PAPER CHROMATOGRAPHIC SEPARATION OF NUCLEOTIDES, NUCLEOSIDES, PURINES, AND PYRIMIDINES*

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During the course of studies of nucleotide catabolism in kidney slices and in ischemic organs[1] numerous nucleotides and their dephosphorylated degradation products (nucleosides, purines, pyrimidines) had to be separated completely by means of paper chromatography. Among the different separation procedures described so far (see refs. 10–12), none was found to be quite so satisfactory for this particular purpose. Some of the methods known involved too many chromatographic steps or gave inadequate separations of the compounds under study, other ones caused, owing to the solvents used, serious disturbances during the final spectrophotometric quantitation. A new method of paper chromatography was therefore developed for the separation, identification and quantitation of mononucleotides, nucleosides, purines, and pyrimidines; the solvents, originally developed for chromatography of phosphorylated compounds[2], could be used with some modifications in the mode of application.

METHODS AND RESULTS

Chromatography was carried out in glass jars; Whatman No. 1 papers (20 × 50 cm) were washed by immersion in 1 N HCl for 60 min and then in distilled water until neutral. This method of washing gave reproducible and low blanks for U.V. absorption.

Reference compounds were obtained from commercial sources. Compounds were dissolved in water or dilute acid except for uric acid which was dissolved in 0.03 M $NaHCO_3$. In this $NaHCO_3$ solution, uric acid was stable for at least one week. Concentrations used were approximately 2 mg/ml and test spots were 1–10 μg.

Preparation of tissues and media

Tissues were ground at the temperature of liquid nitrogen and 0.3–0.5 g were extracted in 1–2 ml of 0.3–0.6 N perchloric acid at 0° by high-speed stirring for 3 min. Media from *in vitro* experiments were chilled to 0° and, after the removal of tissues, were centrifuged. The media supernatants were made up to 0.6 N acidity by the addition of 10 N perchloric acid, and then centrifuged. The acid-soluble fractions, after centrifugation, were neutralized by the addition of 6 N KOH and, after half an hour, potassium perchlorate was removed by centrifugation.

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Solvents for chromatography

I. Isopropyl ether–*n*-butanol–98 % formic acid (30:30:20 by vol.). Run in downward direction at 20–24° for 5 h.

II. *n*-Butanol–*n*-propanol–ethanol–25 % ammonium hydroxide–water (40:40:10:45:15 by vol.). Run in downward direction at 26° for 12 h in solvent-saturated atmosphere.

III. 80% (v/v) formic acid–*n*-butanol–*n*-propanol–acetone–30% (w/v) trichloroacetic acid (25:40:20:25:15 by vol.). Run in ascending direction at 20–24° for 18 h in solvent-saturated atmosphere.

IV. Methanol–isopropanol–25 % ammonium hydroxide–water (45:30:15:10 by vol.). Run in downward direction at 20–24° for 12 h in partially water-saturated atmosphere.

V. Water–*n*-propanol–25 % ammonium hydroxide (90:10:1 by vol.). Run in upward direction at 20–24° for 3–4 h.

R_F values for test compounds are summarized in Table I.

TABLE I

R_F VALUES OF NUCLEOTIDES, PURINES, PYRIMIDINES, AND RELATED COMPOUNDS*

Compounds	*Solvents*				
	I	*II*	*III*	*IV*	*V*
Adenine	0.34	0.46	0.74	0.55	0.48
Adenosine	0.17	0.54	0.59	0.54	0.55
AMP	0.17	0.16	0.38	0.17	0.81
Deoxyadenosine	0.31	0.63	0.66	0.64	0.58
Deoxy-AMP	0.26	0.18	0.50	0.22	
Hypoxanthine	0.23	0.43	0.52	0.62	
Inosine	0.12	0.42	0.36	0.66	0.79
IMP	0.14	0.10	0.24	0.10	0.89
Deoxyinosine	0.20	0.49	0.47	0.69	0.82
Guanine	0.12	0.29	0.45	0.43	
Guanosine	0.10	0.30	0.41	0.53	0.76
GMP	0.12	0.08	0.26	0.10	
Deoxyguanosine	0.18	0.41	0.48	0.55	0.79
Deoxy-GMP	0.17	0.09	0.36	0.13	
Xanthine	0.19	0.33	0.38	0.51	0.64
Xanthosine	0.35	0.31	0.33	0.62	0.75
Uracil	0.38	0.48	0.60	0.70	0.80
Uridine	0.18	0.40	0.46	0.72	0.86
UMP	0.22	0.15	0.36	0.26	0.92
Deoxyuridine	0.29	0.45	0.51	0.73	0.88
Cytosine	0.31	0.51	0.73	0.64	
Cytidine	0.14	0.46	0.58	0.65	0.75
CMP	0.15	0.13	0.36	0.22	0.88
Deoxycytidine	0.22	0.56	0.66	0.73	0.77
Deoxy-CMP	0.22	0.15	0.48	0.28	
Thymine	0.52	0.56	0.71	0.72	
Thymidine	0.42	0.54	0.67	0.77	0.85
TMP	0.36	0.17	0.50	0.32	
Orotic acid	0.22	0.31	0.54	0.49	0.88
Uric acid	0.19	dec.	0.19	dec.	
Allantoin	0.27	0.32	0.28	0.62	

* All values determined on Whatman No. 1 paper using solvents and running times as given in the text. Partial decomposition in a solvent indicated by dec.

Chromatography of extracts

Up to 1 ml of extract was usually applied to the paper as a band (5–10 cm) using an airstream to speed drying. Initial chromatography was done in solvent III resulting in separation into several mixed fractions (Table II). These mixed fractions were then eluted, rechromatographed as shown in Table II, and quantitated.

Nucleoside di- and tri-phosphates were determined in a second sample of extract using methods described elsewhere[2].

TABLE II

PAPER CHROMATOGRAPHIC SEPARATION OF NUCLEOTIDES, NUCLEOSIDES, PURINES, AND PYRIMIDINES FROM TISSUE EXTRACTS

Initial separation solvent III		*Further separation*
R_F values	*Fractions*	*Solvent and running time*
0.68–0.77	Adenine*	V (3 h)
0.57–0.67	Adenosine, adenine*	II (12 h)
0.47–0.55	Hypoxanthine, uracil, adenosine*	II (12 h)
0.38–0.45	Uridine, xanthine	II (12 h)
0.27–0.37	Inosine, AMP, xanthine	II (12 h)
0.22–0.26	CMP, UMP, uric acid**	II (2 × 24 h)
0.17–0.21	IMP, GMP, uric acid**	II (2 × 24 h)
0.00–0.11	Tri- and diphosphonucleotides of adenine, guanine, uridine and cytidine with NAD, NADP, nucleotide coenzymes, etc.	

* Since adenosine and adenine each appear in 2 fractions when extracts are chromatographed, their final quantitation is done after a third chromatographic separation of the appropriately combined eluates. For this purpose, solvent V is used for 3 h.

** Uric acid is unstable in alkaline solvents and rechromatography of a second sample is done with solvent I (2 × 8 h) instead of solvent II prior to quantitation.

Detection of compounds

All purine and pyrimidine compounds could be detected on the chromatograms by viewing with transmitted U.V. light at 253.7 nm using a Mineralight. Photoprints, according to the method of MARKHAM AND SMITH[3], were used to locate trace quantities. Allantoin was detected on chromatograms by spraying with 0.25 % mercuric acetate in 95 % ethanol, drying, and then spraying with 0.05 % diphenylcarbazone in 95 % ethanol[4].

Elution

Elution was done according to the method of SANGER AND TUPPY[5] using water or *N* HCl. The eluate was collected from the tip of the filter paper in a glass vial or test tube. Elution was ordinarily complete after 0.2–0.4 ml eluate had been collected. Recoveries of known amounts after chromatography ranged from 90 % for uric acid to 100 % for more soluble compounds.

Identification and quantitation

Nucleotides, nucleosides, purines and pyrimidines were identified by use of the following methods: U.V.-spectra (220–300 nm) at different pH, comparison of ratios

of optical densities at different wave lengths (250/260, 280/260, 290/260), estimation of the content of phosphorus for nucleotides, comparison of R_F values against known compounds in different solvents, and the specific color reaction of GERLACH AND DÖRING[6] for adenine-containing compounds.

After separation, all substances except allantoin were quantitated by U.V. absorption using known molecular absorption coefficients[7,8]. The application of solvents increased the blank U.V. absorption value of the paper depending on the distance from the starting line (Fig. 1). Thus, the U.V. absorption values of eluates had to be corrected by subtracting the U.V. absorption given at the same wave length by the eluate from an equivalent amount of paper taken from a blank region at the same R_F as the isolated compound.

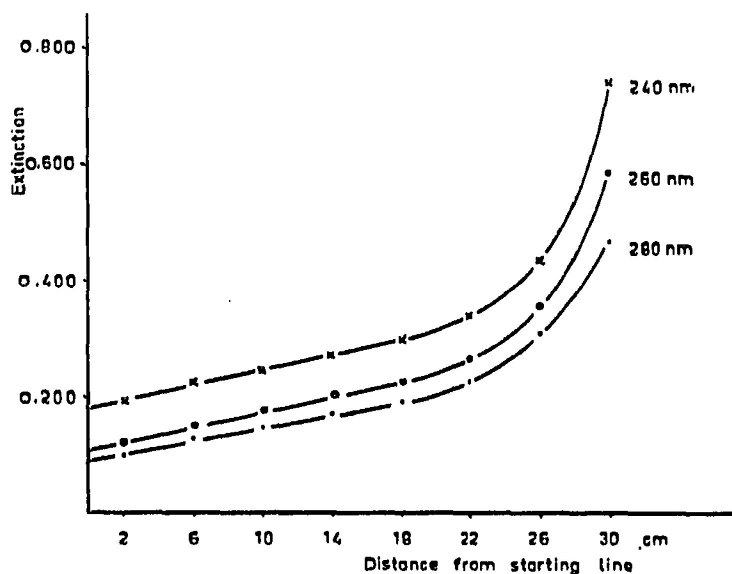

Fig. 1. Blank values *vs.* R_F for acid-washed Whatman No. 1 paper run in solvent II (see text). Paper cut into 4 cm wide strips centering at the indicated distance from the starting line, eluted in 0.3–0.4 ml distilled water, diluted to 0.65 ml and read at the indicated wavelength. The individual extinction values were corrected to correspond to 150 mg of paper strip.

Allantoin was determined by the method of YOUNG AND CONWAY[9], scaled down to a final volume of 2.5 ml. The method, as modified, was sensitive to 0.005 μM allantoin when read at 520 nm in a spectrophotometer. Only uric acid, besides allantoin, gave appreciable color in this reaction, amounting to one third on a molar basis. Since the determination of allantoin could not be carried out after application of the spray reagent, the position of the allantoin fraction was located by running a separate spot of pure allantoin on the same paper and spraying this as a separate strip after chromatographic development.

SUMMARY

A method of paper chromatography is described for separation, identification, and quantitation of nucleotides, nucleosides, purines and pyrimidines. The method has been used for studies of nucleotide catabolism in tissues *in vivo* and *in vitro*.

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