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Fractionation of DNA on Hydroxyapatite with a Base-Specific Complexing Agent

(chromatography/bacterial DNA/satellites/DNA–dye binding)

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ABSTRACT **We describe a chromatographic technique for separating mixtures of DNA of varying G + C content. The method employs a specially prepared high-capacity hydroxyapatite and a G·C-specific DNA ligand (2-methyl-3-amino-7-dimethylamino-5-phenyl-phenazinium cation, abbreviated PNR). DNA molecules rich in G·C pairs bind larger amounts of this dye and are eluted earlier from the hydroxyapatite column than are molecules rich in A·T pairs. The dye can easily be removed from DNA by dialysis or solvent extraction after the chromatographic separation. The resolution of the method approaches that of CsCl density gradient separation, and the capacity of the column is much larger than that of a typical density gradient experiment. Elution of the DNA is not dependent on molecular weight, so samples of different molecular weight can be separated on the basis of G + C content. The technique should be especially useful for separating G·C-rich minor components from DNA obtained from eukaryotic cells, as demonstrated by a fractionation of DNA from calf thymus.**

The separation of DNA mixtures in which the components differ in their base composition is performed in general by density gradient centrifugation (1). As a further method the preferential precipitation of (A + T)-rich DNA by poly(lysine) has been studied (2). Chromatographic and electrophoretic procedures have also been reported (3–5), in which the physical basis for the resolution is complex and unclear. The main disadvantage of these procedures, however, is the simultaneous fractionation according to base composition and molecular size. Only hydroxyapatite could be used successfully for chromatographic separations of DNAs very different in base composition (6).

The discovery of the high G·C-specificity of a phenazinium dye*, the 2-methyl-3-amino-7-dimethylamino-5-phenyl-phenazinium cation, a phenylated neutral red (PNR), caused us to try its use for chromatographic separations of DNA mixtures. The basic idea was to shift selectively the binding equilibria of DNAs bound to a suitable basic adsorbant by adding the dye cation to the eluting salt gradient. Concerning the basic adsorbant to be used in these experiments, hydroxyapatite seemed the most suitable to use since (*i*) it does not fractionate DNA molecules according to size, (*ii*) it does not interact appreciably with the dye, and (*iii*) the capacity of this adsorbant for nucleic acids is high and the recovery of the bound DNA is complete. During our studies we were soon confronted with the problem of preparing hydroxyapatite with constant properties from batch to batch. Since the flow rate, the capacity and the resolving power can vary strongly with each preparation of the adsorbant, its preparation had to be studied in detail with respect to these parameters.

Abbreviation: PNR, 2-methyl-3-amino-7-dimethylamino-5-phenyl-phenazinium perchlorate (phenyl neutral red).

* W. Müller, H. Bünemann, and N. Dattagupta, in preparation.

MATERIAL AND METHODS

(*a*) 2-Methyl-3-amino-7-dimethylamino-5-phenyl-phenazinium perchlorate (PNR). This dye was prepared by condensing *p*-amino-dimethylaniline, *o*-toluidine, and aniline with dichromate in slightly acidic medium. The detailed synthesis will be described elsewhere.

(*b*) BPES-buffer: 0.007 M $Na_2H\ PO_4$, 0.002 M $Na\ H_2PO_4$, 0.001 M EDTA, 0.185 M NaCl (pH 7.0 at 23°).

(*c*) DNA from *Micrococcus luteus*, *Escherichia coli*, *Bacillus subtilis*, *Proteus mirabilis* and *Clostridium perfringens* was prepared from frozen (wet) bacteria by a variation of the procedure described by Marmur (7, 8). The preparations were sheared to a molecular weight of 1 to 2 × 10^6 by treatment in a Sorvall omnimixer 250-ml steel beaker at 15,000 rpm at 0.5° for 6 hr, extensive dialysis against BPES-buffer and precipitation with 0.66 volumes of isopropanol. Redissolution was in BPES or other buffers after collecting by centrifugation and was followed by redialysis.

(*d*) DNA from calf thymus: Sigma, type I DNA was used and sheared as described for bacterial DNAs. High-molecular-weight DNA (25 × 10^6) was obtained from Boehringer, Mannheim, in solution. Before use it was redialysed against BPES-buffer.

(*e*) The following reagents were used for the preparation of hydroxyapatite purchased from E. Merck, Darmstadt: $CaCl_2 \cdot 2H_2O$, analytical grade; $NaH_2PO_4 \cdot 1H_2O$, analytical grade $Na_2HPO_4 \cdot 2H_2O$, analytical grade; NaOH, analytical grade; Ammonia solution (25%, specific gravity = 0.91), analytical grade.

(*f*) *Hydroxyapatite, type A:* This hydroxyapatite was prepared according to Tiselius' standard procedure (9); variations were introduced by different rates of mixing calcium chloride and sodium phosphate (250 ml/hr to 500 ml/hr; the maximum relative difference of rate of mixing of the two components was about 10%) as well as by different heating times for the transformation of the brushite obtained to hydroxyapatite in sodium hydroxide (25–70 min).

(*g*) *Hydroxyapatite type B:* (The procedure described yields about 200–250 ml of packed hydroxyapatite.) Two liters of 0.5 M $CaCl_2$ solution and 2 liters of 0.5 M Na_2HPO_4 solution were mixed in a 2000-ml beaker (13 cm in diameter). The two solutions were pumped into the beaker at a rate of 8 liters/hr.

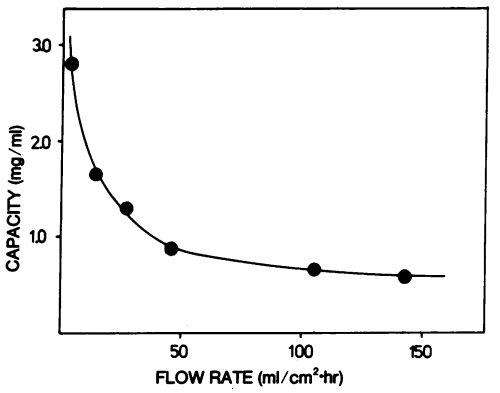

FIG. 1. Simultaneous variation of the binding capacity for calf-thymus DNA of various preparations of hydroxyapatite (type A) and of the flow rate in a column of 1-cm² cross section. The applied pressure corresponds to a pressure drop of 10 (length of the hydrostatic head divided by the length of the column).

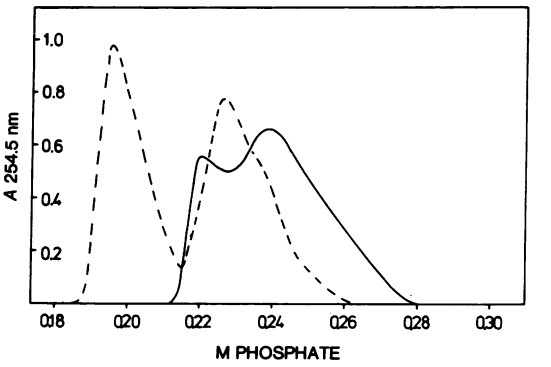

FIG. 3. (——) Elution profile of a mixture of 2 mg of *M. luteus* DNA and 2 mg of *B. subtilis* DNA from hydroxyapatite type A (1.6 cm × 12 cm) of high binding capacity and low flow rate (3 ml/hr). (– – –) Elution of the same DNA mixture from the same hydroxyapatite column in the presence of PNR (A_{548} = 2.6). T = 25° in both cases.

Rapid mixing was obtained by magnetic stirring with a Teflon coated bar (5.5 × 0.8 cm) at about 500–600 rpm. The brushite formed was washed by decantation 5 times with 2 liters of distilled water, resuspended in distilled water to a total volume of 4 liters and collected on a glass-sintered funnel. The moist brushite was suspended in a boiling mixture of 800 ml of water and 80 ml of concentrated ammonia solution placed in a three-necked round bottom flask of 2-liter total volume. The mixture was boiled by a heating mantle for 30 min. During the whole heating process, the suspension was stirred with a glass stirrer of 4-cm stirring radius at about 100 rpm. In a 5-liter beaker the product obtained after decantation was washed 3 times with 2 liters of distilled water each time, the last sediment resuspended in 5 liters of water and the suspension neutralized with dilute phosphoric acid to pH 7.1.

After two further washings with 2 liters of 0.01 sodium phosphate buffer (pH 7.1) at room temperature, two washing procedures at 90° for 5 min and 15 min in 2 liters of this buffer were applied. After two final washing procedures at room temperature by 4 liters of 0.01 M sodium phosphate (pH 7.1) each time, the sedimented product was stored at 4°.

(*h*) Determination of binding capacity of hydroxyapatite for calf-thymus DNA: The binding capacity of the hydroxyapatite preparations was determined on small test columns of known volume to which increasing amounts of DNA in 0.01 M sodium phosphate buffer (pH 7.1) were applied until non-absorbed DNA appeared in the filtrate. Batch experiments in which known amounts of hydroxyapatite were incubated with known amounts of DNA in 0.01 M sodium phosphate gave about 15–20% higher values; the values in Fig. 1 refer to the column experiments.

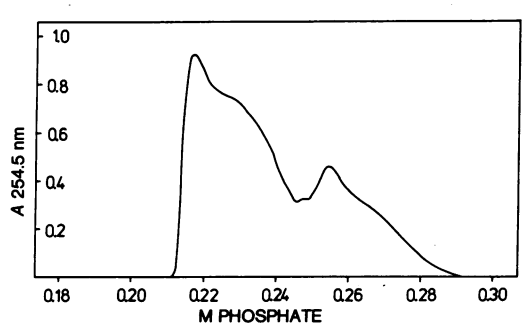

FIG. 2. Elution profile of a mixture of 1.5 mg of *M. luteus* DNA, 1.5 mg of *E. coli* DNA and 1.5 mg of *C. perfringens* DNA from hydroxyapatite type A (1.6 cm × 15 cm) of high binding capacity and correspondingly low flow rate (3 ml/hr) at 25°.

(*i*) *Standard procedure for chromatographic runs on hydroxyapatite:* A thermostated (25°) chromatographic column of 1.6 × 40 cm (Pharmacia, type K 16) equipped with two adaptors was packed to about 12- to 15-cm height with hydroxyapatite suspended on 0.01 M phosphate buffer (pH 7.1). After loading the DNA on the column the elution with a linear sodium phosphate gradient supplied by an "Ultrograd" gradient mixer (LKB) and a peristaltic pump was started. The flow rate for hydroxyapatite B column was about 20 ml/hr. The slope of the gradient was 1.15 mM/ml in all cases. Fractions of 5 ml were collected.

If chromatographic separations in the presence of PNR were carried out, the column was equilibrated after packing with PNR solution of A_{548} about 2.6. This A, which corresponds to a dye-monomer concentration of 4×10^{-5} M, maintains a DNA-loading of 1 dye molecule to about eight base pairs (ratio $r = 0.125$) for calf-thymus DNA at about 0.2 M Na^+. To the DNA to be fractionated the calculated amount for this r value plus the amount for the necessary free dye is added and the complex formed applied to the column. The bound complex can be seen clearly at the top of the column; due to its violet color on a pink background the beginning elution can easily be observed. Relatively sharp violet bands are formed which broaden on migration. The elution was followed by automatic registration of the absorbancy at 254.5 nm (LKB analyser). From the interesting fractions the dye was removed by three to four extractions with isobutanol, followed by two further extractions with chloroform and redialysis against buffer.

(*k*) *Base analysis of DNA fractions:* After removal of the dye the solutions were redialysed against 0.02 M Tris buffer (pH 7.3) containing 0.01 M $MgCl_2$. The DNA content of each fraction was determined by measuring the A_{258} taking $\epsilon_{(P)} = 6550$ as the mean molar absorption coefficient. For base analysis the fractions were treated by DNase I and subsequently by phosphodiesterase as described by Derumez and Biserte (10). The hydrolysates were chromatographed on QAE-Sephadex in a 0.2–0.7 M ammonium acetate gradient (pH 4.6–4.8). The relative amount of each nucleotide was determined from the

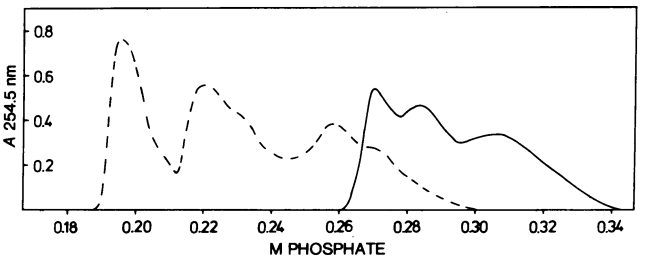

FIG. 4. (——) Elution of *M. luteus* DNA (1.0 mg), *E. coli* DNA (1.0 mg) and *C. perfringens* DNA (1.0 mg) from hydroxyapatite type B (1.6 cm × 12 cm) at 25°. (– – –) Elution of the same DNA mixture from the same column in the presence of PNR (A_{648} = 2.6). The flow rate was 20 ml/hr in both cases.

peak area obtained by recording the eluate on a Zeiss PMQ II spectrophotometer, equipped with an automatic cuvette changing device, a transmission-extinction converter and a recorder (Hartmann u. Braun, type Polycomp 2). The accuracy of the base analysis obtained is about ±0.4% in G + C content.

RESULTS

If hydroxyapatite ("type A") is prepared according to the standard procedure reported by Tiselius *et al.* (9) the flow rate of the material obtained depends strongly on the time of sodium hydroxide treatment of the brushite formed in the first step. Prolonged heating yields finer and finer particles. Normally most of the fine material formed is removed by several sedimentation and resuspension processes in order to obtain a material of high-flow rate. We found, however, that by this fractionation process the capacity for DNA binding decreases. Fig. 1 shows the relationship we obtained between flow rate and DNA-binding capacity. Furthermore, it turned out that the earlier release of a G·C-rich DNA reported by Piperno *et al.* (11) is much more pronounced for the hydroxyapatite of high-binding capacity and correspondingly low-flow rate. The desorption of a mixture of three different DNAs from bacteria is shown in Fig. 2. If, however, a hydroxyapatite of high-flow rate and low-binding capacity is used, the elution profile shows only one broad peak.

The elution profile for a mixture of *M. luteus* and *B. subtilis* DNA from high-capacity hydroxyapatite is shown in Fig. 3; the curve shows two clearly defined maxima, although the separation is still rather poor. Fig. 3 also shows the greatly improved separation of this mixture when the G·C-specific phenyl neutral red (PNR) is added to the eluting phosphate gradient. The two DNAs which differ by about 30% in their G + C content are separated practically completely. The identity of the two DNAs was established, as in all other cases in which bacterial DNAs were fractionated, by base analysis of the fractions.

All attempts to standardize the classical preparation procedure for hydroxyapatite at a reasonable compromise between binding capacity and flow rate failed. We, therefore, checked the conversion of brushite to hydroxyapatite by ammonia as reported by Main *et al.* (12) and obtained a material with much better flow rate and acceptable binding capacity. The latter could be further improved by fast precipitation of the brushite leading to rather fine starlike crystal aggregates with a large surface area. The subsequent ammonia treatment gave a hydroxyapatite ("type B") with a binding capacity of about 3 mg/ml and a flow rate of 25 ml/cm²·hr at

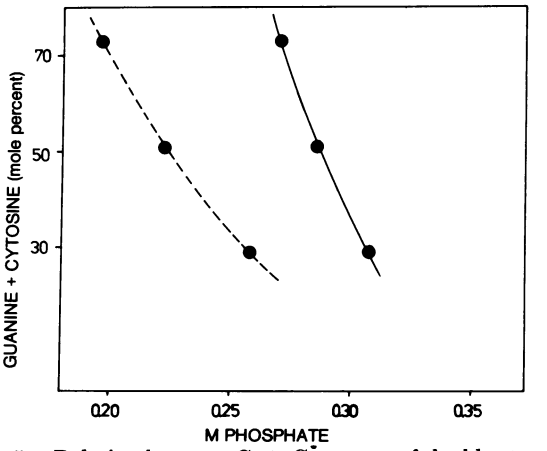

FIG. 5. Relation between G + C content of double stranded DNA and eluting phosphate concentration, hydroxyapatite type B, in absence (——) and in presence (– – –) of PNR (A_{648} = 2.6). T = 25°.

an applied pressure corresponding to a pressure drop of 10 (length of the hydrostatic head divided by the length of the column). The values for hydroxyapatite type A are given in Fig. 1. According to Fig. 4 the intrinsic resolution power of the hydroxyapatite type B is also improved over that of type A. The ternary DNA mixture consisting of *M. luteus* DNA (72% G + C), *E. coli* DNA (50% G + C) and *C. perfringens* DNA (29% G + C) shows clearly three maxima which are strongly spread out by the addition of PNR to the eluting gradient. The separation of the *M. luteus* DNA from the *E. coli* DNA obtained by addition of the dye seems quite remarkable.

In Fig. 5 the shift of the elution positions of the three DNAs in the phosphate gradient caused by the PNR is shown.

The elution diagrams in Figs. 3 and 4 show that the sharpness of the bands increases with increasing G + C content. This finding caused us to try a fractionation of calf-thymus DNA; the G·C-rich satellite fractions should become separable from the bulk DNA. In Fig. 6 it is shown that three different components in front of the main band are detectable. Their positions in the elution gradient correspond roughly to G + C contents of 60, 70, and 80% on the basis of the relations given in Fig. 5.

In all the chromatographic runs described shear-degraded DNA samples with molecular weights in the order of 1 to 2 × 10^6 as determined by viscometry were used. Since the method described here might be of interest for the isolation of larger DNA fragments from eukaryotic cells we checked the procedure with a DNA from calf thymus with a molecular weight

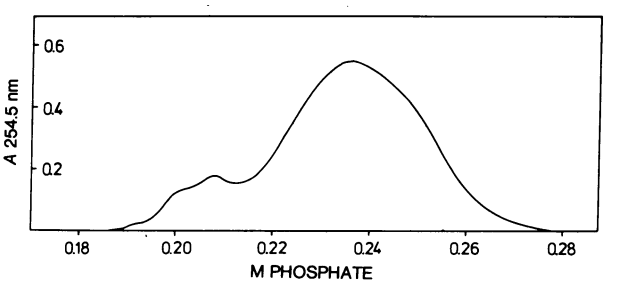

FIG. 6. Elution of 2.5 mg of calf-thymus DNA (molecular weight about 2 × 10^6) from a hydroxyapatite type-B column in presence of PNR (A_{648} = 2.6). T = 25°.

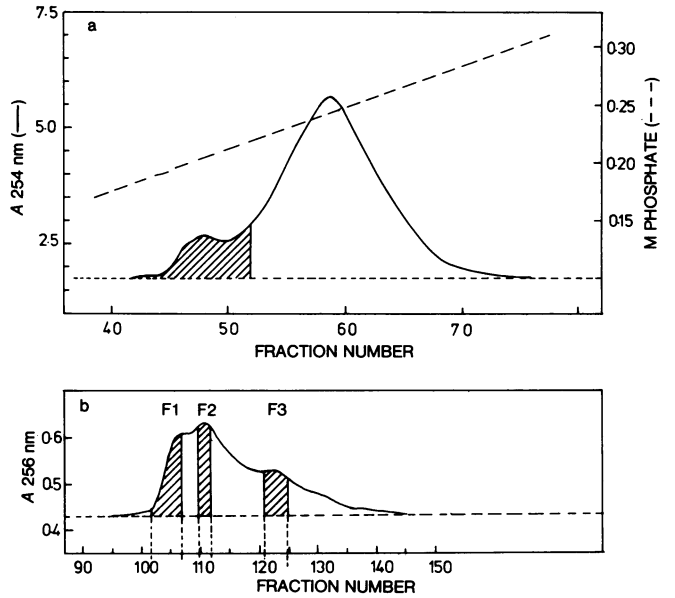

FIG. 7. (*a*) Fractionation of 10 mg of calf-thymus DNA (molecular weight about 25 × 10^6) on a hydroxyapatite–celite column. Flow rate: about 30 ml/hr; fraction size: about 4 ml. T = 25°. (*b*) Rechromatography of the early eluted part (about 15 A_{254} units) from the fractionation shown in Fig. 7*a*. Flow rate: about 20 ml/hr; fraction size: about 2 ml. The broken lines parallel to the abscissae indicate the PNR background (suppressed in 7*b*).

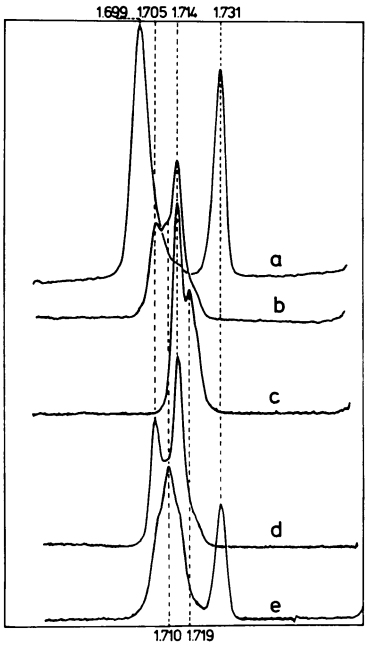

FIG. 8. Analytical cesium chloride density centrifugation of (*a*) DNA from fraction 59 of the elution shown in Fig. 7*a*; DNA from *M. luteus* (ρ = 1.731) is used as marker. (*b*) DNA fraction subjected to rechromatography (hatched area in Fig. 7*a*); (*c*) fraction 1 (Fig. 7*b*); (*d*) fraction 2 (Fig. 7*b*); (*e*) fraction 3 (Fig. 7*b*); DNA from *M. luteus* (ρ = 1.731) is used as marker.

of 25 × 10^6. We found that in some cases such a DNA may plug up the column rather tightly. This can be overcome easily by placing a layer of celite (about 3-mm thickness) on the lower column adapter and mixing the hydroxyapatite with some celite (20 g of celite for about 150 ml of sedimented hydroxyapatite was sufficient).

Furthermore it turned out to be very helpful to adsorb the DNA to be fractionated to some hydroxyapatite–celite mixture in 0.1 M phosphate buffer, transfer this slurry to the prepacked hydroxyapatite–celite column and then apply the eluting dye–phosphate gradient (0.1–0.4 M phosphate). The elution profile of such a run is practically identical to that shown in Fig. 6, except that the amount of material in the first step of the elution profile decreased.

In order to obtain more material for characterizing the fractions richer in G + C content, 10 mg of the high-molecular-weight DNA from calf thymus was chromatographed in the described manner on a column 13 by 2 cm. The elution profile is shown in Fig. 7*a*. The G·C-rich part (about 15 A_{254} units) marked by hatching in Fig. 7*a* was rechromatographed in the same way on a column of 20 by 1.6 cm yielding the elution profile shown in Fig. 7*b*. The fractions 1, 2, and 3 marked in Fig. 7*b* were subjected to analytical density gradient centrifugation and thermal denaturation.

The banding pattern of the main peak of the bulk DNA (Fig. 7*a*), of the mixture subjected to rechromatography and of the three fractions in cesium chloride gradients are shown in Fig. 8. [The densities indicated were determined in a previous centrifugation in which *M. luteus* (ρ = 1.731 g/cm³) and *P. mirabilis* DNA (ρ = 1.700 g/cm³) were used as markers.] For the bulk DNA a density of ρ = 1.699 g/cm³ is found in agreement with other authors (13). Fraction 1 contains two DNAs with ρ = 1.719 g/cm³ and ρ = 1.714 g/cm³, which have been designated as the satellites of calf-thymus DNA (13). The band of the heavier satellite is somewhat broader than that of the lighter one, which could reflect the presence of small amounts of heavier material as detected in chromatographic separations of shear-degraded DNA (Fig. 6).

Fraction 2 contains two sharp bands, with ρ = 1.714 g/cm³ and ρ = 1.705 g/cm³. The heavier of these two also appears in fraction 1. The lighter band, which is just as sharp as the heavier, is a satellite of calf-thymus DNA not easily observed because it is obscured by the main band in DNA that has not been fractionated before centrifugation.

Fraction 3 forms a relatively broad band with a maximum at ρ = 1.710 g/cm³, and shoulders at ρ = 1.705 g/cm³ and 1.714 g/cm³. The band at 1.710 g/cm³ appears to be a new satellite, although the melting experiments described below indicate that it is not as homogeneous as the other satellite bands. It is important to note that this material elutes anomalously late from the PNR–hydroxyapatite column, judged by the banding density. Whereas, the bands with ρ = 1.719, 1.714, and 1.705 g/cm³ elute from the column in that order, the material with ρ = 1.710 g/cm³ elutes after the band with ρ = 1.705 g/cm³. The basis for this effect is not clear, but it points out that a combination of chromatography and density gradient separation can produce fractionations not possible by either alone.

Since the fractions collected in Fig. 7*b* do not correspond to single density species, the satellite bands cannot be completely separated on the PNR–hydroxyapatite column. The satellites with ρ = 1.705 and 1.719 g/cm³ can be separated from each other, but both fractions contain the satellite with ρ = 1.714 g/cm³.

The melting curves for the three fractions of Fig. 7*b*, along with that for the main peak in Fig. 7*a*, were measured in 0.06

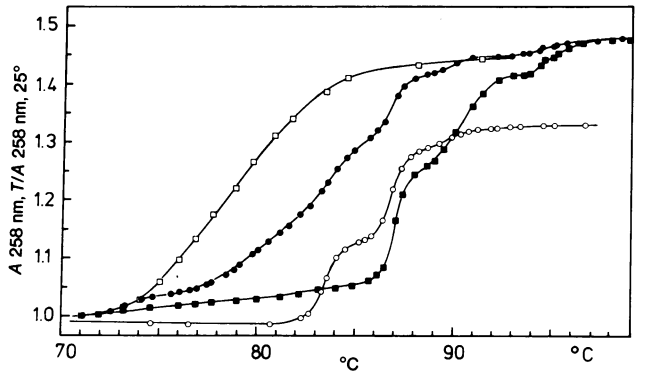

FIG. 9. Melting curves obtained in sodium phosphate–sodium chloride buffer (Na^+ = 0.06 M) for main DNA from calf thymus, fraction 59, Fig. 7*a* (□), fraction 1, Fig. 7*b* (■), fraction 2, Fig. 7*b* (○), fraction 3, Fig. 7*b* (●). In the ordinate label, T indicates the absorbance was measured at the temperatures indicated on the abscissa.

M Na^+ (sodium chloride–sodium phosphate, pH 7) and are shown in Fig. 9. Fraction 1 shows a sharp melting transition at 86.8°, and two broader melting zones at 90.5° and about 95°. Fraction 2 shows primarily two sharp transitions at 83.5° and 86.5°. Only a small step is seen at 90.5°. Fraction 3 shows a broader, only slightly structured, melting curve. The reversibility of these transitions was estimated by cooling the bath from about 100° to 60° over about 15 min, and measuring the absorbance after 5 min of equilibration at 60°. The reversibilities, expressed as the percentage of the total absorbance change that was reversed on cooling, were 54% for fraction 1, 63% for fraction 2, 24% for fraction 3, and 12% for the DNA from the main chromatographic band in Fig. 7*a*.

Comparison of the density gradient and melting-curve analyses of the three fractions permits one to assign a melting temperature, T_m, value to three of the satellite bands. These are 83.5°, 86.8°, and 90.5° for the bands with density ρ = 1.705, 1.714, and 1.719 g/cm^3, respectively. The small melting step at about 95° does not have a corresponding density band, because of the small amount of material present. It contributes to the breadth of the band at ρ = 1.719 g/cm^3 and probably corresponds to the small first elution peak obtained from sheared DNA on the PNR–hydroxyapatite column (Fig. 6).

The melting curve of fraction 3 cannot be unambiguously interpreted. It appears to contain some contribution from the 83.5° and 86.8° transitions, superimposed on a broader melting transition. The latter presumably arises from the material with density 1.710 g/cm^3. The low-melting tail can be ascribed to contamination by the main band DNA; the elution profile of the PNR–hydroxyapatite column is consistent with this interpretation. However, it is not clear why this material should have such a high density.

CONCLUSIONS

It is shown that the base-specific resolving power of hydroxyapatite for double-helical DNA can be substantially improved by use of the G·C-specific PNR. From viscosity and sedimentation studies it can be deduced that this dye binds to DNA by intercalation (14). The structural change produced in the DNA by the intercalation of this agent seems to be the reason for the easier elution of the complex from the hydroxyapatite since cations and anions other than phosphate are rather inefficient as eluting agents (15). The application of the developed method for the fractionation of calf-thymus DNA revealed resolving power similar to that of cesium chloride density gradient centrifugation. The satellites with ρ = 1.719 and ρ = 1.714 become visible in the elution profile with similar clarity. The differences in the basic principles of the two fractionation methods demonstrated by the chromatographic removal of a DNA fraction banding in the satellite region allowed the detection of the further satellite (ρ = 1.705). The combination of both methods should, therefore, be very effective.

It is not clear if the silver–cesium sulfate gradient (16, 17) is more efficient in fractionating DNA mixtures. It is clear however that the chromatographic method presented allows a much easier fractionation of larger quantities and a better fractionation of DNA particles small enough to yield broad, overlapping bands in a density gradient.

Note Added in Proof. In collaboration with W. Goebel we were recently able to work out a procedure for the separation of circular supercoiled DNA from linear duplexes by application of the same principles used in the present work. Ethidium bromide serves as the complexing agent for these separations performed on hydroxyapatite (manuscript in preparation). After acceptance of the present manuscript J. Filipski *et al.* (18) reported a detailed study on the subfractions of the bovine genome obtained by Cs_2SO_4–Ag^+ density gradient centrifugations. The satellites mentioned in the present paper are thoroughly characterized by the authors.

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1. Vinograd, J. (1963) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York and London), Vol. VI, pp. 854–870.
2. Chapiro, J. T., Leng, M. & Felsenfeld, G. (1969) *Biochemistry* **8**, 3219–3232.
3. Sueko, N. & Cheng, T. Y. (1962) *J. Mol. Biol.* **4**, 161–172.
4. Ayad, S. R. & Blamire, J. (1968) *Biochem. Biophys. Res. Commun.* **30**, 207–212.
5. Zeiger, R. S., Salomon, R., Dingman, C. W. & Peacock, A. C. (1972) *Nature New Biol.* **238**, 65–69.
6. Bernardi, G., Faures, M., Piperno, G. & Slonimski, P. P. (1970) *J. Mol. Biol.* **48**, 23–42.
7. Marmur, J. (1961) *J. Mol. Biol.* **3**, 208–218.
8. Thomas, C. A., Jr., Berns, K. I. & Kelly, T. J., Jr. (1966) in *Procedures in Nucleic Acid Research*, eds. Cantoni, J. & Davies, D. (Harper & Row Publ., New York and London), pp. 535–540.
9. Tiselius, A., Hjerten, S. & Levin, Ö. (1956) *Arch. Biochem. Biophys.* **65**, 132–155.
10. Derumez, P. & Biserte, G. (1970) *J. Chromatogr.* **49**, 563–567.
11. Piperno, G., Fonty, G. & Bernardi, G. (1972) *J. Mol. Biol.* **65**, 191–205.
12. Main, R. K., Wilkins, M. J. & Cole, L. J. (1959) *J. Amer. Chem. Soc.* **81**, 6490–6495.
13. Polli, E., Ginelli, E., Bianchi, P. & Corneo, G. (1966) *J. Mol. Biol.* **17**, 305–308.
14. Meyer, H. (1972) Diplomarbeit, University Braunschweig.
15. Bernardi, G. (1968) *Biochim. Biophys. Acta* **174**, 423–434.
16. Jensen, H. R. & Davidson, N. (1966) *Biopolymers* **4**, 17–32.
17. Corneo, G., Ginelli, E. & Polli, E. (1971) *Biochim. Biophys. Acta* **247**, 528–534.
18. Filipski, J., Thiery, J.-P. & Bernardi, G. (1973) *J. Mol. Biol.* **80**, 177–197.