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SEPARATION OF PYRIMIDINE DEOXYRIBOOLIGONUCLEOTIDES ACCORDING TO LENGTH AND COMPOSITION USING THIN-LAYER CHROMATOGRAPHY ON DEAE-CELLULOSE

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SUMMARY

A procedure has been developed for the fractionation of pyrimidine deoxyribooligonucleotides, $Py_n P_{n+1}$, according to length and composition using ascending thin-layer chromatography on DEAE-cellulose. The separation of oligonucleotides according to length ($n = 1–7$) into individual isopliths* was carried out using a linear 0–0.35 *M* sodium chloride gradient in 0.01 *M* sodium acetate buffer of pH 5.1–5.3 and 5 *M* urea. Then the oligonucleotides of individual isopliths were separated according to composition using the same gradient of sodium chloride in 0.01 *M* sodium acetate or sodium citrate buffer of pH 3.2. The procedure can be used for rapid fractionation (2–3 h) and serial determination of the frequencies of pyrimidine sequences differing in length and composition in DNAs of various origin. The procedure can be used for both preparative and analytical applications. It was employed to study the distribution of 5-methylcytosine in pyrimidine isopliths of some DNAs.

INTRODUCTION

Reliable and simple procedures for the fractionation of deoxyribooligonucleotides are needed in order to study the primary structure of DNA and, in particular, to determine the nature and the frequencies of pyrimidine isopliths* differing in length and composition, to study the character of methylation of pyrimidine bases in various DNAs, and so on. Up to now, the method most extensively used for these purposes was the chromatography of mixtures of the respective oligonucleotides on ion-exchange columns[1–9]. However, column chromatography does not offer all of the possibilities of thin-layer chromatography (TLC). In particular, column chromatography so far cannot provide for the rapid simultaneous separation of oligonucleotides in a large number of samples (DNA hydrolyzates) under identical conditions. This can be accomplished by means of TLC or paper chromatography.

In 1967, we developed a procedure for the systematic fractionation of $Py_n P_{n+1}$ deoxyribooligonucleotides according to length and composition[2] by means of sequential chromatography of the respective DNA hydrolyzates on DEAE-Sephadex columns in 7 *M* urea using a linear concentration gradient of sodium chloride, first

* Isopliths: oligonucleotides with an equal number of nucleotide residues.

at pH 7.0 and then at pH 3.0. A similar but somewhat modified fractionation scheme was used in this study of pyrimidine deoxyribooligonucleotides by TLC on DEAE-cellulose.

We have developed a procedure for the separation of pyrimidine $Py_n P_{n+1}$ clusters ($n = 1$–7) according to length and composition using a linear sodium chloride concentration gradient. This procedure was applied to a study of the frequencies of pyrimidine clusters and distribution of minor bases (5-methylcytosine) in various pyrimidine sequences of some DNAs.

EXPERIMENTAL

Isolation of DNA, its hydrolysis to pyrimidine sequences and preparation of the hydrolyzate for chromatography

DNA preparations were obtained from calf thymus and beef hearts according to Marmur[10] and Kirby[11] and freed from RNA by means of treatment with RNAase[10] or alkali (0.5 *N* sodium hydroxide solution at 30° for 17 h). The air-dried DNA was placed in a glass tube, mixed with 66% formic acid containing 2% of freshly recrystallized diphenylamine (1 ml of the mixture per milligram of DNA) and hydrolyzed for 18 h at 30° to pyrimidine sequences according to Burton and Petersen[12]. The hydrolyzate was diluted with water to double the volume and repeatedly extracted with diethyl ether. The final pH of the hydrolyzate after ether extraction was 5.0 and the DNA concentration was about 2–5 mg/ml. The hydrolyzate was then subjected to chromatography or, if required, was stored at −10°.

Preparation of plates with DEAE-cellulose and application of the DNA hydrolyzates

DEAE-cellulose (Reanal, Budapest, Hungary) or DEAE-cellulose SS (Serva, Heidelberg, G.F.R.) was suspended in distilled water (50 g/l) for 1 h and then allowed to stand for 10 min so that large particles could precipitate, whereas a fraction of small particles was obtained from the supernatant by filtration through a paper filter in a büchner funnel. The fraction obtained from the exchanger was mixed with a ten-fold volume of 0.5 *N* potassium hydroxide solution for 10 min using a magnetic stirrer, washed in a büchner funnel with water to neutral pH, suspended for 15 min in 0.5 *N* hydrochloric acid and again washed with water. The washed DEAE-cellulose was suspended in distilled water (2 g of dry cellulose per 20 ml) and applied uniformly over horizontal degreased 5 × 24-cm glass plates (20 ml of the suspension per plate). After being dried at room temperature, the plates were ready for chromatography and were stacked layer-to-layer. If necessary, they could be stored at room temperature for 3 months.

The DNA hydrolyzate was applied to the plate as strips 3–4 cm from the lower edge of the plate and 0.5 cm from the sides (100–700 μg of total DNA material per centimetre length of the starting line). For quantitative determination of oligonucleotides, half of the starting line was left free, so that a free band was left on the chromatogram which could be used later for obtaining control eluates. The hydrolyzate was applied under a flow of warm air in order to dry the starting line. The width of the moist strip of the hydrolyzate did not exceed 0.3–0.5 cm; in this case, the pH of the hydrolyzate being neutral and in the absence of any electrolytes (salts), all of the phosphate-containing pyrimidine clusters are firmly bound with the ion

exchanger as a very narrow band 1–2 mm wide. This facilitates the effective separation of oligonucleotides according to length or composition.

Chromatography of oligonucleotides

The plates containing the DNA hydrolyzate were introduced into a round glass chromatographic chamber so that the layer of ion exchanger faced the wall. The chamber was mounted on a magnetic stirrer unit and covered with a plastic cap. Vapour from a flask containing boiling water passed into the chamber through a glass tube (Fig. 1). The chamber was "saturated" with vapour until the condensation on the walls became dense. After 5–10 min, a certain volume of the starting buffer was passed into the chamber from a funnel through the hole in the cap. For fractionation of the oligonucleotides according to length, that is, into individual isopliths, we used 0.01 *M* sodium acetate buffer, pH 5.1–5.3, in 5 *M* urea as the starting buffer. The urea was previously purified by passing a 7 *M* solution sequentially through Dowex 1-X8, Dowex 50-X8 (100–200 mesh) and DEAE-cellulose columns. For separation of the oligonucleotides according to base composition, we used as the starting buffer 0.01 *M* sodium acetate or sodium citrate buffer, pH 3.2, without urea.

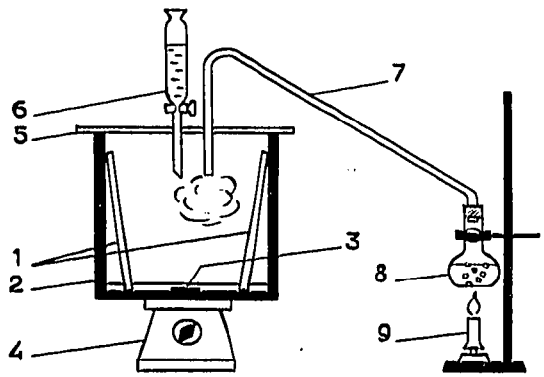

Fig. 1. Device for the thin-layer chromatography of deoxyribooligonucleotides on DEAE-cellulose plates. 1 = Plates with DEAE-cellulose layers and samples applied; 2 = round glass chamber; 3 = magnet; 4 = magnetic stirrer; 5 = cap; 6 = separating funnel; 7 = connecting glass tube; 8 = flask with boiling water; 9 = burner.

When the front of the starting buffer was by 1–2 cm above the application line, the magnetic stirrer was switched on and the elution buffer was fed into the chamber from the funnel. A solution of sodium chloride was used as an elution electrolyte. The eluent feed rate could be controlled by means of a micro-pump.

During the chromatography, as the walls of the chamber were drying, the chamber was filled with vapour a further two or three times. The maximum sodium chloride concentration (0.35 *M*) in the chamber was reached when the solvent front had travelled 17–20 cm. The duration of chromatography was 2–3 h.

After the chromatography, the plates were removed from the chamber and dried in a horizontal position under an air flow at room temperature. Then the positions of the UV-absorbing bands (spots) were marked on the chromatograms.

These bands of the separated oligonucleotides and corresponding zones of the blank area were cut from the plates, placed in test-tubes and eluted with 2–4 ml of 0.5 *N* hydrochloric acid for 5 h at room temperature. The concentration of the oligonucleotides was determined spectrophotometrically[2]: μmoles/ml $= 0.102 \cdot (A_{267.5} - A_{320})$.

RESULTS

Separation of oligonucleotides according to length

As a result of chromatography of the DNA hydrolyzates at pH 5.1–5.3, pyrimidine fragments were clearly separated into mono-, di-, tri-, tetra-, penta-, hexa- and heptanucleotides (Fig. 2). Under these conditions, purines move more or less with the solvent front and can be readily separated from the pyrimidine mono- and oligonucleotides (Fig. 2).

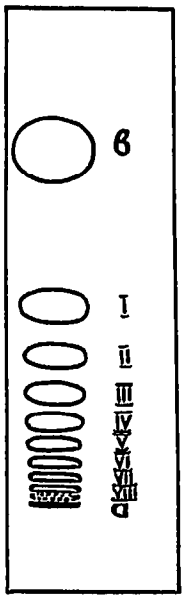

Fig. 2. Separation of $Py_n\ P_{n+1}$ pyrimidine isopliths on DEAE-cellulose plates. a, Starting line; b, purine bases. I = mono-; II = di-; III = tri-; IV = tetra-; V = penta-; VI = hexa-; VII = hepta-; VIII = octapyrimidine isopliths.

The spectra of individual pyrimidine isopliths were similar to those of the corresponding pyrimidine isopliths isolated from calf thymus DNA by column chromatography on DEAE-Sephadex[2].

The frequencies of pyrimidine isopliths isolated from calf thymus DNA by means of thin-layer chromatography on DEAE-cellulose (Table I) are in accordance with reported data. Our results are especially close to the data of Hall and Sinsheimer[13] on the fractionation of $Py_n\ P_{n-1}$ oligonucleotides from calf thymus DNA on DEAE-Sephadex columns.

The first four isopliths (I–IV) can also be readily separated under the condi-

TABLE I

FREQUENCIES OF THE PYRIMIDINE ISOPLITHS IN THE CALF THYMUS DNA

Isoplith	*Oligonucleotide content (mole %)*				
	Experimental data, $\bar{X} \pm \sigma$	*Data reported in literature**			
		Ref. 13	*Ref. 2*	*Ref. 12*	*Ref. 14*
I	10.55 ± 0.15	10.55	10.81	10.15	11.62
II	10.30 ± 0.12	10.00	9.81	9.80	8.60
III	8.22 ± 0.18	7.70	7.55	7.41	7.61
IV	6.42 ± 0.10	6.40	5.82	5.77	6.31
V	4.51 ± 0.20	4.10	3.85	3.81	4.57
VI	4.02 ± 0.22	3.35	3.12	2.61	3.31
VII	2.98 ± 0.12	2.35	2.23	1.23	2.44
⩾VIII	3.00 ± 0.10	4.63	6.33	—	5.52

* Ref. 13: separation of $Py_n\ P_{n-1}$ pyrimidine deoxyribooligonucleotides on DEAE-Sephadex columns, pH 8.7. Ref. 2: separation of $Py_n\ P_{n+1}$ pyrimidine deoxyribooligonucleotides on DEAE-Sephadex columns in 7 *M* urea, pH 7.0. Ref. 12: separation of $Py_n\ P_{n-1}$ deoxyribooligonucleotides by two-dimensional paper chromatography. Ref. 14: separation of $Py_n\ P_{n+1}$ deoxyribooligonucleotides by column chromatography on DEAE-cellulose, pH 5.0.

tions described (5 *M* urea, pH 5.1–5.3) if 0.2 *M* sodium chloride solution is used as eluent, whereas for the fractionation of the longer oligonucleotides a higher sodium chloride concentration is needed. As shown experimentally, for satisfactory separation of the seven pyrimidine isopliths (I–VII), a step-wise sodium chloride gradient can be used instead of a linear gradient. In this case, when the front of the starting buffer had passed the starting line, 5 *M* sodium chloride solution was added with a pipette to the chamber through the hole in the cap up to a final concentration of 0.2 *M*. The solvent front having passed two thirds of the plate, 5 *M* sodium chloride solution was added three times to the chamber so that the concentration in the chamber reached 0.25, 0.30 and 0.35 *M*, respectively. This step-wise increase in the sodium chloride concentration was made every time the solvent front had advanced by about 2 cm. In this way, we managed to accomplish a distinct and reproducible separation of the first seven pyrimidine isopliths of calf thymus DNA. As can be seen from Fig. 2, in addition to these seven isopliths, there are also bands corresponding to longer oligonucleotides ($n \geqslant 8$). Therefore, this chromatography can also be applied to separating and isolating longer pyrimidine fragments, ($n \geqslant 8$), but in this case their direct spectrophotometric determination was not sufficiently accurate as the amounts of the oligonucleotides were rather low.

Separation of the pyrimidine oligonucleotides according to composition

The areas on the chromatograms corresponding to the individual isopliths were cut off, placed in small glass columns (0.6 × 10 cm) with filters and desalted with 95% ethanol or methanol (10–15 ml). The oligonucleotides were eluted with 40% formic acid (1 ml per A_{260} unit) for 30–60 min at room temperature. The eluates were repeatedly extracted with diethyl ether and then applied on to the plates with DEAE-cellulose as narrow strips, being dried with an air flow at room temperature The amount of material applied was 1–3 A_{260} units per plate.

As mentioned above, in the fractionation of the oligonucleotides according to composition, we used as the starting buffer 0.01 *M* sodium acetate or sodium citrate, pH 3.2, without urea. A good separation of the oligonucleotides according to composition was accomplished using a 0.2–0.35 *M* linear sodium chloride concentration gradient.

The front of the starting buffer having passed the starting line, 5 *M* sodium chloride solution was introduced into the chamber up to a concentration of 0.2 *M* and then a 0.2–0.35 *M* linear sodium chloride concentration gradient was established as described above. Under these conditions, the monopyrimidine nucleotides are separated into deoxycytidine diphosphate and deoxythymidine diphosphate (Fig. 3). The dipyrimidine oligonucleotides are separated into three spots: C_2p_3, CTp_3 and T_2p_3. The tripyrimidine nucleotides are separated into C_3p_4, C_2Tp_4, CT_2p_4 and T_3p_4. The tetra- and pentanucleotides are separated into five and six components, respecively, of various composition (Fig. 3).

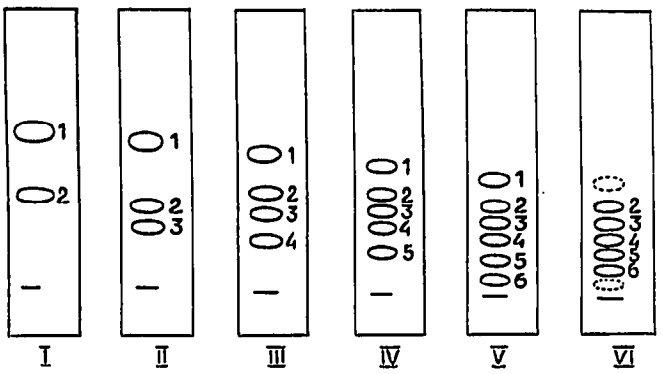

Fig. 3. Separation of individual isopliths (I–VI) into oligonucleotides differing in base composition on DEAE-cellulose plates. Notation of oligonucleotide spots as in Table II.

The data on the quantitative content of various pyrimidine oligonucleotides of calf thymus DNA separated according to composition by means of the above TLC procedure are presented in Table II. We have not included the results for the oligonucleotides of various composition in the VI and VII isopliths, as the amount of the material in them is relatively small and their direct spectrophotometric assay was not particularly effective. However, the oligonucleotides of isoplith VI were separated into seven spots (Fig. 3), which suggests that the procedure may, in principle, be applied for separating the pyrimidine oligonucleotides with six and more nucleotide residues according to composition.

The results of our determinations of the content of the pyrimidine nucleotides of various length and composition in calf thymus DNA (Table II) are in fairly good agreement with the reported data[2,12], especially with the evidence of Burton and Petersen[12] for two-dimensional paper chromatography of $Py_n P_{n-1}$ oligonucleotides dephosphorylated at the 5′- and 3′-ends.

TABLE II

CONTENT OF PYRIMIDINE OLIGONUCLEOTIDES OF DIFFERENT COMPOSITIONS IN CALF THYMUS DNA

Isoplith	*Oligonucleotides*	*Oligonucleotide composition*	A_{280}/A_{260}, *pH 1.0*		*Oligonucleotide content in DNA (mole%)*		
			Experimental values	*Predicted values*[12]	*Found*	*Reported in literature*	
						Ref. 12	*Ref. 2*
I	1	Cp_2	1.78	1.90	4.20	3.92	3.18
	2	Tp_2	0.71	0.68	6.35	6.23	7.63
II	1	C_2p_3	1.90	1.90	2.41	1.99	1.48
	2	CTp_3	1.27	1.25	5.06	5.19	5.59
	3	T_2p_3	0.62	0.68	2.87	2.62	2.74
III	1	C_3p_4	1.78	1.89	0.90	0.76	1.08
	2	C_2Tp_4	1.42	1.46	3.09	2.73	2.67
	3	CT_2p_4	1.05	1.03	2.86	2.64	2.47
	4	T_3p_4	0.68	0.67	1.36	1.28	1.33
IV	1	C_4p_5	1.85	1.92	0.30	0.31	0.20
	2	C_3Tp_5	1.38	1.50	1.20	1.39	1.56
	3	$C_2T_2p_5$	1.24	1.25	2.38	1.99	1.96
	4	CT_3p_5	1.00	0.95	1.94	1.53	1.47
	5	T_4p_5	0.80	0.70	0.60	0.55	0.63
V	1	C_5p_6	1.80	1.89	0.02	0.02	0.04
	2	C_4Tp_6	1.70	1.63	0.35	0.39	0.64
	3	$C_3T_2p_6$	1.30	1.31	1.34	1.06	1.04
	4	$C_2T_3p_6$	1.10	1.07	1.92	1.27	1.15
	5	CT_4p_6	1.00	0.85	0.65	0.82	0.81
	6	T_5p_6	0.69	0.70	0.24	0.19	0.17

DISCUSSION

TLC on DEAE-cellulose in 5 *M* urea, pH 5.1–5.3, using a linear or stepwise sodium chloride concentration gradient, allows, under the conditions described, a clear separation of $Py_n P_{n+1}$ pyrimidine deoxyribooligonucleotides according to length into isopliths. The amount of the long sequences ($n = 4$–7) obtained is greater and the amount of very long sequences ($n \geqslant 8$) is less than when the thymus DNA hydrolyzate is fractionated by means of chromatography on DEAE-Sephadex exchange columns (Table I). In column chromatography, tetra- and longer oligonucleotides often produce partially overlapping peaks. In TLC on DEAE-cellulose under our conditions, the bands corresponding to at least isopliths I–VII give distinct zones without overlapping. This mode of oligonucleotide fractionation seems to yield more correct data than column chromatography on the content of individual isopliths in various DNAs.

When the procedure described is applied to the fractionation of oligonucleotides at pH 3.2, separation occurs according to composition, also without noticeable overlapping. The number of spots on the chromatograms agrees with the predicted number of components of different composition for each of the isopliths studied (Fig. 3). The spectrophotometric characteristics of the separated components are also in agreement with the theoretical values (Table II). It should be noted that in fraction-

ation according to base composition in citrate buffer, cytosine-containing oligonucleotides move as more dense bands than in acetate buffer.

In a less steep salt gradient in citrate buffer, a certain separation of the oligonucleotide mixtures of the same nucleotide composition begins. Thus, if a 0.05–0.2 *M* sodium chloride concentration gradient is used in this buffer, the band (spot) of the CTp_3 oligonucleotides is separated into two components whose compositions and spectra are identical but with different contents of the nucleotide material. Apparently, this peculiarity may be used for a further fractionation of the separated mixtures consisting of the oligonucleotides of identical composition and length but with different sequences. On the other hand, when the aim is to obtain a clear fractionation of the oligonucleotides by base composition only, the salt gradient slopes should not be too steep.

The above fractionation procedure for oligonucleotides can be utilized for both analytical and preparative purposes. Under our conditions, the optimum amount of thymus DNA used for spectrophotometric assays of each of the seven pyrimidine isopliths was 300–400 μg. If a quantitative spectrophotometric assay is to be carried out for oligonucleotides that differ not only in length but also in composition, the starting amount of DNA should be at least a few times higher. If only the contents of the first two or three isopliths are being studied, DNA can be used more economically. Moreover, as various DNAs are characterized by specific frequencies of pyrimidine sequences[2–7,12], the amount of material required for the analysis depends on the nature of the DNA to be analyzed. For instance, a considerably smaller amount of long pyrimidine clusters ($n \geqslant 3$) is contained in bacterial and phage DNA[3–6,12] than in the DNA of animals[2,5,8,12–15] and higher plants[7]. It is evident that when studying the frequencies of pyrimidine sequences by their radioactivity in labelled DNA[3–5], the amount of DNA required for the analysis can be reduced to 10–20 μg. For preparative separation and isolation of pyrimidine deoxyribonucleotides that differ in length and base composition, an 18 × 24-cm plate can give an effective fractionation of 20 mg of hydrolyzate of thymus DNA.

An advantage of the procedure described is that no preliminary removal of purine bases from DNA hydrolyzates[12] is required for fractionation and analysis of the pyrimidine nucleotides. These bases are separated well from all of the pyrimidine components of DNA hydrolyzates (Fig. 2) and can be readily assayed concurrently.

The oligonucleotide separation procedure described here allows a comparatively easy analysis of the composition of isolated pyrimidine fragments and the detection of minor bases in them. To this end, DEAE-cellulose from appropriate bands of the chromatogram were transferred to glass columns (0.5 × 10 cm), desalted with 80% methanol or ethanol and the oligonucleotides were eluted with 50% formic acid and hydrolyzed to bases; the resulting bases were separated by means of TLC on DEAE-cellulose and assayed spectrophotometrically[16]. By analyzing in this way the distribution of 5-methylcytosine over the pyrimidine isopliths in beef heart nuclear DNA, we have found that the fraction of monopyrimidine fragments contains 60% of all the 5-methylcytosine of this DNA. These results are similar to the data on the distribution of 5-methylcytosine in calf thymus DNA reported by Doskocil and Šorm[15], and suggest that in animal DNA, 5-methylcytosine is mostly contained in the purine–5-methylcytosine–purine sequence.

Using large (18 × 24-cm) plates, the fractionation of oligonucleotides accord-

ing to length and base composition can be accomplished on the same plate by means of two-dimensional chromatography. In order to do this, a small spot of DNA hydrolyzate was applied in one of the corners of the plate and the oligonucleotides were separated into individual isopliths at pH 5.1–5.3 as described above. After this chromatography in the first direction, the plates were dried at room temperature and the band containing purines was removed. The layers were desalted by repeatedly washing the plates in a cuvette with 80% methanol or ethanol and drying them at room temperature. Then, in order to separate the oligonucleotides according to base composition, chromatography was carried out in the second direction at pH 3.2 as described above. It should be noted that in this case, the separation of oligonucleotides according to base composition is less satisfactory than when previously isolated individual isopliths were used for this purpose. However, this two-dimensional chromatography is simpler and allows one to obtain fingerprints of hydrolyzates of various DNAs. Also, it allows one to determine quantitatively (spectrophotometrically) the content of the oligonucleotides of varying composition containing at least 1–5 nucleotide residues.

The chromatography of oligonucleotides according to length and base composition described is especially useful for studying DNA hydrolyzates by radioautography. The fractionation procedures described can be applied to studies of the character and specificity of DNA methylation *in vivo* and *in vitro*. Using [methyl-^{14}C]-methionine as a donor of methyl groups *in vivo* or [methyl-^{14}C]-S-adenosylmethionine in DNA methylation reactions *in vitro*, one can localize 5-methylcytosine fairly easily by means of radioautography of the pyrimidine fragments. In particular, we have used TLC on DEAE-cellulose to study the distribution of labelled 5-methylcytosine in pyrimidine isopliths in DNA from beef heart mitochondria after it had been methylated *in vitro* with homologous and heterologous DNA-methylases. This made it possible to establish that mitochondrial DNA differs from nuclear DNA in methylation specificity[17].

Thus, TLC of $Py_n P_{n+1}$ oligonucleotides on DEAE-cellulose is a rapid and simple procedure for the fractionation of DNA pyrimidine fragments both by composition and length. The fractionation procedures developed can be used as a method of studying the primary structure of DNA and its enzymatic modifications. This may be applied, first of all, to a systemic comparative assay of frequencies of various pyrimidine sequences in DNAs of different origin and to the analysis of the character of methylation of cytosine residues in DNA.

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