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Ion-Exchange Chromatography of Amino Acids and Related Amines on[1] Cellulose Sulfate-Impregnated Cellulose Thin-Layers

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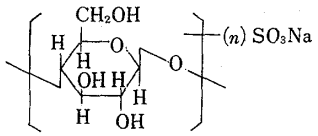
Amino acids, biogenic amines, and local anesthetics could be separated and identified by chromatography on cellulose sulfate-impregnated cellulose thin-layers. Properties of the cellulose sulfate, elution systems, and conditions suitable for the chromatography were investigated. A comparison of chromatographic results obtained for the compounds was made on between the cellulose sulfate-impregnated cellulose and the ordinary cellulose layers.

Recently, paper chromatography of amino acids and biogenic amines employing a cation-exchange paper "cellulose phosphate paper (P-cellulose)" or a cation-exchange resin loaded paper "sulfonated polystyrene resin loaded paper (SPRL paper)," has been prevailed because these ion-exhcange cellulose products share the advantages of paper and column chromatography, permitting simultaneous analysis of many small samples with good resolution and quantitative recoveries.[3,4]

The present paper describes the preparation of cellulose sulfate-impregnated cellulose layers and their application to ion-exchange chromatography of amino acids, biogenic amines, and synthetic local anesthetics.

Cellulose sulfate has been known for a long time, and prepared by various methods.[5,6] According to the degrees of sulfation and depolymerization, properties of the cellulose sulfate

Table I. Properties of Cellulose Sulfate



Sample No.	Sulfur (%)	Sulfate (D.S.)	$[\eta]^{a)}$
1	14.65	1.40	0.004
2	14.67	1.41	0.026
3	14.28	1.34	0.147
4	13.42	1.20	0.365

a) Measured in 0.9% NaCl.

1) This work was presented at the 91st Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, April 1971.
2) Location: *Shirokane 5-9-1, Minato-ku, Tokyo, 108, Japan.*
3) C.S. Knight, in M. Lederer (Editor), "Chromatographic Reviews," Vol. 4, Elsevier Publishing Co., Amsterdam, 1962, p. 69.
4) L.M. Rinaldini, *Anal. Biochem.*, 36, 352 (1970).
5) D.A. Rees, in M.L. Wolfrom and R.S. Tipson (Editor), "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 24, Academic Press, New York and London, 1969, p. 271.
6) R.L. Whistler and W.W. Spencer, in R.L. Whistler (Editor), "Methods in Carbohydrate Chemistry," Vol. III, Academic Press, New York and London, 1963, p. 265.

are very manifold. All of the cellulose sulfate used in this investigation were prepared by a concomitant sulfation and depolymerization of cellulose by the cold concentrated sulfuric acid method.[7] The properties of them are summarized in Table I.

Sodium salt of the cellulose sulfate is freely soluble in water and pH of the solution ranges 5.1—6.0. A titration curve of a cellulose sulfate (Sample No. 4 in Table I) is represented in Fig. 1 in which it can be seen that the pK of this monofunctional strong anionic compound is in the region of 1.7. Thus, because of its chemical structure and properties, cellulose sulfate appears to be applicable to standard stationary phases for making a new type of strong cation-exchange material.

For example, 2.9% (w/v) sodium cellulose sulfate in water gives a moderately viscous solution and forms a homogenous stable suspension with cellulose powder. Cellulose sulfate-treated layers are prepared by coating the suspension on glass plates as usual. Although many types of cellulose material for use in thin-layer chromatography (TLC) were suitable for preparing the layers, Avicel SF (a cellulose product for thin-layer plates) is superior in many respects as stationary phase in this investigation.

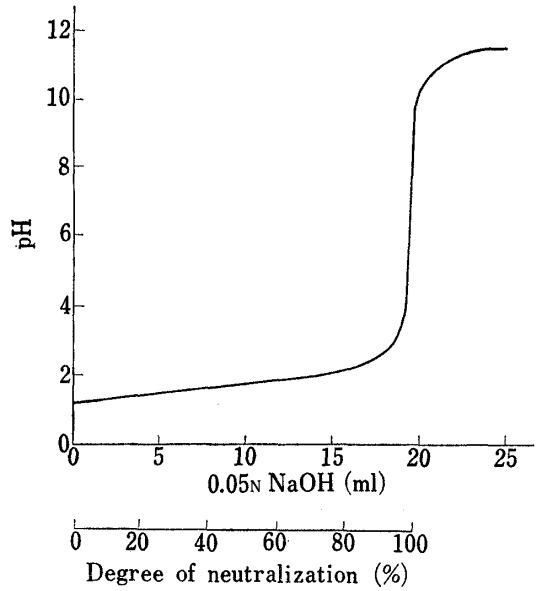


Fig. 1. Titration Curve of Cellulose Sulfate

A solution of sodium cellulose sulfate (sample No. 4, 199.91 mg) was completely decationized by passing through a column of Dowex 50 W (×2, H^+ form). The free cellulose sulfate solution obtained above was titrated with 0.05N NaOH as usual way.

Experimental

Preparation of Cellulose Sulfate (Sample No. 4 in Table I)——The procedure for preparation of cellulose sulfate, which is slightly different from that described in the reference,[7] is presented here. Ten grams of Whatman cellulose powder CF-1 were mixed with 30 ml of concentrated sulfuric acid (sp. gr. 1.84) at about −25° and kept for 100 min at the same temperature, followed by gradually elevating it to 0° with the lapse of 20 min. During this time, an appearance of the reaction mixture was transformed into a homogeneous vitreous paste. The reaction mixture was poured in crashed ice (500 g) in small portions, and the resultant solution was immediately neutralized with powdery calcium carbonate. The precipitate formed was filtered off and washed with water, then the filtrate and washings were combined. To the solution (1.2 liter), 300 ml of ethanol was added with stirring to make its final ethanol concentration to 20% (v/v). The mixture was cooled at 0° for 2 hr and the precipitate was filtered off with the aid of Celite 545. The filtrate was adjusted its pH to about 10 with powdery sodium carbonate and the precipitate formed was removed by filtration. After confirmation of the absence of calcium ions, the filtrate was adjusted its pH to 6.5 with 30% acetic acid. The solution obtained above was dialyzed against tap water for 48 hr, and the dialyzed solution was concentrated to *ca.* 100 ml *in vacuo* and centrifuged. Five volumes of ethanol was added and the precipitate was filtered off, washed and dried over phosphorus pentoxide for 2 hr at 80°. Yield 11.54 g.

Cellulose Powder Avicel SF——Avicel (or Avirin) is a microcrystalline cellulose manufactured by the American Viscose Division of FMC Co. (Marcus Hook, Pa., U.S.A.). Avicel SF, a finely powdered product of Avicel for use in TLC, is obtained from Funakoshi Pharmaceutical Co. and Asahi Kasei Co. (Tokyo, Japan).

Amino Acids, Biogenic Amines, and Local Anesthetics——Amino acids were obtained from Ajinomoto Co., Inc. (Tokyo, Japan). Biogenic amines (hydrochloride form) and monoalkylamines (hydrochloride form) were purchased from Wakō Pure Chemical Ind., Ltd., and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Local anesthetics were commercial products obtained from domestic medicine manufacturers.

Reagents——The solvent used were purified by conventional methods to meet chromatographic standards. All other reagents were prepared from analytical reagent grade materials.

7) K. Nagasawa, Y. Tohira, Y. Inoue and N. Tanoura, *Carbohyd. Res.*, **18**, 95 (1971).

Preparation of Cellulose Sulfate-Impregnated Avicel Plate——Sodium cellulose sulfate (sample No. 4, 2 g) is dissolved in 70 ml of distilled water. It is then filtered on a glass filter. A suspension of Avicel SF (18 g) in the cellulose sulfate solution is homogenized in a galss homogenizer for about 30 sec. After deaeration with suction, the suspension is spread evenly on to glass plates (10×20 cm) with a suitable applicator preset to give 0.25 mm thick layers. The coated plates are kept horizontal and allowed to dry overnight at room temperature, or at 70° for 30 min. The resulting layers which have a capacity of approx. 2.5 μequiv./sq.cm are mechanically very stable and can be stored at least for several months at room temperature.

Chromatography——Sample solutions of amino acids (2 mg/ml), biogenic amines (5 mg/ml), monoalkylamines (5 mg/ml), and local anesthetics (5 mg/ml) in distilled water have been used throughout this experiment. For the determination of *Rf* values, 1 μl of the sample solution is spotted on the starting line 1.5 cm from the edge of the plate. The plate is developed ascendingly at room temperature (22—25°) in a closed tank until the length of run is 10 cm. The development time is variable in the range 120—150 min depending on the composition of eluants and the properties of the cellulose sulfate used. After development, the plate are dried and the compounds resolved are located by using the following methods. Amino acids and primary amines are detected by spraying with Ninhydrin reagent, and synthetic local anesthetics are visualized as orange colored spots with Dragendorff's reagent. The composition of the reagents are as follows:

Ninhydrin Reagent[8]: A solution of 0.2% (w/v) Ninhydrin in ethanol–collidine–acetic acid (50:2:10).

Dragendorff's Reagent[9]: (1) Stock Solution: A mixture of 25 ml acetic acid, 2.6 g basic bismuth carbonate and 7 g sodium iodide is boiled for a few minutes. The copious precipitate of sodium acetate is filtered through a sintered glass filter, after about 12 hr, 20 ml of the clear red-brown filtrate are mixed with 80 ml ethyl acetate and 0.5 ml water is added. This solution must be stored in dark glass bottles. (2) Spray Reagent: A mixture is made of 10 ml stock solution, 100 ml acetic acid and 240 ml ethyl acetate.

Result and Discussion

Examination of Fundamental Conditions for Chromatography of Amino Acids

Fundamental conditions for the chromatography on cellulose sulfate-impregnated Avicel layers were examined on neutral, acidic, and basic amino acids. Four cellulose sulfate preparations which are nearly the same in degree of sulfate substitution (D.S.) but markedly different in viscosity were examined on their chromatographic quality. As can be seen in

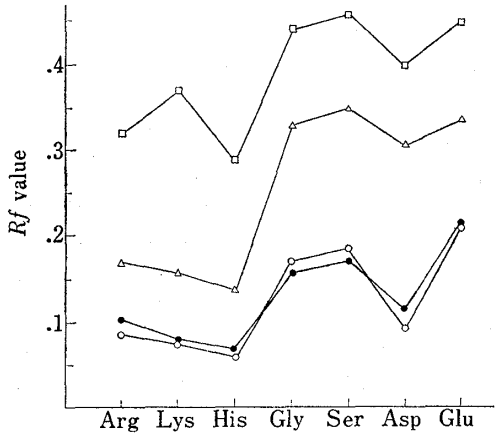


Fig. 2. Influence of the Viscosity of Cellulose Sulfate on the Separation of Amino Acids

Each layer of Avicel SF containing 10% of the indicated cellulose sulfate was developed by ethanol–1/3M CH_3COONa· HCl (pH 3.1) (7:3).
—□—: sample No. 1 [η] 0.004, D.S. 1.40
—△—: sample No. 2 [η] 0.026, D.S. 1.41
—○—: sample No. 3 [η] 0.147, D.S. 1.34
—●—: sample No. 4 [η] 0.365, D.S. 1.20

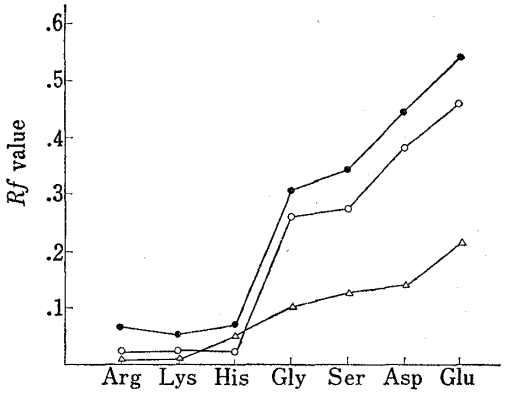


Fig. 3. Influence of the Ethanol Content in Elution Medium (Ethanol–Water) on the Separation of Amino Acids

Each layer of Avicel SF containing 10% of cellulose sulfate (sample No. 4) was developed by the ethanol–water solution with different ethanol concentration.
—●—: ethanol–water (50:50)
—○—: ethanol–water (60:40)
—△—: ethanol–water (70:30)

8) A.L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).
9) D. Vágujfalvi, *Planta Med.*, **8**, 34 (1960).

Fig. 2 the *Rf* values lowered with the increase in viscosity of the cellulose sulfates. This phenomenon is probably attributable to both ionic charactor and viscosity of the cellulose sulfates. It was also shown from another experiment on the cellulose sulfate preparations with different D.S. but nearly the same viscosity, that the decrease in D.S. of the cellulose sulfates resulted in lowering the *Rf* values (data are not shown here). From these results, sodium cellulose sulfate having a $[\eta]$ 0.15—0.37 and D.S.$>$1.20 seems to be suitable for the chromatography, as a cation-exchanger.

Since the cellulose sulfates are water-soluble, the concentration of water in the solvent for elution is greatly restricted. In order to examine the effect of water content in a basal medium, ethanol–water, the amino acids were chromatographed using the ethanol solutions with different water content. For example, the presence of water more than 50% (v/v) in ethanol causes a severe diffusion or tailing of spots on the layers. On the other hand, it can be seen in Fig. 3 that the increase in the content of ethanol results in lowering *Rf* values of the amino acids. Considering the effect of electrolytes to be added, a solution of ethanol–water (70: 30, v/v) was selected as the basal medium for elution.

The result in Fig. 4 shows that the chromatographic behavior depends on the cellulose sulfate content of layers of Avicel SF. As shown in Fig. 4, higher contents of cellulose sulfate ($>$10%, w/w) displays a marked ion-exchange effect of the cellulose sulfate layers on the amino acids, especially on basic amino acids. The Avicel layers containing 10% (w/w) of cellulose sulfate gave the best spot size, separation, and desirable development time, whereas higher concentration ($>$15%, w/w) brought about a severe irregularity in the solvent front and a remarkable increase in development time.

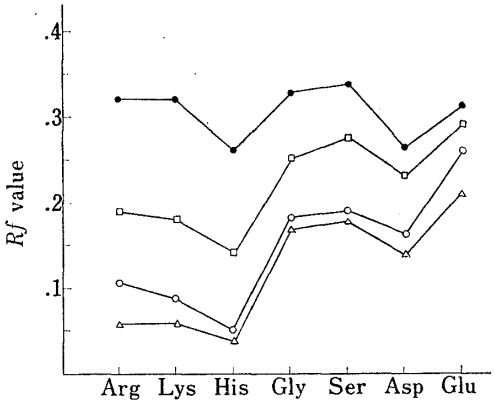


Fig. 4. Influence of the Cellulose Sulfate Content of Layers of Avicel on the Separation of Amino Acids

Each layer of Avicel SF containing different amount of cellulose sulfate (0, 5, 10, and 15% (w/w) of sample No. 4) was developed by ethanol–1/3 M CH_3COONa·HCl (pH 3.1) (7:3).
—●—: Avicel SF only; —□—: 5%;
—○—: 10%; —△—: 15%

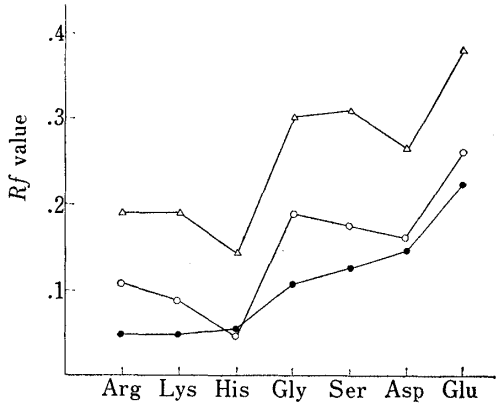


Fig. 5. Influence of the Molar Concentration of Acetate Buffers in Ethanol–CH_3COONa·HCl (pH 3.1) (7:3) on the Separation of Amino Acids

Each layer of Avicel SF containing 10% (w/w) of cellulose sulfate (sample No. 4) was developed by the ethanolic solutions with different molar acetate buffer.
—●—: ethanol–water (70:30);
—○—: 0.1M CH_3COONa·HCl (pH 3.1);
—△—: 0.2M CH_3COONa·HCl (pH 3.1)

Effects of the concentration of electrolytes in elution media on the *Rf* values of the amino acids were examined. As can be seen in Fig. 5, the increase in the salt-concentration in the basal medium, ethanol–water (70: 30, v/v) resulted in an elevation of the *Rf* values.

The detection limits of the spots of the amino acids were determined on the layers of Avicel and the cellulose sulfate-impregnated Avicel. As shown in Table II, the values obtained on both the layers were nearly comparable with each other.

Table II. Detection Limits (μg) of Spots of Amino Acids on the Layers of Avicel and Cellulose Sulfate-Impregnated Avicel

	Amino acid						
	Arg	Lys	His	Gly	Ser	Asp	Glu
Avicel SF	0.025	0.025	0.05	0.025	0.025	0.025	0.025
Cellulose sulfate impregnated Avicel SF	0.025	0.025	0.05	0.025	0.025	0.025	0.05

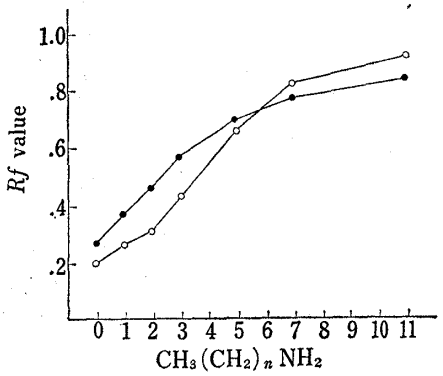


Fig. 6. Separation of Aliphatic Primary Amines on the Layers of Avicel and Cellulose Sulfate-Impregnated Avicel

Each of layers of Avicel SF and Avicel SF containing 10% (w/w) of cellulose sulfate (sample No. 4) was developed by *n*-propanol–1M formic acid (8:2).
—●—: Avicel SF only;
—○—: Avicel SF containing 10% cellulose sulfate

Separation of Aliphatic Primary Amines, Biogenic Amines, and Synthetic Local Anesthetics

A comparison was made between the chromatography of aliphatic primary amines on Avicel layers. In spite of the use of a partition solvent, *n*-propanol–1M formic acid (8: 2), a distribution of the *Rf* values obtained on the cellulose sulfate-impregnated layer is more broad than those on Avicel layer (Fig. 6).

Biogenic amines, especially polyamines, have been hardly separable from each other by chromatography.[4] For the separation of biogenic amines on the cellulose sulfate-impregnated Avicel layers, an alkaline solvent, ethanol–2/3M $CH_3COONa \cdot NaOH$ (pH 10.6) (7: 3) was selected and showed the best resolution of the compounds (Table III).

Thin-layer or paper chromatography of local anesthetics have been carried out on silica gel[10] and alumina layers,[11] and on zirconium phosphate-impregnated papers,[12] but there has been no report on the chromatography of the compounds on ion-exchange materials. On the cellulose

Table III. *Rf* Values of Biogenic Amines and Local Anesthetics on Cellulose Sulfate-Impregnated Avicel Layers

Biogenic amines	Solvent ethanol–2/3M $CH_3COONa \cdot NaOH$ (pH 10.6) (7:3)	Local anesthetics	Solvent isobutanol saturated with 1M formic acid
Tyramine	0.65	procaine	0.22
Tryptamine	0.63	cornecain	0.56
Serotonin	0.50	lidocaine	0.69
Histamine	0.42	carbocain	0.72
Agmatine	0.34	dibucain	0.88
Cadaverine	0.27		
Putrescine	0.13		
Cysteamine	0.31		

Samples were chromatographed on Avicel SF layers containg 10% (w/w) cellulose sulfate (sample No. 4) with the inidcated solvents.

10) I. Sunshine and W.W. Fike, *New Engl. J. Med.*, **271**, 487 (1964).
11) M. Šaršunová, *Pharmazie*, **18**, 748 (1963).
12) K. Tanzawa, O. Hoshino and T. Ukita, *J. Hyg. Chem.* (Japan), **12**, 129 (1966).

sulfate-impregnated Avicel layers, the local anesthetics migrate properly in isobutanol saturated with 1 M formic acid and normally located with Dragendorff's reagent as shown in Table III.

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