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Note

Chromatography and electrophoresis techniques for demonstrating the presence of cellular N^6-(Δ^2-isopentenyl)-adenosine-3'-monophosphate

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Both in whole animals and in mammalian cell culture N^6-(Δ^2-isopentenyl)-adenosine (i^6A), has been reported to have antitumor activity and to exert a transient, suppressive action upon growth[1–13]. This hypermodified nucleoside has been found in the cells of many plants as the free mononucleoside[14,15] and recently in human and mouse cells as the free mononucleotide[16]. The natural function of i^6A or its nucleotide is unknown, except in the case of plants, where the hypermodified nucleoside acts as a hormone cytokinin[15]. In addition, it is known that i^6Ap and its 2-methylthio derivatives (ms^2i^6Ap) occur in specific species of tRNA, *i.e.*, those species responding to codons beginning with uridine, in bacteria, yeast, plants and animals[14,16,17]. Therefore, it is important to be able to demonstrate and quantitate the natural presence of the nucleotide in less than microgram amounts in cells[16] and cellular tRNA[14], and the conversion of exogenously added i^6A to i^6Ap[11–13] in order to elucidate its function.

Analysis of i^6A has been accomplished by taking advantage of the hydrophobic nature of the nucleoside and its biological activity as a plant hormone. Separation and detection of chemically synthesized and naturally occurring isopentenyl-adenosines have been accomplished by Sephadex LH-20 column chromatography of nucleoside mixtures in organic solvents followed by bioassay of the column fractions through their ability to stimulate tobacco callus growth in culture[15]. Unfortunately, this analytical method requires milligram quantities of material for, *e.g.*, the detection of i^6A in tRNA and does not yield a quantitative evaluation of i^6A relative to the amounts of other nucleosides that may be present. A radioactive analysis of ^{32}P-labeled i^6Ap has been shown to be both sensitive and quantitative[16]. The nucleotide has chromatographic and electrophoretic properties distinctively different from those of the nucleoside. Flat-plate electrophoretic methods for the separation of nucleic acid components including some modified nucleotides but not i^6Ap have been reviewed by Smith[18]. The advantage of flat-plate *versus* liquid-immersed paper electrophoresis is in the assessability of buffer solutions. This communication reports in detail sensitive methods of thin-layer chromatography (TLC) and liquid-immersed paper electrophoresis that have been used to demonstrate the presence of i^6Ap as a free cytoplasmic mononucleotide[16] and tRNA component[19,20] within cells in culture and in human leukemia cells[21].

MATERIALS AND METHODS

Materials

Mouse (RAG) cell line was the gift of Dr. F. Ruddle (Yale University). N^6-(Δ^2-Isopentenyl)-adenosine (i^6A) and standard nucleotides, AMP, ADP, ATP, CMP, GMP and UMP, were obtained from Sigma (St. Louis, Mo., U.S.A.); carrier-free [^{32}P]phosphoric acid from New England Nuclear (Boston, Mass., U.S.A.); minimal essential medium (Eagle's with Earls's salts) and horse serum from GIBCO (Grand Island, N.Y., U.S.A.); TLC plates (250 μm thick, microgranular cellulose and silica gel) from Analtech (Newark, Del., U.S.A.); and Whatman No. 1 and DE-81 chromatography paper from H. Reeve Angel (Clifton, N.J., U.S.A.).

Analysis of i^6Ap

The RAG cell line was maintained in minimal essential medium containing [^{32}P]phosphate (0.3 mCi/ml). Transfer RNA was isolated and its nucleotide content was analyzed by ribonuclease digestion and two-dimensional TLC as described previously[19].

After the TLC plates had been subjected to autoradiography, the i^6Ap, identified by its distinctive chromatographic mobilities[14,17,19], was scraped from the cellulose plates and separated from the cellulose by eluting it with ethanol. This i^6Ap was then used for the determination of the mobilities of the compound in other chromatography systems and in electrophoresis. Standard nucleotides, i^6A, and the radioactive i^6Ap isolated from the cell tRNA were then subjected to TLC in the following seven solvent systems: (1) isobutyric acid–concentrated ammonium hydroxide–glass-distilled water (70:1.15:28.85); (2) *tert*-butanol–concentrated HCl–glass-distilled water (70:15:15); (3) isopropanol–concentrated HCl–glass-distilled water (70:10:20); (4) *n*-propanol–concentrated ammonium hydroxide–glass-distilled water (55:10:35); (5) *n*-butanol–concentrated glacial acetic acid–glass-distilled water (40:10:10); (6) *n*-butanol–glass-distilled water (85:15); (7) glass-distilled water. Cellulose plates were used in the first six systems and silica gel in the seventh. The mobilities of each compound were then determined by the quenching of UV light (254 mm) to locate movement of the standard nucleotides and i^6A and by autoradiography to determine movement of the radioactive i^6Ap (see Table I).

The same compounds were run in two electrophoretic systems using Whatman No. 1 (cellulose) and DE-81 (DEAE-cellulose) papers in each of the following systems: (1) 630 ml 89.1% formic acid, diluted to 8 l with water, and resulting in a solution of pH 1.7, 1500 V; (2) 40 ml pyridine, 400 ml glacial acetic acid, diluted to 8 l (with water) that was 1 m*M* in EDTA and resulting in a solution of pH 3.5, 1500 V. A dye, Xylene Cyanol FF, was used in the electrophoresis and the voltage was applied until it had moved 15 cm in the pH 3.5 system or 16 cm in the pH 1.7 system. Again nucleotide migrations were detected using the quenching of UV light and autoradiography. The R_F values were recorded for the electrophoresis in reference to AMP (see Table II).

RESULTS AND DISCUSSION

Radioactive tRNA labeled with [^{32}P]phosphate has been isolated in microgram

nucleotides have subsequently been analyzed[19–21]. However, definite detection of the presence of modified nucleotides, particularly i⁶Ap as the free mononucleotides within the cell cytoplasm or as tRNA components, is of importance for understanding their function and the metabolism of tRNA.

The sensitivity conveyed by the [^{32}P]phosphate label[19] and the chromatographic and electrophoretic analyses described here have enabled us to detect the presence of nanogram quantities of i⁶Ap in the cytoplasms of 10^6 cells or in microgram quantities of tRNA isolated from mammalian cell lines and human leukemia cells[16,19–21]. Chromatographic mobilities of ^{32}P-labeled i⁶Ap isolated from ^{32}P-labeled tRNA as described in Materials and methods, and other nucleic acid components are shown in Table I. Migration of i⁶Ap in the seven systems was consistent with the combined hydrophobicity of the isopentenyl group and the hydrophilicity of the phosphate group. The corresponding nucleoside, i⁶A, migrated faster than the nucleotides in the six primarily organic systems 1–6, but slower in the completely aqueous system[7]. The presence of the 3′-monophosphate group on the hypermodified nucleotide decreased its chromatographic mobility in the organic systems.

TABLE I

MOBILITIES OF i⁶Ap ON TLC IN SEVEN SOLVENT SYSTEMS

The compositions of the solvent systems and the detection of the migration of the components are described in Materials and methods. Mobilities are shown in terms of R_F values; the marker dye used in chromatography was Orange G.

Compound	*Cellulose support*				*Silica gel support*		
	1	*2*	*3*	*4*	*5*	*6*	*7*
i⁶Ap	0.28	0.89	0.88	0.35	0.22	0.00	0.80
i⁶A	0.94	0.94	0.89	0.96	0.94	0.90	0.37
AMP	0.52	0.33	0.29	0.43	0.09	0.02	0.89
ADP	0.35	0.30	0.26	0.41	0.02	0.00	0.90
ATP	0.26	0.29	0.25	0.40	0.00	0.00	0.94
CMP	0.47	0.47	0.40	0.50	0.13	0.02	0.89
GMP	0.15	0.27	0.20	0.33	0.06	0.00	0.88
UMP	0.24	0.66	0.60	0.40	0.15	0.01	0.93
Dye	0.39	0.99	1.00	0.86	0.33	0.17	0.96

The electrophoretic mobilities of i⁶Ap, i⁶A, and the standard nucleotides in two systems on two different supports are shown in Table II. The presence of the phosphate group on i⁶Ap conferred an electrophoretic mobility in relation to the non-mobile nucleoside i⁶A. On Whatman No. 1 paper the mobilities of i⁶Ap in both systems of electrophoresis were only slightly greater than those of AMP; whereas on Whatman DE-81 (DEAE-cellulose) the mobilities were over fourfold those of AMP.

The hypermodified nucleotide i⁶Ap has characteristic chromatographic and electrophoretic mobilities in the nine systems described (Tables I and II). Positive analysis would not require use of all the systems. The authors would, however, put considerable weight on the electrophoretic analysis that has shown distinctively different mobilities of i⁶Ap on cellulose (Whatman No. 1) as compared to DEAE-

TABLE II

MOBILITY OF i^6Ap DURING ELECTROPHORESIS IN TWO SYSTEMS ON TWO SUPPORTS

The compositions of the solvent systems and the detection of the migration of the compounds are described in Materials and methods. Mobilities are shown in terms of R_F values relative to AMP ($R_F = 1.00$). The marker dye used in electrophoresis was Xylene Cyanol FF.

Compound	*Whatman No. 1 paper*		*Whatman DE-81 paper*	
	pH 3.5	*pH 1.7*	*pH 3.5*	*pH 1.7*
i^6Ap	1.58	1.29	4.13	4.68
i^6A	0.49	−0.05	−0.14	not determined
AMP	1.00	1.00	1.00	1.00
ADP	0.83	1.10	2.45	4.23
ATP	0.38	0.38	3.29	6.02
CMP	0.96	1.06	0.72	1.11
GMP	1.05	1.06	1.77	1.49
UMP	1.28	1.43	2.22	5.32
Dye	0.87	0.64	1.56	3.44

cellulose (Whatman DE-81) with reference to AMP. If i^6Ap is isolated from cell cultures grown in a medium containing [^{32}P]phosphate, then its presence in nanogram quantities could be detected by analysis using these systems[16,19–21].

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