprotein ever contains iron. The sperm heads contained no potassium and only 0.097 per cent of calcium. The presence of calcium in small amounts has been observed also in the other sperm heads examined, so that it seems to be generally present. This amount, however, would correspond to less than 1 atom of Ca to 5 molecules of protamine nucleate.

The attempt was now made to isolate the nucleic acid and protamine and to determine the actual amounts of these present in the sperm heads.

In the computations which follow the formula for nucleic acid of Steudel, namely C_{43}H_{77}N_{37}P_{4}O_{20}, has been the one used, and for protamine, namely the coregonine, the formula C_{98}H_{194}N_{54}O_{22}. Kossel and Dakin\(^2\) suggested for salmine the formula C_{98}H_{194}N_{54}O_{21}. Taylor\(^3\) suggested for salmine, C_{96}H_{174}N_{54}O_{12}. The formula which has been adopted above is on the hypothesis that 1 molecule of coregonine yields 12 molecules of arginine, 3 serine, 1 valine, and 2 proline groups, and that 1 molecule of water is eliminated between each two amino-acids. Then the formula should not be that of Taylor but the one given. This would be formed by the following reactions:

\[
12 \text{ arginine } (C_{49}H_{142}N_{54}O_{20}) + 3 \text{ serine } (C_3H_7NO_3) + 1 \text{ valine } (C_5H_11NO_2) + 2 \text{ proline } (C_5H_9NO_2) = 1 \text{ salmine } (C_{96}H_{194}N_{54}O_{22}) + 17 \text{ H}_2\text{O}
\]

This would contain 30.90 per cent N; and, if each of the arginine groups combined with 1 molecule of sulfuric acid, the sulfate C_{96}H_{194}N_{54}O_{22} \cdot 12 \text{ H}_2\text{SO}_4 should contain 20.88 per cent N. I actually found 21.06 per cent. This formula requires for arginine nitrogen 88.9 per cent and for monoamino nitrogen 11.1 per cent; whereas Kossel found in this protamine 87.3 and 9.4 per cent respectively. Coregonine is evidently very closely allied to, if not identical with, salmine. From the Chinook salmon, he obtained 86.2 and 9.4 per cent respectively, and in the Rhine salmon 87.8 arginine N and 9.2 per cent of monoamino-acid N. From salveline, the protamine of the closely allied form of lake trout, he got from the alpha-salveline, 88.9 per cent basic N, and 7.1 per cent monoamino-acid N.

\(^3\)Taylor, A. E., J. Biol. Chem., 1908-09, v, 394.
Furthermore since the sulfate of coregonine shows 1 molecule of sulfuric acid combined with each arginine group, making two acid equivalents to each such group, or 24 acid equivalents in all, the formula adopted for the coregonine nucleate of the sperm head is \( C_{95}H_{164}N_{40}O_{32} (C_{43}H_{57}N_{15}P_{4}O_{30})_4 - 24H_2O \) or \( C_{95}H_{164}N_{40}O_{32} (C_{43}H_{57}N_{15}P_{4}O_{30})_4 \) 100 gm. of sperm heads should yield according to this formula 32.33 gm. of protamine and 73.28 gm. of nucleic acid.

An attempt was made to separate the protamine and nucleic acid and determine them.

**Nucleic Acid.**

The usual method of isolating the nucleic acid from a tissue is by treatment with sodium hydroxide, precipitating the sodium nucleate with alcohol, and reprecipitating it in acid alcohol as the free acid. In the hands of Steudel, this method has yielded as much as 91 per cent of the theoretical amount of nucleic acid calculated from the phosphorus content of the herring sperm. Of the total phosphorus content as much as 95 per cent was actually isolated in the form of nucleic acid, and there is reason to believe that the rest was in this same combination.

Professor Mathews suggested that the nucleic acid might be completely removed from a solution of the sperm heads by precipitation with lead. Since the heads are dissolved in sodium hydroxide, a freshly prepared solution of basic lead acetate (specific gravity 1.25) was employed. Protamine is not precipitated by basic lead acetate.

In order to test the completeness of the separation, a solution of sodium nucleate was precipitated with basic lead acetate, and it was shown by examination of the filtrate that all the phosphorus had been removed. The lead salt was converted into the sodium salt with a final loss of less than 2 per cent.

A sample of the sperm heads weighing 23.33 gm. was shaken for several hours with 1,400 cc. of 1.5 per cent NaOH, and a few resistant particles were dissolved separately with a stronger solution. The nucleic acid was precipitated with an excess of the freshly prepared basic lead acetate, and the precipitate, con-
taining lead nucleate and impurities, was washed until practically free from protamine, as shown by the biuret reaction, and then dried and weighed.

23.33 gm. of sperm heads yielded 59.885 gm. of lead precipitate. (There was a small loss of material in the foregoing analysis so that this figure is not so accurate as it should have been.) This precipitate was found to contain 4.56 per cent of N and 2.46 per cent of P. These low figures show that the precipitate contained a large amount of lead oxide and must have been the basic salt. The precipitate gave a very faint biuret reaction, showing that it was not entirely free from protamine. The ratio of P:N in the foregoing is 1:1.87, whereas nucleic acid requires 1:1.66. This slight excess of N is due probably to the protamine impurity.

If all the P in the precipitate is nucleic acid, we calculate that the 59.885 gm. of precipitate must contain 16.39 gm. of nucleic acid, and as this amount was obtained from 23.33 gm. of sperm 100 gm. of sperm would yield 70.25 gm. of nucleic acid, in place of the 73.28 gm. required. If the nucleic acid is calculated from the nitrogen content, since nucleic acid contains 15.14 per cent of N, this would be 17.65 gm. of nucleic acid from 23.33 gm. of sperm or 73.92 gm. of nucleic acid from 100 gm. of sperm, in place of the 73.28 gm. required. The agreement is therefore very good; a mean of 72.08 gm. being obtained in place of 73.28 gm. calculated.

That the precipitate is nucleic acid is shown also by the close agreement of the ratio of P:N in the precipitate after an attempt to convert it to the sodium salt. 3.2096 gm. were dissolved in sodium hydroxide, partially freed from lead by passing in H₂S for 15 minutes, neutralized with acetic acid, concentrated, and precipitated with alcohol. The precipitate weighed dry 1.1483 gm. Analysis showed that it was chiefly the neutral lead salt containing 8.69 per cent of N and 5.23 per cent of P. The ratio of P:N is, however, 1:1.66, whereas nucleic acid requires 1:1.69.

Protamine.

Some insight into the nature of the protein constituent of this nucleus may be had from the results of the protein reactions upon the extracted sperm heads:
2. Millon. Heated. Also let stand 20 days. Negative.
4. Reduced sulfur. Negative.

The absence of tyrosine, cystine, and tryptophane is in accord with the view that the only protein present is a protamine. The positive xanthoproteic reaction is attributed to the nucleic acid.

A quantitative estimation of the protamine was made in two ways: (1) by determining the nitrogen in the filtrate left after precipitation of the nucleic acid with lead; (2) by extracting the protamine from a sample of sperm with dilute sulfuric acid.

The filtrate left after precipitation of the nucleic acid with basic lead acetate appears to contain no other protein than protamine. It gives the characteristic strong biuret reaction and gives a precipitate with nucleic acid and alkaloïdal reagents, but other protein reactions are negative and phosphorus is absent. A rough estimation of the protamine may therefore be made by determining the nitrogen in this solution, assuming that coregonine, like salmon, contains 30.93 per cent of nitrogen.

\[
\text{Total N of filtrate} \quad 2.2627 \\
\text{Protamine, calculated} \quad 7.315 \\
\]

100 gm. of sperm heads would therefore have yielded 31.36 gm. of protamine. This is nearly that calculated from the above formula of 32.33 gm. In view of the fact that a small amount of protamine was still in the lead nucleinate precipitate, this must be considered as a fair agreement.

A more accurate determination of the protamine is probably presented by the sulfuric acid extraction. 9.2805 gm. of dried extracted sperm heads were extracted in a mortar with three successive portions of 10 per cent H₂SO₄ and the protamine sulfate was precipitated in five volumes of 95 per cent alcohol, allowing the precipitate to stand in the alcohol over night. The white precipitate was washed in 95 per cent alcohol and absolute alcohol and dried in a vacuum desiccator. The yield of prota-
mine sulfate from one-half the sulfuric acid extraction fluid was 2.0851 gm., which on resolution in water and reprecipitation yielded 1.9820 gm. A nitrogen determination on this precipitate showed 21.06 per cent of N. 0.5351 gm. of protamine sulfate gave 0.1127 gm. N = 21.06 per cent. Required for C₉₆H₁₆₄N₅₄O₂₂. 1₂H₂SO₄ is 20.88 per cent.

Thus 9.2805 gm. of sperm heads yielded 3.9640 gm. of protamine sulfate after reprecipitation, or 4.17 gm., as first precipitated. The latter figure would give 2.816 gm. of protamine. 100 gm. of sperm would at this rate have yielded 30.33 gm. of protamine, whereas the formula requires 32.33 gm.

Summarized we have then the following as the composition of the sperm chromatin.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>O</th>
<th>N</th>
<th>P</th>
<th>Nucleic acid</th>
<th>Protamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Required for</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₉₆H₁₆₄N₅₄O₂₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C₄H₅N₄P₂O₂₂)₄</td>
<td>42.54</td>
<td>4.82</td>
<td>24.97</td>
<td>21.11</td>
<td>6.56</td>
<td>73.28</td>
<td>32.33</td>
</tr>
<tr>
<td>Found</td>
<td>42.04</td>
<td>5.77</td>
<td>24.49</td>
<td>21.41</td>
<td>6.29</td>
<td>72.08</td>
<td>30.84</td>
</tr>
</tbody>
</table>

The agreement between these figures is so good as to leave little doubt that the head of the sperm of the whitefish is essentially a chemical compound consisting of the union of nucleic acid and protamine in the proportion of 4 molecules to 1. The union is probably not of the nature of an ammonium salt, but formed with the elimination of water as in an acid amide.

CONCLUSIONS.

1. The dried extracted sperm heads of whitefish consist of about 70 per cent nucleic acid and 30 per cent protamine, coregonine.
2. All the phosphorus, 6 per cent, is combined as nucleic acid.
3. This extracted nucleus does not contain iron.
4. The protamine is in loose combination with nucleic acid, and the nucleus is of the “dissociated” (Kossel) type. The union is probably of the amide, rather than the ammonia, type.
5. The analyses agree with the formula C₉₆H₁₆₄N₅₄O₂₂(C₄H₅N₄P₂O₂₂)₄, and this is the probable formula of this chromatin.
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J. Biol. Chem. 1920, 44:319-328.

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