Biochemical Studies on Carbohydrates

CLXI. Paper Partition Chromatography of Sugars, in particular of Hexosamines and Hexuronic Acids

Second Report

By

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Paper partition chromatography of sugars was attempted this time in the descending way, which served the purpose more than the ascending (Cf. Masamune and Maki¹⁾). Of several solvents examined, *n*-butanol-pyridine-water (5:3:2) of Yosizawa²⁾ and *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1) were found most suitable for irrigation. Since the solvents were let drop from the lower edge of a paper strip, R_r (flow rate of a sugar as compared with rhamnose) values were measured instead of R_f (flow rate of a sugar as compared with solvent front) values. Quite constant R_r values were given within the temperature limits of 22–27°C.

EXPERIMENTAL

Apparatus and Technique

a) In a cylindrical glass jar, 65 cm. long and 18 cm. diametral and having a ground stopper, is placed one of the glass stands which are diagrammatized in Fig. 1. The stands are formed of a 55 cm. rod with a horizontal trident as a foot and a cage with a hexagonal upper margin of 5 cm. sides (Stand I) or a plate (Stand II) attached to the upper end of the rod to support a half Petri dish (diameter 10 cm., height 2 cm.) or a rectangular glass dish (width 7 cm., length 13 cm., height 5 cm.) for the solvent.

b) *Chromatograms.* Toyo filter paper No. 2 is cut into strips of 60 cm. $\times$ 5 cm. for Stand I, but into strips of 60 cm. $\times$ 5–10 cm. for the other stand.

c) *Sampling.* Of a solution, containing a sugar or sugars in 2% or a like concentration with respect to each individual sugar and acidulated to pH 5.8 with a dilute (0.1 N) H_2SO_4 (HCl is used for hexosamine hydrochloride solutions), is pipetted 0.015 cc. on a paper strip at a distance

of 7–10 cm. from the upper edge. Each spot covers an area 0.8 cm. in diameter.

d) 30–50 cc. of a solvent are placed in the trough at first and supplemented from time to time during irrigation.

e) *Solvents for irrigation.* Amyl-alcohol-pyridine-water (7:7:6) of Werner and Odin⁸⁾ (I), *n*-butanol-pyridine-water (5:3:2) of Yosizawa²⁾ (II), *n*-butylacetate-acetic acid-water (3:2:1) of Yosizawa²⁾ (III); III-methanol (6:1) (IV); III-ethanol (6:1) (V); III-*n*-butanol-methanol (6:1:1) (VI); III-*n*-butanol-methanol (6:2:1) of Masamune and Maki¹⁾ (VII).

f) *Indicators.* Aniline hydrogen phthalate reagent of Partridge⁴⁾; 0.2% ninhydrin in butanol. The latter was applied to hexosamines.

g) *Carbohydrates tested.* 1) L-Arabinose, Takeda.

2) Xylose, Takeda.

3) L-Fucose (crystalline), kindly supplied by Prof. N. Hiyama of Hirosaki University.

4) Rhamnose, Takeda.

5) Galactose, E. Merck.

6) Glucose, E. Merck.

7) Mannose, Takeda.

8) Glucosamine hydrochloride (crystalline)

9) Chondrosamine hydrochloride (crystalline)

10) N-Acetylglucosamine, prepared according to Zuckerkancl and Messiner-Klebermass.⁵⁾

11) N-Acetylchondrosamine prepared according to Masamune, Maki and Hiyama.⁶⁾

12) Glucosamine sulfate. i) The crystalline. 1 g. of chitin was refluxed with 30 cc. of 6 *N* H₂SO₄ for 50 hours. The hydrolysate was freed from most of H₂SO₄ with baryta (The solution was retained on the acid side) and distilled in vacuo to about 10 cc., and 2 volumes of alcohol added. The centrifuged supernatant was condensed furtheron. Half-way tetragonal and hexagonal plates began to appear, when the distil-

Fig. 1

lation was stopped and a little additional alcohol was added. The mixture was stood in an ice-chest overnight, and the deposit collected on a funnel with suction and washed with chilled alcohol. The crude product (0.35 g.) was recrystallized once from a dilute H_2SO_4 solution (1%) and three times from water by the aid of alcohol. It browned at about 175°C and decomposed (no liquefaction) at 200°C . Elson-Morgan and Fehling positive. Sulfate ions detected (as BaSO_4). N (micro Kjeldahl) 6.2% (Calc. for $\text{C}_6\text{H}_{13}\text{O}_5\text{N}\cdot\frac{1}{2}\text{H}_2\text{SO}_4$, 6.14%); $[\alpha]_D^{14} = \frac{1.87 \times 100}{2.02 \times 1} = +92.6^\circ$ (at the 3rd minute after dissolution) $\longrightarrow \frac{1.37 \times 100}{2.02 \times 1} = +67.8^\circ$ (at equilibrium).

ii) A condensate. A chitin hydrolysate as the one above was freed from most of H_2SO_4 (Baryta was added to pH 5.8) and distilled in vacuo to a small volume.

13) Chondrosamine sulfate. i) The crystalline. Chondroitinsulfate of Jorpes was electrodialyzed and 2 g. of the free acid recovered were hydrolyzed as above. Then, after liberation from most of H_2SO_4 , the hydrolysate was distilled in vacuo to about 10 cc., and 3 volumes of alcohol added. The deposit here was discarded and the mother fluid was condensed to a syrup, taken up in a mixture of methanol and ethanol and condensed over again to a small volume, whereby crystals in petal form began to appear. After dilution with some methanol, the mixture was stood in an ice chest. The crystalline deposit was separated and washed with chilled methanol. Yield 0.15 g. Elson-Morgan and Fehling positive. Neuberg-Saneyoshi negative. Sulfate ions detected. It turned brown at about 175°C and decomposed at $185\text{--}186^\circ\text{C}$ with a little gas evolution. N (micro Kjeldahl) 6.3% (Calc. for $\text{C}_6\text{H}_{13}\text{O}_5\text{N}\cdot\frac{1}{2}\text{H}_2\text{SO}_4$, 6.14%). $[\alpha]_D^{14} = \frac{1.40 \times 100}{1.67 \times 1} = +83.8^\circ$ (at the 5th minute after dissolution) $\longrightarrow \frac{1.10 \times 100}{1.67 \times 1} = +65.9^\circ$ (at equilibrium). ii) A condensate. The chondroitinsulfate was hydrolyzed and most H_2SO_4 was precipitated with baryta followed by concentration of the filtrate as in 11)ii). iii) The amorphous. An amorphous product was prepared according to Masamune and Maki¹¹⁾ from crystalline chondrosamine hydrochloride.

To prepare chondrosamine sulfate from the hydrochloride, one must be cautious not to use an anion exchange resin to eliminate HCl , because the resin decomposes (probably polymerizes) the hexosamine and the product neutralized with sulfuric acid gives a chromatographic spot with a R_f value even lower than the less mobile spot of chondrosamine sulfate (Cf. Table I). The same was the case with glucosamine.

14) Glucuronic acid (crystalline) was prepared from menthol-glucuronic acid according to Masamune.⁷¹⁾

15) Barium galacturonate. A crude preparation of barium galacturonate from beet pectin was precipitated from water with 5 volumes of alcohol. The deposit once dried was again taken up in water, decolorized with active charcoal and after passing through a cation exchange resin "Amberite IR-120," neutralized with baryta, followed by reprecipitation and washing with alcohol. The product, dried to constant in a vacuum desiccator (H_2SO_4), analyzed:

Ba (micro Pregl)

8.085 mg. substance, 3.627 mg. BaSO_4 .

$\text{C}_6\text{H}_9\text{O}_7 \cdot \frac{1}{2}\text{Ba}$. Found, 26.4%; calc., 26.2%.

The substance was freed from barium by means of the cation exchange resin before use.

16) Barium mannuronate, prepared by combining Nelson-Cretcher⁸⁾ and Link-Nedden⁹⁾ method.

Ba (micro Pregl)

5.251 mg. substance, 2.371 mg. BaSO_4 .

$\text{C}_6\text{H}_9\text{O}_7 \cdot \frac{1}{2}\text{Ba}$. Found, 26.6%; calc., 26.2%.

This was also freed from barium similar to above for the chromatography.

17) Lactose, E. Merck.

18) Mucosin sulfate, prepared as described by Masamune and Maki.¹⁾

Results

Table I and Fig. 2 show the mean R_f value of sugars run by the various solvents at 22–27°C for 24 hours. Within so wide a range of temperature the R_f value of a sugar by one and the same solvent did not vary more than 0.03.

Following findings were particularly to be pointed out:—

On the chromatograms of hexosamine salts. a) Hexosamines were difficultly separated by the solvents when they were applied as hydrochlorides (Cf. Table I).

b) When crystalline glucosamine sulfate (12)i) was run, a spot of higher R_f was obtained by most of the solvents, while the condensate (12) ii) gave a spot of lower R_f in addition. Either of them gave rise to a spot corresponding to free glucosamine barely (These faint spots are not shown in Figs. 3 and 4).

Remark. When the solutions were neutralized or slightly alkalized, the spots of lower R_f became weaker and those of higher R_f as well as those corresponding to free glucosamine stronger.

c) Crystalline chondrosamine sulfate (13)i) gave always only one spot of lower R_f . But the condensate (13)ii) gave two spots, the

TABLE I
Average R_f Values of Sugars run by Various Solvents
Temperature of test, 22-27°C; duration of test 24 hrs. Indicator, aniline-hydrogen phthalate of Partridge

No.	Solvent Composition	Mean movements of rhamnose (spot center) from the start* cm.	Average R _r values of sugars																	
			L-Fucose	Xylose	L-Arabinose	Mannose	Glucose	Galactose	Fructose	Glucosamine hydrochloride†	Chondrosamine hydrochloride†	Glucosamine sulfate†	Chondrosamine sulfate†	N-Acetylglucos- amine	N-Acetylchon- drosamine	Glucuronic acid§	Galacturonic acid	Mannuronic acid§	Mucosin sulfate	Lactose
I	Amyl alcohol	7	0.87	0.86	0.76	0.76	0.67	0.60	0.75	0.58	0.54	0.51	0.47	0.85		0.26	0.22	0.30	0.21	0.49
	Pyridine	7										0.18	0.13			(0.86)				
	Water	6																		
II	n-Butanol	5	0.82	0.81	0.70	0.68	0.59	0.53	0.69	0.43	0.38	0.42	0.34	0.81		0.18	0.12	0.21	0.06	0.28
	Pyridine	3										0.05	0.02			(0.90)				
	Water	2																		
III	n-Butylacetate	3	0.85	0.72	0.68	0.57	0.49	0.46	0.65	0.27	0.26	0.26	0.16	0.83	0.84	0.41	0.38	0.47	0.19	0.21
	Acetic acid	2										0.11	0.06			(0.72)		(0.78)		
	Water	1																		
IV	n-Butylacetate	3	0.83	0.72	0.67	0.55	0.48	0.44	0.62	0.28	0.26	0.27	0.23	0.83	0.81	0.39	0.35	0.48	0.15	0.17
	Acetic acid	2										0.11	0.07			(0.70)		(0.80)		
	Methanol	1																		
V	n-Butylacetate	3	0.86	0.69	0.65	0.55	0.45	0.42	0.59	0.19	0.20	0.23	0.14	0.78	0.77	0.38	0.32	0.46	0.15	0.15
	Acetic acid	2										0.07	0.04			(0.68)		(0.79)		
	Ethanol	1																		
VI	n-Butylacetate	3	0.82	0.71	0.63	0.52	0.44	0.39	0.60	0.24	0.22	0.25	0.22	0.79		0.33	0.27	0.47	0.12	0.14
	Acetic acid	2										0.08	0.06			(0.64)		(0.79)		
	Butanol	1																		
VII	Methanol	1																		
	Water	1																		
	n-Butylacetate	3	0.76	0.61	0.55	0.43	0.34	0.29	0.47	0.16	0.14	0.16	0.14	0.65	0.61	0.24	0.18	0.42	0.04	0.07
	Acetic acid	2										0.04	0.02			(0.57)		(0.76)		
	Butanol	2																		
	Methanol	2																		
	Water	1																		
		1																		

* The variation ± 5 cm. † The spot tailed often by Solvent I. By most of the solvents one spot only was given distinctly with a very weak spot corresponding to free glucosamine besides. ‡ Two spots appeared, though not always depending on the preparation examined, and they tailed often by Solvent I. § The figures in parentheses express R_f values of the lactone.

Fig. 2. A chart showing the average R_f values of sugars in Table I. (1') and (2'): the quicker moving spots respectively of chondrosamine sulfate and glucosamine sulfate, whose slower moving spots are (1) and (2).

spot of higher R_f dominating in the cases of Solvents I and II and the one of lower R_f in the cases of Solvents III–VII. The preparation in 13) iii) resembled that in 13) i) in this connection, although the spot of higher R_f was not missing in chromatograms.

Remark. When the chondrosamine sulfate solutions which were of pH 5.8, were neutralized or slightly alkalized, a spot of higher R_f appeared or enlarged and, besides, a distinct spot of free chondrosamine occurred particularly in case of the preparations in 13) ii) and 13) iii).

On the chromatograms of hexuronic acids. As is demonstrated in Table I, Solvent II is most appropriate for separation of the three hexuronic acids, but Solvent V is also expedient if mannuronic acid which has not as yet been discovered in animal materials, is not present in the unknown

Fig. 3. *n*-Butanol-pyridine-water (5:3:2) chromatograms developed with aniline hydrogen phthalate. Temperature of test: 22–27°C. Duration of test: (A) 24 hrs.; (Ba) and (Bb) 3×24 hrs.; (Bc) and (Bd) 4×24 hrs.

a, b, c, d and e: standard runs. f: traganth (a 1 N H_2SO_4 -hydrolysate). g and h: 1 N H_2SO_4 hydrolysates of mucinogens of toad and frog egg-covering respectively. 1 and 1': chondrosamine sulfate (13)ii), 2 and 2': glucosamine sulfate (12)ii), 3: mucosin sulfate, 4: galacturonic acid, 5: glucuronic acid, 5': glucuronolactone, 6: lactose, 7: galactose, 8: glucose, 9: mannose, 10: L-fucose, 11: rhamnose.

substance (Cf. also Figs. 2–4).

*Traganth*¹⁰⁾ and *mucinogen of toad and frog egg covering*.¹¹⁾ These substances were examined, as examples, by the device in this account. 50 mg. of the substances were heated with 5 cc. of 1 N H_2SO_4 , as sealed in an ampoule, at 100°C for 5 hours. The hydrolysates were neutralized

to pH 5.8 with baryta and filtered, and the filtrates were distilled in vacuo to about 0.5 cc. and readjusted to pH 5.8. 0.02 cc. portions of the condensates were sampled to irrigate with Solvents II and V. The chromatograms are reproduced diagrammatically in Figs. 3A and 4. Those of traganth show spots corresponding to L-fucose, xylose, arabinose, galactose, galacturonic acid, glucose and rhamnose. The rhamnose spot was weak. The spots given by the hydrolysate of the egg jellies were located at the positions of chondrosamine sulfate, galactose, L-fucose and glucosamine sulfate (The glucosamine content was minute).

COMMENTS

The noticeable results mentioned as well as R_f values in Table I indicate that Solvents II and V are useful for separation of not only ordinary sugars but also hexosamines and hexuronic acids in descending manner. The hexosamines in the form of sulfate are divided well from each other by those solvents, but not when applied as hydrochlorides. Solvent II which is alkaline causes partial decomposition of hexosamines as is already known, nevertheless it is advantageous for clear-cut separation of their sulfates by long-timed irrigation (Fig. 3B). Solvents IV-VII are prepared by addition of an alcohol or alcohols to Solvent III. Of the alcohols, methanol was found to spread and obscure spots. Ethanol and butanol contrariwise localized sugars into distinct spots, while the latter retarded the flow of sugars entailing difficult chromatography. Thus Solvent V containing ethanol is most efficient.

By the two solvents II and V, the disaccharide unit, remaining uncleft after hydrolysis of an itin or itinsulfuric acid with 1 N H_2SO_4 , is separated far from the hexuronic acid and hexosamine, especially when the test

Fig. 4. *n*-Butylacetate-acetic acid-ethanol-water (3:2:1:1) chromatograms developed with aniline hydrogen phthalate. Temperature of test, 22–27°C. Duration of test 24 hrs.

Characters and ciphers signify the same as in Fig. 3.

is prolonged.

SUMMARY

A descending method of paper partition chromatography of sugars has been devised, where solutions of sulfate of hexosamines and non-amino sugars are adjusted to pH 5.8 with H_2SO_4 and irrigated with *n*-butanol-pyridine-water (5:3:2) or *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1).

Chromatograms of traganth and mucinogen of toad and frog egg-covering (hydrolysates) were given as examples.

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