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The Mitochondrial Genome of Wild-type Yeast Cells

I. Preparation and Heterogeneity of Mitochondrial DNA

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A new procedure for the preparation of yeast mitochondrial DNA has been developed. Its main advantages over previous methods are its rapidity, its flexibility in scale, and the quantitative yield in mitochondrial DNA.

Several properties of the mitochondrial DNA have been investigated. It has been found that yeast mitochondrial DNA (18% G + C) has a higher buoyant density, in the presence of Ag^+, than *Haemophilus influenzae* (38% G + C), or yeast nuclear DNA (39% G + C). Both the abnormally high buoyant density in Cs_2SO_4–Ag^+ density gradients and the abnormally high elution molarity from hydroxyapatite columns appear to be due mainly to the A + T-rich stretches present in mitochondrial DNA.

G + C-rich "molecules" (22% G + C; 13 s) have been isolated from the bulk of mitochondrial DNA by virtue of their lower elution molarity from hydroxyapatite, thus providing the first evidence that yeast mitochondrial DNA has an "intermolecular" compositional heterogeneity at a size level of about $1{\cdot}5 \times 10^6$ daltons. Fragments having a G + C content of 24% and an $S_{20,w} = 6{\cdot}6$ s could be prepared from these molecules by degradation with spleen acid DNase and centrifugation in a Cs_2SO_4–Ag^+ density-gradient.

1. Introduction

Mitochondria of wild-type (grande colonie) *Saccharomyces cerevisiae* cells contain DNA (Schatz, Haslbrunner & Tuppy, 1964; Tewari, Jayaraman & Mahler, 1965), which is necessary for RNA synthesis in isolated mitochondria (Wintersberger & Tuppy, 1965). DNA–RNA hybridization studies indicate that mitochondrial DNA codes for mitochondrial RNA's (Wintersberger, 1966; Fukuhara, 1967, 1970; Fukuhara, Faurès & Genin, 1969). These include ribosomal RNA's (Wintersberger & Viehauser, 1968), the heavy and light components of which have G + C contents of 25 to 27% or 30 to 33% (Fauman, Rabinowitz & Getz, 1969; Morimoto & Halvorson, 1971), transfer RNA's (Casey, Fukuhara, Getz & Rabinowitz, 1969; Cohen & Rabinowitz, 1970; Halbreich & Rabinowitz, 1971) and, possibly, messenger RNA's.

Chemical and physical investigations on mitochondrial DNA from wild-type yeast (Bernardi, Carnevali, Nicolaieff, Piperno & Tecce, 1968; Bernardi, Faurès, Piperno & Slonimski, 1970; Bernardi & Timasheff, 1970) have shown that: (a) its G + C content calculated from its physical properties (buoyant density, melting temperature, optical rotatory dispersion) does not agree with the analytical value (17·4%); this discrepancy is not due to base methylation or glucosylation; (b) its elution molarity

from hydroxyapatite is much higher than that of bacterial DNA's. These physical and chromatographic "anomalies" of yeast mitochondrial DNA are common in polydeoxyribonucleotides having repetitive sequences, like some synthetic polydeoxyribonucleotides and satellite DNA's (see Discussion). In sharp contrast with these very homogeneous materials, however, yeast mitochondrial DNA shows a striking compositional heterogeneity. Its melting profile is characterized by a main component ($T_m = 72°C$) and by a series of minor components, melting at temperatures up to 90°C. The main component appears to be formed by stretches of alternating dA-T·dA-T‡ containing non-alternating dA·dT structures and/or a low percentage of G·C base pairs. Optical rotatory dispersion and circular dichroism spectra suggest the presence of both alternating and non-alternating dA,T structures in yeast mitochondrial DNA. It should also be noted that the heterogeneity shown by the melting curve is essentially "intramolecular" at a size level of 2×10^6 daltons, as shown by the fact that mitochondrial DNA of this size still shows a single symmetrical peak when sedimented in a CsCl density-gradient. Another anomaly, likely to be connected with the heterogeneity of mitochondrial DNA and the presence of A+T-rich stretches in it, is the dependence of the kinetic complexity of yeast mitochondrial DNA upon its molecular size (Christiansen, Bak, Stenderup & Christiansen, 1971).

All the above results suggested to us that mitochondrial DNA might contain A + T-rich stretches, besides relatively G + C-rich coding stretches such as ribosomal and transfer RNA cistrons. While the latter might have a "normal" DNA structure, like bacterial DNA's, the former might be responsible for the anomalous physical and chromatographic properties of mitochondrial DNA. The two types of segments would be sufficiently intermingled so as not to give any evident intermolecular heterogeneity in DNA preparations of molecular weight as low as 2×10^6 daltons. The present series of investigations was started to study the molecular heterogeneity of mitochondrial DNA and to test the working hypothesis just presented. The long-term aim of this work is to get an insight into the structure and organization of the mitochondrial genome of yeast, which it is hoped might give a first step towards understanding, at the molecular level, the mechanism of the cytoplasmic "petite" mutation.

A pre-requisite for carrying out the present work was the availability of large amounts of mitochondrial DNA. We set up, therefore, a new procedure for the rapid and quantitative preparation of mitochondrial DNA on a large scale. This allowed us to detect a low level of intermolecular compositional heterogeneity in mitochondrial DNA and to recognize, at the same time, the feasibility of fractionating yeast mitochondrial DNA fragments according to their G + C contents on hydroxyapatite columns. In addition, the properties of yeast mitochondrial DNA revealed other anomalies. These investigations are reported in the present article. The following two papers deal, respectively, with the heterogeneity of mitochondrial DNA as studied by enzymic degradation (Piperno, Fonty & Bernardi, 1972) and the pyrimidine tracts of mitochondrial DNA (Ehrlich, Thiery & Bernardi, 1972). The results obtained in this series of investigations appear to support the working hypothesis mentioned above.†

† This work was presented at the International Meeting Les Acides Désoxyribonucléiques des Eukaryotes organized by the Centre National de la Recherche Scientifique at Port-Cros, France, 5 to 9 May 1971.

‡ We have followed the recommendations of the IU PAC-IU B Commission on Biochemical Nomenclature whenever they apply. In addition we have used self-explanatory expressions like "alternating dA-T·dA T sequences", "non-alternating dA·dT sequences", "alternating and non-alternating dA,T sequences", "A + T-rich satellite", "A + T-rich stretches", "G + C-rich stretches".

2. Materials and Methods

(a) *Yeast strain and cell growth*

The *Saccharomyces cerevisiae* strain used in this series of investigations was the respiratory-sufficient, haploid strain D-243-2B-R1, previously indicated as strain A (see Bernardi *et al.*, 1970).

300-ml. cell cultures grown for 14 hr at 28°C (about 8 g of cells, wet weight) were used as inocula for 12-l. cultures; these were grown for 24 hr in a New Brunswick FS300 fermentor (New Brunswick, N.J.) using the antifoam agent 81–35 B (Société Industrielle des Silicones, Paris VIII, France). The medium contained: 1% yeast extract, 5% glucose, 0·12% $(NH_4)_2SO_4$ and 0·01% adenine. Cells were harvested by centrifugation in a Sharples centrifuge (Warminster, Pa.). About 25 g (wet weight) of cells were collected/l. of culture. Cells obtained from 12-l. cultures were then washed with two 600-ml. volumes of 0·15 M-NaCl and centrifuged in a Lourdes centrifuge (Brooklyn, N.Y.) at 7000 ***g***. Each culture was checked for the absence of bacterial contamination and of induced cytoplasmic petite mutants. The level of spontaneous cytoplasmic petite mutants was estimated to be of the order of a few per cent.

(b) *Extraction of nucleic acids*

10-g (wet weight) portions of cells were suspended in 10 ml. of cold (4°C) 0·15 M-NaCl, transferred to a 75-ml. flask containing 50 g of glass beads (0·45 to 0·50 mm in diameter) and homogenized at maximum speed for 70 or 20 sec in a Braun homogenizer (Melsungen, Germany). The 75-ml. flask was cooled before and during homogenization by a jet of liquid CO_2 so that the temperature of the homogenate at the end of the operation was close to 0°C. The homogenate was immediately diluted with 70 ml. of a 0·15 M-NaCl solution, then adjusted to 2·5% sodium dodecyl sulphate, and stirred at room temperature for 0·5 hr. Glass beads and cell debris were centrifuged off at room temperature, washed twice with 30 ml. of 0·15 M-NaCl containing 2·5% sodium dodecyl sulphate. The pooled supernatant and washings were stirred for 2 to 4 hr at room temperature, adjusted to 1 M-NaCl by addition of crystals, stored overnight in an ice-bath with stirring and centrifuged. The supernatant fraction was precipitated with 1 vol. of cold 95% ethanol. The precipitate was collected by centrifugation, dissolved in half its original volume of 0·01 M-NaCl, adjusted to 0·15 M-NaCl and 1·5% sodium dodecyl sulphate and stirred for 0·5 hr. The solution was again adjusted to 1 M-NaCl, stirred in an ice-bath overnight, recentrifuged and precipitated with 1 vol. of 95% ethanol. The precipitate, collected by centrifugation, was dissolved in 10 ml. of 0·01 M-NaCl, adjusted to 0·15 M-NaCl, shaken with 1 vol. of chloroform–isoamyl alcohol (24:1; v/v) and centrifuged for 1 hr at 28,000 rev./min in a Spinco L2 preparative ultracentrifuge using a type 30 rotor. The aqueous phase is the starting nucleic acid preparation from which mitochondrial DNA was extracted.

The absence of pancreatic RNase treatment in the preparation procedure just described was prompted by experiments showing that this avoided the formation of G-rich polyribonucleotides which complicate the purification of mitochondrial DNA on hydroxyapatite columns (Bernardi *et al.*, 1968, 1970; Piperno & Bernardi, 1970). The yeast nucleic acid extracts used in these experiments were prepared by grinding frozen yeast cells with kieselguhr (Hyflo Super-Cel; Johns Manville, New York, N.Y.) as already described (Bernardi *et al.*, 1968). The paste was dispersed in 0·15 M-NaCl–0·05 M-EDTA, pH 8·0 containing 2% sodium dodecyl sulphate and centrifuged to eliminate kieselguhr and cell debris. The extract was then shaken with chloroform–isoamyl alcohol and centrifuged; the aqueous phase was precipitated with 2 vol. of ethanol.

(c) *Purification of mitochondrial DNA*

The purification of preparation IV of Table 1 is described as an example. A scheme of this procedure is shown in Fig. 1. A nucleic acid solution in 0·15 M-NaCl (538 ml.; 118,000 A_{260} units; 106 mg of DNA as estimated by the diphenylamine reaction (Dische, 1955), was adjusted to 0·27 M-sodium phosphate buffer, pH 6·8 and to a final volume of 1500 ml. Packed hydroxyapatite (500 ml.), prepared as described by Bernardi (1971*a*), suspended and equilibrated with 0·27 M-sodium phosphate buffer was added to the nucleic acid solution.

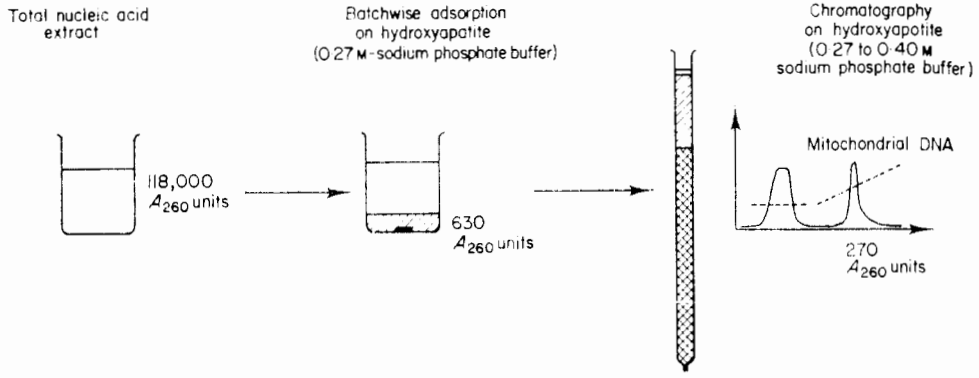

FIG. 1. Scheme of procedure for the preparation of yeast mitochondrial DNA (preparation IV; see Table 1).

The suspension was stirred overnight at room temperature using a magnetic stirrer operated at a speed just great enough to keep the hydroxyapatite crystals in suspension. The crystals were then allowed to sediment and the supernatant fraction was syphoned off. The sediment was washed with two 1000-ml. volumes of 0·27 M-sodium phosphate buffer, suspended in 1000 ml. of 0·27 M-sodium phosphate buffer and loaded on the top of a 4·7 cm × 104 cm hydroxyapatite column equilibrated with 0·27 M-sodium phosphate buffer (the outlet of the column being closed). The supernatant fraction (1030 ml.; A_{260} = 1·08) was syphoned off. The column, now 4·7 cm × 124 cm, was washed with 750 ml. of 0·27 M-sodium phosphate buffer; 360 A_{260} units were washed down during this step. A linear molarity gradient, 0·27 to 0·40 M-sodium phosphate buffer (total volume 6 l.), was then used to elute 273 A_{260} units of mitochondrial DNA. This represented, therefore, less than 0·25% of the starting A_{260} units.

In order to recover any mitochondrial DNA still present in the non-adsorbed fractions, these were pooled and re-extracted with 100 ml. of hydroxyapatite equilibrated with 0·27 M-sodium phosphate buffer. They were then adjusted to 0·25 M and extracted once more with 100 ml. of hydroxyapatite equilibrated with 0·25 M-phosphate buffer. The DNA so obtained was chromatographed on hydroxyapatite columns; fractions containing mitochondrial DNA were processed further (see Results, section (d)); an additional 8% of mitochondrial DNA was thus recoverd.

(d) *Ultracentrifugation experiments*

Analytical caesium chloride density-gradient experiments were done as previously described (Bernardi *et al.*, 1970). A Spinco model E ultracentrifuge equipped with a monochromator, an electronic scanner and a multiplexer were used in most experiments. Scanning was done at 265 nm and at a slit width of 0·11 mm. A scanning speed of 1·25 cm/min was used along with a chart speed of 30 cm/min; this corresponds to an enlargement of 24 along the abscissa. Scannings were also done with a scanning speed of 5·63 cm/min; these scannings, in which the enlargement on the abscissa was only about 5, were used in the Figures.

Preparative caesium sulphate density-gradient experiments were performed in the presence of either Hg^{2+} or Ag^{+} (Davidson *et al.*, 1965) in a Spinco centrifuge model L2-65 B, using a type 65 aluminium rotor. The Ag^{+}/DNA-phosphate molar ratio was 0·34; the Hg^{2+}/DNA-phosphate molar ratio was 0·1. Solutions were spun for 18 hr at 58,000 rev./min at 20 to 25°C. The bottoms of the tubes were pierced and their contents were emptied at a constant flow rate of 12 ml./hr.

Sedimentation velocity experiments were performed as already described (Bernardi *et al.*, 1970).

(e) *Absorbance–temperature experiments*

These were done using the automatic equipment of Rump, Chambron & Roy (1970). The only differences from previous experiments (Bernardi *et al.*, 1970) were that absorbance readings were taken at 0·5 deg. C intervals and differentiation was done on smooth tracings through the experimental points.

(f) *Base composition of DNA*

This was determined by enzymically degrading DNA to nucleosides and by analysing nucleosides on columns of Bio-Gel P2 (Bio-Rad, Richmond, Calif., minus 400 mesh; fraction C) according to the method of Carrara & Bernardi (1968), as modified by Piperno & Bernardi (1971).

(g) *Other DNA's and polydeoxyribonucleotides*

Haemophilus influenzae and *Cancer pagurus* DNA's were prepared using the detergent procedure (Kay, Simmons & Dounce, 1952). Poly[d(A-T)·d(A-T)] was purchased from Miles, Kankakee, Ill.; poly(dA·dT) from Biopolymers, Chagrin Falls, Ohio.

3. Results

(a) *Extraction of nucleic acids and preparation of mitochondrial DNA*

Figure 2 shows the hydroxyapatite chromatogram of a nucleic acid extract from yeast, obtained after grinding cells with kieselguhr. The main peak is formed by ribosomal RNA; this is followed by nuclear and mitochondrial DNA's. The fractions containing mitochondrial DNA were digested with pancreatic DNase; when re-run on the column, no material eluting in the mitochondrial DNA range could be detected. This experiment was used to demonstrate the absence of the G-rich polyribonucleo-

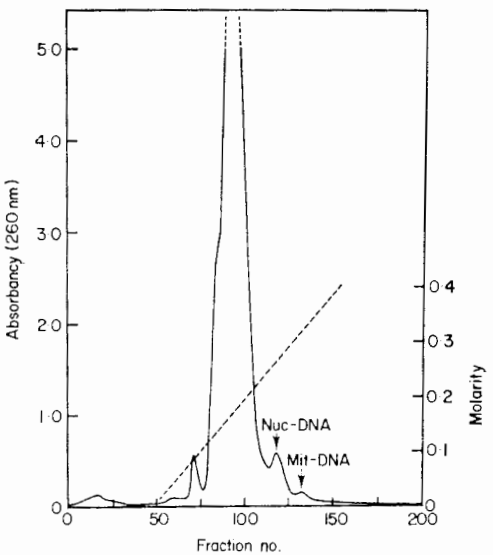

FIG. 2. Chromatography of a nucleic acid extract from yeast.
120 ml. of nucleic acid solution in 0·001 M-sodium phosphate buffer (1650 A_{260} units) were loaded on a 2 cm × 46 cm hydroxyapatite column equilibrated with the same buffer. 200 ml. of this solvent were used to wash the column and a 0·001 to 0·6 M gradient of sodium phosphate buffer (1400 ml.) was applied. 8-ml. fractions were collected. Flow rate was 48 ml./hr. A_{260} recovery was 93%. The absorbancy at the main peak maximum is equal to 16·6. Nuc-DNA, nuclear DNA; Mit-DNA, mitochondrial DNA.

tides (Piperno & Bernardi, 1970), which interfere with the chromatographic purification of mitochondrial DNA (Bernardi *et al.*, 1968,1970). As shown in Figure 2, the direct chromatography on hydroxyapatite of a total nucleic acid extract can be used to prepare mitochondrial DNA; this procedure, however, does not lend itself easily to an important scaling up, because of the excessively large amounts of hydroxyapatite which would be necessary.

Figure 3 shows the hydroxyapatite chromatogram of mitochondrial DNA obtained from preparation IV, using the batchwise adsorption procedure. The material eluted by the molarity gradient showed up as a single peak. This material represents more than 90% of the mitochondrial DNA present in the nucleic acid extract, as judged from the chromatography of the nucleic acids re-extracted with hydroxyapatite (see section (d), below).

Chromatographic fractions were checked for both purity and homogeneity by using the CsCl density-gradient technique. Fractions on the very far left of the chromatogram contained essentially pure nuclear DNA ($\rho = 1{\cdot}698$ g/cm^3; Fig. 3(a)). These were followed by fractions containing decreasing amounts of nuclear DNA (Figs 3(b) and 3(c)) and pure mitochondrial DNA (Fig. 3(d); $\rho = 1{\cdot}682$ g/cm^3). The mitochondrial DNA present in the far left fractions showed higher buoyant densities ($\rho = 1{\cdot}688$ g/cm^3 for fraction 59, Fig. 3(b); $\rho = 1{\cdot}685$ g/cm^3 for fraction 60, Fig. 3(c)) than the bulk of mitochondrial DNA. Further investigations on the "intermolecular" compositional heterogeneity in mitochondrial DNA are presented in section (d), and in the following paper.

Table 1 summarizes the results obtained in four independent extractions of yeast

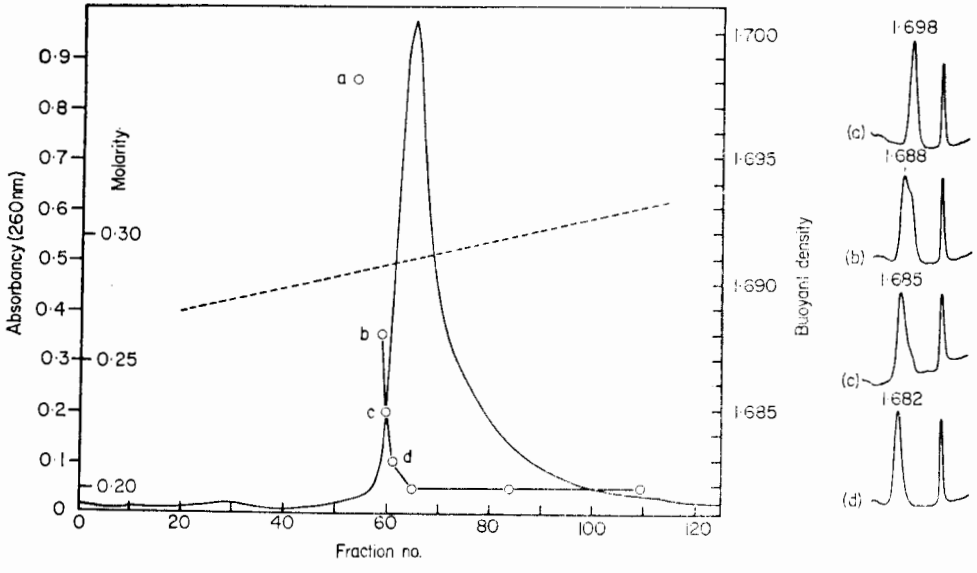

FIG. 3. Chromatography of mitochondrial DNA (preparation IV of Table 1). 21-ml. fractions were collected. The flow rate was 120 ml./hr. All other chromatographic conditions are described in Materials and Methods.

(a), (b), (c) and (d) show the microdensitometer tracings of fractions 54, 59, 60 and 65, respectively. DNA was centrifuged at 44,770 rev./min for 20 hr in a CsCl density gradient. DNA from phage 2C (1·742 g/cm^3) was used as a density marker. Buoyant densities, in g/cm^3, are indicated in the Figure (circles) and in the inserts.

TABLE 1

Extraction of yeast DNA's

Preparation	Wet weight of cells (g)	Homogenization time (sec)	Total DNA† (per g of cells) (μg)	Mitochondrial DNA‡ (per g of cells) (μg)	Total mitochondrial DNA recovered (mg)	Mitochondrial DNA (as % of total DNA)	$S_{20,w}$ of mitochondrial DNA (s)
I	740	70	274	38	56·8	13·8	12
II	755	70	277				
III	530	20	181				
IV	545	20	195	25 (35)§	13·6	12·8	14

† As estimated by diphenylamine reaction.
‡ As obtained from hydroxyapatite chromatography.
§ When corrected for 70% recovery of total DNA from 20-min homogenates.

DNA's. The yields of total (nuclear + mitochondrial) DNA varied according to homogenization time, being lower for shorter times. Microscopy of the homogenates indicated that the latter result was due to incomplete cell breakage. The amount of mitochondrial DNA actually recovered from cells homogenized for 70 minutes was 35 to 38 μg/g of cells (wet weight). This quantity represents 13 to 14% of total DNA and is 6 to 7 times larger than that previously obtained (Bernardi *et al.*, 1970) from the same yeast strain.

(b) *Characterization of mitochondrial DNA*

The important differences between this and the previous preparative procedures prompted a characterization of the mitochondrial DNA preparations obtained in the present work. This characterization was done on the pooled fractions 58 to 115 of the chromatogram shown in Figure 3. This material contained less than 1% of the nuclear DNA as estimated from the densitograms obtained with the early eluting fractions. On the other hand, the mitochondrial DNA not adsorbed in the first batch experiment was not included in the pooled fractions. This represented about 8% of total mitochondrial DNA (see section (d)).

The characterization of mitochondrial DNA showed that its G + C content was 18·2%, its buoyant density 1·682 g/cm^3, and its melting temperature 74·6°C. These results are in agreement with those previously reported (G + C = 17·4%; ρ = 1·683 g/cm^3; T_m = 74·7°C) within experimental error. The differential melting profile was also very similar to that previously reported (Bernardi *et al.*, 1970). The first melting component showed up as a sharper peak in the present work; this is explained by the fact that average data from five independent experiments were used in previous work, thus leading to some flattening of the peak. The amount of the first melting component, as estimated by integration of the area under the peak, was equal to about 50% of total DNA.

A few additional points, not investigated in previous work, were studied using the new preparations. (a) The melting curve of mitochondrial DNA was also investigated at 280 nm in order to obtain an estimate of the G·C contribution to the different parts of the melting curve. It is known from the work of Felsenfeld & Hirschman (1965), that the contribution of G·C base pairs to the hyperchromicity of DNA is more important than that of A·T pairs at 280 nm, whereas both contributions are practically equal at 260 nm. Figure 4 shows the comparison of the differential melting curves, as obtained at both 280 and 260 nm. It is interesting that the 280 nm pattern slightly trails the 260 nm pattern; as expected, the intensity of the 280 nm curve is lower in the low-melting and higher in the high-melting region. (b) A striking property of mitochondrial DNA was discovered when studying its buoyant density in Cs_2SO_4–Ag^+ gradients, namely, that it appeared to become heavier, in the presence of Ag^+, than bacterial DNA's containing higher G + C levels. Figures 5(a) and 8 show that *H. influenzae* DNA (38% G + C) and nuclear yeast DNA (39% G + C), respectively, appear to have a lower buoyant density in Cs_2SO_4–Ag^+ gradient than mitochondrial DNA (18% G + C). In addition, as shown below and in the following paper, when mitochondrial DNA is degraded, the A + T-rich fragments are heavier than the G + C-rich ones. This anomalous behaviour was not found when studying the binding of mercury ions (Fig. 5(b)), since mitochondrial DNA showed the expected higher buoyant density than *H. influenzae* DNA; in this case, however, a heterogeneity of the G + C-rich side of mitochondrial DNA was evident.

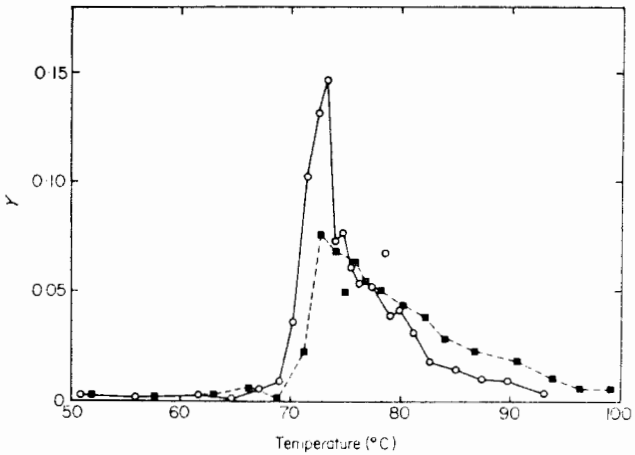

FIG. 4. Differential melting curves of mitochondrial DNA. The ordinate indicates the increment in relative absorbance per degree: $Y = \frac{At_1 - At_2}{A_{100} - A_{25}} / t_1 - t_2$, where At_1, At_2, A_{100}, A_{25}, are absorbances measured at temperatures t_1, t_2, 100 and 25°C, respectively. —○—○—, 260 nm; ——■——■——, 280 nm.

(c) *Chromatographic behaviour of yeast mitochondrial DNA on hydroxyapatite*

Yeast mitochondrial DNA is eluted from hydroxyapatite columns at a much higher molarity than nuclear yeast DNA and other animal cell and bacterial DNA's (Bernardi *et al.*, 1968). Since the very high elution molarity of mitochondrial DNA might be somehow connected with its high A + T level, the elution patterns of the A + T-rich satellite DNA from *Cancer pagurus* (3% G + C) and of biosynthetic poly [d(AT)·d(AT)] and poly(dA·dT) were investigated. The crab d(A-T) satellite was not separated from the main DNA under experimental conditions allowing mitochondrial DNA to be completely separated from nuclear DNA; the high-eluting fractions of total crab DNA were,

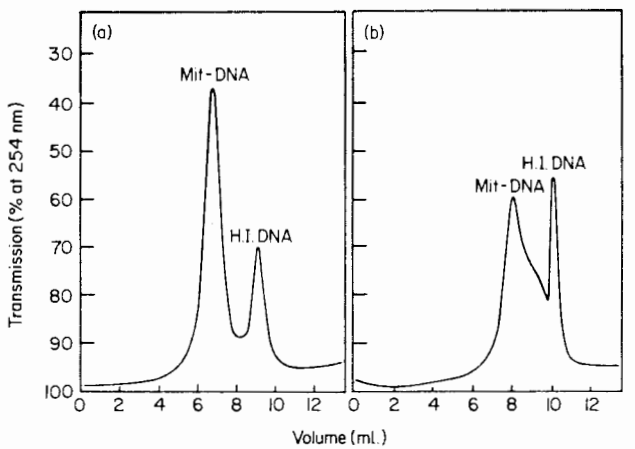

FIG. 5. (a) Ag^+ and (b) Hg^{2+} — Cs_2SO_4 preparative density gradients of a DNA mixture formed by 1·6 A_{260} units of mitochondrial DNA (preparation I + II) and 0·4 A_{260} unit of *Haemophilus influenzae* DNA, as recorded by a Uvicord, using a cell with an optical path equal to 0·3 cm. Bottom of the tube at left.

however, enriched in the A + T-rich satellite component, as shown by CsCl density gradient experiments. In agreement with this finding, the alternating biosynthetic polymer showed a slightly, yet significantly higher elution molarity than *Escherichia coli* DNA, taken as a reference DNA. The non-alternating polymer showed, instead, a very high eluting molarity, 0·5 M-sodium phosphate buffer. Since this molarity is so much higher than that of poly(rA·rU), 0·20 M-potassium phosphate buffer, and is close to that of poly(rA·2 rU), 0·45 to 0·50 M (Bernardi, 1965), the material eluted at 0·5 M was analysed. This showed that the dA/dT molar ratio was equal to 1.

(d) *Heterogeneity of mitochondrial DNA*

The heterogeneity of mitochondrial DNA detected in the low-eluting fractions of the chromatogram shown in Figure 3 was further investigated in the fractions re-extracted from the nucleic acids which had not been adsorbed in the first batch operation. The two fractions, re-extracted at 0·27 and 0·25 M-sodium phosphate buffer, respectively (see Materials and Methods), contained nuclear and mitochondrial DNA; the latter represented 6% and 2%, respectively, of total purified mitochondrial DNA. These fractions were pooled together and rechromatographed once more (Fig. 6). As in the chromatogram of Figure 3, contaminating nuclear DNA was present in the left-hand side of the chromatogram, where the buoyant densities of mitochondrial DNA range from 1·686 to 1·689 g/cm^3.

The densitograms obtained permitted us to estimate the relative amounts of nuclear and mitochondrial DNA's in the low-eluting fractions of the chromatogram (see Fig. 6). Interestingly enough, the densitograms of the nuclear DNA-rich fractions showed the presence of a shoulder having a density of 1·705 g/cm^3 and corresponding to nuclear satellite DNA (this is not evident on the compressed scanning shown in Fig. 6). Another observation made while investigating this chromatogram was that the S value of fraction 36, containing virtually pure nuclear DNA (Fig. 6(a)) was equal to 18 s, whereas the mitochondrial DNA of fraction 84 (Fig. 7(e)) was equal to 13 s.

Fractions 42 to 56 of the chromatogram shown in Figure 6 were re-run on a similar column, the slope of the gradient being half of that of the previous chromatogram. The low-eluting third of this chromatogram, formed by nuclear DNA, was discarded, whereas the higher-eluting fractions were re-run once more using the same conditions as in the chromatogram of Figure 6. Figure 7 shows the results obtained, as well as the CsCl density-gradients of several fractions. The patterns changed along the chromatogram: the early fractions still contained a sizeable amount of nuclear DNA (Fig. 7(a) and (b)); a trace of nuclear DNA is still present in fraction 80 (Fig. 7(c)); this is clearly evident only on the expanded scannings. Finally, fractions 82 and 84 appear to be completely free of nuclear DNA (Fig. 7(d) and (e); see, however, below). The densities of the last three fractions, can therefore be estimated very precisely; they decrease from 1·690 to 1·688. It can be estimated that the amount of mitochondrial DNA having a density higher than 1·688 g/cm^3 is at least 1% of total mitochondrial DNA. This material has a sedimentation coefficient of 13·0 s and therefore, using the relationship of Bernardi & Richards (Bernardi & Sadron, 1964; Bernardi 1968)†, a molecular weight of the order of $1{\cdot}5 \times 10^6$ daltons. A base-composition determination done on fraction 84 showed that its G + C content was 21·1%.

The mitochondrial DNA of the chromatogram shown in Figure 7 was completely

† The relationship $S_{20,w} = 0{\cdot}057\ (\text{mol. wt})^{0{\cdot}382}$ was established by determining sedimentation coefficients and light-scattering molecular weights for calf thymus DNA degraded by acid DNase from hog spleen.

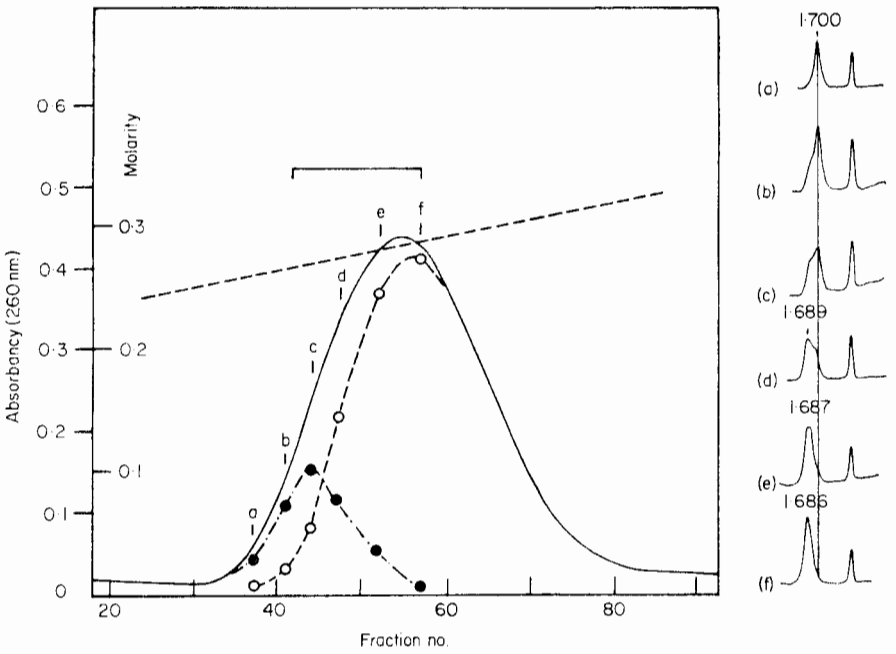

FIG. 6. Chromatography of DNA fractions recovered by re-extraction with hydroxyapatite (see Materials and Methods, section (c)). 1050 ml. of DNA solution adjusted to 0·23 M-sodium phosphate buffer (30 A_{260} units) were loaded on a 1·3 cm × 65 cm column equilibrated with the same buffer. A 0·23 to 0·5 M gradient of sodium phosphate buffer (550 ml.) was applied. Fractions of 2·7 ml. were collected. Flow rate was 40 ml./hr. (a), (b), (c), (d), (e) and (f) show the scanner tracings of DNA from chromatographic fractions 37, 41, 44, 47, 52 and 57, respectively. DNA was centrifuged at 44,000 rev./min. for 21 hr in a CsCl density-gradient. DNA from phage 2C (1·742 g/cm^3) was used as density marker. Buoyant densities in g/cm^3 are indicated in the inserts. Filled and empty circles show the amounts of nuclear and mitochondrial DNA, respectively, in fractions 37 to 57; these were estimated by resolving into Gaussian curves the peaks recorded during the CsCl density-gradient experiments. The horizontal bar indicates the pooled fractions used for the purification of G + C-rich mitochondrial DNA fragments.

freed from nuclear DNA in the following way. The low-eluting half of the chromatogram (fractions 75 to 84), containing material ranging in density from 1·693 to 1·688 g/cm^3 underwent two subsequent centrifugations in Cs_2SO_4–Ag^+ density-gradients (Fig. 8(a) and (b)). This purified material had a G + C content of 22%, a buoyant density of 1·689 g/cm^3 and a molecular weight close to $1{\cdot}5 \times 10^6$ daltons. A single Cs_2SO_4–Ag^+ density-gradient was sufficient to remove nuclear DNA from the high-eluting half of the chromatogram (Fig. 8(c)). The pooled, purified mitochondrial DNA fractions were degraded with spleen acid DNase, an enzyme known for its ability to split both DNA chains at the same level (Bernardi, 1971*b*; Bernardi & Sadron, 1961), to an average S value of 7·8 s. The compositional heterogeneity of degraded DNA was assessed by analysing fractions obtained from a preparative Cs_2SO_4–Ag^+ density-gradient (Fig. 8(d)). About 7% of the degraded material showed a G + C content of 24%; it is interesting that two neighbouring fractions showed the same base-composition, thus confirming the absence of contaminating nuclear DNA on the light side of the peak. The sedimentation coefficient of the 24% G + C material was 6·6 s. An attempt to establish its buoyant density in CsCl failed because of the broadness of the

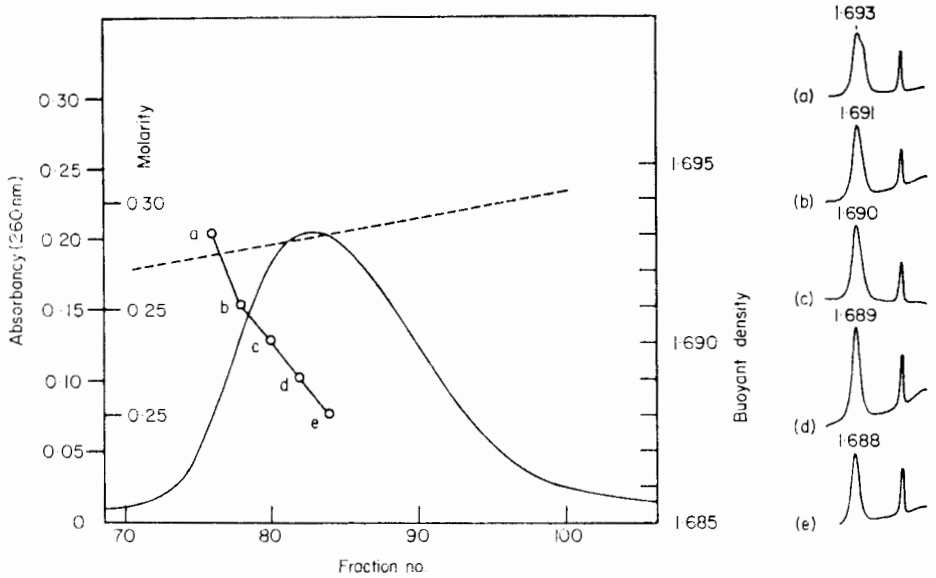

FIG. 7. Chromatography of the G + C-rich mitochondrial DNA fractions from the chromatogram shown in Fig. 6. 130 ml. of DNA solution in 0·23 M-sodium phosphate buffer (9 A_{260} units) were loaded on a hydroxyapatite column. All chromatographic conditions were as in Figure 6. (a), (b), (c), (d) and (e) show the scanner tracings of DNA from chromatographic fractions 76, 78, 80, 82 and 84, respectively. DNA was centrifuged in a CsCl density-gradient as described in Fig. 6. Buoyant-density values in g/cm^3 are shown by the circles and in the inserts.

peak obtained. A side-issue of the experiment shown in Figure 8(d) is the finding that, among mitochondrial DNA fragments, those which are higher in G + C are lighter and *vice versa*; this is in sharp contrast with the behaviour of a normal DNA (see also the following paper).

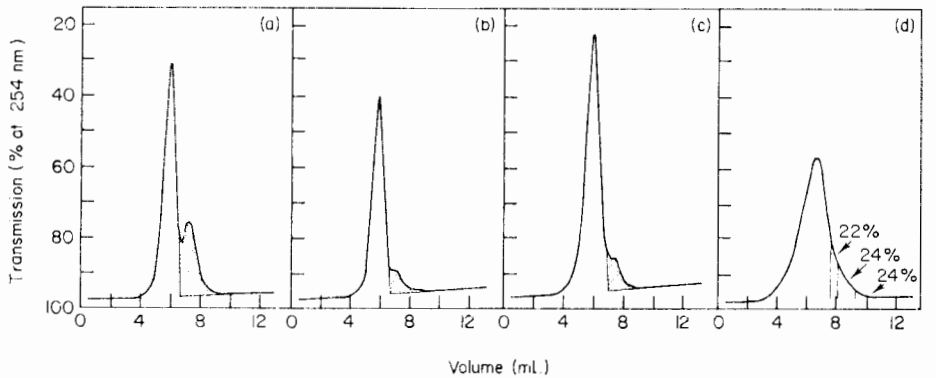

FIG. 8. Ag^+–Cs_2SO_4 preparative density-gradients of G + C-rich mitochondrial DNA fractions as recorded by a Uvicord using a cell with an optical path equal to 0·3 cm. Recordings refer to: (a) the first run and (b) the second run of fractions 75 to 84; (c) the run of fractions 85 to 94 from the chromatogram shown in Fig. 7; (d) pooled purified mitochondrial DNA fractions from gradients (b) and (c) after degradation with spleen acid DNase; the shadowed areas indicate the material which was discarded. Bottom of the tubes at left.

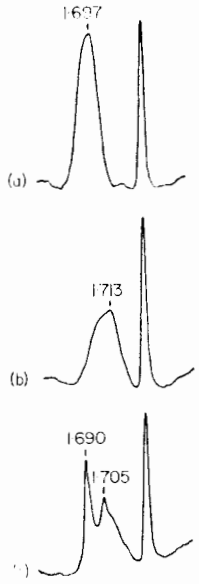

FIG. 9. Microdensitometer tracing of DNA from a chromatographic fraction (see text) containing a mixture of mitochondrial and nuclear DNA centrifuged in a CsCl density-gradient as described in Fig. 3.
(a) Native; (b) heat-denatured; (c) renatured.

The following results show that contaminating nuclear DNA can be distinguished from high-density mitochondrial DNA, even under the most adverse conditions, using a simple denaturation–renaturation procedure. In an experiment similar to that shown in Figure 7, but done on a mitochondrial DNA (preparation I + II of Table 1) having a lower S value (12 S), the low-eluting fractions were predominantly formed by nuclear DNA, as shown by their buoyant density of 1·697 g/cm^3 (Fig. 9(a)). After heat-denaturation, these fractions showed a main peak having the density of denatured nuclear DNA (ρ = 1·713 g/cm^3) and a lighter shoulder (Fig. 9(b)). After renaturation (5 hr at 65°C in 2 × SSC (SSC is 0·015 M-NaCl, 0·15 M-sodium citrate)), the buoyant-density pattern showed a sharp peak of mitochondrial DNA banding at 1·690 g/cm^3, a spike banding at 1·705 g/cm^3 and a heavier shoulder (Fig. 9(c)). This appearance of renatured nuclear DNA had already been seen before (Bernardi *et al.*, 1970) and is due to the presence of a fast-renaturing satellite of native buoyant density equal to 1·705 g/cm^3. The chromatographic fractions following those mainly formed by nuclear DNA, just described, contained a mitochondrial DNA having a density of 1·690 g/cm^3. It is worth noting that the amount of mitochondrial DNA having a density of 1·690 g/cm^3 represented at least 3% of total mitochondrial DNA in this case, whereas in preparation IV only 1% of the material had a density higher than 1·688 g/cm^3. Not unexpectedly, the percentage of high-density mitochondrial DNA is higher when the molecular weight of the preparation is lower.

4. Discussion

(a) *Extraction of nucleic acids and preparation of mitochondrial DNA*

The new procedure for the preparation of yeast mitochondrial DNA described here represents a great improvement over previous methods. Its main advantages are its

rapidity, its flexibility in scale, and the quantitative yield in mitochondrial DNA. The procedure involves three basic steps: (a) cell breakage; (b) nucleic acid extraction; (c) selective adsorption of mitochondrial DNA on hydroxyapatite.

The method of cell breakage mainly used in the present work yielded rather degraded DNA. We have not yet investigated in detail the degradation of DNA during homogenization; but it is probable that this is due not only to mechanical shearing, but also to enzymic attack. It is significant that breakage of cells by grinding with kieselguhr as done both in the present and in previous work (Bernardi *et al.*, 1968) did not yield DNA of higher molecular weight than homogenization with the Braun instrument.

The extraction of nucleic acid was done following the classical detergent procedure of Kay *et al.* (1952); control experiments showed that nucleic acid extraction was complete. As already mentioned, the homogenate was not treated with pancreatic RNase, since this caused the formation of G-rich polyribonucleotides, which have a chromatographic behaviour on hydroxyapatite interfering with that of mitochondrial DNA.

The selective adsorption of mitochondrial DNA on hydroxyapatite is the essential step in the new procedure. It should be noted that, in routine operation, the mitochondrial DNA adsorbed on hydroxyapatite in the second and third batch operation can be pooled with the first extract. A single chromatographic step then permits the quantitative preparation of mitochondrial DNA. It is advisable when using hydroxyapatite and yeast nucleic acid preparations different from those described in the present work, to establish in preliminary experiments the phosphate molarity at which the selective adsorption of mitochondrial DNA takes place.

In the present work the fraction of mitochondrial DNA not adsorbed in the first batch was processed separately for two reasons: (a) by running the two re-extracts on hydroxyapatite columns, it could be seen that only 6% of mitochondrial DNA was present in the first re-extract, and only 2% in the second one; this indicates that, after the two re-extractions, an essentially quantitative yield of mitochondrial DNA is obtained; (b) the higher G + C contents of the low-eluting fractions of mitochondrial DNA suggested that the material not adsorbed in the first batch might be enriched in G + C. This proved to be the case (see section (c), below).

Several data indicate that the G + C-rich mitochondrial DNA "molecules" can be unequivocally distinguished from nuclear DNA: (1) denaturation–renaturation experiments, such as those presented in Figure 9, clearly distinguish the two different DNA's even under the most unfavourable conditions; (2) the buoyant density in Cs_2SO_4–Ag^+ density gradients also discriminates very satisfactorily G + C-rich mitochondrial DNA and nuclear DNA (Fig. 8). It should be noticed that, from a practical point of view, the Cs_2SO_4–Ag^+ density-gradient centrifugation technique is a very powerful tool for resolving minute contamination from nuclear DNA which would escape detection by the conventional analytical CsCl method. This is shown, for example, by a comparison of the densitograms of fractions 82 and 84 of the chromatogram of Figure 7 with the preparative gradient in Cs_2SO_4–Ag^+ of Figure 8(c) (notice that transmittance, not absorbance, was monitored in the latter case); (3) there is a discontinuity in buoyant density between nuclear and mitochondrial DNA; in fact, no densities comprised between that of nuclear DNA (1·699 g/cm^3) and the highest density of mitochondrial DNA (1·691 g/cm^3) were ever found; (4) there is a discontinuity in size at the transition between nuclear and mitochondrial DNA in

hydroxyapatite chromatograms, the largest nuclear DNA molecules (18 s) being those eluted before mitochondrial DNA (13 s). Since the average S value of nuclear DNA is practically identical with that of mitochondrial DNA, the finding that the highest eluting "tail" of nuclear DNA has an S value of 18 s seems to be explained by some fractionation according to DNA size on the column (see the following paper).

(b) *Physical and chromatographic properties of mitochondrial DNA*

Yeast mitochondrial DNA is anomalous, when compared to bacterial DNA's, in all physico-chemical properties investigated so far: adsorption on hydroxyapatite (Bernardi *et al.*, 1968); buoyant density, melting temperature (Bernardi *et al.*, 1970); optical rotatory dispersion, circular dichroism (Bernardi & Timasheff, 1970); and binding of silver ions (present work). In all these respects, yeast mitochondrial DNA shares the anomalous behaviours of synthetic polydeoxyribonucleotides having simple repetitive sequences of mononucleotides (poly(dA·dT); poly(dG·dC)), dinucleotides (poly[d(A-T)·d(A-T)]; poly[d(T-C)·d(G-A)]; poly[d(T-A)·d(G-T)], or trinucleotides (poly[d(T-A-C)·d(G-T-A)]), or complex repetitive nucleotide sequences like the A + T-rich crab satellite and the satellite DNA's from guinea pig and mouse tissues. These "anomalies" are very briefly summarized as follows.

We have just reported that both poly d(A-T)·d(A-T); poly(dA·dT) and the A + T-rich satellite from *Cancer pagurus* show a peculiar behaviour on hydroxyapatite columns, being eluted at slightly or markedly higher phosphate molarities compared to bacterial DNA's. Similar findings have been obtained for the satellite DNA's from calf thymus (Corneo, Zardi & Polli, 1970; M. André & G. Bernardi, unpublished observations) and from mouse and guinea pig liver (Corneo *et al.*, 1970).

The buoyant density, melting temperatures, optical rotatory dispersion and circular dichroism of the synthetic polydeoxyribonucleotides with repetitive mono-, di-, or trinucleotide sequences do not usually agree (Wells & Blair, 1967; Wells, Larson, Grant, Shortle & Cantor, 1970) with those expected on the basis of their composition and the empirical relationship of Schildkraut, Marmur & Doty (1962), Marmur & Doty (1962) and Samejima & Yang (1965), respectively. A similar disagreement exists for the buoyant density and melting temperature of A + T-rich satellite of crab, and for the satellite DNA's from mouse and guinea pig (Corneo, Ginelli, Soave & Bernardi, 1968).

Finally, the binding of silver ions has been found to be anomalous for guinea pig satellite DNA (Corneo *et al.*, 1968) and the A + T-rich satellite of *Cancer pagurus* (unpublished results from our laboratory).

These "anomalous" phenomena appear to be due to the fact that the physical properties of all polydeoxyribonucleotides mentioned above are not simply the average properties of an enormous number of different nucleotide sequences, as in the case of bacterial DNA's, but are those of certain specific nucleotide sequences. In the case of yeast mitochondrial DNA, the anomalous properties appear to be due mainly to the presence of A + T-rich stretches. In fact, the higher eluting molarity from hydroxyapatite columns and the higher binding of Ag^+ of mitochondrial DNA can be explained by its content of A + T-rich structures. More specifically, both anomalies appear to be associated with the presence of non-alternating dA·dT sequences, since alternating dA-T·dA-T structures have only a slightly higher affinity for hydroxyapatite compared to "normal" DNA and do not show any increase in buoyant density in Cs_2SO_4–Ag^+ gradients at the Ag^+/DNA-phosphate ratios used (Davidson *et al.*, 1965).

In agreement with this interpretation, fragments enriched in G + C tend to have a normal chromatographic behaviour and a lower buoyant density in Cs_2SO_4–Ag^+. Additional evidence pointing to the same conclusion is presented in the following paper.

(c) *Heterogeneity of mitochondrial DNA*

Chromatography on hydroxyapatite columns provides the first indication of an "intermolecular" compositional heterogeneity of yeast mitochondrial DNA at a size level of $1{\cdot}5\times10^6$ daltons. At least 1% of the mitochondrial genome is formed by segments having a molecular weight of about $1{\cdot}5\times10^6$ daltons (13 s) and a G + C content of 22% ($\rho = 1{\cdot}689$ g/cm^3). A slight decrease in the molecular weight ($S = 12$ s; preparations I and II) permits the separation of at least 3% of mitochondrial DNA as fragments having a buoyant density of 1·690 g/cm^3. The possibility that the molecules having a higher G + C content derive from spontaneous cytoplasmic petite mutants can be ruled out on the ground that, in this case, practically all the mutants would have to contain a mitochondrial DNA having a buoyant density equal to 1·690 g/cm^3. This is extremely unlikely, since all buoyant densities of mitochondrial DNA's from cytoplasmic petite mutants reported so far in the literature were equal to, or lower than, those of mitochondrial DNA's from wild-type cells.

It must be stressed that since the fragments are formed by a practically random degradation process, and are therefore derived from different regions of the genome, they represent much more than 1% and 3% of the genome, respectively. It is evident, however, that in order to gain further insight into the problem of the heterogeneity of mitochondrial DNA, this should be fragmented to lower sizes and then fractionated according to base composition. When applied to the G + C-rich fractions obtained from preparation IV, this approach produced fragments having a G + C content of 24% and an $S_{20,w} = 6{\cdot}6$ s, corresponding to a molecular weight of $2{\cdot}5\times10^5$. A more detailed investigation of the heterogeneity of mitochondrial DNA using enzyme degradation is presented in the following paper.

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