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SEPARATION OF NATURALLY OCCURRING OR INDUCED METHYLATED NUCLEOBASES OF DNA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Separation of the major bases of DNA along with seven minor methylated ones was obtained by reversed-phase high-performance liquid chromatography using an isocratic elution system. Application of this procedure to DNA treated *in vitro* by N-methyl-N-nitrosourea has allowed identification of two induced minor bases; a third one was resolved using a slightly modified mobile phase. Baseline resolution of 3-methyl- and 5-methylcytosine, detected in *Euglena* DNA hydrolyzates, was also achieved.

INTRODUCTION

Most of the minor bases from nucleic acids are methylated derivatives. Among the rarest are those occurring after treatment of DNA *in vivo* or *in vitro* with alkylating agents. These drugs, such as dimethylnitrosamine or N-methyl-N-nitrosourea, induce modifications of DNA without the intervention of methylating enzymes[1–3]. Such alterations of DNA are often related to the occurrence of malignant tumours. On the other hand, two of the most common minor bases occur naturally in DNA: N^6-methyladenine, characteristic of procaryotic DNA, and 5-methylcytosine which also occurs in some procaryotes and has aroused interest in recent years due to its assigned rôle in the regulation of gene expression[4,5].

Several analytical methods have been used to separate, identify and quantify the minor methylated nucleobases. Apart from recent studies on the proportion of 5-methylcytosine (5-mCyt) in a definite DNA sequence, C^mCGG[6], using differential digestion of DNA with restriction enzymes[7], most of the earlier studies tried to determine, along with the major bases, the whole 5-mCyt residues using either open-column, paper or thin-layer chromatography. As for the other methylated bases, accurate analysis only really began with high-performance liquid chromatography (HPLC)[8,9]. The first attempts to separate the minor methylated bases employed either anion-exchange or cation-exchange resins, but the time required to achieve this separation was at least 2 h[10]. When the analysis was limited to the five natural bases of DNA, the separation was obtained within 40 min by anion-exclusion chromato-

graphy[11]. The reversed-phase system displayed considerable advantages for separating bases and nucleosides on account of the elution speed, reproducibility and resolving capacity[8,9,12]. The analysis of eleven methylated standard bases corresponding to those found in tRNA hydrolysates could be achieved in 30 min using two buffer systems[13]. A two-buffer step-gradient system was used to separate 20 major and modified nucleosides of tRNA in 1 h[14]. A stepwise gradient system also served for quantitative analyses of natural DNA nucleosides, including 5-methyldeoxycytidine, performed in 70 min, but a separate elution with a low-polarity eluent was necessary for determination of N^6-methyldeoxyadenosine[15].

It has been claimed that ion-pairing agents improve the resolution of reversed-phase systems for nucleosides and bases[8,9,16−19]. Cytosine (Cyt) and 5-methylcytosine, when eluted close to the void volume, have been said to be unresolved by the simple reversed-phase system[9,16]. It will be seen that, under our elution conditions, using reversed-phase columns, ion-pairing reagents are unnecessary for separating compounds with short retention times. Besides, a systematic study of the retention times of most methylated purines, and pyrimidines with a methyl group located at C^5, shows that they are easily differentiated by the reversed-phase system[20].

As far as we know, none of the published methods was devised to separate, in a single DNA hydrolyzate, nucleobases occurring naturally in DNA together with the methylated ones induced by alkylating agents. This was achieved using isocratic elution in a reversed-phase system which allows the separation of eleven of the most important nucleobases found in DNA. Two of them, 3-methylcytosine (3-mCyt) and 3-methyladenine (3-mAde), were better resolved using slight modifications of the mobile phase, allowing at the same time the separation of three other bases.

EXPERIMENTAL

A Waters Assoc. Model 6000A solvent-delivery system (Waters, Milford, MA, U.S.A.) equipped with a Model U6K universal injector and a Model 440 absorbance detector operating at 254 or 280 nm were used for HPLC analysis. The absorbance was monitored on an Omniscribe recorder (Houston Instruments).

The reversed-phase columns (300 × 3.9 mm I.D.) were prepacked by Waters Assoc. with μBondapak phenyl (P/N 27198). Insertion of a guard column extends the life of the analytical column. This pre-column was repacked in the laboratory weekly, of after about 30 separations.

Reagents

Free standard bases of analytical grade were obtained from the following sources; adenine (Ade), guanine (Gua), cytosine, thymine (Thy), 1-methyladenine (1-mAde), N^2-methylguanine (N^2-mGua), 7-methylguanine (7-mGua), 3-methylcytosine, 5-methylcytosine (Sigma); 3-methyladenine, N^6-methyladenine (N^6-mAde), 1-methylguanine (1-mGua), 3-methylguanine (3-mGua) (Fluka). O^6-Methylguanine (O^6-mGua) was prepared according to Balsinger and Montgomery[21].

Ammonium dihydrogen phosphate, N-methyl-N-nitrosourea (MNU) and highly polymerized calf thymus DNA (Type I) were from Sigma, formic acid (99%) and methanol Normapur from Prolabo and caesium chloride from Bethesda Research Laboratories. All other chemicals were of the highest purity available.

Preparation of standards and eluents

Stock solutions of standard nucleobases were prepared at concentrations in the range 0.1–1 mg/ml respectively for minor and major bases. All solutions were made in 0.01 M HCl and sterilized by filtration through Millipore membranes, pore size 0.22 μm. These solutions, stored at $-25°$C, were stable for several weeks. Working standard solutions (2–20 μg/ml) were prepared by appropriate dilution with twice distilled water. A 20–200 ng amount of each base was applied to the column in an injection of about 10 μl.

One litre of a stock buffer of 0.5 M $NH_4H_2PO_4$ was prepared weekly. This solution was sterilized by filtration through a filter (pore size 0.22 μm) and stored at 4°C. The elution buffers were prepared daily by diluting aliquots of stock solution in water and appropriate amounts of methanol which has previously been filtered through Celotate membrane filters, Type EH, pore size 0.5 μm (Millipore). The pH of the elution buffers was adjusted to the desired value with a few drops of either 5% NH_4OH (Carlo Erba) or 5% H_3PO_4 (Merck). Prior to use, all buffers were refiltered through 0.22-μm filters and degassed by sonication for 15 min.

Sample preparation procedures

Commercial calf thymus DNA was first purified by CsCl density gradient centrifugation. About 2 mg of DNA, dissolved in 7 ml CsCl in standard saline–citrate (SSC) buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), refractive index 1.3995, was banded at 25°C and 35 000 rpm for 72 h with a 65 rotor in a Beckman ultracentrifuge. The DNA-containing fractions of the gradient were pooled, diluted in three volumes of SSC buffer and the DNA precipitated overnight at $-25°$C with 0.1 volume of 3 M sodium acetate and two volumes of ethanol. The precipitate was collected by centrifugation for 30 min at 15 000 g, washed twice with 70% ethanol, air-dried and allowed to rehydrate for several hours in tris(hydroxymethyl)-aminomethane–ethylenediaminetetraacetate buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). About 200–300 μg of purified DNA are incubated for 1 h at 37°C in 1 ml of Tris–EDTA buffer with 2.5 mg of MNU. The reaction mixture was then dialyzed for 24 h against 1 l of the same buffer (two changes). Before DNA hydrolysis for HPLC analysis, the modified DNA was precipitated and washed as described above.

The method used for the isolation of cellular DNA from *Euglena gracilis* strain Z cells was Dalmon's modification[22] of Marmur's procedure[23] with some minor changes, as follows. A 0.5-g amount of lyophilized cells was ground with two volumes of Fontainebleau sand (Prolabo) for 5 min and suspended in 50 ml SSC buffer. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% and the mixture gently stirred for 3 h at room temperature. After addition of NaCl (1 M final), the mixture was cooled (4°C) and centrifuged at 20 000 g (30 min). One volume of cold ethanol (95%) was added to the supernatant and the resulting precipitate dissolved in 25 ml SSC buffer diluted 1:10 (v/v) before a second detergent extraction step for 1 h. The nucleoprotein pellet was successively digested with pronase (300 μg/ml) and RNases A and T_1 (250 μg and 1 μg/ml respectively) for 3 h at 37°C with a concomitant dialysis against SSC buffer. Between and after these two enzymatic digestion steps, the mixture, made up to 2 M NaCl, was subjected at 4°C to several cycles of deproteinization with 0.25 volume of chloroform–octanol (3:1, v/v). The DNA contained in the aqueous phase was then precipitated with two volumes of

ethanol. It was finally purified by selective precipitation with 0.54 volume of isopropanol and gradient centrifugation in CsCl as described above.

Purified DNA from calf thymus and *Euglena* cells was hydrolyzed in sealed tubes with 90% formic acid (1 μl/μg DNA) at 175°C, for a period not exceeding 30 min, to avoid deamination of cytosine into uracil, as demonstrated with standard compound hydrolyzed for a longer time. Formic acid was then evaporated under vacuum and the dry residue dissolved in 0.01 M HCl before HPLC analysis.

Chromatographic conditions

All runs were made at room temperature in about 15 min under isocratic conditions. Analytical columns were protected from temperature variations by an isolating polyethylene jacket. Before sample injection, they were equilibrated for 30 min with the selected elution buffer, and for 15 min between two different elution buffers. When not in use, columns were stored in methanol–water (70:30, v/v).

Elution system A was used for the complete separation of the major bases and most of the minor methylated derivatives. The buffer composition was 2.3 mM $NH_4H_2PO_4$ containing 6% (v/v) methanol, pH 4.0. For DNA hydrolyzates, which do not contain 3-mGua and 3-mAde, the pH of the eluent was lowered to 3.4 in order to reduce the elution time of adenine (compare Figs. 2, 3 and 6 with Fig. 1). The standard elution settings were: constant initial flow-rate of 1.0 ml/min for 12.5 min, followed by an increase to 2 ml/min to accelerate elutions of O^6-mGua and N^6-mAde (Fig. 1).

Two distinct elution systems B and C were devised to obtain a baseline resolution of some minor compounds with medium (*ca.* 6–8 min) and low (*ca.* 3–5 min) retention times. The compositions of these buffers were: B, 2.0 mM $NH_4H_2PO_4$, 10% (v/v) methanol, pH 5.0; C, 6.5 mM $NH_4H_2PO_4$, 4% (v/v) methanol, pH 5.0*. The flow-rate was increased to shorten the elution of compounds with long retention times: buffer B, initial flow-rate of 1.0 ml/min increased to 2, 4 and 5 ml/min respectively before elution of 3-mAde, O^6-mGua and N^6-mAde (Fig. 4); buffer C, successive increases in the initial flow-rate of 1 ml/min to 2, 3, 4 and 5 ml/min respectively after elution of Thy and N^2-mGua and before those of O^6-mGua and N^6-mAde (Fig. 7).

Absorption was generally monitored at 254 nm with sensitivity from 2.0 up to 0.01 a.u.f.s. for better detection of some minor modified nucleobases in biological samples.

Peak identification was based on retention times, by comparison with standard solutions analyzed before sample analysis. The internal standard method was also used to determine peak identities. In addition, to confirm the presence of 3-methylcytosine in *Euglena* DNA hydrolyzates, the absorbance ratios at 254 and 280 nm were compared with those for 3-mCyt as a standard base.

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* With system C, elutions were carried out on a new column. When an old column is used the buffer molarity should be lowered to 4.0 mM.
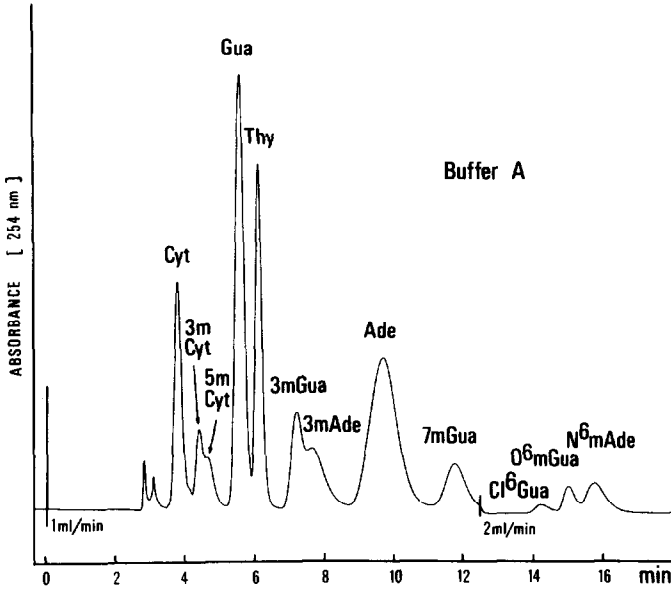
Fig. 1. Separation of a standard mixture of eleven DNA nucleobases. Sample: 200 ng of each major base and 100 ng of each minor base. Column: 300 × 3.9 mm μBondapak phenyl. Eluent: buffer A, 2.3 mM NH$_4$H$_2$PO$_4$, pH 4.0, methanol (6%, v/v). Flow-rates: 1 and 2 ml/min as indicated. Detector: 254 nm, 0.1 a.u.f.s. Temperature: ambient. The peak identified as Cl^{6}Gua corresponds to a residue from the organic synthesis of O^6-mGua.

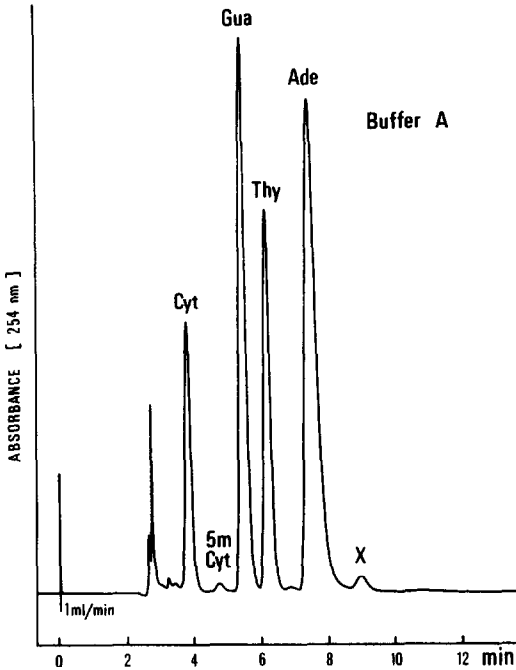

Fig. 2. Elution profile of an acid hydrolyzate of calf thymus DNA. Sample: a base equivalent to 3 μg DNA. Flow-rate: 1 ml/min. Other conditions as in Fig. 1, except that the pH of the elution buffer was adjusted to 3.4.

RESULTS

Separation and identification of standard DNA nucleobases using elution system A;
application to analysis of calf thymus DNA

A chromatogram illustrating the separation of a standard mixture of eleven
nucleobases, using elution buffer A, is shown in Fig. 1. Seven minor methylated bases
along with the four major bases of DNA are sufficiently well separated for correct
individual identification. However, for the pairs 3-mCyt, 5-mCyt and 3-mGua, 3-
mAde, the resolution obtained does not allow accurate quantitation.

Using this elution system, a good separation of the major nucleobases of con-
trol calf thymus DNA, together with the minor base 5-methylcytosine, was obtained
in less than 10 min (Fig. 2). An additional undetermined compound (X) was eluted
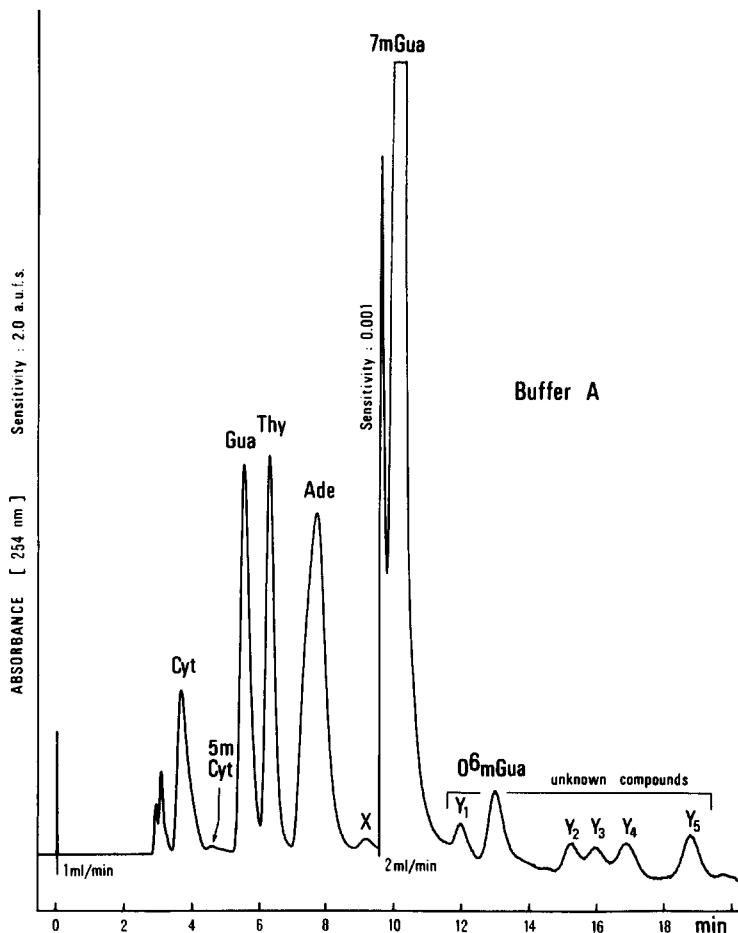just after adenine, at 9.2 min (Fig. 2). It is to be noted that, in the absence of the

Fig. 3. Elution profile of an acid hydrolyzate of calf thymus DNA treated *in vitro* with MNU. Sample:
60 μg of equivalent DNA. Flow-rate: changed from 1 to 2 ml/min at 9.6 min, just before the 7-mGua
elution; at the same time the detector sensitivity was increased from 2.0 to 0.01 a.u.f.s. Other conditions
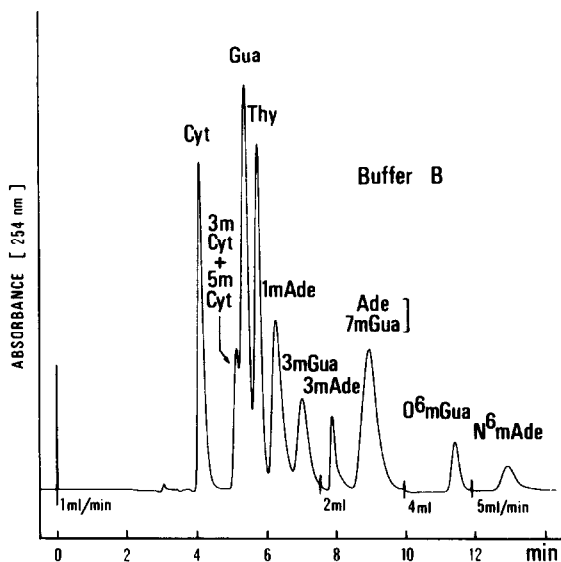as in Fig. 2.

Fig. 4. Separation of a standard mixture of twelve nucleobases. Eluent: buffer B, 2.0 mM NH$_4$H$_2$PO$_4$, pH 5.0, methanol (10%, v/v). Flow-rates: 1, 2, 4 and 5 ml/min as indicated. Other conditions as in Fig. 1.

minor bases 3-mGua and 3-mAde in control calf thymus hydrolyzate, the retention time of adenine, which was eluted in about 10 min with the standard mixture (Fig. 1), was lowered to less than 8 min when the pH of the elution buffer was adjusted to 3.4 (Fig. 2). In contrast, the other major components Cyt, Gua and Thy did not show any difference in their retention time. Such a pH-dependent selective change in the retention time of adenine has already been reported using a reversed-phase C$_{18}$ column[24,25].

Analysis of nucleobases of calf thymus DNA after treatment with N-methyl-N-nitro-sourea

Fig. 3 shows a typical chromatogram of an acid hydrolyzate of calf thymus DNA treated with MNU *in vitro*. As far as the resolution and retention times are concerned, the separation of the unmodified nucleobases with the elution buffer A closely resembles that obtained with control DNA (Fig. 2). However, because of the sample size required to reveal the induced minor derivatives (injection equivalent to 60 μg of MNU-treated DNA), the peaks corresponding to the first eluting nucleo-bases in Fig. 3 are less sharp than those obtained in Fig. 2 for control DNA (injection equivalent to 3 μg DNA). The peaks in series eluted between 9.6 and 20.0 min correspond to the induced derivatives. Among these compounds, 7-mGua and O^6-mGua have been identified by their retention times in comparison with standard compounds, and by the internal standard technique (not shown). The five others, referred to as Y$_1$–Y$_5$, are still undetermined.

The rare minor base, 3 mAde, reported as one of the methylated purines induced by MNU[26], whose elution occurs just before adenine when using the standard mixture (Fig. 1), does not appear in the elution pattern of Fig. 3. The slight asym-

metry present in the ascending part of the adenine peak might be due to cochro-
matography with 3-mAde. Nevertheless, different elution conditions were devised to
obtain a well resolved 3-mAde peak. This was achieved by use of the elution system
B, which not only yielded a good separation of 3-mGua from 3-mAde but also re-
vealed an additional minor base, 1-mAde, between Thy and 3-mGua (Fig. 4). In
return, this better resolution is balanced by the coelution of 3-mCyt with 5-mCyt and
of 7-mGua with Ade.

When calf thymus DNA hydrolyzate (equivalent to 30 µg treated DNA) is
eluted with buffer B, 3-mAde is readily detected (Fig. 5) in accordance with the
standard elution profile (Fig. 4). The identity of 3-mAde was also confirmed by the
internal standard method. Before 3-mAde elution, at 9.7 min, a ten-fold increase of
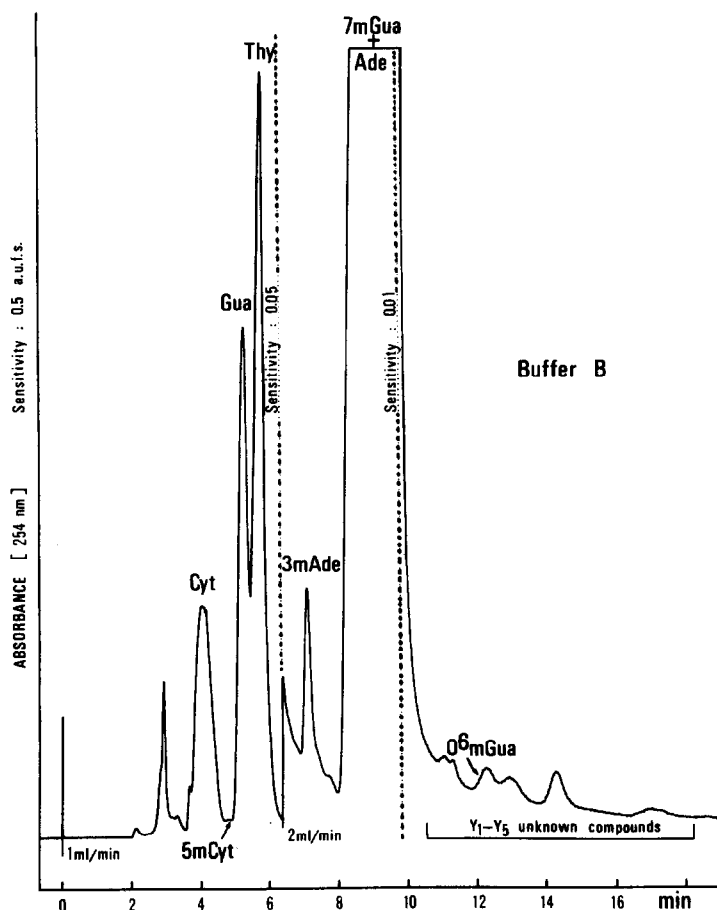the detector sensitivity was necessary to extend the surface area of this minor com-
pound.

Fig. 5. Elution profile of an acid hydrolyzate of calf thymus DNA treated *in vitro* with MNU. Sample:
30 µg of equivalent DNA. Flow-rate: changed from 1 to 2 ml/min at 6.4 min; at the same time the detector
sensitivity was increased from 0.5 to 0.05 to amplify the peak area of 3-mAde; detector sensitivity was
further increased to 0.01 at 9.7 min during the coelution of Ade and 7-mGua for a better detection of
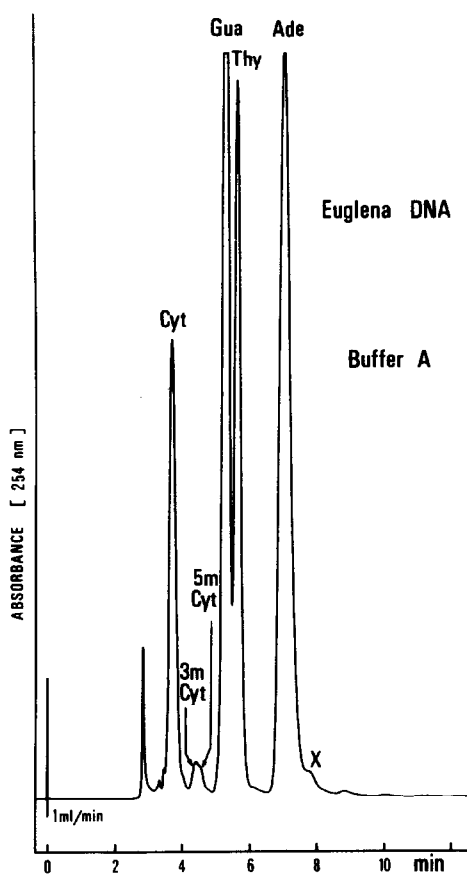O^6-mGua. Other conditions as in Fig. 4.

Fig. 6. Elution profile of an acid hydrolyzate from total cellular DNA of *Euglena gracilis*. Sample: a base equivalent to 7 μg DNA. Flow-rate: 1 ml/min. Other conditions as in Fig. 2.

Analysis of nucleobases of Euglena gracilis DNA

The applicability of our procedure was tested for analysis of the base composition of the total cellular DNA of the unicellular eucaryote *E. gracilis*. The DNA hydrolyzate from *Euglena,* resolved with buffer A (Fig. 6), closely resembles that of calf thymus DNA (Fig. 2), except that a minor compound nearly coelutes with 5-methylcytosine. According to its retention time, and in comparison with the standard mixture separated in Fig. 1, this minor compound may at first sight be identified as 3-methylcytosine. Also, it was shown with a standard mixture that 3-mCyt and 5-mCyt could be fully separated using elution system C (Fig. 7). Moreover, under these conditions, two additional minor bases, 1-mGua and N^2-mGua, could be separated. On the other hand, 3-mGua interferes with 3-mAde as does Ade with 7-mGua.

When the elution system C was applied to *Euglena* DNA hydrolyzates, the two minor compounds were well resolved and have the retention times of 3-mCyt and 5-mCyt (Fig. 8). The identification of the unusual base 3-mCyt in *Euglena* DNA hydrolyzates was further confirmed by the internal standard technique and absor-
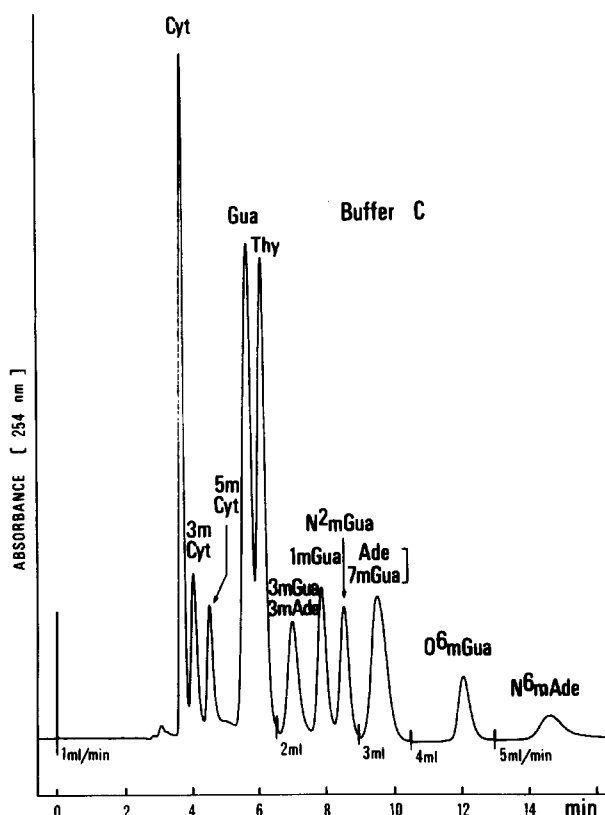
Fig. 7. Separation of a standard mixture of thirteen DNA nucleobases. Eluent: buffer C, 6.5 mM NH$_4$H$_2$PO$_4$, pH 5.0, methanol (4%, v/v). Flow-rates: 1, 2, 3, 4 and 5 ml/min as indicated. Other conditions as in Fig. 1.

bance ratios at 254 and 280 nm. The average values for three analyses were 0.45 ± 0.02 (S.D.) for 3-mCyt in the *Euglena* DNA sample, and 0.47 ± 0.05 for the reference compound; the measurements corresponding to peak areas were obtained with 35–40 ng of DNA in each case.

Reproducibility of retention volume in standard mixtures

In order to examine more easily the reproducibility of the retention behaviour of each base in the different elution systems, we chose to compare the retention volumes. The latter would correspond to the retention times if a uniform flow-rate of 1 ml/min had been maintained during elution. Retention volumes corresponding to an average from 5 to 10 independent analyses are given in Table I.

Standard deviation analysis show that bases with small and medium retention volumes have a reproducible retention behaviour (less than 3% deviation) whatever the elution system, even for two different columns over a period of 15 months. However, retention is less strong with a new column than with an older one. For example, 3-mGua is coeluted with 3-mAde at 6.6 ml on a 1-month old column, while it is partially resolved (7.2 ml) from 3-mAde (7.6 ml) on a 15-months old column (Fig.
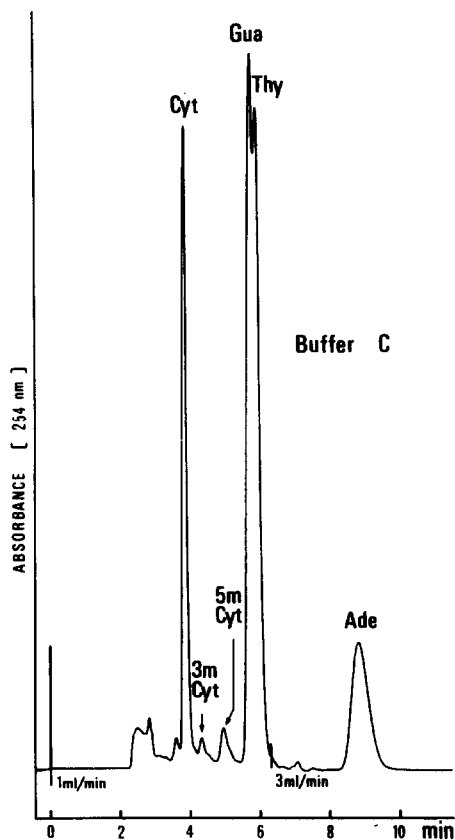
Fig. 8. Elution profile of an acid hydrolyzate from total cellular DNA of *Euglena gracilis*. Sample: 5 μg of equivalent DNA. Flow-rate: 1 ml/min up to 6.3 min, then 3 ml/min. Other conditions as in Fig. 7.

1 and Table I). The accuracy is less good for bases with large retention volumes, especially O^6-mGua and N^6-mAde (deviation 4–22%). Nevertheless, no interferences occur between the retention volumes of these two bases. Finally, the resolution capacity of a column could weaken with time, but can usually be regenerated by extensive washing with methanol.

DISCUSSION

The procedure devised allows simultaneous analysis of the principal purine and pyrimidine bases occurring naturally in DNA or induced by alkylating agents. The main isocratic elution system (A) is able to elute eleven free bases, including the four major ones and seven minor methylated nucleobases. Baseline resolution of bases with low retention times, such as 3-mCyt and 5-mCyt, which are detectable with this elution buffer (A), is obtained by a simple increase of the buffer molarity and a slight diminution of the methanol content (buffer C). The resolution of bases with medium retention times, such as 3-mGua and 3-mAde, is obtained by a small

TABLE I

REPRODUCIBILITY OF RETENTION VOLUMES FOR STANDARD NUCLEOBASES

S.D. = Standard deviation; R.S.D. = relative standard deviation; n = number of analyses.

Bases	Buffer A*		Buffer B*		Buffer C**	
	ml ± S.D. (n = 10)	R.S.D. (%)	ml ± S.D. (n = 10)	R.S.D. (%)	ml ± S.D. (n = 7)	R.S.D. (%)
Cyt	3.7 ± 0.11	1.1	3.8 ± 0.14	1.4	3.7 ± 0.12	1.7
3-mCyt	4.2 ± 0.21	2.1			4.0 ± 0.004	0.06
5-mCyt	4.5 ± 0.10	1.0			4.5 ± 0.053	0.8
3-mCyt, 5-mCyt			4.7 ± 0.15	1.5		
Gua	5.4 ± 0.18	1.8	4.9 ± 0.28	2.8	5.7 ± 0.16	2.3
Thy	5.8 ± 0.23	2.3	5.2 ± 0.23	2.3	6.0 ± 0.14	2.0
1-mAde			5.7 ± 0.23	2.3		
	(n = 5)					
3-mGua***	7.2 ± 0.08	1.6	6.5 ± 0.28	2.8		
3-mAde***	7.6 ± 0.08	1.6	7.4 ± 4.4	4.4		
3-mGua, 3-mAde§	6.6 ± 0.11	2.2			7.4 ± 0.29	4.1
	(n = 10)					
1-mGua					9.1 ± 0.41	5.9
Ade	9.3 ± 0.41	4.1				
7-mGua	10.0 ± 0.46	4.6				
N²-mGua					10.5 ± 0.29	4.1
Ade, 7-mGua			9.4 ± 0.16	1.6	13.2 ± 0.46	6.6
O⁶-mGua	17.2 ± 0.40	4.0	16.3 ± 1.16	11.6	22.2 ± 0.79	11.3
N⁶-mAde	19.1 ± 0.68	6.8	22.5 ± 2.18	21.8	34.3 ± 1.55	22.1

 * Chromatographic conditions described in Figs. 1 and 4, respectively. Elution on two different columns.
 ** Chromatographic conditions as described in Fig. 7. Elution on one new column.
 *** Elution using an old column (15 months).
 § Elution using a new column (1 month).

increase in the concentration of this solvent (buffer B). In both cases, additional rare bases could be separated, such as 1-mAde (B) and 1-mGua and N²-mGua (C).

The question arose as to whether a unique gradient elution system might achieve resolution of all selected nucleobases, including those which were nearly co-eluted. Repetitive assays of gradient elution, using an M 660 programmer (Waters) for two delivery systems, failed to give complete separation of all components. The inertia of continuous changes could explain the difficulties encountered in resolving coeluting bases at several retention times. As could be predicted under our chromatographic conditions, optimum resolution would require a combination of a sudden shift of the buffer molarity with a progressive increase in methanol content.

Since the most frequent modifications induced in DNA by carcinogenic agents occur on purine residues, simplified procedures have been devised to separate adenine and guanine derivatives[2,26], or only the latter[27,28]. Because of the smaller number of compounds involved, these procedures have allowed rapid base analysis in about 15 min, with good resolution. On the other hand, some authors are interested in the

separation of numerous methylated and ethylated purine and pyrimidine derivatives induced in DNA by alkylating agents[29]. Such complex analyses, requiring several appropriate extractions of DNA followed by different HPLC procedures[2,29], have not been related to the complete base composition of DNA, namely to the proportion of 5-methylcytosine.

Indeed, the great majority of papers dealing with DNA base composition are oriented towards the naturally occurring bases and principally to the relative proportion of 5-mCyt[11,15-18], or to the induced methylated derivatives[1-3,19,26-29]. There is no analysis dealing with the naturally occurring nucleobases and at the same time the induced minor ones. The simple procedure described in this paper allows direct and complete analysis of most bases present in a DNA sample.

Detection of 3-mCyt in *Euglena* DNA of normal cells is a delicate question. This minor base has been fortuitously observed, and the elution system C has been devised to obtain its baseline resolution from 5-mCyt. The chromatographic separation of these two bases has previously been described, using cation-exchange resins[30], but the scarce studies relating their co-existence in biological samples only concern tRNA hydrolyzates[10,14,30], and as far as we know, 3-mCyt has never been reported in DNA samples of normal cells. In contrast, mutagenicity was found to correlate with the presence of 3-mCyt formed in DNA[3]. A study is underway to elucidate the problem of this unusual base within *Euglena* DNA.

The compound "X" eluted after adenine in elution system A, first detected in calf thymus DNA hydrolyzates, has since been found in *Euglena* DNA (Fig. 6) and DNA hydrolyzates from hepatocytes. It could either be a normal constituent of DNA or result from degradation in the course of DNA extraction, purification and hydrolysis. Work is now in progress to identify this unknown compound.

Finally, the described procedure proved to be a simple, speedy and inexpensive method of carrying out current analytical work on DNA base composition.

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