THE QUANTITATIVE ESTIMATION OF EXTRACTIVE AND PROTEIN PHOSPHORUS.¹

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The different combinations of phosphorus to be found in a given tissue may be divided into three main groups:

1. *Protein phosphorus* or phosphorus in combination with protein, including nucleoprotein and phospho-proteins or nucleo-albumins, insoluble in water especially after treatment with alcohol;

2. *Lecithin* and *kephalin phosphorus* or phosphorus in combination with fat and a nitrogen complex, soluble in alcohol and ether, but insoluble in acid chloroform water;

3. *Extractive phosphorus*, including inorganic phosphates and the simpler combinations of phosphoric acid, such as glycerophosphoric acid, phytin or diethoxy-diphosphoric acid, and a number of related compounds as yet little investigated, all of which are soluble in water and partly soluble in dilute alcohol.

In a previous paper² a method for the estimation of lecithin and kephalin phosphorus was described. In the following pages are given methods for the determination of extractive and nuclein phosphorus, which can be carried on at the same time and with the same material as the lecithin estimation.

EXTRACTIVE PHOSPHORUS.

A considerable portion of this form is found in the filtrate from the lipoids precipitated with acid chloroform as described in the above mentioned publication. Whether any of this phosphorus

¹These methods were used in the investigation with H. S. Reed, published in vol. iii, p. 49, of this Journal.

²Koch and Woods: This Journal, i, p. 203, 1905.
Extractive and Protein Phosphorus

is inorganic cannot be determined. Schulze in several of his
publications emphasized the fact that absolute alcohol and ether
do not dissolve inorganic phosphates. He is dealing, however,
with relatively dry plant tissues and not with moist animal tissues
which necessarily dilute the alcohol. The separate estimation of
inorganic phosphates has not been attempted in these methods, as
the danger of hydrolyzing simple organic combinations of phos-
phoric acid seemed too great to promise reliable results.

The following table gives an idea of the amount of phosphorus,
not lecithin or kephalin, dissolved out by alcohol and ether from
brain tissues.

**TABLE I.**

<table>
<thead>
<tr>
<th>Number of</th>
<th>In Per Cent of Dry Tissue</th>
<th>In Per Cent of Total Extractive P.</th>
<th>In Per Cent of Total P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34Ω</td>
<td>0.68</td>
<td>37.8</td>
<td>4.8</td>
</tr>
<tr>
<td>35Ω</td>
<td>0.63</td>
<td>32.1</td>
<td>4.5</td>
</tr>
<tr>
<td>44Ω</td>
<td>0.75</td>
<td>36.0</td>
<td>5.4</td>
</tr>
</tbody>
</table>

The remaining portion of the extractive phosphorus is to be
found in the portion of the tissues insoluble in alcohol and ether
and must be removed by treatment with water to which a little
chloroform has been added to prevent bacterial action. Noël
Paton recommends dilute acid for this extraction, but does not
make it clear whether he altogether avoids the possibility of
breaking up more complex substances. Control experiments
have shown that in the case of the brain about five or six extrac-
tions are sufficient to remove all the phosphates that can be
removed. The following table gives the results.

**TABLE II.**

<table>
<thead>
<tr>
<th>Case.</th>
<th>In Per cent of Dry Tissue</th>
<th>In Per Cent of Total Extractive P.</th>
<th>In Per Cent of Total P.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34Ω</td>
<td>1.12</td>
<td>62.2</td>
<td>7.8</td>
</tr>
<tr>
<td>35Ω</td>
<td>1.33</td>
<td>67.9</td>
<td>9.5</td>
</tr>
<tr>
<td>44Ω</td>
<td>1.33</td>
<td>64.0</td>
<td>9.6</td>
</tr>
</tbody>
</table>

PROTEIN PHOSPHORUS.

The phosphorus compounds present in the tissues after extraction with alcohol, ether and water can only be nucleins, phospho-proteins and tricalcium phosphates. The latter compound is not usually supposed to be present in appreciable amount in tissues except under pathological conditions and can therefore be neglected in the case of brain tissues. If calcium is present it would be more likely to exist as a calcium protein compound. Extraction with dilute acid might be used where calcium phosphate is suspected but this procedure so swells the tissues that complete removal of the adhering liquid becomes very difficult. Besides there is the danger of rendering the alcohol-coagulated protein again soluble.

The following table gives some of the results:

<table>
<thead>
<tr>
<th>Case</th>
<th>In Per cent of Dry Tissue</th>
<th>In Per cent of Total P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>34♀</td>
<td>0.81</td>
<td>5.6</td>
</tr>
<tr>
<td>35♀</td>
<td>0.86</td>
<td>6.1</td>
</tr>
<tr>
<td>44♀</td>
<td>0.85</td>
<td>6.1</td>
</tr>
</tbody>
</table>

A comparison of Tables I, II and III will show that about 80 per cent of the total phosphorus remains to be accounted for. This is represented by lipoid phosphorus which, in the case of corpus callosum here analyzed, is present in large amount.

DESCRIPTION OF METHOD.

About 10 grams of the moist tissue are extracted with alcohol and ether as directed in the paper on the "Estimation of the Lecithins." The residue, insoluble in alcohol and ether, is dried at 102°C. to constant weight, transferred to a 300 cc. Jena flask and extracted six times with about 100 cc. of water to each extraction. Every extraction should extend over 24 hours; plenty of chloroform must be added and the mixture occasionally shaken to prevent bacterial decomposition. The filtrates are evaporated in a platinum dish and dried to constant weight. The residue represents the salts and extractives, insoluble in alcohol and ether.
and soluble in water. The dried residue is ignited in the platinum dish, surrounded by an outer larger platinum dish which is heated to bright redness, until a nearly white ash is obtained. If the inner dish does not come in direct contact with the outer dish there is no danger of volatilizing chlorids. This residue is the alcohol-ether-insoluble, water-soluble ash. The difference between this ash and the residue on evaporation gives the alcohol-ether-insoluble, water-soluble, organic extractives. The ash is moistened with 1.5 cc. of nitric acid, dissolved in water, diluted to 100 to 200 cc. and phosphorus estimation made by the molybdate method. This gives the alcohol-ether-insoluble, water-soluble extractive phosphorus. (Table II.)

The residue left above, insoluble in water after six extractions, is burned with nitric and sulphuric acids and the phosphorus estimated. In case calcium is present this must also be estimated in a separate sample. The phosphorus method is described in detail in a previous paper.\(^1\) This phosphorus is called the alcohol-ether-water-insoluble, or protein phosphorus. (Table III.)

The alcohol and ether solutions obtained by the extraction of the moist tissue are treated as directed in the paper above referred to. If the emulsification and precipitation have been properly carried on, the solution in the 100 cc. graduated flask should be clear in two or three days. An excess of fat in the tissue interferes seriously with this clearing and had best be overcome by the presence of a large amount of chloroform (8 to 10 cc.) and the addition of 2 cc. instead of 1 cc. of hydrochloric acid. The amount of chloroform added must be carefully measured and recorded. After the solution has begun to clear and has been made up to the 100 cc. mark of the graduated flask, it is shaken and allowed to stand until the precipitate has settled. After settling the solution is filtered through a dry filter paper into a dry 100 cc. graduated cylinder. As much of the water as possible is decanted from the chloroform, but it is better not to pour any of the chloroform on the filter, as it may pass through and lipoids be thus lost. Instead of washing the chloroform containing the lipoids with acid water as previously directed, it is better to allow the filter to drain and then read the volume of the filtrate,

\(^1\)Koch and Woods: loc. cit.
which should be perfectly clear and transparent. An aliquot part of the filtrate, usually 80 cc., is evaporated in a platinum dish and dried to constant weight at 102° C. This gives the extractives and salts soluble in alcohol, ether and water. The residue is ignited as directed above and the ash is the alcohol-ether-water-soluble ash while the difference between this and the residue obtained at 102° C. represents the alcohol-ether-water-soluble extractives. The ash is again moistened with \( \frac{1}{3} \) cc. of nitric acid, diluted to 100 to 200 cc. and phosphorus estimated. This is the alcohol-ether-water-soluble extractive phosphorus. (Table I.)

**DISCUSSION AND CALCULATION.**

In order to illustrate the method of calculation it is best to take a sample analysis as follows:

**RECORD OF ANALYTICAL RESULTS.**

<table>
<thead>
<tr>
<th>Case 34</th>
<th>Corpus Callosum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of sample</td>
<td>9.9038 grams</td>
</tr>
<tr>
<td>Water*</td>
<td>70.37 per cent</td>
</tr>
<tr>
<td>Weight of residue insoluble in alcohol and ether</td>
<td>0.8866 &quot;</td>
</tr>
<tr>
<td>1. Insoluble residue</td>
<td>8.68 &quot;</td>
</tr>
<tr>
<td>Weight of residue from six extractions</td>
<td>0.0289 &quot;</td>
</tr>
<tr>
<td>2. Lecithins</td>
<td>4.00 &quot;</td>
</tr>
<tr>
<td>3. Kephalins</td>
<td>4.27 &quot;</td>
</tr>
<tr>
<td>4a Extractives</td>
<td>0.15 &quot;</td>
</tr>
<tr>
<td>5a Extractives</td>
<td>1.03 &quot;</td>
</tr>
<tr>
<td>4b Ash</td>
<td>0.14 &quot;</td>
</tr>
<tr>
<td>5b Ash</td>
<td>0.45 &quot;</td>
</tr>
<tr>
<td>4a Extractives</td>
<td>0.0147 &quot;</td>
</tr>
<tr>
<td>6 Sulphur Compound</td>
<td>1.40 &quot;</td>
</tr>
<tr>
<td>Phosphoric acid gave</td>
<td>0.0117 Mg₃P₂O₇</td>
</tr>
<tr>
<td>Protein P</td>
<td>0.024 per cent</td>
</tr>
<tr>
<td>Total Extract P</td>
<td>0.083 &quot;</td>
</tr>
</tbody>
</table>

To precipitate lipoids 3 cc. chloroform were used. The filtrate measured 76 cc.; 70 cc. were evaporated.

Residue | 0.1060 grams |
---|---|
5b Ash on ignition | 0.0623 " |
5a Extractives | 0.0737 " |
Phosphoric acid in this residue gave | 0.0651 " |
Mg₃P₂O₇ |
2' Lecithin P gave | 0.0551 grams |
3 Kephalin P gave | 0.0097 " |

* Separate estimation.
† This essentially represents the total proteids and any glycogen that may be present in the tissues.

The method of calculating the above results is as follows:

For the alcohol-ether-water-soluble extractives and ash are calculated to 97 cc. (thus correcting for the volume of the chloroform)

\[
\frac{0.0737 \times 97 \times 100}{70 \times 9.9038} = 1.03; \quad \frac{0.0323 \times 97 \times 100}{70 \times 9.9038} = 0.45
\]
Alcohol-ether-water-soluble, extractive phosphorus.

\[
\frac{0.0051 \times 97 \times 100 \times 62}{70 \times 9.9038 \times 222.7} = 0.020
\]

The same method applies to the calculation of the alcohol-ether-water-soluble phosphorus. The filtrate obtained was only 74 cc., but as only 5 cc. of chloroform were used, the remaining watery solution must have remained clinging to the rather spongy mass of fat and chloroform. Some little solution goes to moisten the filter paper, but this partly counter-balances whatever chloroform may have gone into solution. This method of calculation is not absolutely accurate, but comes sufficiently near, considering the variations to which the material is liable in any case.

3. The kephalin receives a correction for the amount of phosphorus in the liquid clinging to the lipoid precipitate, in this case 95 - 74 = 21 cc., equivalent to 0.06 milligram of phosphorus or 0.08 per cent of kephalin.

Theoretically the correction should be distributed between the lecithin and kephalin, the values of both of which it affects. As it is not improbable, however, that the water-soluble phosphoric acid derivatives with which we are here dealing form insoluble lead salts in ammoniacal alcohol solution, it was deemed best to apply the whole correction to the kephalin. The kephalin in the case of brain tissues receives a further correction for the phosphorus found in the sulphur compound.

In the light of later results it was found advisable to change the method for the estimation of kephalin outlined in a previous paper as follows:

This Journal, i, p. 208, line 7. Substitute: allow to remain on water-bath until there is no more smell of ammonia. The flask is then set aside to cool, after replacing the alcohol which has evaporated.

p. 208, line 12. Read: and the precipitate washed once with hot alcohol.

p. 208, line 21. Experience has shown that it is preferable to burn the filter paper with the precipitate.

In the muscle tissues of animals containing a solid fat the correction for the kephalin on account of filtrate adhering to lipoid precipitate may become rather large. It can, however, be easily reduced by making the precipitate in a larger flask (200 cc. or 400 cc.) and thus diluting the adhering filtrate.

The method above outlined should be capable of more general application to normal and pathological material. Such a study on the nervous system is soon to be published. This investigation was aided by grant from the Rockefeller Institute for Medical Research.
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W. Koch

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