THE INFLUENCE OF FORMALIN FIXATION ON THE LIPOIDS OF THE CENTRAL NERVOUS SYSTEM.

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During an investigation on lipoid changes of the central nervous tissues under pathological conditions, the necessity for a reliable quantitative method which would allow a differentiation of the manifold lipoids of the tissues arose. At the same time the problem had to be solved whether it was possible to utilize formalin-fixed tissue instead of fresh material.

The enthusiasm which followed the publications of Aschoff and his pupil Kawamura (1) on the differential staining of the lipoids has gradually subsided. It even gave way to a severe criticism of the many histological methods which had been devised based partially on these investigations (Smith, Ciaccio, Dietrich, etc.). Critical observers pointed out that one is not safe in applying test-tube staining methods of isolated lipoids or of their mixtures to the staining of sections of tissue. Kutschera-Aichbergen (2) found that after extraction with warm acetone the extracted sections of suprarenal glands, of brain, and of sclerotic arteries did not stain with the above methods. Besides the neutral fats and cholesterol, acetone also removes a small part of the phosphorus-containing lipids.1 Kaufmann and Lehmann (3) demonstrated recently that the Ciaccio method depends on the presence of oleic acid and that it is not specific for any given lipid. Shapiro (4) reached conclusions similar to those of Kutschera-Aichbergen.

Aside from the present histological methods for the differentiation of the lipoids there remain the quantitative chemical methods for fractional extraction (Fraenkel, MacLean (5)). Many of the figures on the lipoid content of human tissues which are pub-

1 Lipoids = total of fatty substances = neutral fats + cholesterol and its esters + lipids (phosphatides and cerebrosides).
lished in the text-books have been taken from analytical work on formalin-fixed material. This applies especially to the work of Smith and Mair (6) on the development of the brain of growing animals and man, on the comparative studies of gray and white matter, and on the lipoid changes of the nervous system in disease. No attempt seems to have been made to study the influence of the fixing agents on the final results of those methods. It seems to be generally recognized that formalin, the fixing agent which at present is mostly applied for the conservation of nervous tissue, is an excellent preservative for the lipoids (Romeis (7), Lee (8)). Fresh, unfixed material after extraction with acetone does not stain with Sudan III, the Smith, and the Ciaccio methods. Kutschera-Aichbergen, however, demonstrated that formalin-fixed sections of different tissues gave positive staining reactions with these methods after the different steps of extraction with acetone, alcohol, and ether. Spatz (9) has shown that formalin which had been neutralized and which had served for fixation of brains reacted acid after some time. The acidity increased with progressive preservation, while formalin alone without tissue did not undergo similar changes. He thought that the combination of formaldehyde with the amino acids of the proteins and the resulting acid methylene substances were the reason for the constantly increasing acid reaction. The method of Sörensen for the formol titration of amino acids is based on this chemical reaction. Blum who first recommended formaldehyde solutions for fixing purposes has also studied the reaction with the proteins (10).

The aim of the present investigation was to study the behavior of the lipoids of the central nervous system following formalin fixation. The method of fractional extraction which was applied is based on the work of H. and I. S. MacLean (5). The different steps of the extraction may be outlined in the accompanying scheme. A more detailed description has been given elsewhere (11).

1. The fresh substance is extracted with acetone at room temperature.

2. The dried tissue is extracted with absolute alcohol in a Soxhlet apparatus.

3. The extract is concentrated and excess of acetone added.
4. The precipitate is extracted with cold alcohol.

Soluble part; Insoluble part;
lecithin chiefly cephalin chiefly also

Treated with ether

Solution contains
lecithin

White remainder consists of traces
of sphingomyelin
and galactolipids

Solution contains
White remainder contains sphingomyelin
and galactolipids

Treated with pyridine

Soluble part, Insoluble part,
galactolipids sphingomyelin
chiefly chiefly

I am obliged to Dr. J. P. Simonds and Dr. W. W. Brandes for the four human brains which were used for this study and all of which were removed within 5 to 10 hours after death. The central nervous system of these cases did not show anatomical signs of disease. One brain was obtained 6 hours after death from an accident and served as control. In the accompanying tabular matter average figures are given because the present investigation does not attempt to establish standard figures for brains of different age and sex. The intention was to demonstrate the changes of the different lipoid fractions during the progressing process of formalin fixation.

First the amount of phosphorus which was found in the fixing fluid after different stages of fixation was determined. It was found that with 2 liters of 10 per cent formalin (4 per cent solution of formaldehyde) per 1000 gm. of brain 110 mg. of phosphorus per 1000 gm. of fresh brain had passed into the fixing fluid after 24 hours. This formalin was renewed after 24 hours and phosphorus analyses of this second formalin (Neumann’s method) were made at different intervals with the following results.

<table>
<thead>
<tr>
<th>Days of fixation</th>
<th>22</th>
<th>27</th>
<th>38</th>
<th>63</th>
<th>93</th>
<th>109</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg. P per 1000 gm. fresh brain</td>
<td>340</td>
<td>378</td>
<td>400</td>
<td>429</td>
<td>476</td>
<td>532</td>
</tr>
</tbody>
</table>
TABLE I.

Fractional Extraction of Fresh and Formalin-Fixed Nervous Tissue in Per Cent of Dry Substance. Water in Per Cent of Fresh Tissue.

Averages from the central nervous systems of six cats (fresh), five cats (formalin), and three human brains.

<table>
<thead>
<tr>
<th></th>
<th>Cats.</th>
<th>Human brains.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brains</td>
<td>Spinal cords.</td>
</tr>
<tr>
<td>Days</td>
<td>0 22</td>
<td>0 22</td>
</tr>
<tr>
<td>Water in per cent of fresh tissue</td>
<td>75.2 77.2 67.8 68.7 71.1</td>
<td>82.0 85.3 84.3 68.6 72.7 73.1</td>
</tr>
<tr>
<td>I. Acetone extract</td>
<td>10.0 12.1 17.3 20.3 22.6</td>
<td>15.4 19.8 16.3 21.3 23.7 20.0</td>
</tr>
<tr>
<td>II. Alcohol extract</td>
<td>45.5 36.1 55.3 43.8 42.8</td>
<td>31.0 21.3 20.4 47.6 40.0 40.4</td>
</tr>
<tr>
<td>Total</td>
<td>55.5 48.2 72.6 64.1 65.4</td>
<td>46.4 41.1 36.7 68.9 63.7 60.4</td>
</tr>
<tr>
<td>Precipitate</td>
<td>18.0 15.7 28.0 25.3 22.5</td>
<td>12.2 7.8 6.9 30.6 22.1 19.2</td>
</tr>
<tr>
<td>III. Alcohol-soluble</td>
<td>5.0 4.9 7.8 8.4 9.4</td>
<td>2.7 1.8 2.5 5.2 9.1 6.9</td>
</tr>
<tr>
<td>IV. Ether-soluble</td>
<td>6.9 4.3 9.8 7.3 6.5</td>
<td>5.6 2.8 1.7 11.3 5.5 3.2</td>
</tr>
<tr>
<td>V. Pyridine-soluble</td>
<td>2.4 3.1 4.6 5.5 4.6</td>
<td>2.0 2.1 2.0 9.9 6.1 7.6</td>
</tr>
<tr>
<td>VI. Pyridine-insoluble</td>
<td>3.7 3.4 6.1 4.1 2.0</td>
<td>1.9 1.1 0.7 4.2 1.4 1.5</td>
</tr>
<tr>
<td>Rest of extraction</td>
<td>44.5 51.8 27.4 35.9 34.6</td>
<td>53.6 58.9 63.3 31.1 36.3 39.6</td>
</tr>
</tbody>
</table>

TABLE II.

Distribution of Phosphorus in Different Extracts. Averages of Three Human Brains.

<table>
<thead>
<tr>
<th></th>
<th>Gm. P per 1000 gm. dry substance.</th>
<th>Gm. P per 1000 gm. fresh substance.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 22</td>
<td>102</td>
</tr>
<tr>
<td>Acetone extract</td>
<td>1.89</td>
<td>2.75</td>
</tr>
<tr>
<td>Alcohol extract</td>
<td>7.52</td>
<td>6.87</td>
</tr>
<tr>
<td>Total extract</td>
<td>9.41</td>
<td>8.62</td>
</tr>
<tr>
<td>Rest of extract</td>
<td>4.72</td>
<td>4.28</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>14.13</td>
<td>14.19</td>
</tr>
</tbody>
</table>
In order to determine whether this increase in phosphorus was due to a gradual extraction of formalin-soluble phosphorus-containing substances from the brain, to an incomplete fixation with continuing autolysis, or to a direct action of the formalin on the brain the following experiment was made. Fresh brain tissue was ground in a mortar and for 3 hours extracted in a Soxhlet apparatus with boiling water. By this process all fermentative action was interrupted and autolysis prevented. Furthermore all the water-soluble phosphorus-containing substances were extracted. This thoroughly extracted and denatured brain substance was preserved in 10 per cent formalin. After 22 days 256 mg. of P per 1000 gm. of fresh brain were found, after 63 days this amount had increased to 453 mg. per 1000 gm. of brain. This result demonstrates that the third assumption which was stated above is the reason for the increase of phosphorus in the fixing fluid.

To control the results obtained from human material, brains and spinal cords of cats were removed immediately after death and preserved in 10 per cent formalin. After 22 and 92 days respectively they were treated with the same method of fractional extraction that had been applied to the human fresh or formalin-fixed material.

Tables I and II give the values obtained.

**DISCUSSION.**

Both the material obtained from human brains and the central nervous system of cats showed similar changes under the influence of formalin fixation. The increase in water, with swelling, in formalin solutions has been frequently noticed and reported (12). The influence of the lipoid content on the swelling of the brain has been studied earlier (13). The acetone extracts containing neutral fats, cholesterol, and its esters, and small amounts of phosphatides are not changed except in the spinal cords of cats. In the human material there seems to be an increase of the phosphorus-containing substances which are dissolved by acetone after 22 days; after 102 days this amount is the same as in the non-fixed material. The alcohol extracts containing the lipids are considerably diminished and consequently the amount of phosphorus-containing lipids is in the human material reduced by about 30 per cent at the end of 102 days. At the same time
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the amount of lipids which can be precipitated from the alcohol extract by acetone (precipitate in Table I) has decreased correspondingly. In analyzing the different fractions of this precipitate it will be found, that the alcohol-soluble part (containing mainly lecithin) has not changed or is even increased. The same is true of the pyridine-soluble part (containing mainly the galactolipids or cerebrosides). On the other hand the ether-soluble part (containing mainly cephalin) and the part which is not soluble in alcohol, ether, or pyridine (mainly sphingomyelin) are reduced to one-third of the original value.

An analysis of Table II demonstrates that the total content of phosphorus calculated for the fresh substance is diminished by 10 per cent in the gray and by 23 per cent in the white matter of the brain. The amount of phosphorus in mg. which was dissolved by the formalin is calculated from these values as 551 mg. per 1000 gm. of brain. In order to be able to carry out the calculation of the phosphorus content of the whole brain, one has to know the relative part of white and gray substance. This relationship can be established by weighing the separated gray and white substance of a brain, which is a task difficult to perform. A second way is to determine this relationship from the water content of gray matter, white matter and the total brain. Two formulas have to be applied: (1) \(a x + b y = 100 c\); and (2) \(x + y = 100\) (\(x\) = white matter, \(y\) = gray matter in per cent of total brain; \(a\), \(b\), and \(c\) = water content of white matter, gray matter, and total brain respectively in per cent of fresh substance). From the averages of a large number of normal brains the relation of 43 per cent white matter: 57 per cent gray matter has been established (14). The number of 551 mg. of phosphorus per 1000 gm. of brain which was thus calculated corresponds very closely to the amount (532 mg. of P) actually found in the formalin after 102 days.

From these data one may reconstruct the action of formaldehyde solution on the lipoids of the central nervous system as follows. Preservation in formalin does not fix the lipoids. After thorough extraction with alcohol in the Soxhlet apparatus the amount of phosphorus which remains in the residues is the same in fresh tissues as in formalin-fixed tissues. The amount of acetone-soluble substances (neutral fats, cholesterol, and its esters) also remains approximately the same. On the other hand the phos-
phatides which can be extracted with alcohol in the Soxhlet apparatus have been hydrolyzed under the influence of the formaldehyde. The phosphoric acid component is split off and is finally found in a combination which is water-soluble. This hydrolyzing process seems to affect mainly the cephalin and the sphingomyelin fractions. Between the beginning of formalin preservation and this final stage there seems to be a stage in which first intermediary products are formed. These are not yet soluble in water but easily soluble in acetone and alcohol. The galactolipids (cerebrosides) which do not contain phosphoric acid are preserved.

Many facts which by microscopical study are already empirically known to the pathologist may be explained on the basis of the above findings.

1. Galactolipids (cerebrosides) form a considerable part of the white matter. The white matter of the brain and the spinal cord is mainly composed of the conductive elements of the nervous tissue, i.e., axons and their myelin sheaths, while the gray matter contains mainly the ganglion cells. The other fractions of the lipids are twice as large in the white matter and in the spinal cord as in the gray matter. The amount of galactolipids, however, is 5 times as large. The galactolipids are not soluble in alcohol and ether at room temperature, but are soluble in pyridine. This may explain the favorable effect of the staining of the axons after pyridine treatment in the methods of Cajal, Ranson, and Bielschowsky. The removal of the galactolipids makes the subsequent penetration with silver nitrate easier.

2. Metachromatic staining bodies which also stain with mucicarmine have been described in formalin-fixed nervous tissue, which had been treated with alcohol (Buscaino's "grape-like bodies", "mucocitic degeneration of the neuroglia" of Grynfeltt and Pelissier, "mucine-like bodies" of Lhermitte, Kraus, and Bertillon (15)). Bailey and Schaltenbrand showed similar staining reactions in swollen oligodendroglia and assumed that the bodies mentioned above may originate from the decomposition of these diseased glia cells (16). Mucicarmine not only stains mucine, i.e. combinations of proteins with glucosamine, but also other substances, e.g., galactolipids. It does not stain the rest of the lipids, as may easily be demonstrated by the following experiments. Chloroform solutions of the different fractions which were
obtained by the extraction of brain tissue (Fractions I, III, IV, V, and VI of Table I) are spread on glass slides and allowed to dry. They are simultaneously stained for 2 minutes in a 1 per cent solution of mucicarmine and then rinsed in water. Only the slide with Fraction V (containing galactolipids) is stained red. Toluidine blue, however, stains all the different fractions with the exception of Fraction I (neutral fats and cholesterol) blue; Fraction III is stained lighter than the three others. Treatment with 95 per cent alcohol for a sufficient length of time will produce a distinct shade of pink color in Fraction V, while Fractions III, IV, and VI stain more blue.

The mucine bodies are not soluble in alcohol and ether at room temperature but are soluble in chloroform. They cannot be removed by ammonia or dilute acids, but they disappear in hot water at 60°. Galactolipids have similar physical qualities; besides they are relatively increased in the white matter after formalin treatment. By treatment of formalin tissue with alcohol the remainder of the phosphatides which was not hydrolyzed is removed (sphingomyelin having for the greater part been previously destroyed), while galactolipids remain. Ferraro states that he found grape-like areas in the white matter of frozen sections from formaldehyde-fixed material only after treatment with alcohol.

Considering all these different facts one might advance the hypothesis that the staining and other physical qualities of these different areas or bodies are due to the presence of free galactolipids (cerebrosides). Their seemingly frequent appearance in the white matter of diseased brains fixed in formalin may be explained by the assumption of lipid changes intra vitam which favored the following liberation of the galactolipids from the rest of the lipids by formalin and alcohol treatment. Preliminary experiments seem to indicate that the appearance of phosphorus in the fixing fluid occurs much more rapidly in pathologic brains than in normal ones.

**Conclusions.**

1. The present opinion that formaldehyde solutions are a fixative for the preservation of lipoids should be revised.
2. In a 10 per cent solution of formalin (4 per cent solution of
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formaldehyde) the phosphatides are hydrolyzed and the liberated phosphoric acid is found in a water-soluble composition in the fixing fluid. This process of decomposition proceeded gradually and was still found after 90 days fixation.

3. Cholesterol and galactolipids (cerebrosides) are not appreciably changed. Consequently the resulting mixture of lipids after formalin fixation contains more galactolipids than the original tissue.

4. The preservation and relative increase of galactolipids in formalin-fixed tissue have been utilized to explain some empirically known histological facts; namely, the effect of pyridine in silver staining methods of nerve fibers and the staining and physical qualities of areas of so called "mucoid degeneration" which are found in formalin material after alcohol treatment.

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