129. Paper Chromatography of N-Acetylneuraminic Acid using *n*-Butanol-Pyridine-Water (6:4:3) Solvent

By Seiichi OHKUMA and Toshiaki SHINOHARA

Laboratory of Biochemistry, National Research Institute
of Police Science, Tokyo

(Comm. by Tanemoto FURUHATA, M.J.A., Sept. 12, 1964)

A provisional identification of sialic acids can be performed by paper chromatography using *n*-butanol-acetic acid-water (4:1:5),^{1),2)} secbutanol-acetic acid-water (4:1:5),¹⁾ ethyl acetate-acetic acid-water (3:1:3),¹⁾ *n*-butanol-*n*-propanol-0.1 N hydrochloric acid (1:2:1),³⁾ *n*-butanol-pyridine-water (6:4:3),^{1),2)} ethyl acetate-pyridine-water (2:1:2),¹⁾ and/or ethyl acetate-pyridine-acetic acid-water (5:5:1:3).¹⁾

However, during the course⁴⁾ of the isolation and identification of N-acetylneuraminic acid from mucoprotein in human ovarian cyst fluid in this laboratory, it has been found that two spots sometimes appear in chromatography of N-acetylneuraminic acid on Whatman No. 1 paper in *n*-butanol-pyridine-water (6:4:3). This paper describes a property of N-acetylneuraminic acid in paper chromatography using *n*-butanol-pyridine-water (6:4:3) as a solvent.

Experimental. Crystalline N-acetylneuraminic acid (m.p. (decomp) 184–5°, $C_{11}H_{19}NO_9$, calculated, C 42.74%, H 6.19%, N 4.53%, found, C 42.67%, H 5.90%, N 4.71%, $[\alpha]_D^{23.5^\circ}$ –31.6°) was prepared from mucoprotein in human ovarian cyst fluid by the procedure⁵⁾ of sulfuric acid-hydrolysis. Whatman No. 1 paper (2 cm × 40 cm) and 1% hydrochloric acid-treated Whatman No. 1 paper (2 cm × 40 cm) were used for chromatography of N-acetylneuraminic acid. 1% Hydrochloric acid-treated Whatman No. 1 paper was prepared as follows: Five of Whatman No. 1 paper (24 cm × 40 cm) were piled on a glass plate (0.5 cm × 30 cm × 34 cm) of a washing apparatus shown in Fig. 1, and the ends of the strips were dipped in 1% hydrochloric acid in box A (depth 4.5 cm, width 6 cm, and length 35 cm), and the other ends of the strips were hung in air, and one liter of 1% hydrochloric acid allowed to run. Box B is a receiver for 1% hydrochloric acid which ran from box A. The strips were then washed with distilled water to remove the excess of acid until the water washings were free from chlorine ion. The strips were dried in air at room temperature and cut in a piece (2 cm × 40 cm). One-dimensional and ascending chromatography of N-acetylneuraminic acid was carried out on Whatman No. 1 and 1% hydrochloric acid-treated Whatman No. 1 papers in *n*-butanol-pyridine-water (6:4:3) for 20 hours at 7–9°. The

Fig. 1. Washing apparatus.

spray reagents used were: The indirect Morgan-Elson (The paper was first sprayed with the potassium tetraborate-ethanol spray reagent (this reagent was prepared freshly before use by mixing equal parts of 0.1 M potassium tetraborate and ethanol)⁶⁾ and

steamed for 10 to 15 minutes in a steam oven. The steamed paper was then sprayed with the p-dimethylaminobenzaldehyde spray reagent⁷⁾ and allowed to develop at room temperature), orcinol-trichloroacetic acid,⁸⁾ p-anisidine phosphate,⁹⁾ periodate-thiobarbiturate,¹⁰⁾ periodate-iodide-starch (The paper was sprayed lightly and evenly with the periodate spray reagent (230 mg of H_5IO_6 was dissolved in 100 ml of water), allowed stand for 8–10 minutes at room temperature and then sprayed with the potassium iodide-starch spray reagent (this reagent was a solution of saturated sodium tetraborate solution containing 0.8% potassium iodide, 0.9% boric acid, and 3% soluble starch, boiling to bring the starch in solution)¹¹⁾, aniline hydrogen phthalate¹²⁾ (at 105° for 5 minutes), ninhydrin⁹⁾ (at 105° for 5 minutes), ammoniacal silver nitrate,⁸⁾ bromothymol blue¹³⁾ and/or 2,6-dichlorophenolindophenol¹⁴⁾ spray reagents.

Results and discussion. When 50 γ , 100 γ , and 200 γ of N-acetylneuraminic acid were submitted to chromatography on untreated-paper, these samples were separated each into two spots (spots A and B). Both spots gave positive reactions with the indirect Morgan-Elson, orcinol-trichloroacetic acid, p-anisidine phosphate, periodate-thiobarbiturate, and periodate-iodide-starch spray reagents and did not react with the aniline hydrogen phthalate, ninhydrin, and ammoniacal silver nitrate spray reagents. The spot with large R_f value (spot A) gave a positive reaction with the bromothymol blue or 2,6-dichlorophenolindophenol spray reagent, whereas the spot with small R_f value (spot B) did not react with the same pH spray reagent. The color reactions and R_f values of both spots are summarized in Table I. Under the same chromatographic conditions, 5 γ , 10 γ , and 20 γ of N-acetylneuraminic acid gave each one spot (spot C). The spot C gave positive reactions with the indirect Morgan-Elson, orcinol-trichloroacetic acid, and periodate-thiobarbiturate spray reagents and did not react with the bromothymol blue, aniline hydrogen phthalate, ninhydrin, and ammoniacal silver nitrate spray reagents. The color reactions and R_f values of the spot C are summarized in Table II. The results given in Tables I and II indicate that the spot B is identical with the spot C and that the spots A, B, and C have the same N-acetylneuraminate structure, and the spot A is a free acid form, and the

Table I. Chromatography of N-acetylneuraminic acid on Whatman No. 1 paper in *n*-butanol-pyridine-water (6:4:3)

Spray reagent	N-acetylneuraminic acid						
	Spot	50 γ		100 γ		200 γ	
		Color	Rf	Color	Rf	Color	Rf
Indirect Morgan-Elson	A	P	0.17	P	0.18	int-P	0.19
	B	l-P	0.09	l-P	0.09	l-P	0.09
Orcinol-trichloroacetic acid	A	GyV	0.17	GyV	0.18	int-GyV	0.19
	B	l-GyV	0.09	l-GyV	0.09	l-GyV	0.09
p-Anisidine phosphate	A	GyV	0.17	GyV	0.18	int-GyV	0.19
	B	l-GyV	0.09	l-GyV	0.09	l-GyV	0.09
Periodate-thiobarbiturate	A	O	0.17	RO	0.18	OR	0.19
	B	l-O	0.09	l-O	0.09	l-O	0.09
Periodate-iodide-starch	A	Wht	0.17	Wht	0.18	Wht	0.19
	B	Wht	0.09	Wht	0.09	Wht	0.09
Bromothymol blue	A	Y	0.17	Y	0.18	Y	0.19
	B	non	—	non	—	non	—
2,6-Dichlorophenolindo-phenol	A	BrR	0.17	BrR	0.18	BrR	0.19
	B	non	—	non	—	non	—

l: light, int: intense, Br: Brown, Gy: Gray, O: Orange, P: Purple, R: Red, V: Violet, Wht: White, Y: Yellow.

Table II. Chromatography of N-acetylneuraminic acid on Whatman No. 1 paper in *n*-butanol-pyridine-water (6:4:3)

Spray reagent	N-acetylneuraminic acid						
	Spot	5 γ		10 γ		20 γ	
		Color	Rf	Color	Rf	Color	Rf
Indirect Morgan-Elson	C	l-P	0.08	l-P	0.08	l-P	0.09
Orcinol-trichloroacetic acid	C	l-GyV	0.08	l-GyV	0.08	l-GyV	0.09
Periodate-thiobarbiturate	C	l-O	0.08	l-O	0.08	l-O	0.09
Bromothymol blue	C	non	—	non	—	non	—

spots B and C are a non-acidic form, probably a salt.

When chromatographed on treated-paper, 5 γ , 10 γ , 20 γ , and 50 γ of N-acetylneuraminic acid gave one spot (spot D) on each chromatogram. The spot D gave positive reactions with the indirect Morgan-

Table III. Chromatography of N-acetylneuraminic acid on 1% hydrochloric acid-treated Whatman No. 1 paper in *n*-butanol-pyridine-water (6:4:3)

Spray reagent	N-acetylneuraminic acid								
	Spot	5 <i>r</i>		10 <i>r</i>		20 <i>r</i>		50 <i>r</i>	
		Color	Rf	Color	Rf	Color	Rf	Color	Rf
Indirect Morgan-Elson	D	l-P	0.23	l-P	0.23	P	0.20	int-P	0.21
Orcinol-trichloroacetic acid	D	l-GyV	0.23	l-GyV	0.23	l-GyV	0.20	GyV	0.21
Periodate-thiobarbiturate	D	l-O	0.23	l-O	0.23	l-O	0.20	O	0.21
Bromothymol blue	D	non	—	Y	0.23	Y	0.20	Y	0.21

Elson, orcinol-trichloroacetic acid, periodate-thiobarbiturate, and bromothymol blue spray reagents, and did not react with the aniline hydrogen phthalate, ninhydrin, and ammoniacal silver nitrate spray reagents. The color reactions and Rf values are summarized in Table III, and the results indicate that the spot D is free N-acetylneuraminic acid.

It has been shown by Schute¹⁵⁾ that many of the abnormalities occurring in paper chromatography of ionizable acidic substances may be caused by the ion exchange of the hydrogen ions of solute with the cations of the paper and further that the rather common observation of obtaining two spots for one organic acid is probably due to the acid and its salt, and in such cases it may be necessary to wash the paper with hydrochloric acid prior to chromatography. From the present results and Schute's report,¹⁵⁾ it is probable that the formation of multispots in chromatography of N-acetylneuraminic acid on Whatman No. 1 paper using *n*-butanol-pyridine-water (6:4:3) may be due to the ion exchange of the hydrogen ions of the N-acetylneuraminic acid with the cations (such as, sodium, potassium, calcium, and magnesium, etc.) of the paper. Therefore, it is concluded that it is necessary to wash the paper with hydrochloric acid to obtain normal chromatogram of the N-acetylneuraminic acid.

Acknowledgement. The authors wish to thank Prof. T. Furu-hata, director of this institute, for his guidance and encouragement and to thank Dr. M. Watanabe and Dr. E. Fujiwara, for their support in this work.

Summary. Two spots do sometimes arise from authentic sample of N-acetylneuraminic acid, in chromatography of Whatman No. 1 paper using *n*-butanol-pyridine-water (6:4:3). Normal chromatogram of the acid is obtained by the use of Whatman No. 1 paper washed with 1% hydrochloric acid and water. The abnormalities occurring

in chromatography may be caused by the ion exchange of the hydrogen ions of the acid with the cations of the paper.

References

- 1) F. Zilliken and M. W. Whitehouse: *Advances in Carbohydrate Chemistry*, **13**, 237 (1958).
- 2) A. Gottschalk: *The Chemistry and Biology of Sialic Acids and Related Substances*, Cambridge University Press, p. 61 (1960).
- 3) E. Svennerholm and L. Svennerholm: *Nature*, **181**, 1154 (1958).
- 4) S. Ohkuma and T. Shinohara: *Proc. Japan Acad.*, **40**, 604-609 (1964).
- 5) A. Gottschalk: *Chemistry and Biology of Sialic Acids and Related Substances*, Cambridge University Press, p. 40 (1960).
- 6) S. Ohkuma and T. Shinohara: *Proc. Japan Acad.*, **39**, 400, 534 (1963).
- 7) S. M. Partridge: *Biochem. J.*, **42**, 238 (1948).
- 8) R. Klevstrand and A. Nordal: *Acta Chem. Scand.*, **4**, 1320 (1950); L. Svennerholm: *Acta Soc. Med. Upsaliensis*, **61**, 75 (1956).
- 9) J. Cerbulis and C. A. Zittle: *Anal. Chem.*, **33**, 1131 (1961).
- 10) L. Warren: *Nature*, **186**, 237 (1960).
- 11) S. Ohkuma and C. Miyauchi: *Seikagaku*, **34**, 166 (1962).
- 12) S. M. Partridge: *Nature*, **164**, 443 (1949).
- 13) F. Brown and L. P. Hall: *Nature*, **166**, 66 (1950).
- 14) J. W. H. Lugg and B. T. Overell: *Nature*, **160**, 87 (1947).
- 15) J. B. Schute: *Nature*, **171**, 839 (1953).