THE EFFECT OF CALCIUM SULFATE AS THE BINDER IN DEAE-CELLULOSE THIN-LAYER CHROMATOGRAPHY FOR SEPARATING NUCLEIC ACID DEGRADATION PRODUCTS*

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RANDERATH has investigated the separation of purine bases, nucleosides, and ribo nucleotides by thin-layer chromatography[1-3]. His systems for nucleotides appear most appropriate for the separation of a mixture of mono-, di-, and tri-phosphates of a given nucleotide. The separation of the mono-, di-, and tri-phosphates of adenosine also has been accomplished using an ammonium formate solvent system with diethylaminoethyl (DEAE)-Sephadex as the stationary phase by WIELAND AND DETERMANN[4].

In our laboratory it was desirable to find a system which would separate the 2'- and 3'-phosphates of the four principal nucleotides resulting from a basic hydrolysis of ribonucleic acid (RNA), and the 5'-phosphates of the four principal deoxynucleotides resulting from an enzymic hydrolysis of deoxyribonucleic acid (DNA), involving 100 to 1000 μg of sample. Previous work has been concerned primarily with separation of the 5'-phosphates of ribonucleotides[1,3], with the exception of the ammonium sulfate–sodium acetate–isopropanol system to separate 2'- from 3'-phosphates[3]. This system, however, does not separate the phosphates of cytidine from those of uridine, and the high salt content of the solvent requires that the eluant be desalted if further chromatographic separation or enzymic hydrolysis is desired.

MATERIALS AND METHODS

Materials

MN-Cellulose Powder 300 DEAE and 300 G/DEAE were obtained from Macherey, Nagel & Co., Düren, Germany. The latter preparation contains 10% $CaSO_4 \cdot 1/2\ H_2O$ as a binder. All purine and pyrimidine compounds were obtained from Sigma Chemical Corp. except for the following: adenosine-2'- and 3'-phosphates, guanosine-2'- and 3'-phosphates, adenosine, and cytosine from Mann Chemical Company; guanosine from Schwarz Biochemicals; and cytidine and 2-deoxy-adenosine-5'-phosphate from California Corp. for Biochemical Research.

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Preparation of plates

9.5 g of DEAE-Cellulose powder was mixed with 62 ml H_2O and spread onto three 8 in. × 12 in. plates at a thickness of about 375 μ, using a spreader apparatus obtained from Desaga, Brinkmann Corp. The glass plates were dried at 50° for 2 hours. Standards were spotted at 2 cm from the lower edge of the plate and air-dried prior to chromatography. Chromatograms were developed in closed chromatography jars of a diameter of about 21 cm or in open glass trays when a second dimension was desired.

The preparation containing 5% $CaSO_4$ was made by mixing equal volumes of the powder containing 10% $CaSO_4$ and that containing no $CaSO_4$. Thorough washing of the 300 G/DEAE cellulose resulted in removal of $CaSO_4$, leaving a preparation which gave chromatographic results identical to those of the originally $CaSO_4$-free powder (MN-Cellulose 300 DEAE).

Solvent systems

Solvents were: (1) isobutyric acid–ammonia (s.g. 0.90)–water (66:2:32, v/v); (2) distilled water, pH 5.7; (3) hydrochloric acid, pH 2.3; and (4) saturated ammonium sulfate–1 *M* sodium acetate, pH 7.5–isopropanol (80:18:2, v/v)[3]. Times required for the movement of the solvent a distance of 6 in. were, respectively: 6 h, 35 min, 35 min and 2 h.

The isobutyric acid should be redistilled to remove impurities that migrate with the solvent front and interfere with detection and assay of compounds of R_F greater than 0.95.

Preparation of samples

A solution of nucleotides was dried *in vacuo* in a test tube, dissolved in 0.10 ml of 0.10 *N* NH_4OH, and spotted onto the cellulose prior to chromatography. Nucleosides were used from a neutral solution, and purines and pyrimidines from a solution of 0.1 *N* hydrochloric acid.

In order to separate ribonucleotides from a basic RNA hydrolysate, it was first necessary to desalt the mixture. This was done by absorbing the nucleotides onto a small column of charcoal (20 mg Darco G previously washed with 0.1 *N* HCl and distilled water and then made into a 0.3 × 0.6 cm column) to remove salts, and then they were eluted quantitatively with 5 ml of a solution of 95% ethanol–ammonia (s.g. 0.90)–water (5:2:3)[5].

Elution from chromatogram

The spots were located by ultraviolet light and outlined by use of a thin glass rod. The cellulose surrounding the spot was removed with a razor blade, and then the spot itself was removed by scraping with a razor blade held at a 30° angle to the glass. This results in a small cylinder of cellulose which is then placed in a 3 ml conical glass centrifuge tube. To the tube is added 2.5 ml of 0.01 *N* HCl. This is thoroughly mixed and centrifuged at full speed in a clinical centrifuge for a few minutes, and the supernatant decanted through a micro funnel, using Schleicher & Schuell No. 589 blue ribbon filter paper, which had been previously washed with H_2O. This was adequate to trap the cellulose "fines". An alternative successful method was to use a fine

grade sintered glass filter, omitting the micro funnel and filter paper. The elution was repeated twice and gave 95–100 % recovery of the nucleotides.

RESULTS AND DISCUSSION

In Table I are listed the R_F data for the ammonium isobutyrate and the hydrochloric acid solvents. Since the data for distilled water were so similar to those for HCl, and HCl effected slightly better separations in nearly all cases, this data was omitted. When the percent $CaSO_4$ in the cellulose layer is varied, large differences in the R_F of almost all compounds are found with the HCl solvent. Lesser differences are seen with the ammonium isobutyrate solvent.

The best separation of ribonucleotides is obtained with the ammonium isobutyrate solvent. Addition of $CaSO_4$ to the DEAE cellulose tends to separate the 2′- and 3′-phosphates of guanosine, eventually rendering an overlap with the uridylic acid

TABLE I

SEPARATION OF PURINES, PYRIMIDINES, NUCLEOSIDES, AND NUCLEOTIDES

	Compound	R_F in solvent system					
		Isobutyric acid–NH_3 (s.g. 0.90)–H_2O (66:2:32)			0.005 N HCl		
		% $CaSO_4$			% $CaSO_4$		
		0	5	10	0	5	10
Nucleotides	Adenosine-2′-P	0.63	0.62	0.66	0.00	0.04	0.32
	Adenosine-3′-P	0.63	0.62	0.66	0.00	0.04	0.24
	Guanosine-2′-P	0.30	0.29	0.33	0.00	0.02	0.18
	Guanosine-3′-P	0.30	0.24	0.29	0.00	0.02	0.09
	Cytidine-2′-P	0.55	0.55	0.55	0.00	0.09	0.61
	Cytidine-3′-P	0.51	0.50	0.51	0.00	0.05	0.50
	Uridine-2′-P	0.36	0.35	0.37	0.00	0.02	0.48
	Uridine-3′-P	0.36	0.35	0.37	0.00	0.02	0.42
	2-Deoxy-adenosine-5′-P	0.64	0.62	0.69	0.00	0.04	0.28
	2-Deoxy-guanosine-5′-P	0.35	0.32	0.36	0.00	0.02	0.15
	2-Deoxy-cytidine-5′-P	0.60	0.57	0.59	0.00	0.08	0.48
	Thymidine-5′-P	0.47	0.46	0.47	0.00	0.05	0.48
Nucleosides	Adenosine	0.90	0.92	0.91	0.68	0.58	0.62
	Guanosine	0.63		0.59	0.13	0.31*	0.39*
	Cytidine	0.79	0.73	0.78	0.96	0.79	0.94
	Uridine	0.65		0.63	0.30	0.68	0.87
	2-Deoxy-adenosine	0.98	0.96	0.97	0.66	0.70	0.70
	2-Deoxy-guanosine	0.73	0.69	0.73	0.12	0.40	0.50
	2-Deoxy-cytidine	0.91	0.88	0.90	0.93	0.82	0.90
	Thymidine	0.83	0.79	0.81	0.49	0.81	0.90
Bases	Adenine	0.98	0.95	0.94	0.20	0.33	0.44
	Guanine	0.73		0.73	0.10	0.31	0.40
	Cytosine	0.92	0.90	0.94	0.26	0.80	0.90
	Uracil	0.74	0.79	0.73	0.26	0.66	0.75
	Thymine	0.83	0.80	0.83	0.41	0.74	0.85

* Streaking of the spot over an R_F value of 0.1.

spot. If separation of each 2'- from the 3'-phosphate isomer is desired, 0.005 *N* HCl solvent and 10 % $CaSO_4$-cellulose layer is best, giving at least 0.06 difference in the R_F's of the two isomers of each nucleotide. The uridine-2'-phosphate and cytidine-3'-phosphate spots definitely overlap. It is recommended that a mixture of monoribonucleotides be prepared in 0.10 *N* NH_4OH before spotting, since, below pH 10, guanylic acid tends to streak badly. This streaking is more predominant in the HCl solvent than in the isobutyrate solvent. In the ammonium sulfate–sodium acetate–isopropanol solvent, the length of the streak varies almost directly with pH, as seen from Table II.

TABLE II

EFFECT OF pH OF THE INITIAL NUCLEOTIDE SOLUTION ON THE SEPARATION

The R_F's of the upper and lower limits of the spot are given. Stationary phase: 10 % $CaSO_4$–DEAE-cellulose powder.

Compound	*pH of initial solution*	*R_F in solvent*		
		Saturated ammonium sulfate–1 M sodium acetate–isopropanol (80:18:2, v/v)	*0.005 N HCl*	*0.01 N HCl*
Guanosine-2'-phosphate + guanosine-3'-phosphate	2.8	0.08–0.60	0.01–0.36	0.04–0.21
	5.8	0.08–0.63		
	6.5	0.20–0.63		
	7.5	0.30–0.63		0.04–0.21
	9.9	0.35–0.65		
	10.2	0.43–0.65	0.08–0.16	0.11–0.21
	11.0	0.50–0.65		
Adenosine-2'-phosphate + adenosine-3'-phosphate	2–11	0.26–0.32		
Cytidine-2'-phosphate + cytidine-3'-phosphate	2–11	0.73–0.82		
Uridine-2'-phosphate + uridine-3'-phosphate	2–11	0.80–0.87		

The best separation of deoxyribomononucleotides is obtained using the 10 % $CaSO_4$ preparation with ammonium isobutyrate. Whereas 2-deoxy-cytidine-5'-phosphate and 2-deoxy-adenosine-5'-phosphate tend to overlap when no $CaSO_4$ is present; the addition of this binder results in a separation in which the R_F difference increases from 0.04 to 0.10. It is also advisable to prepare a mixture of deoxyribonucleotides in base, since the compounds are more soluble in base, and the purine-deoxyribotides are quite labile in acid.

Dilute HCl affects the best separation of ribonucleosides as well as deoxyribonucleosides (Table I). The $CaSO_4$-free preparation should be used, as the uridine and thymidine spots move up into the range of the cytidine and 2-deoxy-cytidine spots, respectively, when $CaSO_4$ is present. Guanosine tends to streak badly, in the presence of $CaSO_4$, unless the nucleosides are prepared in either a strongly acidic or a strongly basic solution. When spotted from acidic or basic solutions, the R_F of guanosine is increased from 0.39 to 0.52 in 10 % $CaSO_4$. Although ammonium isobutyrate gives a moderately good separation of deoxyribonucleotides, one should be cautioned against use of this solvent if elution of a compound near the solvent front is desired. Impurities

present in commercially available isobutyric acid move with the solvent front and absorb ultraviolet light. A slight tendency to streak was observed when deoxyribonucleotides are prepared at a low pH. Since they are apparently as soluble in neutral or basic pH as in acid, it is desirable to spot these compounds from a solution of pH 7 or higher.

The free bases resulting from RNA hydrolysis are best separated using the ammonium sulfate–sodium acetate–isopropanol system of RANDERATH[3]. Apparently, the solvent has such a high salt content that little difference in R_F's are observed, depending on the presence or absence of $CaSO_4$ in the layer (See Table III). This solvent does not adequately separate thymine from cytosine, but the HCl solvent will

TABLE III

SEPARATION OF FREE BASES WITH SATURATED AMMONIUM SULFATE–1 *N* SODIUM ACETATE–ISOPROPANOL (80:18:2, v/v)

Stationary phase: DEAE-cellulose powder plus $CaSO_4$ as indicated.

Compound	R_F	
	0% $CaSO_4$	*10% $CaSO_4$*
Adenine	0.14	0.13
Guanine	0.23	0.00
Cytosine	0.50	0.56
Uracil	0.62	0.68
Thymine	0.52	0.54

separate the four bases resulting from DNA hydrolysis if the $CaSO_4$-free DEAE-cellulose is used. Addition of $CaSO_4$ causes a one to four-fold increase in the R_F's of all free bases, but moves the guanine spot into the lower range of the adenine spot. Although the isobutyrate solvent effects a fair separation of the bases of DNA, the adenine spot is too near the solvent front to permit direct ultraviolet analysis of the eluant. This solvent cannot be used to separate the bases of RNA because guanine and uracil spots overlap. Free bases should be prepared in 0.1 *N* HCl, owing to their greater solubility at a low pH, and the lessened tendency to streak at low pH.

Two-dimensional chromatograms are easily developed, allowing 1–2 h for drying between solvents. The ammonium sulfate–sodium acetate–isopropanol solvent was unsatisfactory as a first solvent because the salt solvents create a salt gradient over the chromatogram which interferes with the second solvent, Ammonium formate solvents have proven unsatisfactory for either first or second dimensions. However, both HCl and distilled water function nicely as a first solvent, and water, but not HCl, will work as a second solvent when isobutyrate was used in the first dimension. Since the ammonium isobutyrate solvent changes composition in an open system, the R_F's were observed to change somewhat when it was used as the second solvent in an open system. In fact, it has been found that even in a well-sealed chromatography jar, this solvent changes composition slightly from day to day. The effect of aging this solvent on changes in R_F's can be duplicated by preparing a fresh solvent with a greater percentage of ammonia. The desirability of using a two-dimensional system will depend on the extent of separation desired and the number of compounds

in a mixture to be separated. The 8 in. × 8 in. glass plates obtained especially for thin-layer work would be advantageous for two-dimensional chromatograms.

If one wishes to assay the eluted compounds quantitatively by ultraviolet spectrometry, it is advisable to observe two precautions:

(1) The eluant should be filtered as described to avoid the fines which interfere with ultraviolet spectrometry. Glass wool and filter papers not designed for extremely fine particles have proven inadequate. Alternatively, one could wash the cellulose repeatedly in distilled water before use. This procedure, however, will remove the $CaSO_4$ present as "binder", and if $CaSO_4$ is desired, it must be added back. We have not found the presence of $CaSO_4$ necessary for its "binding" properties; both the cellulose preparations described will adhere well to the glass plates in all solvent systems investigated. (2) If isobutyrate were used, the chromatogram should be well-dried to remove this solvent, which absorbs ultraviolet light. If acid-labile nucleotides are present, 24 h at room temperature is best, since this is adequate for drying and destruction of nucleotides does not occur. Otherwise, 1 h at 50–60° is sufficient.

The limitations of the quantities of material which can be handled by the thin-layer methods appear to be few. As little as 10 μg of material can be detected in a spot of 1 cm diameter, and no smearing has been observed when 1 to 2 mg of material was chromatographed.

In Table IV is given a summary of the best systems for separating the compounds of each group of nucleic acid degradation products. Since thin-layer chromatography is

TABLE IV

BEST SOLVENT SYSTEMS AND DEAE-CELLULOSE PREPARATIONS FOR EACH GROUP

Group	*Solvent*	*% $CaSO_4$*
Ribonucleotides, according to bases	Isobutyrate	0
Ribonucleotides, 2′- from 3′-phosphate	HCl	10
Deoxyribonucleotide-5′-phosphates	Isobutyrate	10
Ribonucleosides	HCl	0
Deoxyribonucleosides	HCl	0
Free bases from RNA basic hydrolysis	Ammonium sulfate	no difference
Free bases from DNA enzymic hydrolysis	HCl	0

similar to paper chromatography in most respects, it should be emphasized that the cleanest separations are obtained only if the mixture is first desalted. For purine and pyrimidine compounds, this step is easily accomplished by use of the charcoal column. Serious streaking has been observed in all solvents attempted in the presence of salts.

DISCUSSION

The addition of $CaSO_4$ to the cellulose resulted in better movement of various nucleic acid degradation products. Although the effect of $CaSO_4$ in these studies is not completely understood, at least two explanations are reasonable: (1) a physical change in the tertiary structure of the DEAE-cellulose in the presence of calcium and sulfate ions; (2) the sulfate ions may bind electrostatically with nitrogen moieties of the

diethylaminoethyl groups. The nitrogen of this compound would be in the ammonium (R_3N^+) form at an acidic pH. Electrostatic binding of this ion by sulfate would tend to prohibit a similar bonding by the hydroxyl groups of nucleotide, and, to a lesser extent, by the hydroxyl and amino groups of the purine and pyrimidine bases. This would permit the developing solvent to carry the nucleotides (or possibly their calcium salts) along the cellulose layer in accordance with less powerful interactions, such as Van der Waals' forces and hydrogen bonding.

Several experimental observations tend to support the second suggestion: (1) With the ammonium sulfate solvent a $CaSO_4$ effect is not observed; (2) the $CaSO_4$ effect is less pronounced with nucleosides and free bases; (3) the effect is negligible for adenosine which does not have an hydroxyl group. The fact that all the bases travel to some extent when $CaSO_4$ is absent may be attributed to the predominance of keto and imino forms over the hydroxyl and amino forms, the latter being more capable of binding to the R_3N^+ ion.

SUMMARY

Thin-layer techniques involving layers of diethylaminoethyl-cellulose, with and without $CaSO_4$, are presented for the complete separation and quantitative elution of the major degradation products of ribonucleic acid and deoxyribonucleic acid. Emphasis is given to the effects of the pH of the material to be separated and to the percent $CaSO_4$ present in the cellulose as "binder".

REFERENCES

1 K. RANDERATH, *J. Chromatog.*, 6 (1961) 365.
2 K. RANDERATH, *Nature*, 194 (1962) 768.
3 K. RANDERATH, *Angew. Chem.*, 74 (1962) 484.
4 T. WIELAND AND H. DETERMANN, personal communication to R. R. BECKER.
5 K. KIMURA, *Biochim. Biophys. Acta*, 55 (1962) 22.