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Mitochondrial DNA's from Respiratory-sufficient and Cytoplasmic Respiratory-deficient Mutant Yeast

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The mitochondrial DNA's from two "grande" yeast strains and three cytoplasmic "petite" mutants were isolated by hydroxyapatite chromatography and investigated in their chemical and physical properties.

The buoyant densities of all DNA's were equal to 1·683 g/cm^3, except for one from a petite strain which had a density equal to 1·678 g/cm^3. The mitochondrial DNA's from the petite mutants were "reversible", their buoyant densities and their ultraviolet absorptions showing very little or no increase after heating and fast cooling. In contrast, the DNA's from the grande cells showed an increase in buoyant density of 0·010 to 0·011 g/cm^3 and a residual hyperchromicity of 10 to 12% upon the same treatment. In this case, both density and residual hyperchromicity were lowered by thermal treatment at 65°C.

The guanine plus cytosine contents of the DNA's from the grande strains were found to be 17·4 and 16·8%. Those of the DNA's from the petite mutants were 15·5, 15·6 and 12·6%. For all mitochondrial DNA's, the base compositions calculated from their buoyant densities and T_m values did not fit these obtained by analysis.

A striking compositional heterogeneity was indicated by the ultraviolet melting curves of all mitochondrial DNA's. The distribution of the melting components of the DNA's from the grande strains was remarkably different from that of the DNA's from the petite strains. The first one was broad and asymmetric, the second one was multimodal with a small number of components. Furthermore, the DNA's from the different petite mutants showed distinct melting patterns.

1. Introduction

During the past few years, mitochondrial DNA has been the subject of intensive investigations (see Borst & Kroon, 1969, for a recent review of the literature). These are justified by the interest raised by its physico-chemical properties, its replication and its genetic functions. In these respects, yeast mitochondrial DNA is particularly interesting, compared to the mitochondrial DNA's from higher organisms, because the irreversible loss of a functional respiratory system in cytoplasmic mutants was found to be accompanied by modifications of its buoyant density and base composition (Mounolou, Jakob & Slonimski, 1966; Mounolou 1967; Bernardi, Carnevali, Nicolaieff, Piperno & Tecce, 1968; Mehrotra & Mahler, 1968).

The advent of a chromatographic technique for the separation of yeast nuclear and mitochondrial DNA's (Bernardi *et al.*, 1968) enabled us to prepare relatively large amounts of the latter and to characterize them in their physical properties and base

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composition. In the present work, these investigations have been carried out on mitochondrial DNA's from two grande yeast strains and three cytoplasmic petite mutants, in an attempt to correlate the changes in the DNA structure with the changes in its genetic information. We have refrained from suggesting here a general model for the cytoplasmic mutation, feeling that more information is needed to do so. For some of the models previously proposed the reader is referred to Slonimski (1968), Borst & Kroon (1969) and Carnevali, Morpurgo & Tecce (1969).

2. Materials and Methods

(a) *Yeast strains*

Two "grande", respiratory sufficient haploid strains of *Saccharomyces cerevisiae* were used in this work: strain D-243-2B-R_1 and strain C-982-19d; they will be designated here as strain A and strain B, respectively. These two strains were chosen because they derive from different stocks and it is assumed that their chromosome gene-pools are widely separated. Three respiratory-deficient cytoplasmic "petite" mutants were also investigated. Two of them, D-243-2B-106 and D-243-2B-13, derived (like the wild type strain D-243-2B-R_1) from strain D-243-2B; they will be designated here as strain a_1 and strain a_2, respectively. The third petite strain, C-982-19dp 6/6-M15-1B-2/16, derived from the other wild type strain C-982-19d; it will be indicated here as strain b. The genetic properties and the relationships among these strains belonging to two isogenic families, A, a_1, a_2 and B, b, respectively, are summarized in Table 1. Further information about these strains can be found elsewhere (Mounolou *et al.*, 1966; Mounolou, 1967).

In view of the relatively large volumes of cultures necessary for obtaining sufficient amounts of cells it was not convenient to use chemostat-grown cultures. It was found that well-aerated batch cultures which had been in stationary phase at least overnight, permitted the preparation of workable amounts of mitochondrial DNA. Cells were inoculated at about 50 mg (dry weight)/litre in a medium containing: 1% yeast extract (Difco), 5% glucose, 0·12% $(NH_4)_2SO_4$ and 100 μg adenine/ml. Cells were harvested by centrifugation when they had reached a concentration of about 2g (dry weight)/litre and washed first with cold distilled water, and then with 2×10^{-2} M-Tris–HCl, pH 7·8, containing 10^{-2} M-EDTA and 15% saccharose. Cells were then suspended in the same solvent at a concentration of 0·4 g (wet weight)/ml. (about 0·1 g dry weight/ml.). Absence of reversions towards grandes in cultures of petites was checked by plating on glycerol media. Cultures of grandes always contain a small proportion (<5%) of spontaneously arising petites.

(b) *DNA preparation*

Cells were disrupted by mixing with glass beads (0·45 mm diameter; 20 g/40 ml. of cell suspension) in a Braun (Melsungen, Germany) homogenizer at 0 to 5°C operated at maximum speed for 1 min. The supernatant was decanted and the glass beads were washed with 2 vol. of 2×10^{-2} M-Tris–HCl, pH 7·8, containing 10^{-2} M-EDTA and 1% sodium lauryl sarcosinate (Sarkosyl NL30 Geigy, Basle, Switzerland). The homogenate was then made 1 M in $NaClO_4$ by addition of 5 M-$NaClO_4$, shaken for 3 hr at 25°C with 1 vol. of chloroform–isoamyl alcohol (24:1; v/v) and centrifuged. The clear supernatant solution was added to 1 vol. of cold (−20°C) ethanol. The precipitate which formed was dissolved in a small volume of 0·1 × SSC (SSC is 0·15 M-NaCl − 0·015 M-sodium citrate, pH 7·2) and incubated at room temperature first with 20 μg of pancreatic RNase/ml. (Calbiochem, Los Angeles, Calif.; the enzyme solution was heated up to 80°C for 10 min before use) and 100 μg of pancreatic α-amylase/ml. (Worthington, Freehold, N.J.), and subsequently with 100 μg of pronase/ml. (Calbiochem.; preincubated for 24 hr at 28°C). The digest was treated with chloroform–isoamyl alcohol for 30 min and centrifuged. The aqueous phase was precipitated with 1 vol. of ethanol as above. The precipitate was dissolved in a small volume of NaP (10^{-3} M-sodium phosphate buffer, pH 6·8), treated once more with chloroform–isoamyl alcohol and centrifuged. The aqueous phase was dialysed against 10^{-3} M-NaP at 4°C for 18 hr to eliminate citrate completely. About 30% of the A_{260} of the solution was

TABLE 1

Genetic relationships among the yeast strains used

D 243-2B
chromosomal mutation p_7
$(a, p_7, ad_1, lys_2, \rho^+)$

chromosomal spontaneous reverse mutation† → D 243-2B-R$_1$ (A) (a, P, ad_1, ρ^+) grande

cytoplasmic spontaneous mutation → D 243-2B-13 (a_2) $(a, p_7, ad_1, lys_2, \rho^-)$ suppressive (95%) petite

cytoplasmic spontaneous mutation → D 243-2B-106 (a_1) $(a, p_7, ad_1, lys_2, \rho^-)$ suppressive (78%) petite

C 982-19 d (B)
$(\alpha, P, tr_1, his_1, \rho^+)$

cytoplasmic spontaneous mutation → C 982-19d p 6/6-M15-1B-2/16 (b) $(\alpha, P, tr_1, his_1, \rho^-)$ suppressive (95%) petite

For more details on the strains used, see Mounolou *et al.*, 1966; Mounolou, 1967.

† The $p_7 \rightarrow P$ revertant may not be a back mutation *sensu stricto* but a suppressor mutation (Mounolou, 1967).

eliminated upon this dialysis and the dialysed solution was submitted to chromatographic purification. The DNA samples, as obtained from hydroxyapatite chromatography, were stored at −20°C.

(c) *Chromatography of nucleic acids on hydroxyapatite columns.*

This was performed according to Bernardi (1965,1969 *a,b,c*). The experimental conditions used in the present work are similar to those previously reported (Bernardi *et al.*, 1968) and are given in detail in the legends to the Figures. In all chromatograms, recoveries of the loaded material from the columns were higher than 95% as judged from the ultraviolet absorption. Columns were generally used three times and then discarded.

(d) *Caesium chloride density-gradient*

This was done at 25°C as previously described (Bernardi *et al.*, 1968) using phage 2C DNA ($\rho = 1{\cdot}742$ g/cm^3; Szybalski, 1968) as a density marker. DNA solutions were in 0·001 M-NaP except for heat-denatured samples which were in SSC/3. The densities of the DNA samples were determined as described by Mandel, Schildkraut & Marmur (1968). Alkaline CsCl density gradients were performed using solutions having a density equal to 1·740 g/cm^3 and a pH close to 12·5. Poly dT from poly (dA : dT) was used as a density marker; its buoyant density was taken as equal to 1·771 g/cm^3 (Wells & Blair, 1967). With the gradients used, poly dA, which has a buoyant density equal to 1·622 g/cm^3 (Wells & Blair, 1967), banded near the meniscus.

(e) *Sedimentation velocity analysis*

A Spinco model E instrument, equipped with ultraviolet optics, was used. Nucleic acid concentrations were close to 25 μg/ml. Solvents were SSC, or SSC adjusted to pH 12·5 with N-NaOH. Median S-values were calculated.

(f) *Enzymic digestion of DNA to nucleosides.*

This was done as described by Bernardi *et al.* (1968), using acid DNase (Bernardi, Bernardi & Chersi, 1966), exonuclease (Bernardi & Bernardi, 1968), and acid phosphomonoesterase II (G. Bernardi, A. Chersi & A. Bernardi, manuscript in preparation) from hog spleen.

(g) *Nucleoside analysis on polyacrylamide gel columns.*

This was done by a modification of the method of Carrara & Bernardi (1968*a*). Nucleosides were loaded on a 0·8 cm × 120 cm column of Bio-Gel P2 (minus 400 mesh; Bio-Rad, Richmond, Calif.) equilibrated with 0·002 M-ammonium carbonate, pH 10·3. Elution was carried out with this solvent at a constant flow rate of about 6 ml./hr (see also legend to Fig. 11). A detailed description of this procedure will be published elsewhere.

(h) *Absorbance–temperature profiles*

DNA solutions in SSC having an A_{260} close to 0·5 were used in these experiments, which were done at least in triplicate. Absorbance–temperature profiles were determined using a Beckman DB spectrophotometer equipped with a cuvette heater, whose rise in temperature was programmed to be linear with time. Absorbances at 260 mμ and the temperature in the sample cuvette (as measured with a thermocouple) were recorded and, at the same time, digital values were automatically printed. Recordings were also taken when the temperature was lowered to 25°C (by cutting off the heater) and during a second heating cycle. A detailed description of the automatic set-up will be given elsewhere. Data were corrected for the volume changes of the solvent and for the non-linearity of the thermocouple response using a computer program. T_m values were calculated according to Marmur & Doty (1962). The absorbance–temperature profiles were analysed by differentiating them. Results obtained by differentiation were satisfactory because of the relatively high DNA concentrations used in the melting experiments and also because of the high degree of precision and reproducibility of the automatic measurements.

(i) *Renaturation experiments*

DNA solutions in SSC (10 μg/ml.), heated to 100°C and fast-cooled, were adjusted to 2 × SSC and kept, in sealed tubes, at 65°C for 5 hr.

3. Results

(a) *DNA fractionation on hydroxyapatite columns*

The chromatographic fractionation on hydroxyapatite columns of the nucleic acid preparations from the five strains investigated in the present work is shown in Figure 1. In all cases, five main fractions, characterized by a typical range of molarity of the eluting phosphate buffer, can be seen in the chromatograms:

(1) a fraction formed by material not retained by the columns equilibrated with 0·001 M-NaP; this elution behaviour is that found for nucleotides and small oligonucleotides (Bernardi, 1964,1965,1969*a*,*b*,*c*);

(2) a fraction eluted between 0·02 and 0·2 M-NaP and characterized by the presence of a large number of partially resolved peaks which are severely compressed in the transmission recordings shown in Figure 1; the elution profiles shown by the different preparations studied in this region are very similar, particularly as far as the high-eluting region is concerned; the material eluted in the 0·02 to 0·2 M-NaP region is likely to be formed by large RNA fragments (Bernardi 1965,1969*a*,*b*,*c*; Mundry, 1965);

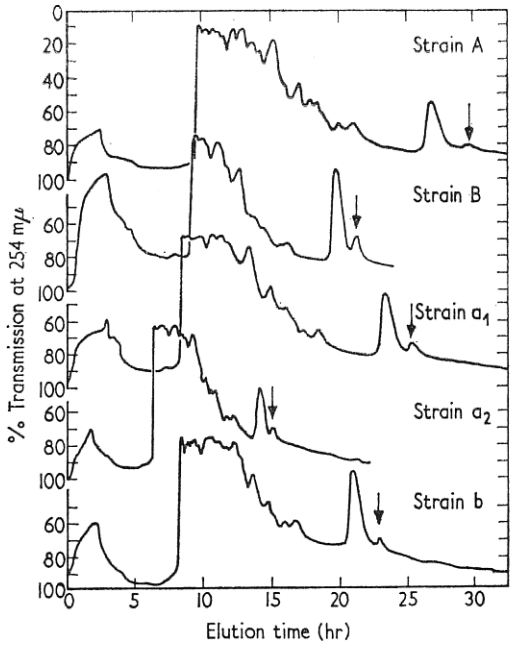

FIG. 1. Chromatograms of DNA preparations from yeast cells, as recorded by a Uvicord (LKB, Stockholm, Sweden) using a cell with an optical path equal to 1 cm. In each case, 100 ml. of nucleic acid solutions in 0·001 M-NaP (415 to 515 A_{260} units) were loaded on 2 cm × 40 cm hydroxyapatite columns equilibrated with the same buffer, 200 ml. of this solvent were used to wash the columns and a 0·001 to 0·5 M gradient of NaP (1120 ml.) was applied. Flow rates varied between 32 and 60 ml./hr. Arrowed peaks correspond to mitochondrial DNA.

(3) a fraction eluted as a single peak at 0·27 to 0·29 M-NaP which is formed by nuclear DNA, as indicated by the previous work of Bernardi *et al.* (1968) and confirmed here by the determination of its buoyant density;

(4) a small fraction eluted as a single peak at 0·29 to 0·33 M-NaP, which is formed by mitochondrial DNA; this identification is based on the previous results of Bernardi *et al.* (1968), and was confirmed by the finding that the buoyant densities of the DNA present in the 0·29 to 0·33 M fractions, as obtained from different strains, were extremely close or identical to those of DNA's isolated from mitochondrial preparations previously obtained from the same strains by Mounolou *et al.* (1966) and Mounolou (1967)†.

(5) a fraction eluting as a broad band between 0·33 and 0·50 M-NaP; this fraction is formed by a small polyribonucleotide extremely rich in guanine; its properties will be reported elsewhere (Bernardi *et al.*, 1968; Piperno & Bernardi, manuscript in preparation).

† The buoyant densities of mitochondrial DNA from strain A (D-243-2B-R_1) was found in previous investigations to be equal to 1·687 (Mounolou *et al.*, 1966), 1·685 (Mounolou, 1967), $1{\cdot}686_5$ (Fukuhara, 1968) and 1·683 g/cm³ (Guérineau, Grandchamp, Yotsuyanagi & Slonimski, 1968). In spite of these variations, the difference found in those experiments between the buoyant density of mitochondrial DNA and that of nuclear DNA was quite reproducible and constant (0·015 ± 0·001 g/cm³).

The buoyant density value of the DNA from strain a_2, as determined in the present work (1·683 g/cm³), is considerably different from that previously reported (1·695 g/cm³) by Mounolou *et al.* (1966) and Mounolou (1967) for DNA extracted from cellular fractions enriched in mitochondria.

The patterns shown in Figure 1 refer to small-scale experiments. They are presented to illustrate the over-all patterns shown by the nucleic acid preparations used in this work. The actual chromatographic purification of mitochondrial DNA has generally involved four subsequent steps. Figure 2 shows these steps in the case of the purification of mitochondrial DNA from strain B, as an example.

The final yields in mitochondrial DNA's from the different strains were close to 30 (strain A), 12 (strain B), 14 (strain a_1), 17 (strain a_2) and 25 (strain b) μg DNA/g (dry weight) of cells. These yields, although equal to, or higher than, those obtained by other authors (Mehrotra & Mahler, 1968) are relatively low since the total amounts of mitochondrial DNA's from strains A and B were estimated to be of the order of 200 μg/g (dry weight) of yeast (P. Clertant, manuscript in preparation). It should be pointed out, however, that the low yields are essentially due to incomplete extrac-

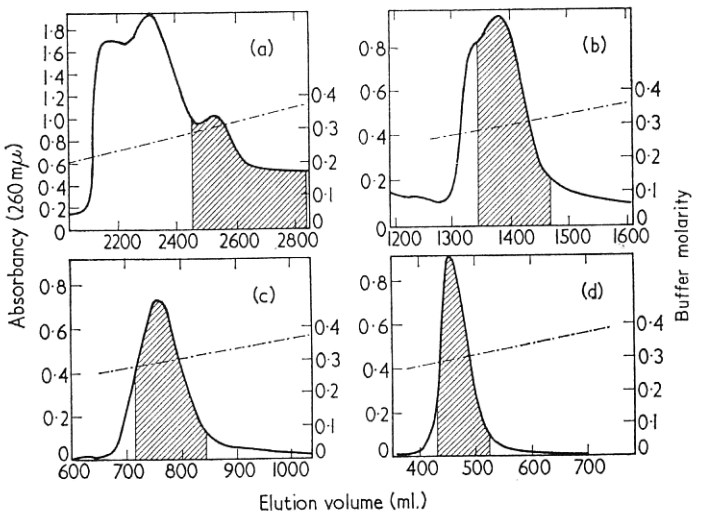

FIG. 2. Chromatographic purification of mitochondrial DNA from strain B.

(a) 500 ml. of DNA solution in 0·2 M-NaP, A_{260} = 38 units, were loaded on a 3 cm × 57 cm hydroxyapatite column equilibrated with the same buffer. 1200 ml. of this solvent were used to wash through all material which was not retained (not shown in the Figure). A gradient (0·2 to 0·5 M) of NaP (1650 ml.) was used to elute adsorbed nucleic acids. Fraction volume was 7·5 ml.; flow rate was 60 ml./hr. The hatched area indicates the fractions containing mitochondrial DNA which were further purified. All materials preceding the mitochondrial DNA were adjusted to 0·20 M-NaP and rechromatographed; the fractions eluted between 0·28 and 0·34 M-NaP were also further processed.

(b) Fractions from the chromatogram shown in (a) (388 ml.; A_{260} = 1·437) and from the rechromatography of the early fractions (313 ml.; A_{260} = 0·552) were adjusted to 0·27 M-NaP and loaded on a 2 cm × 59 cm hydroxyapatite column. 350 ml. of this solvent were used to wash through all material which was not retained. A gradient (0·27 to 0·52 M) of NaP (900 ml.) was used to elute adsorbed DNA. Fraction volumes were 3·8 ml. All other indications as above.

(c) 122 ml. of DNA solution (A_{260} = 0·690) from the previous chromatogram and 88 ml. of DNA solution (A_{260} = 0·115) from the chromatogram of the DNA preparation from strain B shown in Fig. 1, were adjusted to 0·27 M-NaP and loaded on a 2 cm × 57 cm hydroxyapatite column. 230 ml. of this solvent were used to wash through all material which was not retained. Fraction volumes were 3·8 ml. All other indications as above.

(d) 120 ml. of DNA solutions (A_{260} = 0·493), from the previous chromatogram, were adjusted to 0·27 M-NaP and loaded on a 2 cm × 38 cm hydroxyapatite column. 140 ml. of this solvent were used to wash through all material which was not retained. Fraction volumes were 3·8 ml. The hatched area shows the fractions which were pooled and used for chemical and physical studies. All other indications as above.

tion of DNA from the cells and to losses during the preparative steps preceding the chromatographic purification. In fact, the over-all recoveries from the series of hydroxyapatite columns can be estimated as higher than 70%, the losses being due to the elimination of the side fractions of mitochondrial DNA peaks (Fig. 2).

(b) *Physical properties of mitochondrial DNA's*

The physical properties of yeast mitochondrial DNA's investigated here are reported in Table 2, which also shows the results obtained with nuclear DNA.

(i) *Caesium chloride density-gradient centrifugation*

Figures 3 and 4 show the CsCl density gradient centrifugations of yeast mitochondrial DNA's. The banding properties of nuclear DNA are also shown for the sake of comparison.

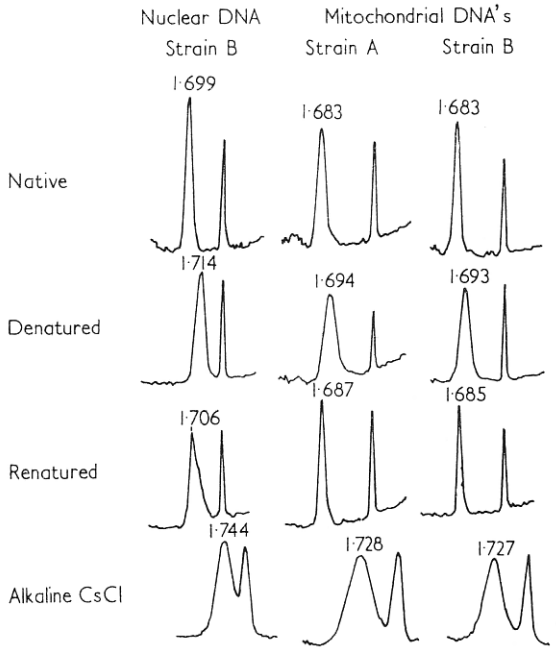

FIG. 3. Microdensitometer tracings of DNA preparations from grande yeast cells centrifuged at 44,770 rev./min for 21 hr in a CsCl density gradient. DNA's from phage 2C (1·742 g/cm^3) and poly dT (1·771 g/cm^3; from synthetic poly dA : dT) were used as density markers for the neutral and alkaline gradients, respectively. Buoyant densities, in g/cm^3, of DNA's are shown in the Figure.

In the native state, all DNA's showed a single band. The densities found were 1·683 g/cm^3 for the DNA's from the two grande cells and for those from strains a_1 and a_2; the density of the DNA from strain b was equal to 1·678 g/cm^3; the nuclear DNA showed a buoyant density equal to 1·699 g/cm^3.

The densities of heat-denatured, fast-cooled mitochondrial DNA's were found to be different for the preparations from the grande cells and for those from the petite mutants. The DNA's from the grande strains showed an increase in buoyant density of 0·010 to 0·011 g/cm^3, whereas the DNA's from the petite mutants showed no change

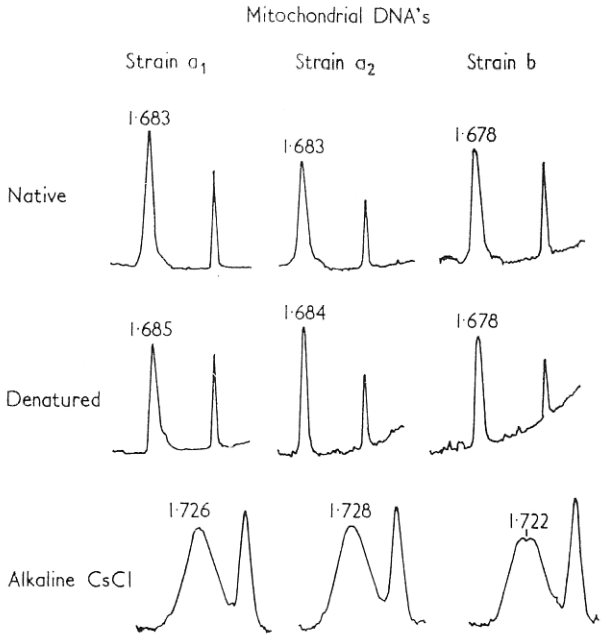

FIG. 4. Microdensitometer tracings of mitochondrial DNA preparations from petite mutant yeast cells. All other indications as in Fig. 3.

in their buoyant densities after heat-denaturation and fast-cooling, except for the DNA from strain a_1 which showed a very small increase of 0·002 g/cm^3. Upon heat-denaturation and fast-cooling, nuclear DNA showed the expected increase in buoyant density of 0·015 g/cm^3.

Upon renaturation, the mitochondrial DNA's from the grande strains showed a decrease of their buoyant densities to values close, yet not identical, to those of the native samples, since they were equal to 1·687 and 1·685 g/cm^3 for the DNA's from strains A and B, respectively. The bands obtained with renatured DNA's from the grande strains were sharper than those of the native samples, probably owing to intermolecular aggregation. A similar phenomenon was also observed for the DNA from the petite strain a_1 (not shown in the Figure).

Nuclear DNA showed, upon renaturation, the presence of a sharp peak having a density equal to 1·706 g/cm^3 and of a heavier shoulder, whose buoyant density is close to that of denatured nuclear DNA (1·714 g/cm^3). It is possible that the sharp peak is due, in part at least, to the contribution of renatured satellite DNA. In the native state, this appears as a shoulder (not always clearly visible on the microdensitometer tracings) having a density equal to 1·704 to 1·706 g/cm^3 (Corneo, Moore, Sanadi, Grossman & Marmur, 1966; Moustacchi & Williamson, 1966).

Finally, when centrifuged in alkaline CsCl density gradients, mitochondrial DNA's showed bands with densities equal to 1·726 to 1·728 g/cm^3, except in the case of DNA from strain b where the density was equal to 1·722 g/cm^3 (median value). In all cases, bands were broader than that of nuclear DNA (ρ = 1·744 g/cm^3); a clearly bimodal distribution could be detected in the case of DNA from strain b.

(ii) *Sedimentation velocity*

The nuclear and mitochondrial DNA's used in this work showed single boundaries having sedimentation coefficients of between 15 and 17 s, except in the case of DNA from strain B, which showed two sedimenting boundaries associated with S-values of 17 and 9 s, respectively. In a control experiment performed after several months, the fast-sedimenting material showed a decrease in its S-value, from 17 to 13 s and a decrease in amount from 70 to 50%, whereas the slow-sedimenting component, at the same time, increased in amount without showing any significant change in its S-value. Further investigations on the two sedimenting components were not done because, when sedimented in alkali, the DNA from strain B showed a single boundary having an S-value equal to 10 s.

(iii) *Absorbance–temperature profiles*

Figures 5 and 6 show the absorbance–temperature curves obtained with the yeast DNA samples.

The hyperchromic shifts obtained upon heating the DNA samples to 100°C ranged from 45 to 50% for the mitochondrial DNA's, whereas they were equal to 44, 50 and 53% for the nuclear yeast DNA, poly (dA : dT) and poly (dAT : dAT), respectively. The high hyperchromic shifts exhibited by mitochondrial DNA's are far from surprising in view of the fact that native DNA is more hypochromic when its A + T content is higher (Fresco, Klotz & Richards, 1963; Mahler, Kline & Mehrotra, 1964). A similar observation has been made on the hyperchromic shifts caused by the combined DNase–exonuclease digestion of mitochondrial DNA's. These were also higher (75%) than that obtained for calf thymus DNA, 70% (Carrara & Bernardi, 1968*b*).

As shown in Figure 5, nuclear DNA exhibited a $T_m = 85$°C; its residual hyperchromicity upon fast-cooling was equal to 16%; when re-heated up to 100°C, only a very small region of sharp melting above 75°C (probably due to renatured satellite DNA) could be seen. The two mitochondrial DNA's from grande strains A and B

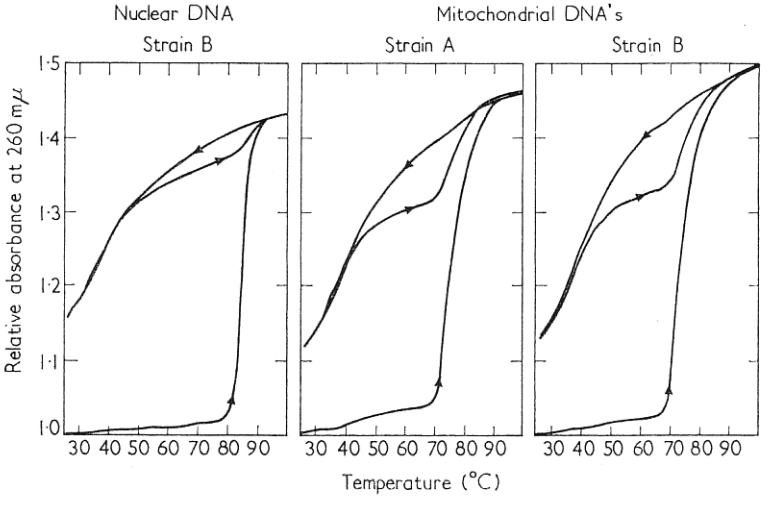

FIG. 5. Ultraviolet melting curves of DNA's from grande yeast cells. Curves indicate the relative optical densities at 260 mμ as measured during the first heating, the cooling and the second heating of the solution (see text).

showed T_m values equal to 74·7 and 73·7°C, respectively. The melting curves showed that the thermal transitions were very broad. The residual hyperchromicities upon fast-cooling were close to 10 to 12% in both cases; they could, however, be decreased by a factor of almost two by heating the DNA's to 65°C in 2 × SSC. When the first heating was followed by a second heating cycle (Fig. 5), both samples showed a considerable region of sharp melting above 65°C.

The mitochondrial DNA's from the petite mutants showed T_m values close to 73·4°C (Fig. 6 and Table 2). The recordings of the melting curves (Fig. 8) showed that these were multimodal, this being particularly striking in the case of the DNA from strain b. When fast-cooled, the mitochondrial DNA's from the petite mutants showed residual hyperchromicities comprised between 0 and 6%, the highest value being exhibited by the DNA from strain a_1. A second heating cycle (Fig. 6) caused these

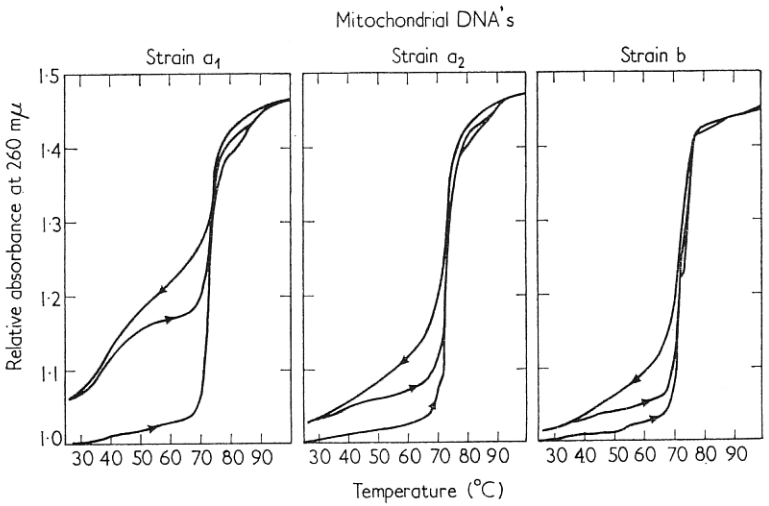

FIG. 6. Ultraviolet melting curves of mitochondrial DNA's from petite mutant yeast cells. All other indications as in the previous Figure.

TABLE 2

Physical properties of mitochondrial DNA's

Strain	Buoyant density (g/cm³)			Alkaline CsCl	T_m† (°C)	% Hyperchromic shift
	Native	Denatured	Renatured			
B (Nuclear DNA)	1·699	1·714	1·706‡	1·744	85·0	44
A	1·683	1·694	1·687	1·728	74·7	47
B	1·683	1·693	1·685	1·727	73·7	50
a_1	1·683	1·685	1·684	1·726	73·4	47
a_2	1·683	1·684	—	1·728	73·5	47
b	1·678	1·678	—	1·722§	73·3	45

† Average of 3 to 5 replicate determinations.
‡ Density of the sharp light peak, probably due to satellite DNA (see Fig. 3).
§ Median value (see Fig. 4); two partially resolved bands are present.

samples to melt very sharply again; the behaviours of the DNA from strain a_2 and, more so, of that from strain b being very close to those of the corresponding native DNA's.

The melting curves of poly (dAT : dAT), poly (dA : dT) and of crab (*Cancer pagurus*) DNA, a DNA containing a satellite formed essentially by alternating (dAT : dAT) (Swartz, Trautner & Kornberg, 1962) were also investigated (Fig. 7) to provide a useful comparison of the A + T-rich yeast mitochondrial DNA's with polynucleotides formed only (or almost only, in the case of the crab satellite DNA) by A and T and having different sequences.

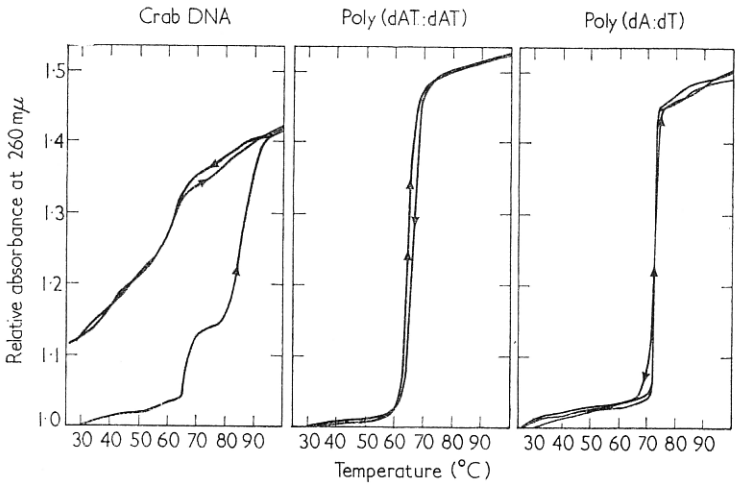

FIG. 7. Ultraviolet melting curves of crab DNA, poly (dAT : dAT) and poly (dA : dT). All other indications as in the previous Figure.

The light satellite of crab DNA, and the biosynthetic polymers poly (dAT : dAT) and poly (dA : dT) showed T_m values equal to 67·0, 65·0 and 73·0°C respectively, in good agreement with previous data by other authors (Marmur & Doty, 1962; Smith, 1964; Davidson *et al.*, 1965; Riley, Maling & Chamberlin, 1966). The biosynthetic polymers showed no residual hyperchromicity upon cooling.

(iv) *Analysis of melting curves*

The broadness and asymmetry of the thermal transitions exhibited by the mitochondrial DNA's from the grande strains and the multimodality of the thermal transitions of the DNA's from the petite strains prompted a finer analysis of the melting curves. This was done by studying the first derivatives of the curves.

Figure 9 shows the results obtained with the two biosynthetic polydeoxyribonucleotides and with nuclear yeast DNA; the data obtained with the mitochondrial DNA from the grande strain B are also presented to show how this DNA strikingly differs from both the sharply melting synthetic polymers and the nuclear DNA. Figure 10 shows a detailed comparison of the differential melting curves obtained with mitochondrial DNA's, in the 65 to 90°C temperature range, upon both a first and a second heating cycle.

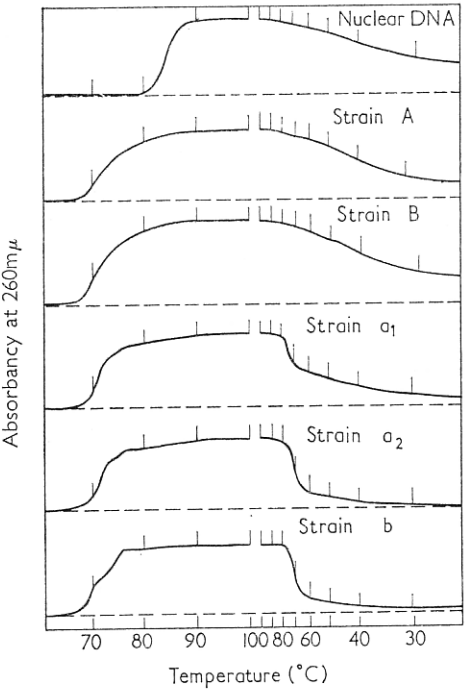

FIG. 8. Recordings of the absorbancy at 260 mμ of yeast DNA solutions in SSC during heating (left-hand curves) and cooling (right-hand curves). The broken lines indicate the A_{260} of the starting sample, to show the different behaviour of various DNA's upon cooling. The chart speed used during the cooling process was 2·5 times slower than during heating.

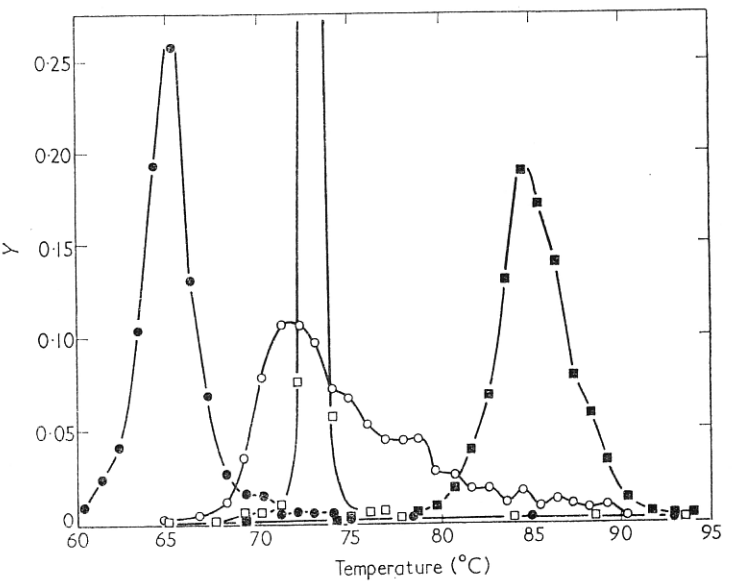

FIG. 9. Differential melting curves obtained with poly (dAT : dAT) (—●—●—), poly (dA : dT) (—□—□—), mitochondrial DNA from strain B (—○—○—) and nuclear DNA from strain B (—■—■—). The ordinate indicates the increment in relative absorbance per degree: $Y = \dfrac{A_{t_1} - A_{t_2}}{A_{100} - A_{25}} \Big/ (t_1 - t_2)$, where A_{t_1}, A_{t_2}, A_{100}, A_{25} are absorbances measured at temperatures t_1, t_2, 100°C and 25°C, respectively. The abscissa values are equal to $t_1 + t_2/2$. Y_{max} of poly (dA : dT) had a value of 0·71.

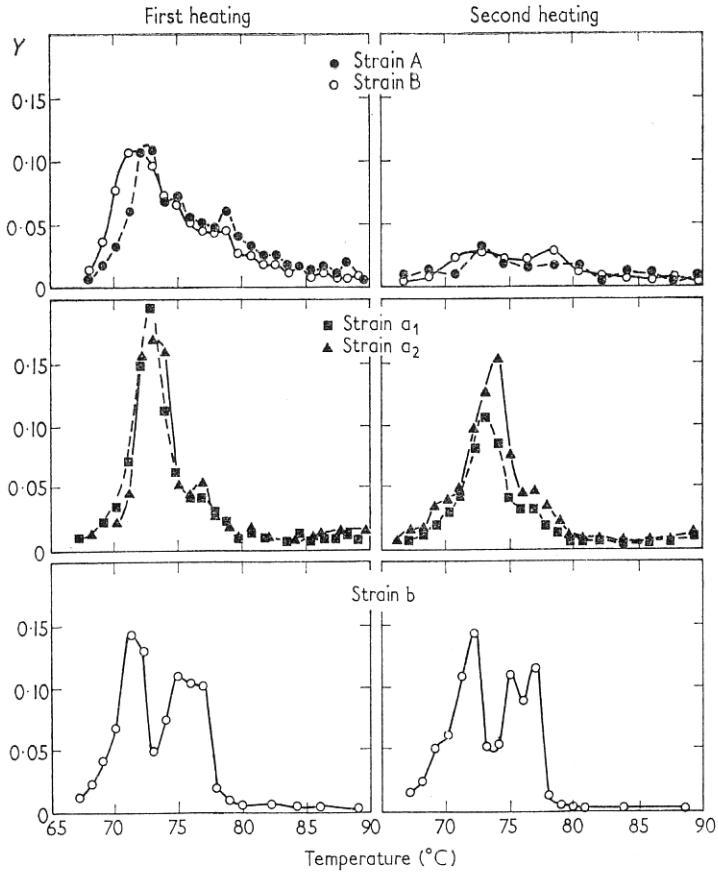

FIG. 10. Differential melting curves obtained with mitochondrial DNA's. Every curve is an average from 2 to 5 replicate experiments. See Fig. 9 for other indications.

The comparison of the differential melting curves of mitochondrial DNA's shows several interesting features.

(a) The two DNA's from the grande cells show a main component, which has a slightly different melting temperature in the two cases (72·5 for A and 71·5°C for B) followed by a series of components of decreasing importance, which appear at the same temperatures in both DNA's; the extent of the thermal transition is exceptionally broad and asymmetric in both cases; upon a second heating, both DNA's show a rather similar pattern characterized by a broad and low transition.

(b) The two DNA's from the petite strains a_1 and a_2 show very similar patterns; in the first heating cycle one main component, melting at about 73·0°C (a value close to the melting temperature of the main component of the DNA from strain A), is present, followed by a minor component melting at about 77°C; in contrast to the DNA's from the grande strains, very little material melts at temperatures above 80°C. The second heating cycle shows again the presence of two components having the same melting temperatures as shown in the first cycle.

(c) The DNA from the petite strain b shows two components of almost comparable importance melting at 71·5°C (a melting temperature identical to that of the main

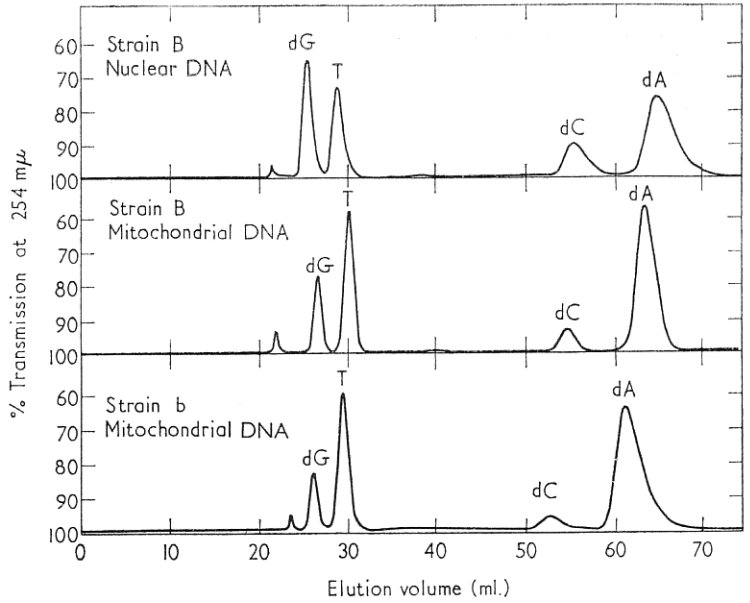

FIG. 11. Chromatography on Bio-Gel P2 columns of the deoxyribonucleosides obtained by enzymic digestion from three yeast DNA samples, as recorded by a Uvicord, using a cell with an optical path equal to 0·3 cm. The minor peak preceding the deoxyguanosine peak contains deoxyinosine (derived by deamination of deoxyadenosine).

component of the parent B strain), and 76·0°C, respectively; these two components reappear in the second heating cycle; also in this case, in contrast to the DNA's from the grande strains, very little material melts at temperatures above 80°C.

Incidentally, the first derivatives of melting curves provide good evidence that the mitochondrial DNA's, as obtained by hydroxyapatite chromatography, do not contain detectable amounts of nuclear DNA, since this would appear as a peak at 85°C.

(c) *Base composition*

The base compositions of mitochondrial DNA's are summarized in Table 3, which also shows the results obtained with nuclear DNA for the sake of comparison. Figure 11 shows three typical chromatograms obtained with the enzymic hydrolysates of DNA's.

TABLE 3

Base composition of mitochondrial DNA's

Strain	dA	T	dG	dC	% G + C
B (Nuclear DNA)	29·5	30·8	20·1	19·6	39·7
A	42·0	40·6	8·7	8·7	17·4
B	41·7	41·6	8·5	8·3	16·8
a_1	42·5	42·0	7·9	7·6	15·5
a_2	42·2	42·1	8·2	7·4	15·6
b	44·1	43·5	6·4	6·2	12·6

The results are given in molar fractions. All analyses were done in duplicate.

The base compositions found for the mitochondrial DNA's from the two grande strains A and B indicated a G + C content of 17·4 and 16·8% respectively. Slightly lower values, 15·5 and 15·6% were found for the mitochondrial DNA's from the petite strains a_1 and a_2, respectively, whereas a much lower value, 12·6%, was found for the DNA from strain b.

Table 4 shows the base compositions of mitochondrial DNA's as obtained by direct analysis or as calculated from buoyant densities, melting temperatures, and optical rotatory dispersions, using the empirical equations of Schildkraut, Marmur & Doty (1962), Marmur & Doty (1962), and Samejima & Yang (1965), respectively. Table 4 also reports the G + C contents as calculated by summing over the 65 to 90°C range the products of the Y-values ($Y = \dfrac{A_{t_1} - A_{t_2}}{A_{100} - A_{25}} \Big/ (t_1 - t_2)$, where $A_{t_1}, A_{t_2}, A_{100}, A_{25}$, are absorbances measured at temperatures t_1, t_2, 100 and 25°C, respectively), taken at different temperatures, by the G + C contents (determined for these temperatures using the empirical relationship of Marmur & Doty, 1962), $\sum_{i\,=\,65°C}^{i\,=\,90°C} Y_i \cdot GC_i$. This procedure was used in order to take into account the relative contributions of the melting components present in the complex transition patterns exhibited by mitochondrial DNA's.

TABLE 4

Percentage guanine plus cytosine content of mitochondrial DNA's

Strain	From: Analysis	Buoyant density	T_m	Y†	$[\alpha]_{max}$‡
B (Nuclear DNA)	39·7	40·0	38·5	40·0	34·3
A	17·4	23·5	13·2	17·6	30·7
B	16·8	23·5	10·7	14·7	20·4
a_1	15·5	23·5	10·0	14·7	36·2
a_2	15·6	23·5	10·2	16·3	38·3
b	12·6	19·0	9·8	12·3	27·8

† For the definition of Y see legend to Fig. 9.

‡ From Bernardi & Timasheff (1970). $[\alpha]_{max}$ is the optical rotatory dispersion at the maximum (290 to 287 mμ).

4. Discussion

(a) *DNA fractionation on hydroxyapatite columns*

The two major differences between the present and the previous results (Bernardi *et al.*, 1968) concern the multipeak region formed by material eluting between 0·02 and 0·2 M-NaP, and the material eluting between 0·33 and 0·50 M-NaP. The multipeak region found in this work was replaced by a single peak eluting at about 0·20 M in the previous work, where the material eluting after mitochondrial DNA was practically absent. An explanation for these differences is that the preparations examined in the previous work had been purified more extensively by a procedure involving several alcohol precipitations. These treatments probably eliminated most of the RNA fragments, leaving only one class of them, which was eluted by 0·20 M-phosphate. It is also possible that, in addition, the RNase treatments gave different levels of RNA degradation in the two cases. As far as the high-eluting material (the G-rich poly-

ribonucleotide) is concerned, it was already remarked in the previous work that its amount diminished very considerably upon repeated ethanol and isopropanol precipitation. The lower purity and the larger amounts of the nucleic acids preparations to be purified explain why four chromatographic steps were necessary in this work to obtain mitochondrial DNA instead of only two in the previous work.

(b) *Physical properties*

(i) *Buoyant densities*

Except for the DNA of one petite mutant, which showed a very low buoyant density (1·678 g/cm^3), the densities of the DNA's from the grande and the petite cells were found to be equal to 1·683 g/cm^3, showing that a buoyant density change does not necessarily accompany the cytoplasmic mutation as previously suggested (Mounolou *et al.*, 1966,1968). In spite of these identical values of buoyant densities, the denaturation–renaturation properties of mitochondrial DNA's are strikingly different in the cases of the samples from grande and petite cells, respectively. In fact, while the DNA's from grande cells show an increase in buoyant densities upon denaturation and fast-cooling which is equal to 0·010 to 0·011 g/cm^3, the DNA's from the petite mutants show no increase at all in two cases and a very small increase (0·002 g/cm^3) in the third one (a_1).

It may be interesting to remark that the buoyant density of mitochondrial DNA from strain b (1·678 g/cm^3) is the same as that of poly (dAT : dAT); the effect of the G + C contents (12·6%) of this DNA must, therefore, be offset by the presence of sequences lighter than those of (dAT : dAT), namely (dA : dT) sequences (the buoyant density of the non-alternating polymer is 1·647 g/cm^3). Similar conclusions can be reached for the other mitochondrial DNA's investigated here and for the very light mitochondrial DNA ($\rho = 1{\cdot}672$ g/cm^3)† from another petite mutant previously investigated (Bernardi *et al.*, 1968), if the contribution of the G : C pairs to their buoyant densities obeys the relationship of Schildkraut *et al.* (1962).

The buoyant density results are therefore in agreement with the conclusion, derived from optical rotatory dispersion and circular dichroism spectra, that both alternating (dAT : dAT) and non-alternating (dA : dT) sequences are present in yeast mitochondrial DNA's (Bernardi & Timasheff, 1970).

(ii) *Melting curves*

The most striking feature of the derivatives of the melting curves of yeast mitochondrial DNA's is that they deviate strongly from a Gaussian distribution. The left-hand parts (up to 74°C) of the first derivatives of the melting curves approximate a single Gaussian distribution characterized by σ_{left} values comprised between 1 and 1·8 deg.C. This is not the case for the right-hand parts (above 74°C) which trail far into the 85 to 95°C region and show different profiles according to the strains; fitting the data to a single Gaussian distribution would lead to a value of σ_{right} larger than 10 deg. C.

These features are quite different from those shown, for instance, by bacterial DNA's‡. In fact, these may show slightly asymmetrical denaturation curves (using

† Values of 1·672 g/cm^3 and 1·675 g/cm^3 were reported earlier (Bernardi *et al.*, 1968). The higher value was due to the presence of 0·3 M-NaP in the DNA solutions used.

‡ The comparison with nuclear DNA's is complicated by the presence of satellites. The comparison with bacterial DNA's may also be of interest in view of the suggestion of a bacterial origin for mitochondrial DNA (Nass, 1969).

Y versus t^0 plots), yet each half, above and below T_m, is nearly perfectly Gaussian; the degree of skewness, given by the ratio $\sigma_{left}/\sigma_{right}$, is quite small and ranges from 1·0 to 1·6 deg.C; furthermore the average value $\sigma_{left} + \sigma_{right}/2$ does not depend upon the average G + C content of DNA and ranges between 1·6 and 2·5 deg.C (De Ley, 1969).

It appears, therefore, that mitochondrial DNA's are formed by segments of quite different average base composition. That this heterogeneity is intramolecular and not intermolecular is suggested by the single and essentially symmetrical bands formed by mitochondrial DNA's in CsCl density gradients. A similar situation seems to exist in the light satellite DNA of *Trypanosoma cruzii* (Riou & Paoletti, 1967).

As far as the compositional heterogeneity of mitochondrial DNA's is concerned, it is interesting to remark that the first derivatives of the melting curves clearly indicate that the cytoplasmic mutation causes drastic changes in the distribution of melting components. In fact, the continuous, asymmetric distribution shown by the DNA's of the grande strains is replaced by a small number of melting components in the DNA's of the petite strains. Furthermore, the melting patterns of the latter show various degrees of dissimilarity. Interestingly enough, all mitochondrial DNA's showed a first melting component having a T_m between 71 and 73°C. This component certainly is not formed by pure alternating (dAT : dAT), since this has a much lower T_m, (65°C), and is probably formed by alternating (dAT : dAT) stretches interspersed with a small number of G : C pairs† and/or a relatively large amount of non-alternating (dA : dT), which has a $T_m = 73$°C (see also below).

As far as the denaturation–renaturation behaviour of mitochondrial DNA is concerned, the results obtained in the melting experiments lead to a picture quite similar to that arrived at from the buoyant density studies. In fact, the mitochondrial DNA's from the grande strains show a significant residual hyperchromicity which can, however, be decreased by heating the DNA's for five hours at 65°C. The behaviour of the DNA's from the three petite mutants also parallels that shown in CsCl, since the samples from strains a_2 and b, which show after denaturation the same buoyant density as in the native state, also show a negligible residual hyperchromicity and a "second melting" curve, which is essentially identical to the "first melting" one. The DNA from strain a_1, which shows a very small increase in its buoyant density after heating and fast-cooling, also shows a small yet significant extent of residual hyperchromicity, thus exhibiting a behaviour which has some resemblance to those of the DNA's from the grande cells.

The simplest explanation for the increase in the renaturation rate observed in mitochondrial DNA's from the petite mutants compared to those from the grande cells is that it is due to the relative increase in AT sequence reiteration. Two alternative explanations for the "reversibility" (Geiduschek, 1961) of mitochondrial DNA's from the petite strains should be mentioned here: (a) that they are double-stranded circles with no interruptions; (b) that their strands are cross-linked. The first explanation may be ruled out on the basis of the low molecular weights of all mitochondrial DNA's investigated here, which implies that some extent of enzymic degradation has taken place in the early extraction steps. The percentage of small circular molecules in preparations of the kind examined here (Bernardi *et al.*, 1968; Guérineau *et al.*, 1968)

† It may be recalled here, in this connection, that the crab dAT satellite, which contains 3% G + C in an otherwise perfectly alternating poly (dAT : dAT) structure (Swartz *et al.*, 1962) has a melting temperature 2 deg.C higher than the alternating biosynthetic poly (dAT : dAT).

is too small (<5% of the total DNA) to account for the phenomenon. The second explanation seems unlikely to us, but renaturation kinetics are needed to disprove it.

(c) *Base composition*

An important conclusion derived from our analytical results is that the cytoplasmic mutation, which leads in some cases (Bernardi *et al.*, 1968; Mehrotra & Mahler, 1968; and strain b of the present work) to a large decrease in G + C contents of mitochondrial DNA's, may cause, in other cases, (strain a_1 and a_2) only a very small, yet significant, decrease in G + C contents.

The agreement between the base composition estimated by analysis and that calculated from the physical parameters is satisfactory in the case of nuclear DNA. In contrast, no such agreement exists in the case of mitochondrial DNA's (Table 4; see also Bernardi & Timasheff, 1970).

This disagreement does not appear to be caused by the presence of rare bases or by glucosylation. Although the analytical method used in this work does not give clear separations of nucleosides like 5-methyldeoxycytidine or 5-hydroxymethyldeoxyuridine from deoxycytidine and thymidine, respectively, it may be ruled out that the important deviations of the calculated base compositions are due to their presence, since correct spectral ratios were found for the isolated nucleosides. Glucosylation may be ruled out since 3′-P-oligonucleotides from mitochondrial DNA's could be degraded by acid spleen exonuclease, an enzyme unable to degrade the T2 glucosylated 3′-P-oligonucleotides. The comparison of optical rotatory dispersion results obtained with T2 DNA and mitochondrial DNA's (Bernardi & Timasheff, 1970) also leads to the same conclusion.

We conclude therefore that, in all likeliness, these deviations are due to the fact that all mitochondrial DNA's, from both grande and petite cells, are conformationally slightly different from DNA's having a balanced base composition, a fact which would also explain their separation on hydroxyapatite columns.

It may be relevant to note here that the empirical relationships between physical properties and base compositions are also not obeyed by synthetic polynucleotides (dAT : dAT, dA : dT, dG : dC, etc., Schildkraut *et al.*, 1962), by DNA-like polymers of repeating sequences (Wells & Blair, 1967), and also by some DNA's of "normal" G + C content (and probably containing repetitive sequences), like the satellite DNA's from mouse and guinea pig tissues (Corneo, Ginelli, Soave & Bernardi, 1968). It is not at all surprising, therefore, that yeast mitochondrial DNA's which probably contain reiterative sequences involving alternating and non-alternating A,T, also do not obey the empirical relationship mentioned above.

It may be interesting to observe that, if the G + C contents calculated from T_m values of yeast mitochondrial DNA's are in disagreement with the analytical values, this is not the case if the G + C contents are calculated from the Y values (Table 4). This implies that the empirical relationship of Marmur & Doty (1962) leads to G + C contents in satisfactory agreement with the analytical data, if applied to the melting components shown by the first derivatives of the melting curves. This agreement is unexpected because of the high A + T contents of mitochondrial DNA's, since it is known that both alternating and non-alternating A,T polymers do not obey the relationship of Marmur & Doty (1962). A possible explanation for this agreement is that alternating and non-alternating A,T stretches are present to similar extents in mitochondrial DNA's. In fact, the extrapolation to zero G + C contents of the straight

line given by the relationship of Marmur & Doty (1962), 69·3, is very close to the arithmetical mean (69·0°C) of the T_m values of alternating and non-alternating A,T polymers.

In conclusion, the major findings of the present work are: (a) the demonstration of a large compositional heterogeneity in the mitochondrial DNA's from both grande cells and petite mutants, the melting patterns of the DNA's from the grande cells and from the different petite mutants being different from each other; (b) the variable decrease in G + C contents in the mitochondrial DNA's from the different petite mutants. Both results support the idea that the cytoplasmic factor responsible for the petite mutation resides in mitochondrial DNA.

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