3 E. CERRAI AND C. TESTA, *J. Chromatogr.*, 8 (1962) 232; E. CERRAI AND G. GHERSINI, *J. Chromatogr.*, 13 (1964) 211; 15 (1964) 236; 18 (1965) 124.
4 G. ALBERTI, V. CAGLIOTI AND M. LEDERER, *J. Chromatogr.*, 7 (1962) 242.
5 G. ALBERTI, F. DOBICI AND G. GRASSINI, *J. Chromatogr.*, 8 (1962) 103.
6 J. PRÁŠILOVÁ AND F. ŠEBESTA, *J. Chromatogr.*, 14 (1964) 555.
7 D. COZZI, P. G. DESIDERI AND L. LEPRI, *J. Chromatogr.*, 40 (1969) 130.
8 T. YAMABE, *Rep. Inst. Ind. Sci.*, 6 (1956) 7.
9 E. C. BATE-SMITH AND R. G. C. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
10 M. LEDERER AND S. KERTES, *Anal. Chim. Acta*, 15 (1956) 226.
11 T. IIDA AND T. YAMABE, *J. Chromatogr.*, 41 (1969) 163.

Received December 11th, 1970

J. Chromatogr., 56 (1971) 373–378

CHROM. 5206

Ion-exchange chromatography of dinucleoside-3'→5'-phosphates on chitosan-impregnated cellulose thin layers

In a preceding paper[1], we presented evidence that a number of nucleic acid constituents, such as 5'-mononucleotides, nucleosides, and nucleic bases, can be resolved by ion-exchange chromatography on chitosan formate-impregnated cellulose thin layers. The present communication will describe the separation of dinucleoside-3'→5'-phosphates, which are a phosphodiester type of nucleotide, on the layers.

Materials

Chitosan. The same chitosan as was used in the preceding experiments* was used again in the present investigation. The intrinsic viscosity $[\eta]$ of the chitosan was 8.25, which was determined in 0.5% formic acid solution at 25 ± 0.1°.**

Cellulose powder. Avicel SF, a finely powdered product of microcrystalline cellulose "Avicel" for use in TLC, was obtained from Funakoshi Pharmaceutical Co. and Asahi Kasei Co. (Tokyo, Japan).

*Dinucleoside-3'→5'-phosphates.**** All sixteen dinucleoside-3'→5'-phosphates were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio).

---

* The commercial chitin purified by HACKMAN's method[2,3] was deacetylated with concentrated hot alkali by the procedure of WOLFROM *et al.*[4–6]. Quantitative analysis of the chitosan thus prepared gave 7.95% N, 1.71% N-acetyl and 1.15% ash; while 8.75% N and 0% N-acetyl were calculated for $C_6H_{11}NO_4$. These analytical data show that about 92% of the total nitrogen in the chitosan is present as the free amino group.

** The viscosity of chitosan markedly influences the chromatographic behavior in this procedure. An examination of the viscosity range of chitosan optimum for the chromatography is in progress, and the results obtained will be reported later.

*** The following abbreviations will be used: ApA, ApG, ApC, ApU = 3'-adenylyl esters of adenosine-5', guanosine-5', cytidine-5', uridine-5'; GpA, GpG, GpC, GpU = 3'-guanylyl esters of adenosine-5', guanosine-5', cytidine-5', uridine-5'; CpA, CpG, CpC, CpU = 3'-cytidylyl esters of adenosine-5', guanosine-5', cytidine-5', uridine-5'; UpA, UpG, UpC, UpU = 3'-uridylyl esters of adenosine-5', guanosine-5', cytidine-5', uridine-5'.

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

Preparation of chitosan formate-impregnated Avicel plates

Six glass plates (20 × 20 cm) were coated with a homogenized suspension of 15 g Avicel SF in 60 ml of 0.8% (w/v) chitosan solution in 0.5% (w/v) formic acid using a suitable applicator (slit width 0.25 mm). Details of the procedure were given previously[1].

Chromatography

For the determination of R_F values, 1 μl of 1 mM solution of the nucleotide is spotted on the plate which is developed ascendingly at 25° in a closed tank until the length of run is 10 cm. After development, the plate is dried well and the nucleotides resolved are located by examining the plate in transmitted UV light.

TABLE I

R_F VALUES FOR DINUCLEOSIDE-3′ → 5′-PHOSPHATES IN 0.25 M PYRIDINE–FORMIC ACID SYSTEMS
Development time: 80 min (pH 2.6), 100 min (pH 3.0), 120 min (pH 4.4).

Compound	*Solvent*			*Compound*	*Solvent*		
	pH 2.6	*3.0*	*4.4*		*pH 2.6*	*3.0*	*4.4*
ApA	0.61	0.45	0.33	CpA	0.63	0.63	0.50
ApG	0.37	0.30	0.41	CpG	0.53	0.59	0.47
ApC	0.61	0.56	0.44	CpC	0.71	0.71	0.57
ApU	0.47	0.41	0.43	CpU	0.66	0.58	0.53
GpA	0.39	0.34	0.36	UpA	0.54	0.52	0.49
GpG	<0.16[a]	<0.32[a]	<0.32[a]	UpG	0.24	0.28	0.44
GpC	0.52	0.48	0.41	UpC	0.66	0.62	0.58
GpU	0.27	0.26	0.38	UpU	0.38	0.43	0.57

[a] Tailing.

Results and discussion

The separation of dinucleoside-3′→5′-phosphates on our layers was achieved using both the pyridine–formate system and ammonium formate system as was the case for 5′-mononucleotides[1].

The R_F values obtained using three 0.25 M pyridine–formate systems (pH 2.6, 3.0, and 4.4) are listed in Table I. As can be seen in Table I, 0.25 M pyridine–formate (pH 2.6) gave the best separation of the nucleotides in these solvents.

0.25 M ammonium formate systems (pH 3.0, 4.0, and 6.7) also gave good separations of the nucleotides. The R_F values obtained by them are listed in Table II. As can be seen from Table II, both the solvents at pH 3.0 and 6.7 are better than that at pH 4.0.

For the separation with acid materials, a formic acid–water system was selected as the most suitable one. The R_F data for the formic acid–water systems (0.05, 0.1, and 0.2 M) are listed in Table III. Development with 0.2 M formic acid gave the

TABLE II

R_F VALUES FOR DINUCLEOSIDE-3′ → 5′-PHOSPHATES IN 0.25 *M* AMMONIA–FORMIC ACID SYSTEMS
Development time: 130 min (pH 3.0), 100 min (pH 4.0), 80 min (pH 6.7).

Compound	*Solvent*			*Compound*	*Solvent*		
	pH 3.0	*4.0*	*6.7*		*pH 3.0*	*4.0*	*6.7*
ApA	0.50	0.29	0.28	CpA	0.67	0.43	0.48
ApG	0.31	0.24	0.43	CpG	0.49	0.40	0.45
ApC	0.63	0.40	0.41	CpC	0.73	0.53	0.61
ApU	0.45	0.39	0.44	CpU	0.62	0.54	0.65
GpA	0.36	0.27	0.31	UpA	0.50	0.44	0.50
GpG	< 0.25[a]	< 0.24[a]	< 0.28[a]	UpG	0.26	0.40	0.49
GpC	0.46	0.38	0.40	UpC	0.66	0.54	0.62
GpU	0.26	0.35	0.43	UpU	0.36	0.59	0.68

[a] Tailing.

TABLE III

R_F VALUES FOR DINUCLEOSIDE -3′ → 5′-PHOSPHATES IN FORMIC ACID–WATER SYSTEMS
Development time: 115 min (0.05 *M*), 170 min (0.1 *M*), 400 min (0.2 *M*).

Compound	*Solvent*			*Compound*	*Solvent*		
	0.2 M	*0.1 M*	*0.05 M*		*0.2 M*	*0.1 M*	*0.05 M*
ApA	0.67			CpA	0.73	0.65	0.35
ApG	0.39			CpG	0.52		
ApC	0.76			CpC	0.76		
ApU	0.41			CpU	0.56		
GpA	0.39	0.29	0.14	UpA	0.49	0.37	0.20
GpG	< 0.1[a]			UpG	0.11	0.05	0.03
GpC	0.50			UpC	0.58	0.52	0.35
GpU	0.10			UpU	0.13		

[a] Tailing

TABLE IV

THE DIFFERENCE (ΔR_F) BETWEEN R_F VALUES OF TWO DINUCLEOSIDE-3′ → 5′-PHOSPHATES DIFFERENT IN THE ORDER OF NUCLEOSIDE LINKAGE

Compounds paired	*0.25 M ammonium formate*		*0.25 M pyridine–formate, pH 2.6*
	pH 6.7	*pH 3.0*	
ApG–GpA	0.12	0.05	0.03
ApC–CpA	0.07	0.04	0.02
ApU–UpA	0.06	0.05	0.07
GpC–CpG	0.05	0.03	0.01
GpU–UpG	0.06	0	0.03
CpU–UpC	0.03	0.04	0

best separation, but it required a rather long time for development. On the other hand, a decrease in the concentration of formic acid results in a narrow R_F range for the nucleotides resolved. Judging from the data of Table III, the 0.1 M formic acid is probably the most suitable for separation within a reasonable development time.

Various solvent systems containing boric acid or urea were examined for an effective separation of two dinucleoside-3'→5'-phosphates, different only in the order of nucleoside linkage, but no satisfactory result was obtained with these solvents. The differences (ΔR_F) between the R_F values of each pair of the nucleotides obtained by the three solvents recommended above are summarized in Table IV. These data show that 0.25 M ammonium formate (pH 6.7) is obviously the most effective for discriminating between these nucleotide pairs.

School of Pharmaceutical Sciences,
Kitasato University, Shirokane 5-9-1,
Minato-ku, Tokyo 108 (Japan)

KINZO NAGASAWA
HIROKO WATANABE
AKIRA OGAMO

1 K. NAGASAWA, H. WATANABE AND A. OGAMO, *J. Chromatogr.*, 47 (1970) 408.
2 J. N. BEMILLER in R. L. WHISTLER (Editor), *Methods in Carbohydrate Chemistry*, Vol. 5, Academic Press, New York, 1965, p. 104.
3 R. H. HACKMAN, *Australian J. Biol. Sci.*, 7 (1954) 168.
4 D. HORTON AND D. R. LINEBACK, in R. L. WHISTLER (Editor), *Methods in Carbohydrate Chemistry*, Vol. 5, Academic Press, New York, 1965, p. 405.
5 M. L. WOLFROM, G. G. MAHER AND A. CHANEY, *J. Org. Chem.*, 23 (1958) 1990.
6 M. L. WOLFROM AND T. M. SHEN HAN, *J. Amer. Chem. Soc.*, 81 (1959) 1764.

Received December 22nd, 1970