**148. Chromatography of Erythrocyte Sialoglycopeptides
on DEAE-Cellulose Paper and DEAE-Sephadex
Thin-Layer Plate**

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Previously, it was reported from this laboratory that treatment of human red blood cells with crystalline trypsin,¹⁾ chymotrypsin,²⁾ or pronase³⁾ resulted in destruction of the I,¹⁾ F_y^{a, 2)} or M³⁾ agglutinin on their cells and simultaneous liberation of a sialoglycopeptide with the I, F_y^a, or M blood-group activity. This communication describes the differentiation of these blood-group active erythrocyte sialoglycopeptides by chromatography on DEAE-cellulose paper and DEAE-Sephadex thin-layer plate.

Materials. (1). I,¹⁾ F_y^{a, 2)} and M-N^{3), 4)} (M-N denotes a group of M, N, and MN) blood-group active sialoglycopeptides released from human erythrocytes by the treatment with crystalline trypsin, chymotrypsin, and pronase were used as samples. The carbohydrate composition, ultraviolet absorption, and paper electrophoretic behavior of the sialoglycopeptides are summarized in Table I. (2). Pronase-digested M-N-sialoglycopeptides were prepared as follows: A 0.6 % solution of the M-, N-, or MN-sialoglycopeptide in 0.005 M·CaCl₂, adjusted to pH 7.0 with 0.5 M·NaHCO₃, was incubated at 37°C with pronase (*Streptomyces griseus* protease, Pronase-P, Kaken Chemical Co., Tokyo) at a substrate to enzyme ratio of 50:1, in a dialysis tube (Visking Co.) immersed in 90 vol. of the same buffer with stirring. At intervals of 8h, the outside buffer of the tube was changed and half of the amount of enzyme originally taken was added to the tube. Incubation was carried out for 48 h. The digest was cooled, centrifuged, and dialyzed against 4 changes of distilled water at 3–4°C for 2 days. The dialyzed solution was concentrated in a rotary evaporator at 30°C and lyophilised. The dry residue was purified by preparative paper electrophoresis.^{1)–4)} The yields of the pronase-digested sialoglycopeptides (which were crystalline matters with slight brown-yellow color) obtained from 30 mg each of M-, N-, and MN-sialoglycopeptides were 21.6 mg, 22.7 mg, and 23.7 mg, respectively. Some properties of the pronase-digested peptides are presented in Table I. (3). Dinitrophenylsialoglycopeptides which used

Table I. Analysis of blood-group active erythrocyte sialoglycopeptides

Blood-group activity	N-Acetyl-neuraminic acid *1 %	Hexosamine *2 % (As galactosamine)	Hexose *3 % (As galactose)	Ultraviolet absorption λ max ($m\mu$)	Mobility (cm)*4 in PE *5
F _y ^a	22.20	16.10	15.60	262 268.5	2.2
I	25.90	18.10	18.00	262 268.5	2.9
M *6	34.30	14.60	18.90	257-263	3.5
PD-M	31.80	16.70	18.80	none	3.6
N *6	35.00	15.50	19.10	257-263	3.6
PD-N	33.70	17.00	19.00	none	3.8
MN *6	32.50	15.20	21.50	257-263	3.5
PD-MN	32.70	16.00	21.60	none	3.6

*1 Determined by the direct Ehrlich reaction.⁸⁾ *2 Determined by the modified Morgan-Elson reaction.⁹⁾ *3 Determined by the phenol-sulphuric acid reaction.¹⁰⁾ *4 All sialoglycopeptides migrated towards the anode. *5 Whatman No. 3MM paper strip (32 cm $\times$ 46 cm), 300V, 6h, the horizontal open strip method, the methylene blue staining.⁷⁾ Pyridine-acetic acid-water-acetone, 10: 0.4: 70: 20, by vol. *6 These analytical data were quoted from a previous paper.⁴⁾ PD denotes pronase-digested.

as markers, were prepared from M-N-sialoglycopeptides^{3),4)} by the 2,4-dinitrofluorobenzene method of Sanger.⁵⁾ The yields of dinitrophenyl-M-N-sialoglycopeptides were 93.8 % (M), 94.0 % (N), and 97.3 % (MN), respectively. (4). Ovarian cyst glycoprotein was prepared from human ovarian cyst fluid by a modification of the method of Aminoff *et al.*⁶⁾

Separation methods. (1). *DEAE (Diethylaminoethyl)-cellulose paper chromatography.* A strip of DEAE-cellulose TD 85 paper (Tohoku Pulp Co. Ltd., Tokyo) with dimensions as shown in Fig. 2, was immersed in 0.1 M $\cdot$ CH₃COONa for 1 h. The excess solution was removed by placing the strip on a filter paper. Sample solution containing 0.4 to 0.6 mg. of the erythrocyte sialoglycopeptide or glycoprotein was applied as a spot on an original line of the strip. After the strip was hung in air for 15 to 25 min at room temperature, the L part of the strip was immersed in 0.1 M $\cdot$ NaCl-0.1 M $\cdot$ CM₃COONa which used as a developing solvent. Ascending chromatography was allowed to proceed for 1.5 h in a chromatography chamber. After development the strip was dried in a stream of air at room temperature. The dry strip was immersed in the methylene blue solution⁷⁾ for 1 h and the excess of the dye was removed by

washing with methanol until the background had only a light blue tint. The strip was then dried in air. In this stain, the erythrocyte sialoglycopeptides used gave blue spots, whereas acid mucopolysaccharides gave each a blue violet spot. (2). *DEAE-Sephadex thin-layer chromatography*. To 5 g of DEAE-Sephadex A-25 (fine type) (Pharmacia Fine Chemical Co., Uppsala), 200 ml of 0.1 M·CH₃COONa was added. The mixture was mechanically stirred for 3 h at room temperature and allowed to stand for overnight. The supernatant solution was removed by aspirating and DEAE-Sephadex was mixed with equal volume of 0.1 M·CH₃COONa. The slurry was applied to eight glass plates with an adjustable applicator to a thickness of 0.5 mm. The development of the gel plates was carried out by the descending method in an air-tight box as shown in Fig. 1. Previous

Fig. 1. Development box for DEAE-Sephadex thin layer chromatography.

to the application of the sample, the gel plates were flowed with 0.1 M·CH₃COONa for overnight. Sample solution containing 0.5 mg of the sialoglycopeptide or glycoprotein as applied as a spot on an original line of the gel plate and development was allowed to proceed for 12 to 22 h in 0.4 M·CH₃COONa-0.4M·NaCl. After development the gel plates were each covered with a Na₂CO₃-pyridine mixture-treated paper (This paper was prepared as follows: Whatman No. 1 paper of the same dimensions as the gel plate was immersed in 1 W/V % Na₂CO₃-30 V/V % aqueous pyridine for 1 min and pressed slightly between two sheets of filter paper) and a glass plate of the same dimensions as the gel plate was placed on the paper. After 3 h the glass plates and papers were removed from the gel plates. The stripped papers were dried at room temperature, immersed in the methylene blue solution⁷⁾ for 30 min, washed with 90 % methanol, and dried. The sialoglycopeptides visualized as blue spots on papers. (3). *Paper electrophoresis*. Electrophoresis of the erythrocyte sialoglycopeptides on paper was performed according to the method in a previous paper.⁴⁾

Fig. 2. DEAE-cellulose paper chromatography.

- M: M-active sialoglycopeptide.
 N: N-active sialoglycopeptide.
 MN: MN-active sialoglycopeptide.
 I: I-active sialoglycopeptide.
 F_y^a : F_y^a -active sialoglycopeptide.
 f: faint int : intense 1 : light B : Blue.
 PD: Pronase-digested.

Results and discussion. The I-, F_y^a -, M-N-, and pronase-digested M-N-sialoglycopeptides were subjected to chromatography on DEAE-cellulose paper. The results shown in Figs. 2 and 3 indicate that DEAE-cellulose paper chromatography separates these erythrocyte sialoglycopeptides into 4 to 3 components which react with the methylene blue and that this chromatographic method differentiates the I-, F_y^a -, and M-N- sialoglycopeptides, but not the M-, N-, MN-, and pronase-digested M-N- sialoglycopeptides. The results of DEAE-Sephadex thin-layer chromatography of the erythrocyte sialoglycopeptides shown in Figs. 4 and 5, indicate that the erythrocyte sialoglycopeptides give 1 or 2 main methylene blue positive spots under the conditions of the thin-layer chromatography and that this chromatography differentiates the I-, F_y^a -, and M-N- sialogly-

Fig. 3. DEAE-cellulose paper chromatography.

Fig. 4. DEAE-Sephadex (A-25) thin layer chromatography
Time: 11 h DNP: Dinitrophenyl.

Fig. 5. DEAE-Sephadex (A-25) thin layer chromatography Time: 12 h.

Summary. Chromatography on DEAE-cellulose paper and DEAE-Sephadex thin-layer plate was described for the analytical separation and differentiation of erythrocyte sialoglycopeptides with the blood-group activity.

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References

- 1) Ohkuma, S., and Ikemoto, S.: *Proc. Japan Acad.*, **41**, 482 (1965)., *Nature*, **212**, 198 (1966).
- 2) Ohkuma, S., Ikemoto, S., Miyauchi, C., and Furuhata, T.: *Proc. Japan Acad.*, **42**, 275 (1966).
- 3) Ohkuma, S., Ikemoto, S., Shinohara, T., and Furuhata, T.: *Ibid.*, **42**, 661 (1966).
- 4) Ohkuma, S., and Shinohara, T.: *Biochim. Biophys. Acta*, **147**, 169 (1967).
- 5) Sanger, F.: *Biochem. J.*, **39**, 507 (1945).
- 6) Aminoff, D., Morgan, W. T. J., and Watkins, W. M.: *Ibid.*, **46**, 426 (1950).
- 7) Ohkuma, S., Shinohara, T., and Miyauchi, C.: *Nature*, **207**, 527 (1965).
- 8) Werner, I., and Odin, L.: *Acta Soc. Med. Upsalien.*, **57**, 230 (1952).
- 9) Ohkuma, S., Shinohara, T., and Furuhata, T.: *Proc. Japan Acad.*, **42**, 970 (1966).
- 10) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F.: *Anal. Chem.*, **28**, 350 (1956).

copeptides, but not the M-, N-, and MN-sialoglycopeptides and their pronase-digest. The glycoproteins from human ovarian cyst fluid and bovine submaxillary gland did not migrate under the same conditions of both chromatography. The erythrocyte sialoglycopeptides each gave only single spots in paper electrophoresis with volatile buffer as presented in Table I. It is therefore concluded that both chromatographic methods described here are more useful than the paper electrophoresis for the analytical separation and differentiation of the erythrocyte sialoglycopeptides and that these two methods may be applicable to the analysis of various sialoglycopeptides.