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STAPHYLOCOCCAL NUCLEASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF DIAMINOOCTANE-MODIFIED DNA AND ITS BIOTIN AND FLUORESCEIN DERIVATIVES

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SUMMARY

DNA was subjected to bisulfite-catalyzed transamination at the N^4 sites of its cytosine residues with 1,8-diaminooctane (DAO). The product, DNA–DAO, was non-specifically degraded with a cloned staphylococcal nuclease (Nase). The products from the Nase digestion were determined by high-performance liquid chromatography (HPLC) to define the extent of reaction with DAO. Mostly, nucleoside 3'-monophosphates were obtained, along with four Nase-resistant dinucleotides: TpdGp, dApdGp, TpdCp–DAO and dApdCp–DAO. The addition of spleen phosphodiesterase II gave a faster hydrolysis and left no dinucleotides. A mixture of Nase, snake venom phosphodiesterase I and alkaline phosphatase gave a fast hydrolysis as well but two dinucleotides, apparently TpdC–DAO and dApdC–DAO, persisted. Further modification of the diaminooctyl side chains with fluorescein isothiocyanate or biotin N-hydroxysuccinimide ester was similarly investigated. Interestingly, derivatization of the DAO side chain with biotin eliminates the resistance of TpdCp–DAO and dApdCp–DAO to Nase digestion. This work provides some guidelines for using Nase, alone or with other nucleases, along with HPLC, for characterizing alkyldiamine DNA products, and should be similarly useful for studying other modifications of DNA.

INTRODUCTION

Chemically modified polynucleotides are used to detect or characterize the biomolecules with which they interact. For example, DNA hybridization probes are used to detect trace quantities of DNA associated with infectious disease[1]. These probes contain a sensitive signal group such as radioactive phosphate, an enzyme or biotin.

To facilitate the attachment of multiple signal groups and other reagents to DNA, bisulfite-catalyzed transamination at the N^4-cytosine sites with alkyldiamines[2] is used. The resulting aminoalkyl-nucleic acid possesses reactive primary amino groups, suitable for further modification with a variety of reagents. Using this ap-

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proach, nucleic acids have been labeled with biotin[3,4], alkaline phosphatase[5], fluorescent dyes[6] and protein-affinity labeling reagents[2,7]. Also, the S-peptide and S-protein fragments of ribonuclease were coupled to diaminooctane-modified polycytidylic acid[8].

In the present work, we evaluated a convenient enzyme high-performance liquid chromatographic (HPLC) method for characterizing diaminoalkane-derivatized DNA. Further modification of the diaminooctane (DAO) side chains with biotin-N-hydroxysuccinimide ester or fluorescein isothiocyanate was also monitored. The primary enzyme is staphylococcal nuclease (Nase) which degrades DNA to nucleoside 3′-monophosphates with little base specificity[9]. These nucleotides can then be resolved and quantified by reversed-phase HPLC. Currently, nucleases such as snake venom phosphodiesterase[10], DNase 1 or nuclease P1[11] are commonly used, either alone or in combination, for the non-specific degradation of DNA. We were particularly interested in evaluating the usefulness of Nase, both alone and in combination with other enzymes, since it has been cloned[12], and large quantities of it can be easily obtained in a highly purified form[13].

EXPERIMENTAL

Chemicals and reagents

Wild-type staphylococcal nuclease (E.C. 3.1.4.7) was isolated in our laboratory by a previously described procedure[13]. An engineered strain of *Escherichia coli* carrying the expression plasmid pF0G405[14], was utilized. This bacterium was kindly provided by David Shortle (John Hopkins University, Baltimore, MD, U.S.A.). Biotin N-hydroxysuccinimide ester was prepared in our laboratory according to a previously described procedure[15].

Highly polymerized calf thymus DNA, *E. coli* alkaline phosphatase type III (E.C. 3.1.3.1), calcium chloride, magnesium chloride and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma (St. Louis, MO, U.S.A.); snake venom phosphodiesterase I (E.C. 3.1.4.1) and bovine spleen phosphodiesterase II (E.C. 3.1.4.18) from Worthington (Freehold, NJ, U.S.A.); sodium sulfite, sodium metabisulfite and HPLC-grade monobasic potassium phosphate and solvents from Fisher (Bedford, MA, U.S.A.); silylation grade dimethylsulfoxide (DMSO) from Pierce (Rockford, IL, U.S.A.); fluorescein isothiocyanate from Aldrich (Milwaukee, WI, U.S.A.); *d*-biotin from Boehringer Mannheim (Indianapolis, IN, U.S.A.); and PD-10 columns from Pharmacia (Piscataway, NJ, U.S.A.).

High-performance liquid chromatography

The equipment consisted of a Series 4 liquid chromatograph from Perkin-Elmer (Norwalk, CT, U.S.A.), a Varian 9060 Polychrom diode array detector (Walnut Creek, CA, U.S.A.), a FS970 fluorescence detector from Schoeffel (Westwood, NJ, U.S.A.), and a CI-10B integrator from LDC (Bloomfield, CT, U.S.A.). The fluorescence and diode array detectors were connected in series.

HPLC was carried out on a Brownlee 22 × 0.46 cm RP-8, Spheri-5 cartridge column purchased from LDC. Solvent A was 0.1 *M* potassium dihydrogenphosphate (pH 4.6 inherently), and solvent B was acetonitrile. The nucleoside 3′-monophosphates (Nase digests) were analyzed with a 30-min gradient from 0 to 15% B. For

the nucleosides (Nase, alkaline phosphatase, and phosphodiesterase I digests) and the free bases (formic acid hydrolysates) the gradients were from 5 to 40% B in 30 and 15 min, respectively. The Nase digests of biotin and fluorescein-labeled DNA–DAO were analyzed with an 8-min gradient from 0 to 4% B, followed by a 22-min gradient from 4 to 35% and from 4 to 45% B, respectively.

Enzymatic digestion

For exhaustive digestion with Nase, DNA or DNA–DAO and its derivatives were dissolved at a concentration of 0.5–2.0 mg/ml in 0.025 *M* Tris–HCl buffer (pH 8.8), containing 10 m*M* calcium chloride. To this solution were added 500 μg (1000 units) of enzyme per milliliter, and the digestion was allowed to proceed at 37°C for at least 48 h. To obtain a partially digested sample, the Nase was decreased to 50 μg/ml and the incubation time to 24 h.

Digestion with Nase and spleen phosphodiesterase II was carried out in two steps: 1 h after the addition of Nase (200 μg/ml), the solution was combined with an equal volume of 0.1 *M* sodium acetate buffer, (pH 5.5), containing phosphodiesterase II (1 unit/ml) and incubated for 2 h at 37°C.

Digestion with Nase (130 μg/ml), alkaline phosphatase (3 units/ml) and phosphodiesterase I (3 units/ml) was carried out at 37°C in 0.05 *M* Tris (pH 8.6), containing 20 m*M* calcium chloride, 15 m*M* magnesium chloride and DNA or DNA–DAO (1.0 mg/ml).

DNA–DAO

The calf thymus DNA was denatured by dissolving 115 mg in 20 ml of water and heating the solution in a boiling water bath for 30 min. After rapidly cooling it in an ice-bath, the solution was sonicated at 0°C for 40 min, using a Heat Systems (Melville, NY, U.S.A.) sonication probe and a Branson Sonic Power (Danbury, CT, U.S.A.) source (setting 5). This solution was added to 30 ml of water, containing 3.15 g sodium sulfite, 7.15 g sodium metabisulfite, and 7.2 g of DAO. The pH of the solution was adjusted to 7.0 with concentrated hydrochloric acid prior to the addition of DNA. The final sodium hydrogensulfite and DAO concentrations were 2 *M* and 1 *M*, respectively.

The mixture was clarified by centrifugation (3000 *g*) and then stirred at 60°C for four days. Any precipitate that formed was removed by centrifugation, and the mixture was purified in 25-ml portions on a 320-ml BioRad (Richmond, CA, U.S.A.) P-4 gel filtration column (2.6 × 60 cm bed) with 0.02 *M* sodium chloride, containing 0.2 m*M* EDTA (pH 8). The DNA–DAO was eluted at the void volume and was dialyzed extensively against water and then lyophilized (72% yield, based on absorbance at 260 nm).

Nucleobase composition of Nase-resistant peaks

DNA or DNA–DAO digests were chromatographed as described above, except that 0.1 *M* acetic acid, adjusted to pH 4.5 with triethylamine was used in place of potassium phosphate. Individual, unidentified peaks were collected, and the mobile phase was evaporated under vacuum. The residue was redissolved in 88% formic acid and hydrolyzed to yield the free bases by heating under nitrogen at 150°C for 2 h. The formic acid was evaporated and the residue was reconstituted in 0.1 *M* potassium phosphate buffer (pH 4.5) prior to analysis by HPLC.

Sequencing of Nase-resistant peaks

Following HPLC isolation as just described, Nase-resistant peaks 11 and 12 were redissolved in 0.4 ml of 0.05 *M* Tris–15 m*M* magnesium chloride buffer (pH 8.5) and 0.5 unit of alkaline phosphatase was added. The solutions were incubated for 2 h at 37°C and separated by HPLC. The peak fractions were collected, and the mobile phase was evaporated under vacuum. After redissolving the residues in 100 μl of 0.1 *M* sodium acetate buffer (pH 5.5), 0.3 unit of phosphodiesterase II was added. The solutions were incubated for 2 h at 37°C, and then analyzed by HPLC.

Biotin-labeled DNA–DAO

DNA–DAO (6.0 mg, containing 2.6 μmol cytosine–DAO residues) was dissolved in 1 ml of 0.1 *M* potassium phosphate buffer (pH 7.6)–1 m*M* EDTA. Biotin N-hydroxysuccinimide ester (13.7 mg, 40 μmol) dissolved in 0.5 ml of DMSO was added. After 2.5 h at room temperature, the mixture was purified by gel chromatography on a PD-10 column, using 0.025 *M* Tris buffer (pH 8.8)–1.0 m*M* EDTA for elution. The high-molecular-weight fraction, which contained the biotinylated DNA–DAO, was stored at 4°C.

Fluorescein-labeled DNA–DAO

DNA–DAO (1.6 mg; 0.7 μmol of cytosine–DAO residues) was dissolved in 1.0 ml of 0.2 *M* potassium phosphate–1 m*M* EDTA (pH 9.5). The solution was cooled in an ice-bath, and 1.0 ml of DMSO, containing 3.0 mg (7.7 μmol) of fluorescein isothiocyanate, was added. After 16 h on a rocking plate at room temperature, the DNA–DAO–fluorescein was purified twice on PD-10 columns with the same buffer as above and a third time with 0.025 *M* Tris–1 m*M* EDTA (pH 8.8) as eluent. The high-molecular-weight fraction, containing the yellow DNA–DAO–fluorescein, was stored at 4°C.

RESULTS AND DISCUSSION

Based on the work of others[2], we followed the scheme shown in Fig. 1 for preparing DNA transaminated at its N^4-cytosine sites with DAO, to yield DNA–DAO. We labeled the DAO side chains further with either biotin or fluorescein. Calf thymus DNA was used because of its commercial availability and low cost. To enhance the solubility of the DNA–DAO and its biotin and fluorescein derivatives, we sonicated the DNA prior to these modifications. This reduced the average chain length of the DNA, as demonstrated in Fig. 2.

We examined the relative usefulness of several combinations of enzymes along with variations in conditions to hydrolyze these DNA derivatives to mononucleotides or mononucleosides for analysis by HPLC. The identities and designations of these hydrolysis products, including some intermediate dinucleotides, are presented in Table I. Peaks were identified throughout this work by the simultaneous injection of authentic standards and by their diode array UV spectra (Fig. 3).

Exhaustive Nase digestion

The chromatographic separation of the four major nucleotide 3′-monophosphates obtained from the exhaustive digestion of DNA with Nase is shown in Fig.

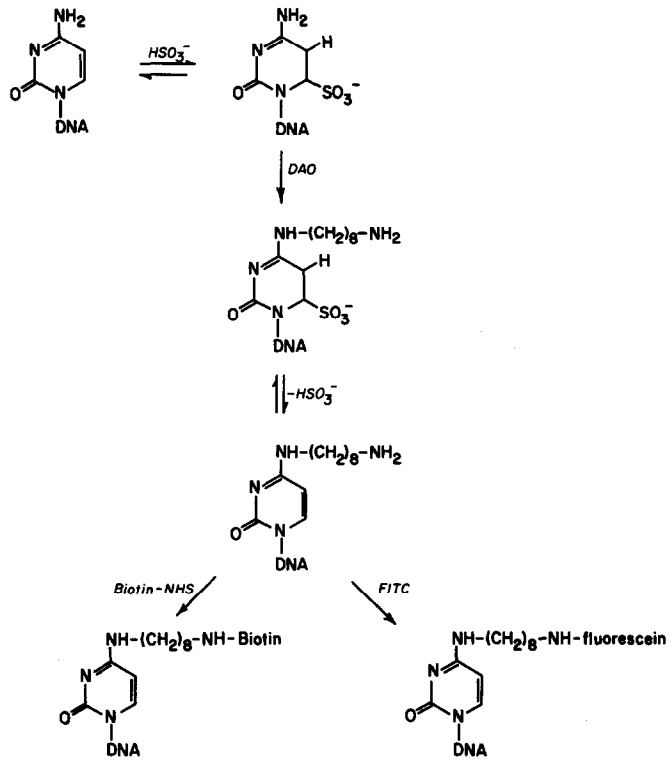

Fig. 1. Scheme for the preparation of DNA–DAO and subsequent derivatization with biotin or fluorescein.

4A. Peak 3 is 5-methylcytidine 3′-monophosphate, which in most mammalian species represents about 1 mol-% of the total nucleotide residues in DNA[16]. HPLC after partial (Fig. 4B) and exhaustive (Fig. 4C) digestion of DNA–DAO with Nase showed that 94% of the cytosine residues had been modified with diaminooctane. The extent of this modification is conveniently regulated by time and temperature[17]. Approximately 5% of the cytosine residues are converted to dUp (peak 2), due to deamination of the bisulfite adduct by reaction with water. This side reaction is minimized at or above pH 7[2]. The remaining cytosine residues can be accounted for as dCp–DAO (peak 10) and two later-eluted peaks (11 and 12). The UV spectrum (Fig. 3) and extinction coefficient at the wavelength of detection of dCp–DAO are essentially unchanged compared to that of dCp[17]. The variations in retention times shown in Fig. 4 are typical of the variations observed with chromatograms run on different days. Column temperature was not regulated and changes in the ambient temperature, along with aging of the column and the precision of the mobile phase composition, probably account for this variation. Nevertheless, the elution order of the nucleotides was always the same and the retention times were consistent on a given day.

Peaks 7 and 9 (Fig. 4B) are dinucleotides, composed of thymine and guanine (peak 7) and adenine and guanine (peak 9) residues. We established this by hydrolyzing the collected peak fractions with formic acid to the bases which were then

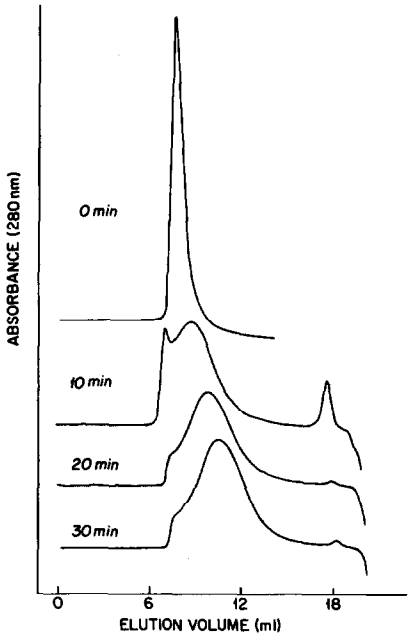

Fig. 2. Size-exclusion chromatography of sonicated DNA. Calf thymus DNA was sonicated for the period indicated and analyzed on a Pharmacia Superose-12 HR 10/30 high-performance gel filtration column. The eluent buffer was 0.05 *M* potassium phosphate, (pH 7), containing 6 *M* guanidine hydrochloride.

determined by HPLC. Individual bases were identified by the simultaneous injection of authentic standards. The molar ratios of the bases were determined from the ratio of peak areas after correcting for differences in molar extinction coefficients[18]. The sequences of these dinucleotides are clear from the work of Mikulski *et al.*[19], who established that TpdGp and dApdGp are particularly resistant to Nase digestion. However, after more exhaustive digestion, the amount of these compounds becomes insignificant (Fig. 4A and C).

Peaks 11 and 12, representing 9% of the total peak areas, were resistant to Nase digestion even at the highest enzyme concentration and an incubation period exceeding 72 h (Fig. 4C). To identify these peaks, they were collected and treated, first, with alkaline phosphatase to remove the 3′-terminal phosphate, followed by phosphodiesterase II. The products of this digestion were Tp and dC–DAO in a 1:1 ratio (from peak 11) and dAp and dC–DAO (from peak 12), also in a 1:1 molar ratio. Thus, peaks 11 and 12 are TpdCp–DAO and dApdCp–DAO, respectively. This assignment was also consistent with the base composition determined by formic acid–HPLC analysis (see above) of peaks 11 and 12. Incubation at elevated temperatures (50–60°C) or high salt concentration (0.8 *M* sodium chloride) had little or no influence on the rate of disappearance of these dinucleotides.

Nase and phosphodiesterase II

The incubation times for thorough hydrolysis of DNA–DAO can be dramatically reduced by including a second exonuclease along with Nase. DNA–DAO was

TABLE I

LIST OF ABBREVIATIONS FOR NUCLEIC ACID FRAGMENTS

No.	*Compound*	*Abbreviation*
1	2′-Deoxycytidine 3′-monophosphate	dCp
2	2′-Deoxyuridine 3′-monophosphate	dUp
3	2′-Deoxy-5-methylcytosine 3′-monophosphate	d5meCp
4	2′-Deoxyguanosine 3′-monophosphate	dGp
5	Thymidine 3′-monophosphate	Tp
6	Unknown	–
7	Thymidyl-(3′→5′)-2′-deoxyguanosine monophosphate	TpdGp
8	2′-Deoxyadenosine 3′monophosphate	dAp
9	2′-Deoxyadenosyl-(3′→5′)-2′-deoxyguanosine monophosphate	dApdGp
10	N^4-(8-Aminooctyl)-2′-deoxycytidine 3′-monophosphate	dCp–DAO
11	Thymidyl-(3′→5′)-N^4-(8-aminooctyl)-2′-deoxycytidine monophosphate	TpdCp–DAO
12	2′-Deoxyadenyl-(3′→5′)-N^4-(8-aminooctyl)-2′-deoxycytidine monophospohate	dApdCp–DAO
13	2′-Deoxycytidine	dC
14	2′-Deoxyuridine	dU
15	2′-Deoxy-5-methylcytidine	d5MeC
16	2′-Deoxyguanosine	dG
17	Thymidine	T
18	2′-Deoxyadenine	dA
19	N^4-(8-Aminooctyl)-2′-deoxycytidine	dC–DAO
20	Thymidyl-(3′→5′)-N^4-(8-aminooctyl)-2′-deoxycytidine	TpdC–DAO
21	2′-Deoxyadenyl-(3′→5′)-N^4-(8-aminooctyl)-2′-deoxycytidine	dApC–DAO
22	N^4-(8-Biotinylaminooctyl)-2′-deoxycytidine 3′-monophosphate	dCp–DAO–biotin
23	Unknown	–
24	N^4-(8-Fluoresceinylaminooctyl)-2′-deoxycytidine 3′-monophosphate	dCp–DAO–fluorescein

incubated for 1 h with Nase, followed by 2 h incubation with phosphodiesterase II[20]. The procedure is best carried out in two steps, because the optimum pH ranges of Nase (pH 8–10) and phosphodiesterase II (pH 5–6) are different. The hydrolysis is essentially complete within this 3-h period, and yields 3′-monophosphates, as shown in Fig. 4D. As seen, the Nase-resistant dinucleotides 11 and 12 are completely digested.

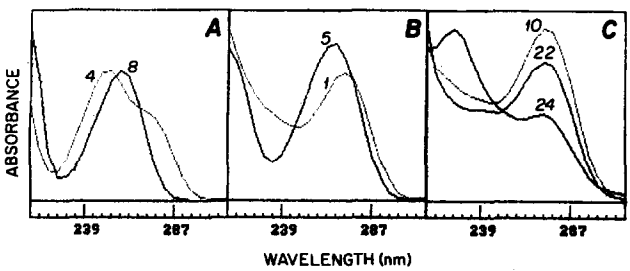

Fig. 3. Diode array spectra of HPLC peaks listed in Table I.

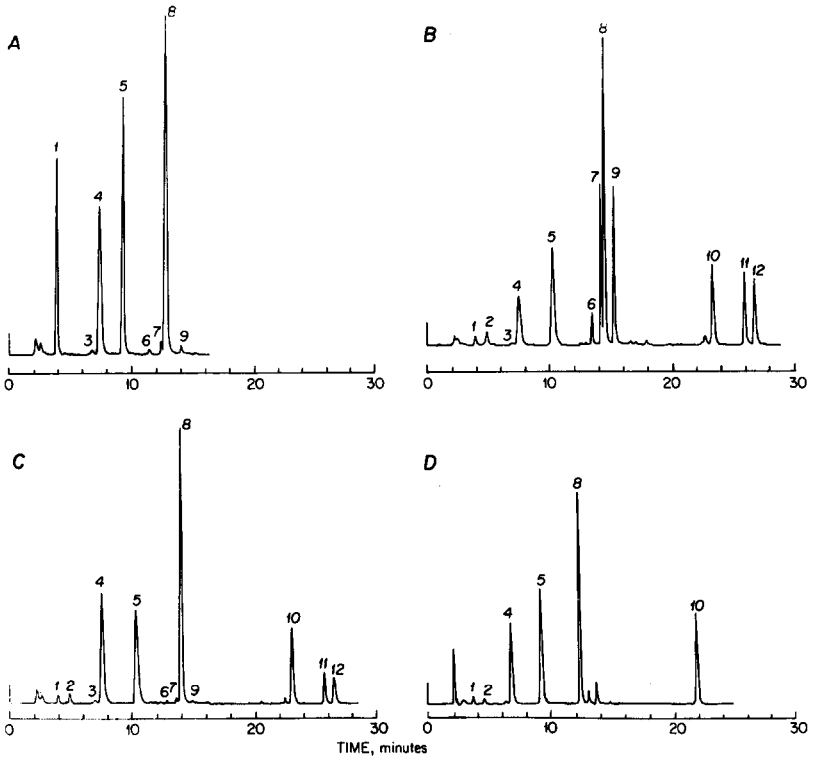

Fig. 4. HPLC chromatograms of nucleoside-3′-monophosphates after enzymatic hydrolysis. (A) Exhaustive digestion of DNA with Nase. (B) Partial and (C) exhaustive digestion of DNA–DAO with Nase. (D) Digestion of DNA–DAO with Nase and phosphodiesterase II. Detection was at 263 nm (0.1–0.5 a.u.f.s.).

Nase, alkaline phosphatase and phosphodiesterase I

We also used a mixture of Nase, alkaline phosphatase and phosphodiesterase I[21] to digest DNA and DNA–DAO. Phosphodiesterase I removes 5′-mononucleotides from 3′-hydroxy terminated oligonucleotides. Thus, alkaline phosphatase was included to remove the terminal 3′-phosphates generated by Nase. Judging from HPLC of the resulting mixture of nucleosides (Fig. 5), the digestion was nearly complete after 1 h with the exception of peaks 20 and 21 (Fig. 5B). Based on their UV spectra (data not shown), these correspond to dinucleotides 11 and 12, and they are just as resistant to the three-enzyme mixture as they are to Nase alone.

Biotin and fluorescein derivatives of DNA–DAO

DNA–DAO was further derivatized with an excess of biotin N-hydroxysuccinimide ester or fluorescein isothiocyanate. The Nase digests are presented in Fig. 6. As seen, the cytosine–DAO residues are completely modified in both cases, and the dCp–DAO is quantitatively converted to the biotin derivative which appears as a single late-eluted peak (Fig. 6A, peak 22). The UV spectrum of this peak (Fig. 3, spectrum 22) is similar to that of dCp–DAO. Interestingly, masking of the positively charged diaminooctyl side chain with biotin eliminates the resistance to Nase diges-

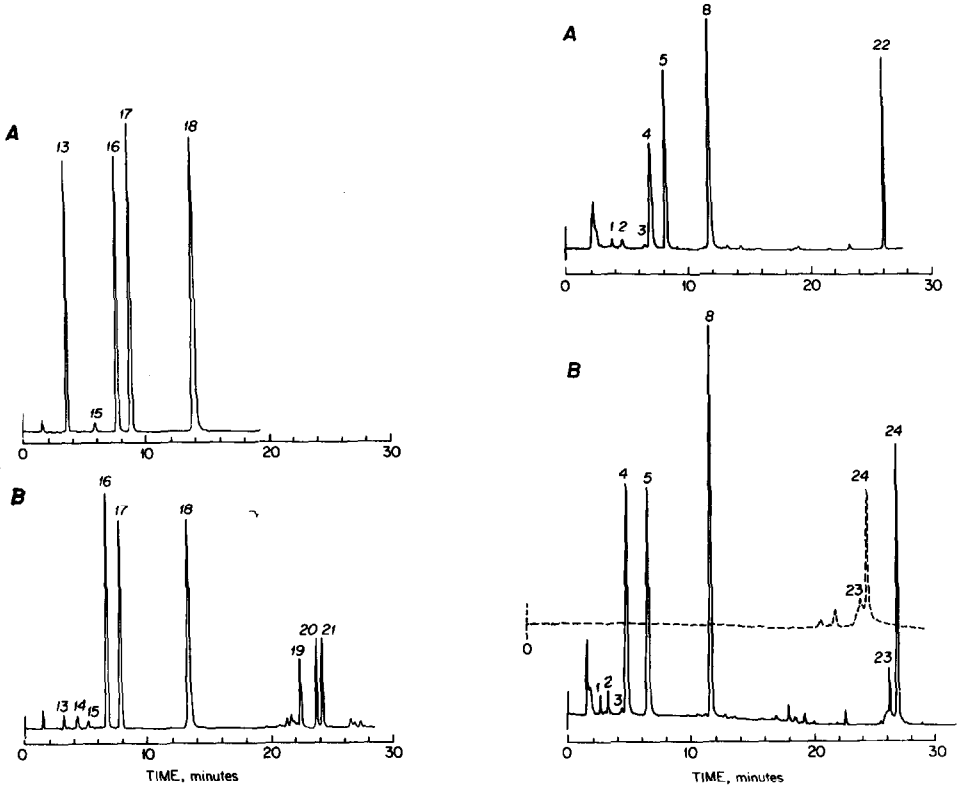

Fig. 5. HPLC chromatograms of nucleosides obtained from the hydrolysis of DNA (A) or DNA–DAO (B) with a mixture of Nase, alkaline phosphatase, and phosphodiesterase I. Detection was at 263 nm (0.1 a.u.f.s.).

Fig. 6. HPLC chromatograms of nucleoside 3'-monophosphates from the Nase hydrolysis of (A) biotin or (B) fluorescein-labeled DNA–DAO. UV detection (——) was at 263 nm; fluorescence detection (– – –) was at 520 nm (excitation at 480 nm).

tion previously observed with fractions 11 and 12. The fluorescein derivative of dCp–DAO (Fig. 6B, peak 24) retains the UV maximum at 270 nm, characteristic of cytosine and its derivatives (Fig. 3, spectrum 24), and also is appropriately fluorescent (Fig. 6B, fluorescence detection). We did not investigate the identity of the minor fluorescent and UV-absorbing compound (peak 23). Given this incomplete understanding of the latter chromatogram, the effect of fluorescein derivatization on the Nase resistance of dinucleotides 11 and 12 remains to be determined.

Other applications of Nase/HPLC characterization

Nase digestion, followed by HPLC analysis is a suitable technique for the study of modified DNA in general, largely because it combines the lack of base specificity of this enzyme with the separating power of HPLC. The methodology presented here could facilitate, *e.g.*, the study of DNA adducts. The latter are modifications of DNA, arising from exposure to injurious chemicals or conditions[22]. Minute damage of this kind needs to be detected. Cloned Nase is potentially useful for this purpose. The

use of a highly purified preparation of Nase for the hydrolysis of DNA could help to minimize the introduction of contaminants, including related enzymes, into the sample.

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