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Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA*

Kenneth C.Kuo, Roy A.McCune and Charles W.Gehrke
Department of Biochemistry, Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO 65211, and

Rosemarie Midgett and Melanie Ehrlich
Department of Biochemistry, Tulane Medical School, New Orleans, LA 70112, USA

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ABSTRACT

We have developed a method to accurately determine (< 3% RSD) the complete major and modified base composition of a few micrograms of unlabeled DNA. The DNA samples were quantitatively hydrolyzed with DNase 1, Nuclease P1, and bacterial alkaline phosphatase. The resulting deoxyribonucleosides were directly separated in 70 min by reversed-phase high performance liquid chromatography with detection by ultraviolet absorption at 254 nm and 280 nm (RP-HPLC). The highly sensitive and selective dual wavelength quantitation greatly enhances the precision and accuracy of the chromatographic analysis. Contamination of DNA preparations with RNA does not interfere with the DNA analysis due to the high resolution of the chromatography. We have used this method for the quantitation of m^5dCyd in 5 µg of calf thymus and salmon sperm DNA in which the m^5dCyd comprises only 1 to 2% of the total bases. This method should be a useful research tool in studies on various DNAs and DNA subfractions and should help to elucidate the functions of methylation of DNA.

INTRODUCTION

Most DNA molecules contain minor amounts of bases which are methylated after DNA replication (1). In procaryotes and some non-vertebrate eucaryotes, 5-methylcytosine (m^5Cyt) and/or N^6-methyladenine (m^6Ade) are present as minor bases (1,2,3,4,5). In all examined DNAs from vertebrates or higher plants, m^5Cyt is present as the only minor base (1). These minor bases may comprise less than 0.05 mole percent of the bases in some insect and bacterial DNAs or as much as 7 mole percent of the bases in higher plant DNAs. Typically mammalian DNA has 0.5-1.5 mole percent m^5Cyt (1,3).

Several different analytical techniques have been used in the determination of the major and minor base composition of DNA. One of the earliest techniques used was paper chromtography of DNA digests with quantitation by ultraviolet absorption (6,7). Paper and thin layer chromatographic methods have also been applied to digests of DNA which were labeled in vivo with radioactive precursors (8,9,10,11). This approach has high sensitivity but quantitation of radiolabeled methylated bases using [^{3}H]

or [^{14}C-methyl] methionine has had limited success due to the difficulty in determining the sizes of the appropriate methionine pools (12,13,14). More advanced chromatographic techniques including gas-liquid chromatography (15,16), cation-exchange chromatography (17,18,19,20,21,22,23,24, 25), reversed-phase HPLC (26), paired-ion reversed-phase HPLC (27) and gas chromatography - mass spectrometry (28) have also been applied to analysis of the major, minor or total base composition of DNAs.

Mass spectral (29,30,31) and immunological (32,33) methods have also been applied to DNA analysis. However these methods have one or more of the following limitations; the ability to measure only the major bases or one of the modified bases, exposure of the DNA to harsh conditions, and dependence of the determination on the conformation of the DNA and distribution of the modified bases.

This article describes a reversed-phase high performance liquid chromatographic (RP-HPLC) method for the analysis of major and minor deoxyribonucleosides in DNA. The method, which is based on our studies of the chromatographic parameters for the analysis of major and minor ribonucleosides in RNA and other biological materials, (34,35,36,37,38) offers good sensitivity, selectivity, precision and accuracy for the determination of all six deoxyribonucleosides without the use of harsh hydrolysis conditions, large DNA samples, difficult sample preparation procedures or *in vivo* labeling of DNA.

MATERIAL AND METHODS

For these studies an automated HPLC system was used which included an M-6000A solvent delivery system, a WISP 710-A automatic sample injection system, a model 440 fixed wavelength UV detector (Waters Assoc. Inc.), and a Model 3352B data handling system (Hewlett-Packard). The detector was set to monitor the absorption at both 254 and 280 nm. The buffer supplied to the pump was controlled by a model MV-4 four port valve with timer control (MER Chromatographic). This timer-valve system was modified by the addition of a 24 volt power supply and relay to allow interfacing of the timer controlled valve with the automated injector. With this arrangement a complete, automated recyclable 3 buffer stepwise gradient system was obtained.

An internal standard method was used for quantitation with 8-bromoguanosine (Br^{8}Guo) as the internal standard (IS). The nucleoside standards used were obtained from several sources (Sigma Chemical Co., Mann Research Labs, P & L Biochemicals, and Vega-Fox Biochemicals).

The HPLC separation of deoxyribonucleosides was performed on two reversed-phase μBondapak C_{18} 300 x 4 mm columns (Waters Assoc., Inc.) connected in series and surrounded by a water jacket as we have described previously (35). For chromatography, $NH_4H_2PO_4$ solutions were mixed with the appropriate amount of methanol (Burdick and Jackson) and adjusted to the desired pH with 0.25 Molar NH_4OH. All HPLC buffers were made with water purified by reversed osmosis, passage through charcoal and deionizing cartridges (Barnstead Co.), and then distillation. Just before use the buffers were filtered through sterile 0.22 μm membrane filters (Millipore).

Complete separation of the six deoxyribonucleosides (dCyd, dUrd, m^5dCyd, dGuo, dThd, dAdo) from their corresponding ribonucleosides was achieved in 70 minutes using a two buffer step gradient system on two μBondapak C_{18} reversed-phase columns (Waters Assoc. Inc.). Buffer A (2.5% v/v methanol, 0.01 M $NH_4H_2PO_4$, pH 5.3) was pumped for the first 30 min of the run, followed by 40 min of Buffer B (8% v/v methanol, 0.01 M $NH_4H_2PO_4$, pH 5.1). During automated operation 70% v/v methanol/H_2O was used for 20 min to flush strongly retained material from the column prior to a 20 min re-equilibration with buffer A. A flow of 1.0 ml/min and a column temperature of 45°C were maintained throughout the run. Absorption was measured simultaneously at 254 nm and 280 nm with sensitivity of 0.01 a.u.f.s. The method was calibrated based on the absorption of known concentrations of standard deoxyribonucleosides in the same buffer.

A separate isocratic run with a less polar eluent (1 ml/min, 45°C, 20% v/v methanol, 0.01 M $NH_4H_2PO_4$, pH 5.1) was required for separation of m^6dAdo from the other nucleosides. If insufficient DNA is available for a separate m^6dAdo analysis, then the m^6dAdo together with the other nucleosides can be measured from a single chromatographic run by adding the buffer containing 20% methanol following buffer B.

Typically 50 to 150 μl of a DNA solution containing up to 100 μg of DNA in 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.2) were made to 40 μg/ml of DNase I (DP 200 Kunitz Units/mg, Worthington) and 4 mM $MgCl_2$, and incubated for 18 h at 37°C. Then two volumes of 30 mM NaAc (pH 5.2), $ZnSO_4$ to a final concentration of 50 μg/ml and nuclease P1 (200 U/mg, Boehringer-Mannheim) to a final concentration of 50 μg/ml were added. After incubation at 37°C for 7 h, 0.1 volume of 2.2 mg/ml Escherichia coli alkaline phosphatase (40 U/mg Type III, Sigma) was added. The phosphatase had been pre-incubated at 95°C for 10 min in 50 mM Tris-HCl pH 8.0 to inactivate deoxyadenosine deaminase. Incubation at 37°C was continued for 16 h and then the samples were

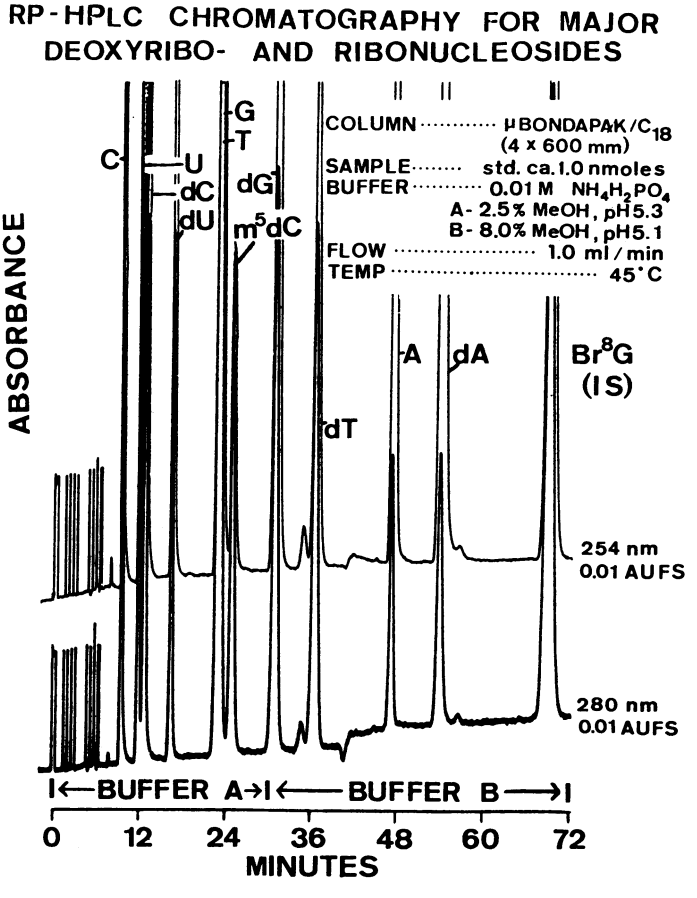
frozen until analysis.

RESULTS AND DISCUSSION

Separation, identification, and quantitation of deoxyribonucleosides

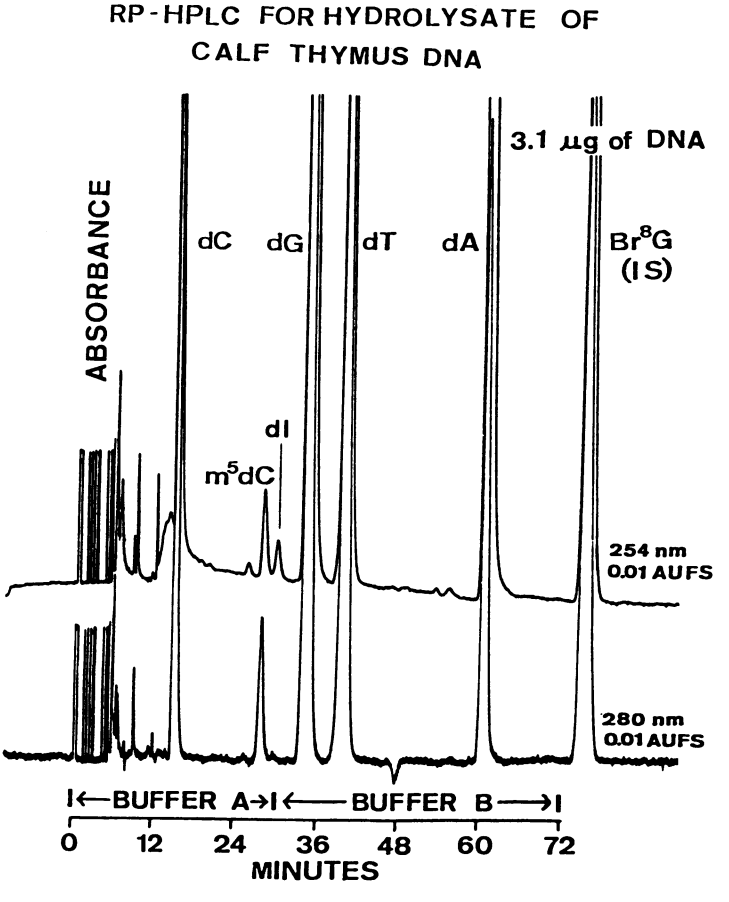
A chromatogram illustrating the separation of a standard mixture of deoxyribonucleosides and ribonucleosides is presented in Figure 1. Six deoxyribonucleosides, four ribonucleosides, and Br^8Guo as internal standard for quantitation were all well separated. The precision of retention time ranged from only 0.3 to 0.8% standard deviation for 15 independent runs over a period of two days.

The use of two HPLC columns in series is needed to obtain the baseline separation of Urd and dCyd and Guo from m^5dCyd. For analysis of DNA digests without any contaminating RNA, one C_{18} HPLC column is sufficient to achieve



the separation. When two columns are used, deoxyribonucleosides in enzymatic hydrolysates of DNA can be precisely quantitated even in the presence of moderate levels of ribonucleosides from RNA contaminating the original DNA preparations.

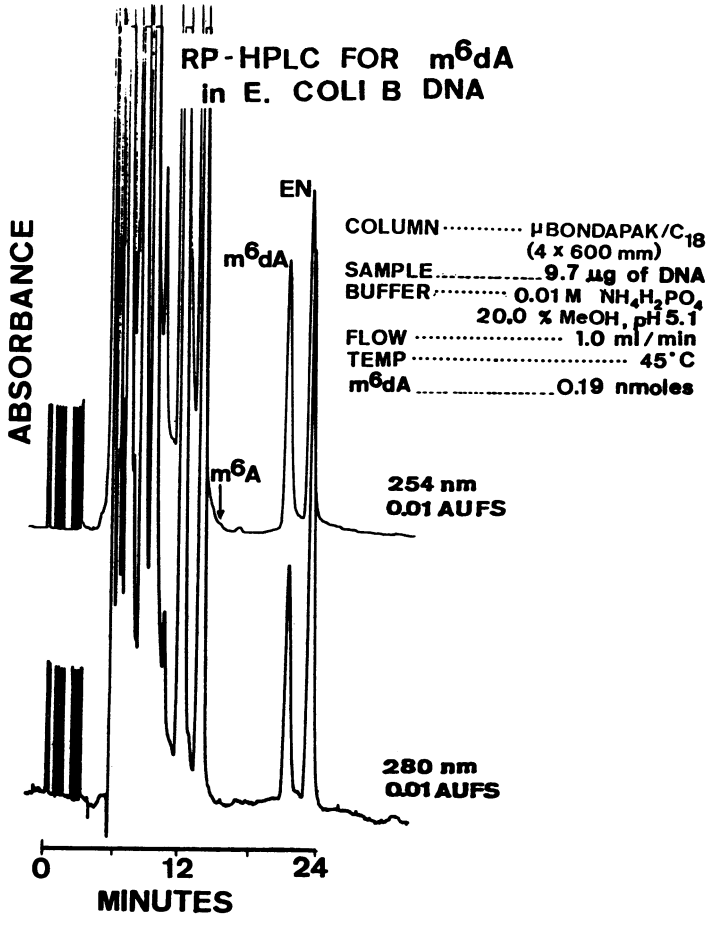
The absorbance values were monitored simultaneously, at both 254 and 280 nm (Figure 1). From examination of the A_{254}/A_{280} absorbance ratios, interfering substances present in significant amounts or slight errors in integration may be detectable as a difference in the absorbance ratio for a given peak and its corresponding deoxyribonucleoside. For example, the large peak under dCyd (Figure 2) prevented an accurate integration of dCyd at 254 nm. This interference peak originating from reagent background had no detectable 280 nm absorption, thus an accurate dCyd value could be obtained from measurement at 280 nm.



Separate chromatography is required for m^6dAdo because it is very strongly retained on the C_{18} column. The separation of m^6dAdo is illustrated with a chromatogram of an enzymatic hydrolysate of E. coli B DNA (Figure 3). Using our chromatographic conditions the ribonucleosides eluted faster than the less polar corresponding deoxyribonucleosides so that a readily available m^6Ado standard was used to predict the elution position for m^6dAdo, which was not commercially available. Unlike the other deoxyribonucleosides, m^6dAdo was quantitated without an internal standard, therefore the hydrolysate volume had to be known exactly.

Preparation of digests

Nuclease P1 is an endonuclease which quantitatively hydrolyzes both DNA and RNA to 5'-mononucleotides with little specificity for the type of base



(39,40). Single-stranded DNA or oligonucleotides are hydrolyzed approximately 200-fold faster than double-stranded DNA (40). To take advantage of this difference in digestion rates, DNA was either heat denatured or digested to oligonucleotides with DNase I prior to digestion with nuclease P1. The incubation of DNA with DNase I cleaved more than 98% of the nucleotide residues in native DNA to acid-soluble material. Subsequent digestion with nuclease P1 and bacterial alkaline phosphatase converted more than 99.8% of the oligonucleotides to the deoxyribonucleosides as determined by thin layer chromatography of Norit treated digests of [^{3}H-thymidine] labeled DNA (41).

The commercially available bacterial alkaline phosphatase was found to be contaminated with enough deoxyadenosine deaminase activity to give as much as 2 mole % of deoxyinosine in the final digest. Pre-incubating the phosphatase at 95°C for 10 minutes reduced the deaminase activity more than ten-fold with no loss of phosphatase activity. This pre-treatment had also been used to reduce contaminating phosphodiesterase activity in preparations of alkaline phosphatase (42).

Precision, accuracy, and linearity of HPLC chromatography of free deoxyribonucleosides

A series of experiments were made to determine the precision, accuracy and linear range of the HPLC measurement of six deoxyribonucleosides and their corresponding ribonucleosides. The percent relative standard deviation (RSD) obtained from three analyses for each deoxyribonucleoside at 2500, 500, 250 and 50 pmol injected were as follows: above 250 pmole the RSD was less than 1% and at 50 pmol a range of 2-5%. When smaller amounts of deoxyribonucleosides are injected (< 50 pmoles ≃ 15 ng or less) the RSD % increases. The increase in the RSD at these lower levels is due to the decrease in the signal/noise ratio. We are working on a number of these parameters to improve still further the precision of the method at the lower levels of deoxyribonucleosides injected. An excellent average precision for the 50 to 2500 pmole range of less than 2% RSD indicates that the method is linear over the range used in this investigation.

Calibration of the method for quantitation of m^6dAdo had to be accomplished using the corresponding ribonucleoside since a pure standard of m^6dAdo was not available. The loss of a hydroxyl group from the ribose portion of a nucleoside should not affect the UV absorbing part of the molecule. The good agreement of the relative molar response, where relative molar response is: $[(area_{NUC})/(nmol/ml_{NUC})] \times [(nmol/ml_{IS})/area_{IS})]$,

for deoxyribonucleosides and their corresponding ribonucleosides shows this to be the case. This agreement allows an additional check on the purity of the standard compounds and on the accuracy of preparation of the standard solutions. A standard was prepared from pure m^6Ado and used to determine the 254 and 280 nm response factors for m^6dAdo. In addition, the A_{254}/A_{280} absorbance ratio for m^6Ado was used to confirm the identity of the m^6dAdo peak in hydrolysates of E. coli DNA. The m^6dAdo values obtained form duplicates injections of DNA hydrolysates gave good precision.

Precision of the Total Method for Deoxyribonucleic Acids

Due to the difficulties encountered in isolating large quantities of DNA the precision and accuracy of the total method when applied to low microgram amounts of DNA is of great importance.

To determine the precision and linearity of the total method for analysis of DNA, multiple analyses were made of two model DNAs, calf thymus DNA and salmon sperm DNA. Triplicate samples of DNA were subjected to enzymatic hydrolysis at levels ranging from 150 µg to 5 µg of DNA per hydrolysate. We found that the mole fraction values obtained at all levels were essentiality the same (Table I).

The precision obtained was excellent, in general giving 2% RSD or less for all deoxyribonucleosides (Table I). No decrease in precision was seen at lower levels even for m^5dCyd which comprises only about 1.5% of the bases in calf thymus and salmon sperm DNA. The reagent background in the hydrolysates and HPLC buffers can become the limiting factor in precision if care isn't taken to prevent external contamination.

Applications

This method for analyzing the deoxyribonucleoside composition of DNA by HPLC of enzymatic digests gives the total base composition of DNA including quantitation of m^5dCyd and m^6dAdo down to 25 pmol of a given nucleoside. This methodology is also applicable to examining the content of chemically or physically altered nucleosides in DNA providing that these alterations do not interfere with the enzymatic hydrolysis. Such an application is described in the accompanying article on the bisulfite-induced deamination of DNA (45). Given the relatively low base specificity of the two enzymes necessary for the hydrolysis, namely, nuclease P1 and E. coli alkaline phosphatase, digests of other types of experimentally modified DNAs ought to be analyzable by this HPLC procedure.

Table II and III present the results of analyses of the base composition of six different types of DNA by our method and by various other

TABLE I. PRECISION OF TOTAL METHOD FOR DNA ANALYSIS

DNA Source	µg DNA	N	Mole Percent[a] dCyd	m^5dCyd	dGuo	dThd	dAdo
Calf[c] Thymus	113	3	21.1 (0.1)[b]	1.41 (2.3)	22.8 (0.1)	27.2 (0.3)	27.3 (0.4)
	23	3	21.0 (0.8)	1.42 (0.7)	23.1 (0.2)	26.9 (1.2)	27.5 (1.1)
	4.5	3	21.0 (1.8)	1.42 (3.5)	22.8 (0.4)	28.0 (0.6)	28.0 (0.1)
	Total[d]	9	21.0 (1.3)	1.42 (2.2)	22.9 (0.8)	27.4 (1.9)	27.6 (1.4)
Salmon[c] Sperm	167	3	20.6 (0.2)	1.56 (2.6)	22.5 (0.0)	27.4 (0.2)	27.9 (0.1)
	33	3	20.5 (0.8)	1.58 (1.3)	22.9 (0.7)	27.4 (0.7)	27.6 (0.6)
	6.7	3	20.3 (1.6)	1.57 (1.3)	22.6 (0.4)	27.5 (0.7)	28.0 (1.4)
	Total[d]	9	20.4 (1.2)	1.57 (1.7)	22.7 (1.0)	27.4 (0.6)	27.8 (1.1)

a/(Nanomoles of deoxyribonucleoside/total nmoles deoxribonucleosides) x 100.

b/( ) desiginates RSD, %

c/Hydrolyzed with Nuclease P1 and BAP

d/Average for all levels

TABLE II: DETERMINATION OF THE NUCLEOSIDE COMPOSITION OF SEVERAL EUCARYOTIC DNA SAMPLES

Source of DNA		N[a]	Mole percent						Pu/Py
			dCyd	m^5dCyd	dGuo	dThd	dAdo	m^6dAdo	
Calf Thymus	Authors'	9	21.0	1.42	22.9	27.4	27.6	-	1.01
	Range		20.8-21.4	1.37-1.47	22.7-23.2	26.7-28.1	27.1-28.2	-	1.00-1.04
	Literature[b]	18	21.2	1.50	21.7	28.2	28.5	-	1.01
	Range		20.0-22.1	1.1-1.9	19.5-23.9	26.6-31.2	27.1-30.3	-	0.94-1.12
Salmon Sperm	Authors'	9	20.4	1.57	22.7	27.4	27.8	-	1.02
	Range		19.9-20.7	1.52-1.60	22.5-23.1	27.2-27.7	27.5-28.4	-	1.01-1.04
	Literature[c]	7	21.0	1.67	21.3	28.6	28.6	-	1.00
	Range		19.8-22.1	1.61-1.73	20.4-22.4	27.1-30.4	27.6-29.7	-	0.96-1.05
Human Liver	Authors'	5	20.5	0.88	21.2	28.4	29.0	-	1.01
	Literature[d]	1	19.9	NR[e]	19.5	30.3	30.3	-	0.99

[a]Authors' values are the average of N independent analyses while literature values are the average of N reported values

[b]Eleven investigators reported only the 4 major bases (43), five gave the complete composition (43), and two determined only m^5dCyt (16,28)

[c]Six investigators reported only the 4 major bases (26,31,43), one gave the complete composition (43), and one reported only m^5dCyt (28)

[d]reference (43)

[e]NR is not reported

TABLE III: DETERMINATION OF THE NUCLEOSIDE COMPOSITION OF PROCARYOTIC AND VIRAL DNA SAMPLES

Source of DNA		N[a]	Mole percent						Pu/Py
			dCyd	m^5dCyd	dGuo	dThd	dAdo	m^6dAdo	
XP-12 Phage	Authors'	13	0.74[d]	34.1	34.7	14.9	15.6	-	1.01
	Literature[b]	1	NR[e]	33.4	33.8	16.0	16.8	-	1.02
Xanthomonas oryzae	Authors'	5	32.4	0.09	32.6	17.3	17.7	-	1.01
	Literature[b]	1	32.6	NR	31.8	17.4	18.2	-	1.00
E. coli B	Authors'	2	26.4	ND[f]	26.7	23.4	23.6	2.5	1.01
	Literature[c]	1	26.3	ND	26.8	23.1	23.8	2.0-1.49[g]	1.02

[a]Authors' values are the average of N independent analyses while literature values are the average of N reported values

[b]reference (44)

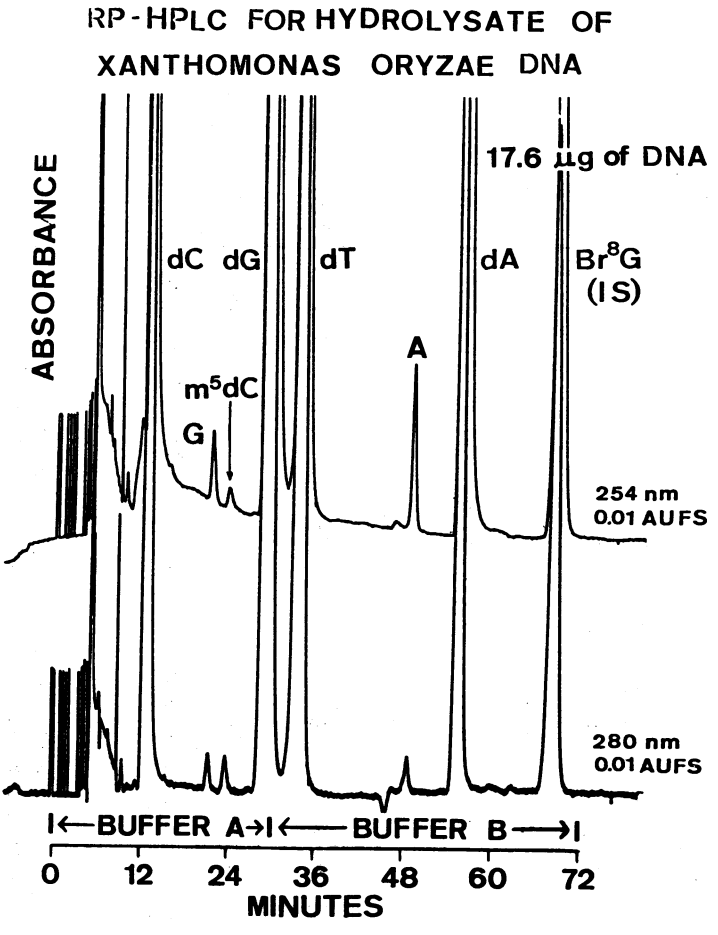
[c]reference (43)

[d]This dCyd may be derived from small amounts of host DNA packaged in phage particles because phage DNA labeled after infection contained < 0.5 % as much radiolabel in Cyt residues as in m^5Cyt residues (45).

[e]NR is not reported

[f]ND is not detected

[g]references (23,43)



methods as reported in the literature. In most cases the average literature values agreed fairly closely with our data, and, in general, the disagreement was less than 4 percent for the major deoxyribonucleoside values in all DNAs compared. However, the range for the reported literature values is quite large. The purine to pyrimidine ratios from our analysis were 1.01 for all the DNAs indicating that the method has essentially no bias.

The deoxyribonucleoside content of four DNAs having a widely different m^5dCyd content was examined to test the applicability of this method for a variety of samples. m^5dCyd was not detected in E. coli B DNA (Table III). A very low level of m^5dCyd was found in the DNA from Xanthomonas oryzae, 0.09 mole % (Table III; Figure 4); whereas calf thymus DNA (Figure 2) has

a 15 fold higher level of m^5dCyd (1.42 mole percent). In bacteriophage XP-12 DNA almost all of the dCyd is present as m^5dCyd (Table III). There is currently much interest in the as yet ill defined roles of methylated bases in DNA, especially m^5dCyd in eucaryotic DNA. This type of sensitive analysis of various types of DNA and of subfractions of DNA should help advance our understanding of the relationship of methylation to DNA function.

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