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Note

Chromatography of the components of the purine reutilization pathway

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Several procedures are available for the separation and identification of nucleic acid components. Good separations can be obtained with column chromatography[1] and high-pressure liquid chromatography[2], but thin-layer chromatography (TLC) methods are most frequently used. TLC has advantages in that many samples can be run simultaneously, it is available to many investigators, and it can be applied readily to studies using radioactive nucleotides.

Separations by TLC are frequently designed for specific problems involving a single purine and its derivatives, or for the separations of the bases from the nucleotides and nucleosides. When separations involve more compounds, two-dimensional TLC is used to obtain the necessary separations, but fewer samples can be analyzed per unit time.

For the study of purine reutilization pathways, procedures involving only one-dimensional chromatography were developed to permit the separation of many samples and the quantitation of the radioactive purines and their nucleosides and nucleotides. For this purpose one paper chromatographic (PC) and three TLC separations are involved, three if one is not interested in XTP. All chromatography is at room temperature. The spots are visualized in UV light, and the radioactivity measured by scintillation counting.

EXPERIMENTAL AND RESULTS**

Separation 1

Support: Whatman No. 1 paper

Solvent: *n*-butanol–propionic acid–water (45:23:32)

(A) 124.6 ml *n*-butanol + 8.4 ml water

(B) 62.0 ml propionic acid + 79 ml water

This PC method is one part of a two-dimensional system described by Calvin and Benson[3] and Meikle *et al.*[4]. Equal volumes of Solvents A and B were mixed just before

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** Abbreviations: Guan = guanine, Xan = xanthine, Hx = hypoxanthine, Aden = adenine, G = guanosine, X = xanthosine, I = inosine, A = adenosine, GMP, etc. = the nucleotides.

TABLE I

R_F VALUES OF PURINES AND THEIR DERIVATIVES

The R_F values of most value in the complete study of purine metabolism are printed bold face. Other R_F values are given for other possible uses.

Compound	*R_F value*			
	Separation 1	*Separation 2*	*Separation 3*	*Separation 4*
Guanine	Streak		**0.25**	0.83
Xanthine	**0.36**		**0.52**	0.70
Hypoxanthine	**0.48**			0.84
Adenine	**0.70**			0.68
G	0.32		**0.61**	0.76
X	**0.26**		**0.70**	0.61
I	**0.32**			0.79
A	**0.56**			0.95
GMP	0.11	**0.55**	**0.87**	**0.41**
XMP	0.09	**0.63**		0.33
IMP	0.10	**0.71**		0.45
AMP	**0.13**	**0.87**		0.01
GDP	0.03	**0.17**	0.86	0.29
IDP	0.04	**0.28**		0.31
ADP	0.06	**0.45**		0.65
GTP	0.02	**0.03**	0.85	**0.20**
XTP	0.01	0.05		**0.12**
ITP	0.02	**0.06**		0.22
ATP	0.03	**0.12**		0.46
cAMP	0.20	0.87	0.71	0.84

use. The mixed solvent had to be a single phase at room temperature. After equilibrating for 120 min in a Chromatocab (Scientific Products, McGaw Park, Ill., U.S.A.), the spotted papers were chromatographed for 16 h by descending chromatography. In this time the solvent front migrated about 40 cm. The R_F values for the useful components separated are given in Table I in boldfaced numbers. R_F values of other components that might be of use in specific cases are also given.

This method of separation is useful for the bases and the nucleosides. It cannot be used if guanine is present, as guanine streaks over most of the separation range. An attempt to use cellulose TLC plates rather than paper was unsuccessful, as the components were not adequately separated.

Separation 2

Support: PEI-cellulose. Pre-coated sheets of Polygram CEL-300 PEI (Brinkmann, Westbury, N.Y., U.S.A.). Before use, the plates were washed according to the method of Randerath and Randerath[5] to remove background fluorescence. They were soaked for 1 min in 10% NaCl, air dried, soaked for 5 min in water, and air dried. The plates were given a final wash by ascending chromatography in water until the solvent had reached the top. They were stored at 4° face to face, wrapped in aluminium foil.

Solvent: Methanol–water (1:1), overnight

Buffers: (1) 0.5 *M* sodium formate, pH 3.4, 30 sec
(2) 2.0 *M* sodium formate, pH 3.4, 2 min
(3) 4.0 *M* sodium formate, pH 3.4, until the migration front is 15 cm

The PEI plates were used essentially by the method described by Randerath and Randerath[5], who used the above solvent as the second solvent in a two-dimensional system. A wick of Whatman 3MM filter paper was stapled to the top of the plate. After the samples had been applied to the washed plates, they were developed overnight in the solvent. The free bases and the nucleosides migrated into the wicks. After drying, the plates were developed in the formate buffers for the times indicated above without drying between the buffers. The R_F values of the various components are given in Table I.

Separation 3

Support: ECTEOLA-cellulose plates (Brinkmann)
Solvent: 0.2 *M* NaCl + 0.25 ml NH_4OH/100 ml

The method was modified from one described by Randerath[6] and uses commercial plates. A better resolution was obtained by increasing the NaCl concentration to 0.2 *M* from the 0.15 *M* described by Randerath. It was found that the commercial plates were too acid to give good separations and a small amount of ammonium hydroxide had to be incorporated in the solvent. Different batches of plates may require other amounts of NH_4OH. Without the NH_4OH both guanine and xanthine streaked, so they could not be estimated. With the above concentration of NH_4OH, only guanine streaked and as it was the last component to migrate, its quantitation was adequate. Streaking of guanine could be prevented by further increasing the NH_4OH concentration, but other components were not so well separated. The development was for 90 min.

Separation 4

Support: cellulose plates (Eastman-Kodak, Rochester, N.Y., U.S.A.)
Solvent: isobutyric acid–2 *N* NH_4OH (66:34)

The method was adapted from the PC system developed by Schwarz/Mann[7,8]. The same solvent system was used but cellulose-TLC plates were used in place of paper. Instead of a single development, the plates were developed three times for 5 h each, with drying between each run. The same solvent was used for each development. The repeat chromatography gave a better separation of the nucleotides, and it was the only system that gave a clear separation of XTP. It was usually used only for XTP, but it has advantages in the identification of GMP and GTP. The R_F values reported in Table I are higher than those reported by Schwarz/Mann because of the manner of development and include R_F values for substances not previously identified.

Sequence of use

The metabolic pathways of purine reutilization are studied by including a radioactive substrate in the incubation mixture containing the enzyme systems extracted from leukocytes. The resulting radioactive products are chromatographed with the proper inactive carrier mixture of compounds to be separated and identified. Separations 1 and 2 are used for the isolation of adenine and hypoxanthine bases and

their nucleoside and nucleotide derivatives. The guanine and xanthine derivatives are separated with Separations 2 and 3.

In Separation 1 with PC and butanol–propionic acid–water as the solvent, the carrier mixture contains AMP, X, I, Xan, Hx, A, and Aden. After development, the papers are air dried and the position of the various compounds located with UV light. The spots are cut from the paper and quantitated by scintillation counting. The AMP spot and the area between it and the origin represent all the nucleotides. The nucleotides are separated on PEI plates (Separation 2) including ITP, ATP, IDP, ADP, XMP, and AMP as carriers. Each isolated spot is again quantitated by scintillation counting, and the yield of each product calculated by the formula:

$$\% \text{ of each nucleotide} = \frac{\% \text{ AMP from paper}}{\text{Total count on PEI}} \times \text{Count in each spot}$$

In a similar manner, the carrier mixture for the ECTEOLA-cellulose plates (Separation 3) contains Guan, Xan, G, X, and GMP (represents nucleotides). A separate PEI plate (Separation 2) is used to separate the nucleotides GTP, GDP, GMP, and XMP, and the yield of each is calculated by the above formula. Separation 4 is usually used only for the identification of XTP.

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