SEPARATION OF SUGAR NUCLEOTIDES, PHOSPHORIC ESTERS AND FREE SUGARS BY PAPER CHROMATOGRAPHY WITH SOLVENTS CONTAINING BORATES OF ORGANIC BASES*

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Methods based on the formation of borate complexes have been used with some success for the separation of free sugars and sugar phosphates by paper chromatography[1–4], paper electrophoresis[5–7] and ion-exchange[8–16]. However, none of these methods will separate mixtures of some of the nucleoside diphosphate sugars, such as UDP-glucose and UDP-galactose, which differ only in the orientation of a hydroxyl group.

By using boric acid salts of organic bases, which are more soluble in the solvents used for paper chromatography, it was possible to separate glucose, galactose and mannose nucleotides of the same base. The method has also been applied with good results to the separation of free sugars or their phosphates.

EXPERIMENTAL

Materials

GDP-α-mannose, UDP-α-glucose, UDP-α-acetylglucosamine, UDP and UMP were isolated from yeast as reported by PONTIS *et al.*[17] and further purified by chromatography on Whatman No. 17 paper with ethanol–ammonium acetate as solvent[18].

UDP-α-mannose, UDP-α-xylose, UDP-α-galactose, ADP-α-glucose, ADP-β-glucose, TDP-α-mannose, ADP-α-galactose, ADP-α-mannose, GDP-α-glucose, GDP-α-galactose, deADP-α-glucose, deUDP-α-glucose and ADP-P-glyceric acid were prepared according to ROSEMAN *et al.*[19] with slight modifications.

All the sugar phosphates and free sugars employed were of the D-series, with the exception of L-arabinose. Xylose-1-P and mannose-1-P were synthesized according to MEAGHER AND HASSID[20] and POSTERNAK[21], respectively. Psicose was prepared as described elsewhere[22]. All other chemicals were commercial products.

Chromatography

In the analytical runs, Schleicher and Schüell No. 2043 B paper, cut in the manner described by MATTHIAS[23], was found to give the best resolution.

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For preparative purposes, Whatman No. 3 MM paper was used, which was sewn to a piece of Whatman No. 1 in order to slow down the solvent flow[24]. Chromatograms were developed by the descending technique.

The following solvents were used:

(A) Ethylene glycol dimethyl ether–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA (70:20:30).

(B) Ethanol–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA (70:20:30).

(C) Butanol–pyridine–piperidinium borate buffer of pH 10.5 (6:4:3). The molarity of both components in the buffer is 0.35 M.

(D) Butanol–pyridine–0.05 *M* morpholinium tetraborate, pH 8.6 (7:5:2).

(E) Methanol–1 *M* ammonium acetate, pH 3.8 (70:35). This solvent is a modification of that described by PALADINI AND LELOIR[18].

0.5 *M* morpholinium borate buffer was prepared by dissolving 123.4 g of boric acid and 87 g of morpholine ($\sigma = 0.99$) in 0.01 *M* EDTA up to 1 l. For use with solvent D, EDTA was omitted and the buffer was diluted 10-fold.

Piperidinium borate buffer was prepared mixing 21.60 g of boric acid and 29.75 of piperidine ($\sigma = 0.86$) and adding water up to 1 l.

When solvents A, B, or E were used, the papers were dipped in 0.01 *M* EDTA, pH 7.0, and dried before use, as described elsewhere[25]. For use with solvent C, the papers were dipped in a 7-fold dilution of the piperidinium borate buffer.

Detection procedure

Nucleotides were detected with a Mineralight lamp. Phosphate-containing compounds were revealed according to BURROWS *et al.*[26] and sugars by the silver nitrate technique[27].

Radioactivity was located with an automatic scanner (Nuclear Chicago Corp. model D-47 gas flow counter fitted to a C-100 A actigraph II).

Recovery of the nucleotides

To free the samples from borate after chromatography with solvent C or D, the procedure described by ZILL *et al.*[10] was followed. In the case of solvents A or B, two different methods were used:

(1) The sample was eluted with water and brought to pH 6.0 with acetic acid; Norit A was added (50 mg/μmole), the suspension was shaken occasionally and filtered. After washing with water, the nucleotides were eluted from the charcoal with ethanol–concentrated ammonia–water (25:0.5:75). The total recovery was about 80 %.

(2) Alternatively a simpler method was used: the eluted sample was neutralized with acetic acid using bromothymol blue as internal pH indicator, and chromatographed with solvent E to remove borate.

RESULTS AND DISCUSSION

Nucleoside diphosphate sugars

Small differences in the mobilities of sugar nucleotides were obtained with the system of HARRAP[1] employed by NIKAIDO[28] to resolve a mixture of galactose-1-P and glucose-1-P. This solvent (methyl cellosolve–methyl ethyl ketone–3 *N* ammonia

(70:20:30), saturated with boric acid), however, has the disadvantage of changing its composition during the chromatographic run, due to precipitation of borate. To overcome this difficulty, different bases, more soluble in the organic phase, were tested instead of ammonia. Morpholine gave reproducible results and better resolution. Another improvement was obtained when methyl cellosolve was replaced by ethylene glycol dimethyl ether or ethanol.

The pH was found to be critical. At high pH values (above 10), galactose- and xylose-nucleotides decomposed almost completely, while low pH values cannot be used because no borate complexes are formed. A compromise was reached with a buffer of pH 8.6.

Solvent B gives a clear separation between galactose- and glucose-containing nucleotides; the running time necessary for the resolution depends upon the base involved (Table I). The corresponding mannose nucleotide runs in an intermediate position and it is difficult to obtain a complete separation among the three compounds, especially in the case of guanosine derivatives.

Deoxyribonucleotides run much faster than the other substances tested. This is understandable because the elimination of one of the hydroxyl groups decreases the ability to form a borate complex.

TABLE I

MOBILITIES OF SUGAR NUCLEOTIDES AND SUGAR PHOSPHATES IN SOLVENT B*

Running time: 70 h		*Running time*: 60 h		*Running time*: 20 h	
Compound	$R_{glucose\text{-}1\text{-}P}$	*Compound*	$R_{glucose\text{-}1\text{-}P}$	*Compound*	$R_{glucose\text{-}1\text{-}P}$
GDP-galactose	0.20	ADP-galactose	0.30	deADP-glucose	1.07
GDP-mannose	0.23	ADP-P-glyceric acid	0.33	TDP-mannose	1.28
GTP	0.25	ADP-mannose	0.35	TTP	1.36
GDP	0.27	ATP	0.36	de-UDP-glucose	1.40
GDP-glucose	0.28	ADP	0.42	TDP-glucose	1.49
GMP	0.32	ADP-α-glucose	0.46	TMP	1.52
		ADP-β-glucose	0.46	cyclic 3′,5′-AMP	1.90
		AMP	0.49		

Running time: 50 h			
Compound	$R_{glucose\text{-}1\text{-}P}$	*Compound*	$R_{glucose\text{-}1\text{-}P}$
UDP-galactose	0.32	Fructose-1,6-P_2	0.33
UDP-mannose	0.43	2,3-P_2-glyceric acid	0.51
UTP	0.45	Glucose-6-P	0.72
UDP	0.48	Galactose-1-P	0.75
UDP-glucose	0.51	Mannose-1-P	0.80
UDP-xylose	0.59	PP_i**	0.93
UMP	0.62	P_i	0.98
UDP-acetylglucosamine	0.71	Glucose-1-P	1.00
		Xylose-1-P	1.04
		3-P-glyceric acid	1.05

* Ethanol–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA (70:20:30). Mobilities are referred to glucose-1-P.

** Tailing of this compound is often observed.

The highest mobility was observed with cyclic 3',5'-AMP, which has only one acid group and one hydroxyl group.

With solvent A the separation pattern is similar although the spots are smaller and sharper than with solvent B. Solvent A has the disadvantage of requiring longer development times (Fig. 1).

Mixtures of sugar nucleotides and nucleoside mono-, di- or tri-phosphates of the same base cannot be resolved with these systems. Good results were obtained by first submitting the sample to preliminary separation with the neutral ethanol–ammonium acetate solvent.

To counteract the tendency of several compounds to give diffuse spots with solvents A or B, the paper was impregnated with 0.01 *M* EDTA.

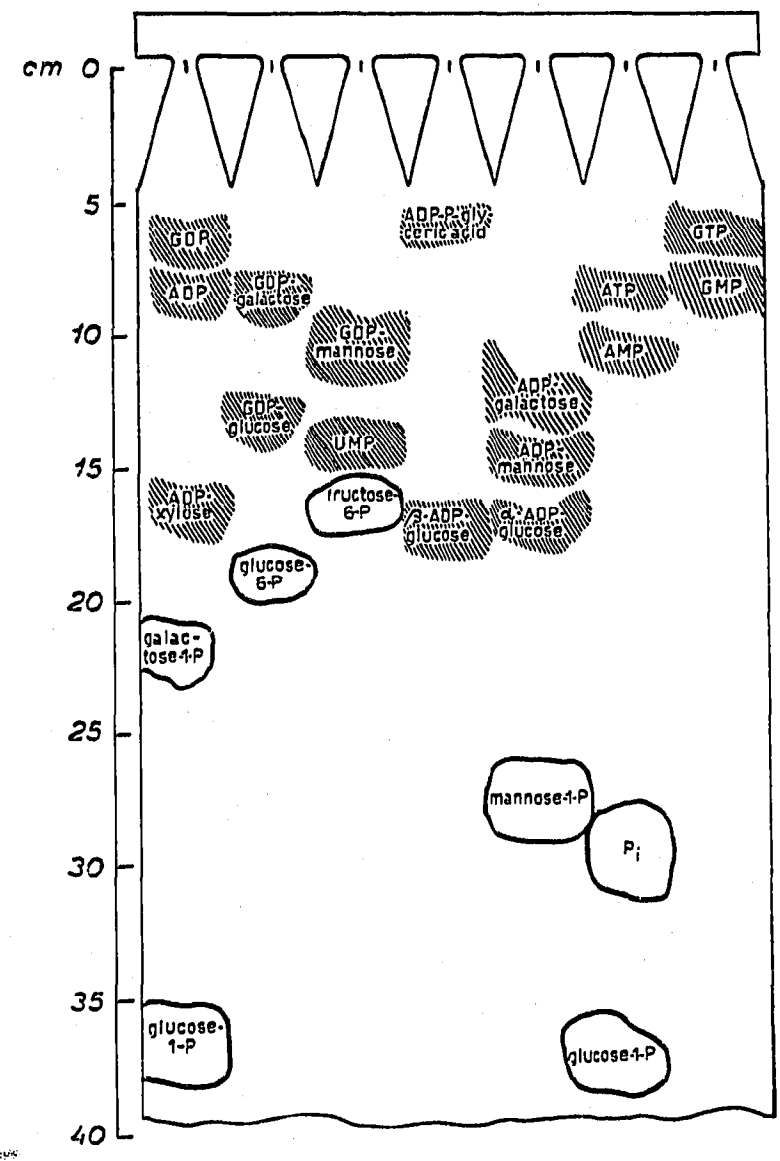

Fig. 1. Separation of nucleotides and phosphoric esters by paper chromatography for seven days in solvent A (ethylene glycol dimethyl ether–methyl ethyl ketone–0.5 *M* morpholinium tetraborate, pH 8.6, in 0.01 *M* EDTA, 70:20:30). Shaded areas correspond to ultraviolet-absorbing substances.

Solvent systems A and B have been successfully used to resolve a mixture of ADP-glucose, ADP-mannose and ADP-galactose[29] as well as UDP-acetylglucosamine and UDP-acetylgalactosamine from corn grains.

Another application of this chromatographic technique was the separation of a mixture of UDP-^{14}C-glucose and UDP-^{14}C-galactose prepared with an enzymatic extract from *Saccharomyces fragilis*[30] as shown in Fig. 2.

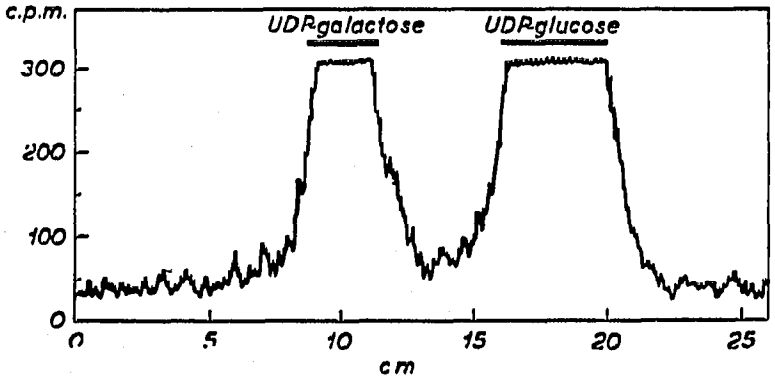

Fig. 2. Distribution of radioactivity in a chromatogram with solvent A of a mixture of labeled uridine nucleotides prepared with an enzymatic extract from *Saccharomyces fragilis*[30].

Sugar phosphates

With the exception of xylose-1-P, which runs very close to glucose-1-P, both solvents A and B gave a good resolution of the different sugar 1-phosphates tested.

The separation pattern is similar to that obtained with the sugar nucleotides: mannose-1-P running between galactose-1-P and glucose-1-P.

Free sugars

Some groups of sugars are very difficult to separate in a single run with the usual solvents; this is the case of glucose, fructose, mannose and arabinose.

TABLE II

MOBILITIES OF SUGARS IN SOLVENT D*

Sugar	$R_{glucose}$	*Sugar*	$R_{glucose}$
Glucuronic acid	0.18	Glucosamine	0.90
Psicose	0.36	Erythrose	0.95
Sorbitol	0.37	Glucose	1.00
Mannitol	0.42	Mannose	1.15
Lactose	0.43	Arabinose**	1.22
Sorbose	0.44	Ribose	1.29
Trehalose	0.51	Acetylglucosamine	1.36
Tagatose	0.60	Xylose	1.38
Maltose	0.62	Acetylgalactosamine	1.52
Fructose	0.64	Fucose	1.59
Galactosamine	0.69	Dihydroxyacetone	1.82
Sucrose	0.73	Deoxyglucose	1.98
Galactose	0.89	Deoxyribose	2.22

* Butanol–pyridine–morpholinium tetraborate 0.05 *M* (7:5:2). Mobilities are referred to glucose.

** Produces a diffuse spot.

The principle of complex formation with borate led to the development of solvent D, which allows the separation of glucose, fructose and mannose after an 18 h run[31] (see Table II).

TABLE III

MOBILITIES OF SUGARS IN SOLVENT C*

Sugar	$R_{mannose}$	*Sugar*	$R_{mannose}$
Galactosamine	0.21	Fructose	0.58
Glucosamine	0.28	Trehalose	0.68
Glucose	0.38	Arabinose	0.71
Sorbose	0.38	Maltose	0.78
Sorbitol	0.42	Psicose	0.78
Xylose	0.48	Sucrose	0.98
Galactose	0.48	Mannose	1.00
Lactose	0.49	Ribose	1.05
Tagatose	0.54	Acetylgalactosamine	1.75
		Acetylglucosamine	2.40

* Butanol–pyridine–piperidinium borate buffer, pH 10.5 (6:4:3). See "Methods". Mobilities are referred to that of mannose.

A more alkaline solvent (solvent C), obtained by substituting morpholine by the stronger base piperidine, was also found useful. It has the advantage of separating the four above-mentioned sugars, but it needs about 70 h of development (Table III). In addition, the buffer concentration is very critical as it is shown in Fig. 3. Slight changes of its molarity produce significant modifications in the relative mobilities of the compounds.

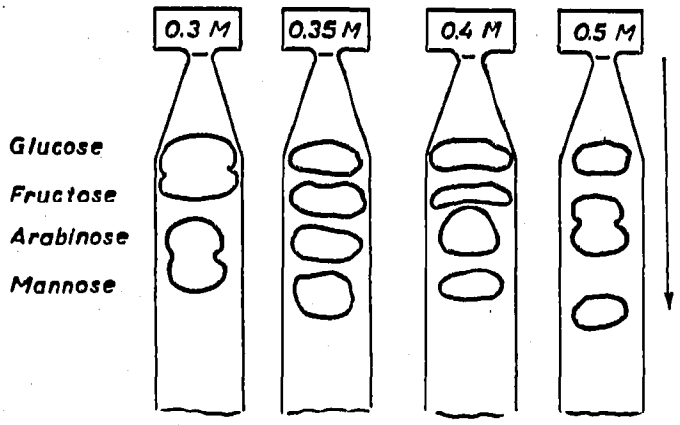

Fig. 3. Changes in the mobilities of glucose, fructose, arabinose and mannose, using solvent C prepared with increasing molarities of both components of the buffer, piperidine and boric acid.

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SUMMARY

Four different chromatographic solvents containing borates of organic bases are described. Two of them permit the separation of sugar nucleotides differing only in the sugar moiety and give also a good resolution for some sugar 1-phosphates. The other two systems permit, in a single run, the separation of a group of sugars which is usually difficult to achieve.

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