CHROM. 4610

ION-EXCHANGE CHROMATOGRAPHY OF NUCLEIC ACID CONSTITUENTS ON CHITOSAN-IMPREGNATED CELLULOSE THIN LAYERS

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(Received January 15th, 1970)

SUMMARY

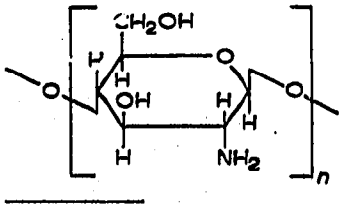
A number of nucleic acid constituents, such as 5'-nucleotides, nucleosides and nucleic bases, could be separated and identified by chromatography on chitosan formate-impregnated cellulose thin layers. Suitable solvent systems and conditions for the chromatography were investigated, and R_F data obtained for the compounds are given.

INTRODUCTION

Recently, thin-layer chromatography of nucleic acid constituents employing three ion-exchange celluloses*, DEAE-[1], ECTEOLA-[2] and PEI-cellulose[3], has been widely used because of the excellent and rapid separation of minute amounts of these constituents possible using these celluloses. The properties of these materials were assessed by RANDERATH[4]. The unique chromatographic results obtained on PEI-cellulose thin layers have especially attracted our attention.

The present paper describes the preparation of chitosan-impregnated cellulose layers and their application to ion-exchange thin-layer chromatography of nucleic acid constituents.

Chitosan is a polysaccharide derivative, "β-1,4-linked D-glucosamine polymer", and is prepared from chitin by deacetylation. As can be seen from its partial structure, there are a primary amino group and two hydroxylic groups per D-glucosamine unit. Chitosan is solubilized in acidic media such as formic acid, acetic acid, hydrochloric acid, etc., to form its ammonium ion but is insoluble in neutral or alkaline medium. Thus, because of its chemical structure and properties, chitosan appears to be applicable to standard stationary phases for making a new type of anion-exchange material.



* The following abbreviations will be used: DEAE-cellulose, diethylaminoethyl cellulose; ECTEOLA-cellulose, a cellulose anion-exchange material obtained by treating sodium cellulose with epichlorohydrin and triethanolamine; PEI-cellulose, a cellulose anion-exchange material obtained by impregnating cellulose for chromatography with poly(ethyleneimine).

For example, 0.8 % (w/v) chitosan in 0.5 % (w/v) formic acid gives a moderately viscous clear solution and forms a homogeneous stable suspension with powdery materials such as starch, cellulose powder, etc. Chitosan-treated layers for ion-exchange chromatography are prepared by suspending cellulose powder in an appropriate chitosan–formic acid solution and by coating the resultant suspension on glass plates as usual. Avicel (a cellulose product for thin-layer plates, see section *Materials*) is known to be superior in many respects to the cellulose products usually used for thin layers[5]. During preparation and characterization of the chitosan-impregnated cellulose layers, the superiority of Avicel as the stationary phase in this investigation was also confirmed.

Separation of nucleic acid constituents on the chitosan-impregnated Avicel layers was successfully achieved by using volatile materials such as carbonic and formic acids, ammonia, triethylamine and pyridine as electrolytes. The use of such volatile electrolytes will give some additional advantages for two-dimensional or multiple development of the chromatoplates and elution of the materials separated from the chromatoplates. UV detection of nucleic acid constituents separated on the chitosan-impregnated Avicel layers was much more distinct than of those separated on DEAE- and ECTEOLA-cellulose layers because of the bright background of the chitosan-treated layers.

EXPERIMENTAL

Materials

Chitosan. Commercial chitin purified by Hackman's method[6] was deacetylated with hot concentrated alkali by the procedure of WOLFROM *et al.*[7–9]. The quantitative analysis of chitosan thus prepared gave 7.95 % N and 1.71 % N-acetyl; while 8.75 % N and 0 % N-acetyl were calculated for $C_6H_{11}NO_4$. These analytical data show that 92 % of the total nitrogen in the chitosan is present as the free amino group.

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).

*Nucleic acid constituents** . Nucleic bases, ribo- and deoxyribonucleosides, 5'-ribo- and 5'-deoxyribonucleotides were commercial products obtained from Schwarz BioResearch, Inc. (Orangeburg, N.Y.).

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

Powdered chitosan (2.4 g) is dissolved in 60 ml of 2.5 % (w/v) formic acid and diluted to 300 ml with distilled water. It is then filtered on a glass filter. A suspension of Avicel SF (15 g) in 60 ml of the (0.8 %, w/v) above-mentioned chitosan formate

* The following abbreviations will be used: AMP, IMP, GMP, CMP, UMP, TMP = 5'-monophosphates of adenosine, inosine, guanosine, cytidine, uridine, thymidine; ADP, ATP = 5'-diphosphate and triphosphate of adenosine, respectively; d-AMP, d-GMP, d-CMP = 5'-monophosphates of deoxyadenosine, deoxyguanosine, deoxycytidine.

solution is homogenized in a glass homogenizer for about 25 sec. After deaeration with suction, the suspension is spread evenly on 6 glass plates (20 × 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 before being stored in the desiccator containing silica gel. The resulting layers which have a capacity of approx. 0.2 mequiv. N/g cellulose are mechanically very stable and can be stored at least for several months at room temperature. If necessary, layers of lower or higher capacity ($<$ or $>$ 0.2 mequiv. N/g cellulose) can be prepared with the corresponding chitosan formate solution according to the procedure described above.

Layers impregnated with free-type chitosan are prepared by soaking the previously prepared chitosan formate layers in 1% ammonia for about 1 h, by washing with water and then by drying in air. The free-type layers are also stable for storage, and their chromatographic properties are nearly identical with those of the formate-type layers.

Chromatography

In order to avoid edging, 5 mm on opposite sides of the plates are scraped off. 1 m*M* solutions of the nucleotides (sodium or ammonium salt), nucleosides and nucleic bases in distilled water have been used throughout this experiment. For the determination of R_F values, 1 μl of the test solutions (1 mμmole) is spotted on the starting line 2.5 cm from the edge of the plate. The plate is developed ascendingly at 25° in a closed tank until the length of run is 10 cm. The development is carried out perpendicular to the coating direction. The development time is variable in the range 60–240 min depending on the composition of eluants, the concentration of the chitosan formate solution used, and the thickness and size of the layers. After development, the plates are well dried, particularly in the case of the eluants containing pyridine, in a stream of hot air. The compounds resolved are located by examining the plates in transmitted UV light.

RESULTS AND DISCUSSION

Separation of nucleotides

Neither the lithium chloride systems used on PEI-cellulose thin layers by RANDERATH AND RANDERATH[3], nor the dilute hydrochloric acid systems used successfully on DEAE- and ECTEOLA-cellulose layers[4] were suitable for the separation of nucleotides on the chitosan formate-impregnated layers. The separation of nucleotides on our layers was achieved using a pyridine formate system, which properly resolved each of the nucleotides as a small dense spot. As shown in Fig. 1, an examination of the molar concentration of the pyridine formate system revealed that 0.25 *M* pyridine formate (pH 4.4) resulted in the best spot size and migration rate for each nucleotide tested. 0.25 *M* ammonium formate systems (pH 4.0 and 6.5) also gave good separations of the nucleotides although the spots were slightly elongated. The R_F values obtained using these solvent systems are listed in Table I.

Separation of nucleosides and nucleic bases

Three neutral solvent systems containing no electrolyte (solvents A, B and C in Tables II and III), an acidic solvent (solvent D), and two alkaline solvents (sol-

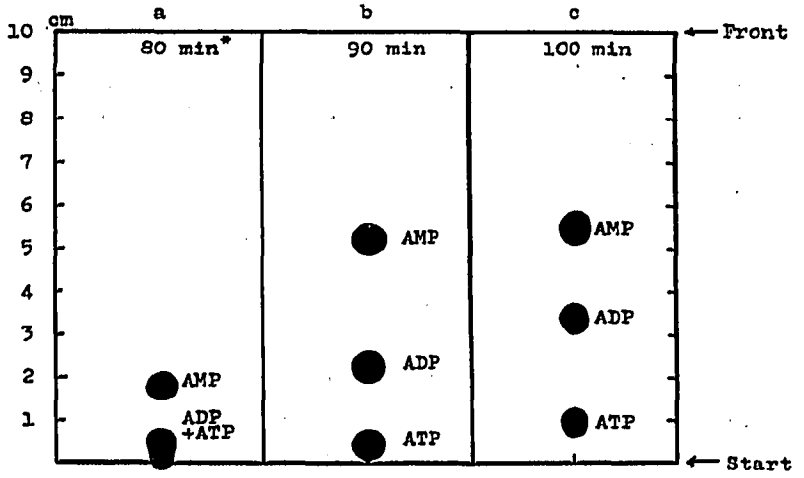


Fig. 1. Influence of the molar concentration of pyridine formate buffers on the separation of nucleotides. The nucleotides (each 1 mμmole) were chromatographed on 0.8% chitosan formate-impregnated Avicel SF layers (0.25 mm thick) with 0.05 *M* (a), 0.25 *M* (b), and 0.5 *M* (c) pyridine formate buffers. *Development time.

TABLE I

R_F VALUES FOR NUCLEOTIDES IN THE SOLVENTS CONTAINING FORMATE

Compound	*Solvent*		
	0.25 M pyridine formate pH 4.4	*0.25 M ammonium formate pH 4.0*	*0.25 M ammonium formate pH 6.5*
AMP	0.50	0.42	0.35
IMP	0.49	0.44	0.45
GMP	0.40	0.36	0.27
CMP	0.60	0.47	0.55
UMP	0.54	0.47	0.57
ADP	0.24	0.17	0.17
ATP	0.08	0.05	0.09
d-AMP	0.50	0.46	0.39
d-GMP	0.40	0.32	0.31
d-CMP	0.60	0.50	0.61
TMP	0.57	0.56	0.65

vents E and F) were selected for the separation of nucleosides and nucleic bases. Each nucleoside and nucleic base appeared as a small circular spot using solvents A–F, and their R_F values are listed in Tables II and III. Among the results shown in Table II, it is seen that a discrimination between ribo- and deoxyribonucleosides could be achieved by using solvents B or C. This separation is presumably due to the difference in solubilities of the ribo- and deoxyribonucleosides in these solvents rather than to the ion-exchange properties of the materials.

Separation of nucleotides from nucleosides and nucleic bases

On the chitosan formate layers, the nucleosides and nucleic bases migrate properly in the neutral media (solvents A, B and C in Tables II and III), whereas all the nucleotides remain at the origin using these solvents (data not shown). On the

TABLE II

R_F VALUES FOR NUCLEOSIDES IN VARIOUS SOLVENTS

Solvent: A = water; B = methanol–water (1:1); C = ethanol–water (4:1); D = 0.05 *M* formic acid, pH 2.5; E = 0.5 *M* pyridine, pH 9.2; F = 0.5 *M* triethylammonium carbonate, pH 7.3.

Compound	*Solvent*					
	A	*B*	*C*	*D*	*E*	*F*
Inosine	0.60	0.45	0.40	0.64	0.63	0.74
Adenosine	0.42	0.39	0.51	0.43	0.51	0.44
Guanosine	0.45	0.37	0.31	0.46	0.54	0.63
Cytidine	0.63	0.56	0.47	0.71	0.70	0.69
Uridine	0.71	0.57	0.60	0.75	0.72	0.77
Deoxyadenosine	0.40	0.44	0.63	0.43	0.51	0.45
Deoxyguanosine	0.46	0.43	0.50	0.48	0.56	0.64
Deoxycytidine	0.63	0.62	0.67	0.74	0.69	0.69
Deoxyuridine	0.70	0.61	0.61	0.77	0.70	0.77
Thymidine	0.70	0.66	0.66	0.79	0.72	0.72

TABLE III

R_F VALUES FOR NUCLEIC BASES IN VARIOUS SOLVENTS

Solvent: A = water; B = methanol–water (1:1); C = ethanol–water (4:1); D = 0.05 *M* formic acid, pH 2.5; E = 0.5 *M* pyridine, pH 9.2; F = 0.5 *M* triethylammonium carbonate, pH 7.3.

Compound	*Solvent*					
	A	*B*	*C*	*D*	*E*	*F*
Adenine	0.28	0.46	0.73	0.29	0.32	0.33
Hypoxanthine	0.42	0.55	0.62	0.50	0.50	0.58
Guanine	0.21	0.33	0.39	0.23	0.29	0.21
Cytosine	0.49	0.64	0.67	0.70	0.64	0.65
Uracil	0.58	0.66	0.80	0.70	0.63	0.67
Thymine	0.58	0.71	0.92	0.70	0.65	0.65

other hand, the R_F values of all the nucleotides are >0.78 in 0.5 *M* triethylammonium carbonate (data not shown), while R_F values of the nucleosides and nucleic bases range from 0.21 to 0.77 in the same solvent (Tables II and III). The phenomena described above suggest that it is possible to separate the nucleotides from the nucleosides and nucleic bases by using these solvents.

Separation of ribonucleotides and ribonucleosides from their deoxy analogues

As mentioned above, an effective separation between the ribo- and deoxyribonucleosides was performed, particularly using solvent C. It was also found that the ribonucleosides and ribonucleotides can be separated from their deoxy analogues using *n*-butanol–saturated 0.5 *M* ammonium formate containing H_3BO_3 (2 %, w/v). The R_F values listed in Table IV show that this solvent could satisfactorily differentiate the ribo-type compounds from their deoxy analogues.

Influence of the content of chitosan formate

Fig. 2 shows that the chromatographic behavior depends on the concentration of the chitosan formate solution used in preparing the layers. As can be seen in Fig. 2,

TABLE IV

R_F VALUES FOR NUCLEOTIDES AND NUCLEOSIDES IN THE SOLVENT CONTAINING BORATE

Solvent: *n*-Butanol–saturated 0.5 *M* ammonium formate containing H_3BO_3 (2%, w/v), pH 6.2.

Nucleotide	R_F	*Nucleoside*	R_F
IMP	0.44	Inosine	0.61
AMP	0.36	Adenosine	0.45
d-AMP	0.51	Deoxyadenosine	0.55
GMP	0.25	Guanosine	0.47
d-GMP	0.46	Deoxyguanosine	0.57
CMP	0.49	Cytidine	0.68
d-CMP	0.63	Deoxycytidine	0.78
UMP	0.53	Uridine	0.67
TMP	0.67	Deoxyuridine	0.78
ADP	0.20	Thymidine	0.77
ATP	0.15		

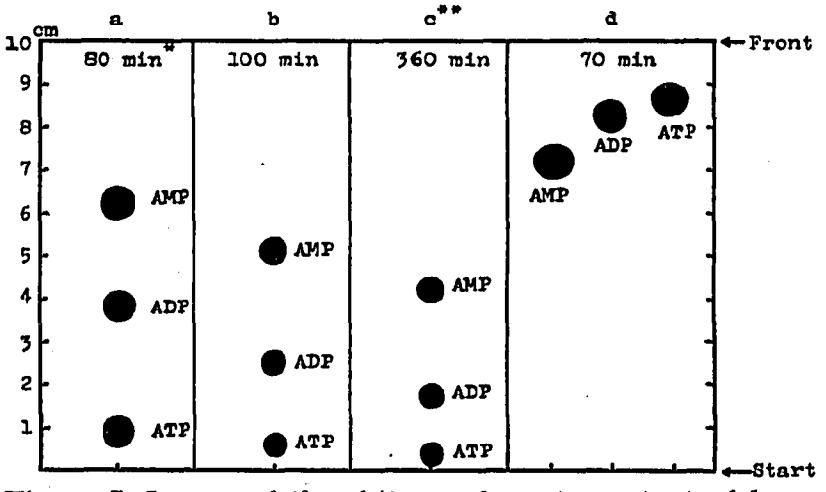


Fig. 2. Influence of the chitosan formate content of layers of Avicel SF on the separation of nucleotides. The nucleotides (each 1 mμmole) were chromatographed on layers (0.25 mm thick) prepared with 0.4% (a), 0.8% (b), and 1.6% (c) chitosan formate solutions and 0.25 *M* pyridine formate buffer (pH 4.4). (d) Avicel SF thin layer (0.25 mm thick). *Development time. **In this case, a severely irregular solvent front was formed, so that the R_F values could not be calculated.

lowering the concentration of chitosan formate results in the formation of larger, more elongated spots and higher R_F values. The 0.8% (w/v) chitosan formate solution gave the best spot size, separation, and desirable development time (Fig. 2b), whereas higher concentrations (>1.5%, w/v) brought about a severe irregularity in the solvent front and a remarkable increase in development time (Fig. 2c).

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