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Note

The use of high-pressure liquid cation-exchange chromatography for determination of the 5-methylcytosine content of DNA

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Besides thymine, methylated bases, the minor components of nucleic acids, have become an important feature in programmed synthesis on account of their suspected participation in translational and transcriptional processes[1].

The elucidation of the function of such minor bases as 5-methylcytosine (5MeCyt) in DNA, however, requires sensitive methods for the separation and determination of these compounds. In the most common methods so far used, the free bases in formic acid or perchloric acid hydrolysates of DNA are separated by paper and thin-layer[2–6] or column[7] chromatography. With DNA containing [methyl-^{14}C]5MeCyt residues, the determination is easy, even with 5MeCyt still bound to oligodeoxyribonucleosides, as in 5MeCyt-containing pyrimidine isostichs of Novikoff hepatoma DNA according to Sneider[8].

As Vanyushin *et al.*[2] reported, there appears to be a relationship between the 5MeCyt content of an individual's DNA and its age, since the 5MeCyt content in male albino rats decreases with age in brain, heart and spleen, increases in the kidneys, and does not significantly change in liver and lungs.

We applied high-pressure liquid cation-exchange chromatography as a highly sensitive device for nucleic acid analysis to the separation of 5-methyldeoxycytidine (5MedCyd) in DNA hydrolysates to ensure that even small differences in the 5MeCyt content of DNAs could be detected quantitatively by routine analysis. Although acid hydrolysis is mainly used for the estimation of DNA base ratios and of minor-base concentrations, the errors inherent in this procedure are still under discussion, as 5MeCyt is said to be deaminated or demethylated during hydrolysis[9], these effects varying with the method of prior purification of the DNA[10].

We therefore used enzymic DNA hydrolysis[11,12], thus avoiding deamination or demethylation by hot acid. Since deoxyribonucleotides under these conditions of handling would be less stable than the nucleosides, and since the separation of deoxyribonucleotides would require more tedious gradient elution, our separations were carried out on the deoxyribonucleoside level at constant eluent composition.

MATERIALS

The four major deoxyribonucleosides (dAdo, dCyd, dGuo, dThd) were pur-

chased from Papierwerke Waldhof-Aschaffenburg (Mannheim, G.F.R.), and 5MedCyd from P.L. Biochemicals (Milwaukee, Wis., U.S.A.). Other reagent-grade chemicals were obtained from E. Merck (Darmstadt, G.F.R.).

Strongly acidic cation-exchange resin, Type M71, particle diameter 10–12 μm, was purchased from Beckman (München, G.F.R.).

Deoxyribonuclease I (1000 U/ml) and snake-venom phosphodiesterase (0.5 mg/ml) were obtained from Worthington (Freehold, N.J., U.S.A.), alkaline phosphatase [from calf intestine, highly purified with respect to adenosine and cytidine deaminase, in ammonium sulphate solution (1 mg/ml)] was a gift from Boehringer (Mannheim, G.F.R.).

Herring-sperm *(Clupea harengus)* DNA and rainbow-trout sperm *(Salmo irideus)* DNA isolated according to Zahn *et al.*[13] were a gift from H. Mack (Illertissen, G.F.R.). *Allium cepa* was bought from commercially available stocks, and the moss *Hypnum cupressiforme* was collected in spring.

METHODS

For DNA preparation, the moss was cleaned and minced, and one-third of its weight of silicon carbide grinding powder (500 mesh) was added. This was then mixed in a blender at 60° with an equal volume of 0.01 *M* Tris–HCl (pH 7.4) containing 4% of sodium dodecyl sulphate (SDS) and 0.05 *M* EDTA, and the moss was homogenized in a ball mill (Hormuth, Wiesloch, G.F.R.) for 5 min at maximum speed. After chilling, 0.1 volume of 1.6 *M* potassium chloride was added to insolubilize the dodecyl sulfate as its potassium salt. This, together with other insoluble matter, was removed by centrifugation at 15,000 *g* for 5 min at 0°. To the clear viscous supernatant liquid was added, with stirring, one-third of its volume of 2.0 *M* sodium chloride (pH 7.2) containing 4% of cetyltrimethylammonium bromide ($CtMe_3N^+Br$) The resulting $CtMe_3N^+$ salt of DNA was purified according to Hönig *et al.*[14]. To convert the $CtMe_3N^+$ salt to the sodium salt, the DNA was dissolved in 1 *M* sodium chloride and an equal volume of 2% SDS solution was added; from this solution, the DNA was precipitated with isopropanol–water (75:25) containing 0.5% of SDS. The DNA was then washed in a series of solutions containing isopropanol in concentrations increasing from 75 to 100%; isopropanol was eventually removed with diethyl ether, and the product was finally dried under reduced pressure.

Equilibrium centrifugation of the DNAs was performed for 72 h at 20° in an MSE Superspeed 65 ultracentrifuge at 65,000 *g* in 5.9 *M* caesium chloride buffered with 0.015 *M* Tris–HCl (pH 8.0). The fractions I and II from the DNA of the moss *Hypnum cupressiforme* (see Fig. 1) were collected separately and re-centrifuged once in the same manner.

The deoxyribonucleosides were separated in a Varian LCS 1000 liquid chromatograph equipped with a 254-nm UV detector; a 30-cm × 0.18-cm stainless-steel column was used for DNA base ratio determinations and a 100 cm × 0.18 cm column for the separation of 5MedCyd from the major deoxyribonucleosides. Each column was filled with strongly acidic cation-exchange resin, according to the method of Scott and Lee[15]. Elution was carried out with 0.4 *M* ammonium formate (pH 4.6) at a flow-rate of 13.3 ml/h (flow-velocity 8.7 cm/min) or 16.0 ml/h (10.5 cm/min), respectively, at 50°. The elution diagrams were plotted with a Varian strip-chart

recorder (Model 20) set at 0.02 absorbance units to full scale deflection (0.02 AUFS). The deoxyribonucleosides were determined either by electronic integration or peak-height evaluation; both methods gave identical results, the standard deviation being less than 3%. Reference samples of dAdo, dCyd, dGuo, and dThd were dissolved in 0.1 *M* phosphate buffer (pH 7.0), and 5MedCyd in 0.1 *M* hydrochloric acid; the concentrations were adjusted, by spectrophotometry, to 95–190 μM.

A portion (50–200 μg) of DNA purified by caesium chloride gradient centrifugation was centrifuged to a pellet, and to this were added 35 μl of 0.5 *M* Tris–HCl (pH 8.5), 20 μl of deoxyribonuclease I, 20 μl of snake-venom phosphodiesterase, 20 μl of alkaline phosphatase and 5 μl of 0.07 *M* magnesium chloride. Each mixture was kept at 37° for 12 h for enzymic degradation of the DNA to deoxyribonucleosides; after being frozen at −30°, the mixtures were stored for further use.

RESULTS AND DISCUSSION

Fig. 1 shows the $A_{260\ nm}$ profile of DNA obtained from *Hypnum cupressiforme* by caesium chloride gradient centrifugation. Two fractions were separated; one (I) had a buoyant density of 1.728 and the other (II) a buoyant density of 1.719 g/cm³. In order to obtain pure fractions, the peaks I and II were re-centrifuged separately; two uncontaminated fractions of about 100 μg (I) and 110 μg (II) were cut from the final gradients.

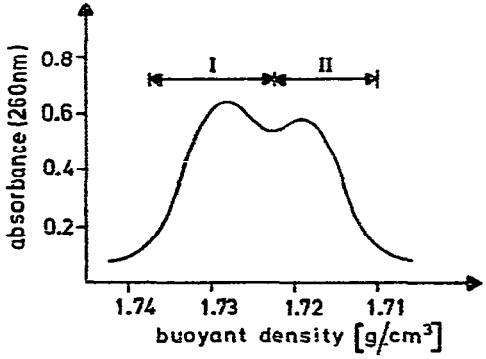

Fig. 1. Absorbance (260 nm) profile of *Hypnum cupressiforme* DNA after the first caesium chloride-gradient centrifugation: I = (G + C)-rich fraction, 1.728 g/cm³; II = (A + T)-rich fraction, 1.719 g/cm³.

Fig. 2 shows the elution patterns obtained from the 30-cm column used for the determination of the (A + T)/(G + C) ratios of the DNA hydrolysates according to Breter and Zahn[12] (see Table I). Fig. 3 gives the patterns obtained from mixtures of deoxyribonucleosides prepared to simulate hydrolysates of DNAs with different 5MeCyt contents (6 and 0.5 mol %) and with different base ratios (1.50, 1.00 and 0.66); a 5MeCyt content of 0.5 mol % is clearly detectable.

Fig. 4 demonstrates the elution patterns of hydrolysates of herring-sperm and rainbow-trout-sperm DNA. The 5MeCyt content of herring-sperm DNA as calcu-

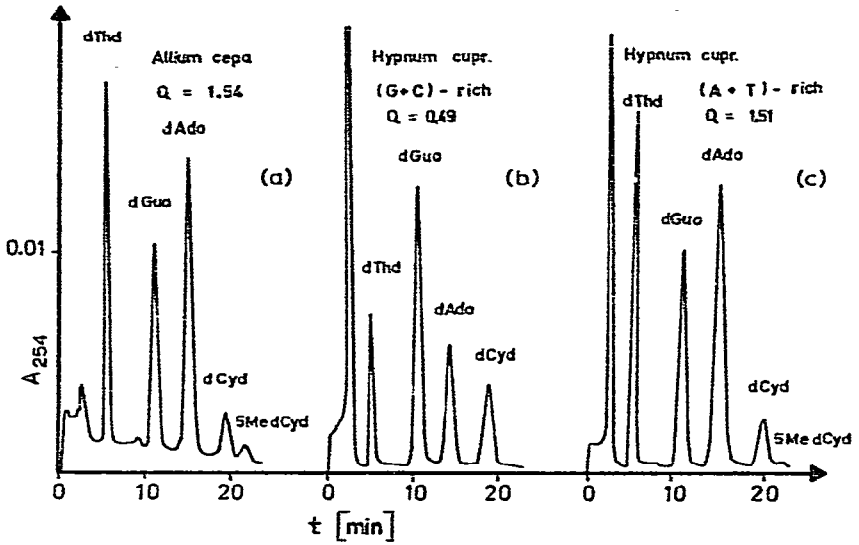

Fig. 2. Elution patterns of: (a) *Allium cepa* DNA hydrolysate; (b) (G + C)-rich fraction of *Hypnum cupressiforme* DNA hydrolysate; and (c) (A + T)-rich fraction of *Hypnum cupressiforme* DNA hydrolysate, each used for the base-ratio determination obtained from the 30-cm cation-exchange column (see Table I and ref. 12). Q indicates the base ratio.

lated from the diagram is in good agreement with the value first reported by Zahn[16] (see Table I). The 5MeCyt content of rainbow-trout-sperm DNA has not so far been reported, but Felix *et al.*[17] qualitatively detected this minor component.

Fig. 5 shows the elution patterns of an *Allium cepa* DNA hydrolysate. These diagrams illustrate the advantage of the method. Quite small amounts of 5MedCyd in DNA hydrolysates can easily be determined in this single-step operation merely by increasing the volume of hydrolysate applied to the column containing a high-capacity exchange resin. Large deviations due to column overloading will, however,

TABLE I

BASE RATIOS AND 5MeCyt CONTENTS OF SOME DNA SPECIES

DNA species	*Base ratio*		*5MeCyt content (mol %)*		*Reference*
	Found	*Literature value*	*Found*	*Literature value*	
Herring sperm	1.31*	1.32	1.83	1.9	16
Rainbow-trout sperm	1.22*	1.24	1.30	—**	17
Allium cepa	1.54	1.72	5.8	5.4	6
Hypnum cupressiforme					
(G + C)-rich fraction	0.49	—***	0.0	—***	—
(A + T)-rich fraction	1.40	—***	3.8	—***	—

* See ref. 12.
** Qualitative estimate.
*** No literature values available.

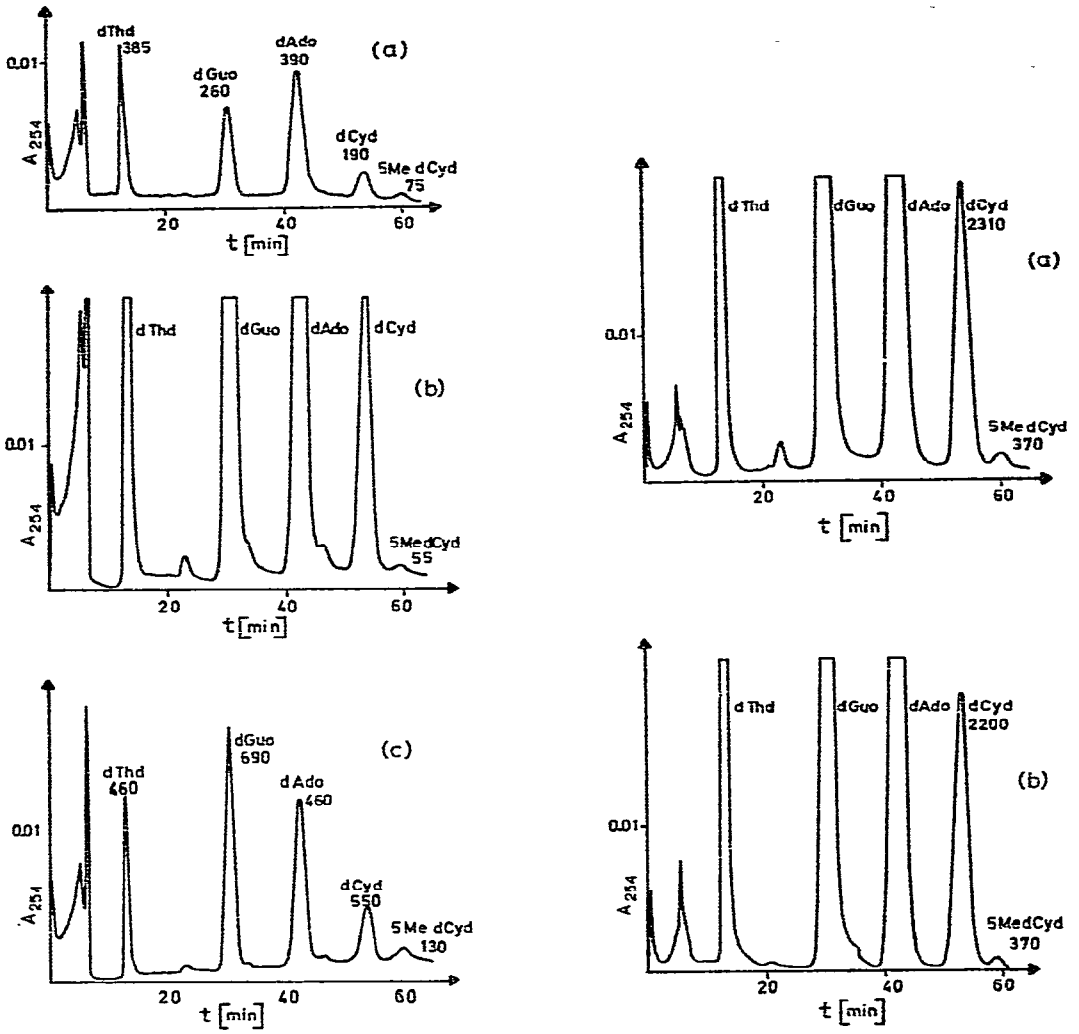

Fig. 3. Elution patterns (100-cm column) of deoxyribonucleoside mixtures corresponding to a DNA hydrolysate with: (a) a base ratio of 1.50 and a 5MeCyt content of 6.0 mol %; (b) a base ratio of 1.00 and a 5MeCyt content of 0.5 mol %; (c) a base ratio of 0.66 and a 5MeCyt content of 6 mol %. The numbers at the peaks refer to the deoxyribonucleoside contents (picomoles) as determined from the diagram.

Fig. 4. Elution patterns (100-cm column) of DNA hydrolysates of: (a) herring-sperm DNA (base ratio 1.24, 5MeCyt content 1.83 mol %); and (b) rainbow-trout-sperm DNA (base ratio 1.32, 5MeCyt content 1.30 mol %). The numbers at the peaks refer to the deoxyribonucleoside contents (picomoles) as determined from the diagram.

be observed if extremely concentrated synthetic mixtures of deoxyribonucleosides exceeding 50 μg of DNA hydrolysate are used; in these circumstances, quantitative determinations are not possible.

Fig. 6 demonstrates the elution patterns of hydrolysates of the (G + C)-rich and (A + T)-rich fractions of *Hypnum cupressiforme* DNA. The (A + T)-rich fraction

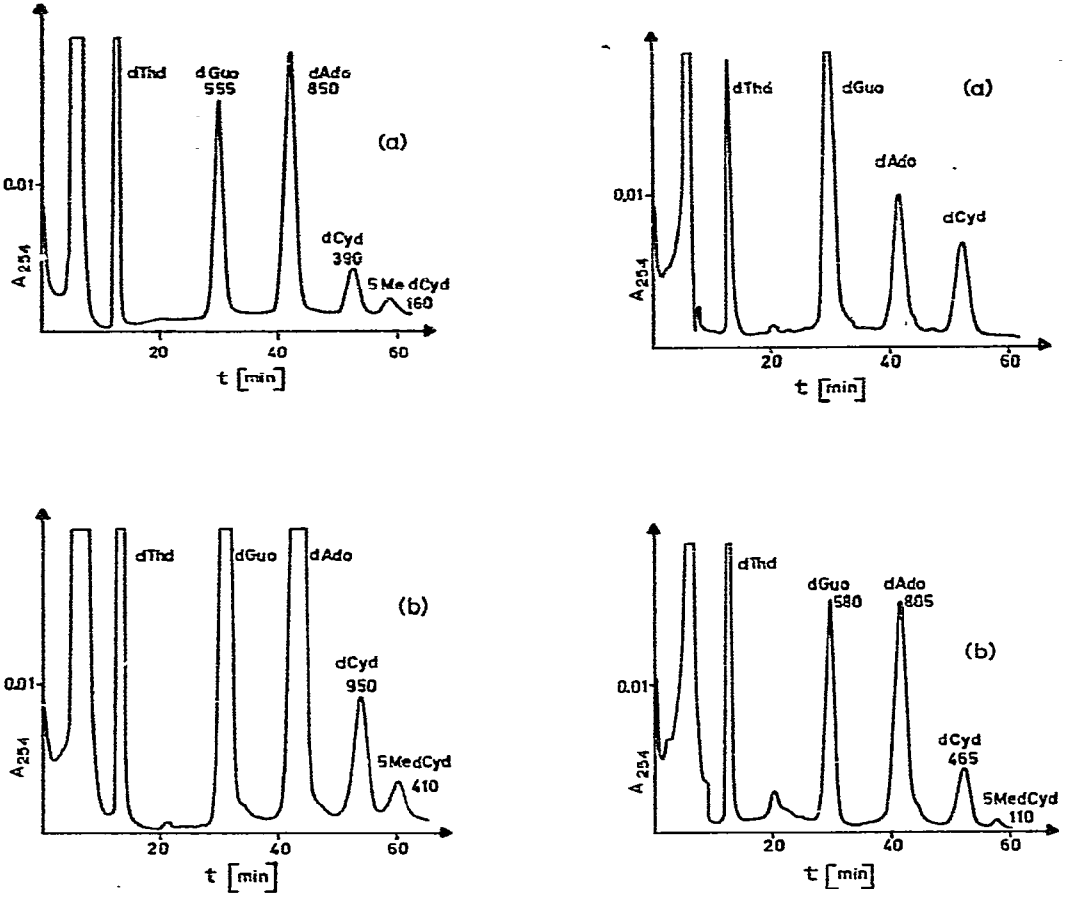

Fig. 5. Elution patterns (100-cm column) of an *Allium cepa* DNA hydrolysate: (a) 10 μl of hydrolysate (dGuo, dAdo and dCyd are within full-scale deflection of the recorder and can be determined quantitatively); (b) 25 μl of hydrolysate (dThd, dGuo and dAdo exceed the chart width, but dCyd and ^{5}MedCyd can be determined quantitatively). The numbers at the peaks refer to the deoxyribonucleoside contents (picomoles).

Fig. 6. Elution patterns (100-cm column) of a *Hypnum cupressiforme* DNA hydrolysate: (a) (G + C)-rich fraction containing no 5MeCyt; (b) (A + T)-rich fraction containing 3.8 mol% of 5MeCyt, detected as 5MedCyd. The numbers at the peaks refer to the deoxyribonucleoside contents (picomoles) as determined from the diagram.

contains 3.8 mol % of 5MeCyt, but this minor component is absent from the (G + C)-rich fraction. Since amounts of 0.5 mol % can be detected in synthetically prepared deoxyribonucleoside mixtures (see Fig. 3), the (G + C)-rich fraction contains less than one 5MeCyt per thousand bases.

The sensitivity of detection may, however, be improved by using a variable-wavelength UV detector set at the optimum absorption wavelength (275 to 280 nm for the pH-range 7.2 to 4.4) instead of at the 254-nm setting used here.

The DNAs investigated were chosen because they had already been thoroughly examined (herring-sperm[16] and rainbow-trout-sperm[17] DNA), or because they con-

tain large amounts of the minor component (*Allium cepa* DNA[6]) or because their 5MeCyt contents were not available to us (rainbow-trout-sperm[17] and *Hypnum cupressiforme*[18] DNA). The separation of 5MedCyd from the major deoxyribonucleosides as described here has been developed from a high-pressure cation-exchange separation used for the rapid determination of DNA base ratios[12]. Improvement in the resolution between 5MedCyd and dCyd is achieved by increasing the column length from 30 to 100 cm. Unfortunately, 5MedCyd is eluted at the very end of each run, which leads to broadening of the 5MedCyd peak and makes quantitation less accurate as compared with peaks having short elution times. Minimal retention of the positively charged compound, however, would call for anion-exchange chromatography. No data have so far been reported to indicate whether or not the major deoxyribonucleosides (particularly dThd) would be separated in such an anion-exchange system from 5MedCyd and dCyd. The various methods described so far for the detection of 5MeCyt in DNA hydrolysates all suffer from poor resolution either between cytosine, uracil and 5MeCyt[3] or between dCyd and 5MedCyd[7]. From this point of view, the cation-exchange method is to be preferred, since it has proved to be applicable to the quantitative determination of such different nucleic acid compounds as deoxyribonucleosides[12], ribonucleosides[11], thymine[19], uracil[20] and thymine dimers[21] merely by varying the column length and the flow-rate. Further, this system enables one to work with standard deviations less than 4% in routine analysis. Other experiments[22] show that this system may also be useful for the rapid and sensitive determination of other methylated purine and pyrimidine deoxyribo- and ribonucleosides.

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