170. Separation and Identification of N-Acetylhexosamines and N-Acetylneuraminic Acid by Two-dimensional Electrophoresis and Chromatography on Paper

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In previous papers\(^1,2\) from this laboratory, it was reported that the separation of N-acetylhexosamines and N-acetylneuraminic acid\(^3\) can be performed by chromatography on paper impregnated with tetraborate in n-butanol-pyridine-water solvent systems and the order of \(R_F\) values of these N-acetylamino sugars was as follows: N-acetylglucosamine\(^4\) > N-acetylmannosamine\(^5\) > N-acetylgalactosamine\(^6\) > SA.\(^7\) On another occasion,\(^4\) paper electrophoresis with tetraborate was found to be a valuable method for the separation of these N-acetylamino sugars; and the order of electrophoretic mobilities of these N-acetylamino sugars was as follows: SA > ManNAc > GalNAc > GlcNAc. These studies\(^4\)-\(^6\) show that paper electrophoresis differs essentially from paper chromatography, in efficiency on the separation of these N-acetylamino sugars. It is therefore expected that separation with higher resolving power may be obtained by a method in which electrophoresis is combined with chromatography on tetraborate-buffered paper.

This paper describes a two-dimensional combination of paper electrophoresis and paper chromatography for the separation and characterization of these N-acetylamino sugars and its application to the identification of free N-acetylamino sugars in a human ovarian cyst fluid.

**Experimental. Buffers, solvents and paper strip.** Potassium tetraborate and sodium tetraborate in concentration of each 0.025M\(^8\) were used as buffers (electrolytes). n-Butanol-pyridine-water solvent systems (solvent A, 2:2:1\(^9\) and solvent B, 6:4:3,\(^10\) by volume) were used as solvents. Whatman No. 3 MM paper strip with dimension as shown in Fig. 1 was used.

**Two-dimensional paper electrophoresis-chromatography.** Before being placed in an electrophoretic apparatus, the paper strip was moistened with a buffer and then pressed between two sheets of clean filter paper to remove the excess of the buffer. The strip was then
placed in the apparatus and the parts A and (another) A of two tags were dipt in each buffer and the part C of the strip was hung in air. Sample solutions were applied to the starting point (origin) and the voltage was connected 5 min. later, in order to allow the diffusion of the buffer. Electrophoresis was carried out by the horizontal open strip method at 200 V for 6 hr. Platinum electrodes were used. After electrophoretic run, the strip was dried at room temperature in a current of air for not less than 2 hr. The tags (A+B and another A+B) were cut off from the strip. The dry paper strip, now 18 cm x 24 cm, was irrigated in a n-butanol-pyridine-water solvent system (solvent A or B) at 7±1°C for 16 hr. by the ascending technique. After development, the strip was dried at room temperature in a current of air.

Detection of spots. Color test I (for the detection of N-acetylhexosamines, SA and chromogens): The strip was first sprayed with the water-saturated n-butanol spray and heated at 95°C for 5 min. and then sprayed with the p-dimethylaminobenzaldehyde spray at room temperature. The N-acetylhexosamines and chromogens gave violet spots on a white background and SA produced slowly a red violet spot which was stable for a few days. Color test II (for the detection of chromogens): The strip was sprayed with the PDBA spray at room temperature. The N-acetylhexosamines and SA did not react under this condition. Chromogens gave violet spots on a white background.

Identification of free N acylhexosamines in a human ovarian cyst fluid by two-dimensional paper electrophoresis-chromatography.

**N-Acetylhexosamines are partly converted into some chromogens which give a positive reaction with the PDBA spray at room temperature, by treatment with the columns of Amberlite IR-120 (H⁺ form) and Amberlite IR-4B (OH⁻ form) or by heating on paper impregnated with tetraborate. These chromogens may be probably anhydro-N-acetylhexosamines and furan derivatives.
Four hundred and fifty ml of a human ovarian cyst fluid (pseudomucinous) was dialyzed in a cellophan tube (Visking CO) against 4.5 l of distilled water at 3–4°C for 4 days. After dialysis, the dialysate was passed through a column (27 mm × 300 mm) of Amberlite IR–120 resin (H⁺ form), 100–200 mesh, and the column was washed with 100 ml of distilled water. The effluent and washings combined. The combined solution was passed through a column (25 mm × 350 mm) of Amberlite IR–4B resin (OH⁻ form), 100–200 mesh, and the column was washed with 100 ml of distilled water. The effluent and washings were combined. The combined solution was concentrated to about 2–3 ml at 40–45°C under reduced pressure and the concentrated solution was evaporated to dryness in a vacuum desiccator. The residue (125 mg) was taken up in 1 ml of distilled water and 0.04 ml of the resulting solution was applied to the origin of paper strip. Two paper strips were used in this experiment and each strip was subjected to the two-dimensional paper electrophoresis-chromatography described above. One of two paper electrodichromatograms obtained was treated by the color test I (Table II and Fig. 6) and another electrodichromatogram was treated by the color test II (Table II and Fig. 7).

Results. When the N-acetylated hexosamines and SA were subjected to the two-dimensional paper electrophoresis-chromatography, the results shown in Table I were obtained. Figs. 2, 3, 4, and 5 show the tracings of typical maps of GlcNAc, GalNAc, ManNAc, and SA,

<table>
<thead>
<tr>
<th>Borate buffer</th>
<th>N-Acetylated sugars*2</th>
<th>The first dimension.</th>
<th>The second dimension.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>General name</td>
<td>Paper electrophoretic mobility</td>
<td>Paper chromatographic mobility</td>
</tr>
<tr>
<td>K₂B₄O₇</td>
<td>GlcNAc</td>
<td>+ 2.5</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>GalNAc</td>
<td>+ 4.4</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>ManNAc</td>
<td>+ 8.2</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>+11.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Na₂B₄O₇</td>
<td>GlcNAc</td>
<td>+ 1.7</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>GalNAc</td>
<td>+ 3.5</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>ManNAc</td>
<td>+ 7.4</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>+10.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*1) The positive sign denotes movement towards the anode. The negative sign denotes movement towards the cathode.  
*2) The N-acetylated amino sugar solution in concentration of 5 mg/ml was used in this work.
applied as a mixture on the paper. These results and maps show that the N-acetylhexosamines and SA can be completely separated from one another under the conditions employed.

In order to examine the applicability of the two-dimensional technique, neutral fraction obtained from the dialysate of a human ovarian cyst fluid was subjected to the two-dimensional paper electrophoresis-chromatography. The experimental results are given in Table II and the tracings of maps obtained are shown in Figs. 6 and 7. Spot 3 in Fig. 6 was identified as of GlcNAc by its electrophoretic and chromatographic mobilities. The electrophoretic and chromatographic mobilities of spots 4 and 7 in Fig. 6 were found to coincide with those of spots 14 and 15 in Fig. 7, respectively. Therefore, spots 4 (or 14) and 7 (or 15) were identified as of chromogens.** Spots 5 and 9 in Fig. 6 are of unidentified 2-acetamido-2-deoxy-hexoses (or 2-acetamido-2-deoxy-aldoses). Spots 1, 2, (or 13), 6, 8, 10, 11, and 12 are of unidentified compounds.

**Conclusion.** From these studies, it is concluded that two-dimensional paper electrophoresis-chromatography described above is useful for the separation and identification of the N-acetylhexosamines, SA
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and chromogens in admixture.

Figs. 2-5. The tracings of maps of two-dimensional paper electrophoresis-chromatography of N-acetylhexosamines and SA (see Table I).
1: GlcNAc, 2: GalNAc, 3: ManNAc, 4: SA.

Figs. 6 and 7. The tracings of maps of two-dimensional paper electrophoresis-chromatography of free N-acylamino sugars in a human ovarian cyst fluid (see Table II).
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References